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Degradation, cytotoxicity and biocompatibility of NIPAAm-based thermosensitive, injectable and bioresorbable polymer hydrogels

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Abstract

A thermosensitive, injectable and bioresorbable polymer hydrogel, Poly(N-isopropylacrylamide-co-dimethyl- γ -butyrolactone acrylate-co-acrylic acid) (poly(NDBA)), was synthesized by radical copolymerization with 7.00 mol.% dimethyl- γ -butyrolactone acrylate in tetrahydrofuran (THF). The chemical composition was determined by acid titration in conjunction with ^1H NMR quantification. The molecular weight and polydispersity were determined by gel permeation chromatography (GPC) in conjunction with static light scattering. The degradation properties of the polymer hydrogel were characterized using differential scanning calorimetry (DSC), percentage mass loss, cloud point test and swelling ratio over time. It was found that the initial LCST of the polymer is between room temperature and body temperature and that it takes about 2 weeks for the LCST to surpasses body temperature under physiological conditions. An indirect cytotoxicity test indicated that this copolymer has relatively low cytotoxicity as seen with 3T3 fibroblast cells. The *in vivo*-gelation and degradation study showed good agreement with *in vitro*-degradation findings and no detrimental effects to adjacent tissues were observed after the complete dissolution of the polymer.

Keywords

thermosensitive; injectable; bioresorbable; polymer hydrogel; N-isopropylacrylamide

Introduction

Significant research has been conducted on the temperature-sensitive polymer, poly(N-isopropylacrylamide) (poly-(NIPAAm)), and its copolymers for biomedical applications, such as drug delivery, cell immobilization, in situ-gelling implantation, and tissue engineering^{1–3}. Many thermosensitive polymers have lower critical solution temperatures (LCSTs), which are temperatures at which these polymers in aqueous solution experience a phase change from a soluble state to an insoluble state. Poly(NIPAAm) exhibits a LCST at 32°C in aqueous solutions^{4,5}. It has been suggested that this temperature-induced phase transition of poly(NIPAAm) in aqueous solution is mainly driven by the thermal destruction of hydrogen bonds between water molecules in a semi-clathrate structure around the hydrophobic structures in NIPAAm, and an increased interaction between the hydrophobic segments on the polymer with increased temperature^{6–11}. The LCST of poly(NIPAAm) copolymers can be controlled by varying the monomer composition. Generally, the

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incorporation of hydrophobic co-monomers leads to a lower LCST, and incorporation of hydrophilic co-monomers leads to a higher LCST. Acrylic acid increases the LCST of poly(NIPAAm) copolymers at neutral pH^{12,13}. 2-Hydroxyethyl methacrylate-monolactate (HEMA-monolactate) lowers the LCST of poly(NIPAAm) copolymers^{14–17}.

One challenge with using poly(NIPAAm) in biomedical applications such as controlled drug delivery is that poly(NIPAAm) is not resorbable, limiting its application in temporary implantation applications. The existence of a non-degradable polymer in the human body may cause a chronic inflammatory response and make multiple doses difficult, so bioerodable polymers for a controlled drug release system are preferred. In response, several groups have been working to make NIPAAm copolymers biodegradable. Neradovic et al. reported the synthesis of a new type of thermosensitive NIPAAm copolymer with hydrolysable lactate ester side groups in which the LCST increases after the hydrolysis of the ester groups^{14–17}. In this system, a low-molecular-weight by-product of the degradation is lactic acid. Synthesis of NIPAAm with the cyclic monomer 2-methylene-1,3-dioxepane (MDO) and discussion of its biodegradable properties were done by Sun, et al.¹⁸. Yoshida et al. reported the synthesis of NIPAAm copolymers cross-linked with biodegradable poly(amino acid)¹⁹. Lee et al. and Neradovic et al. reported thermosensitive and bioerodible hydrogels with time-dependent LCST properties^{12,14–17,20,21}. In these studies, water soluble and excretable high molecular weight products are formed after degradation. At the same time, there are various small molecules produced. Although small molecules can be cleared by the kidney, they can also be toxic. For example, decreases in pH in the local environment can be cytotoxic in the case of lactic acid^{22–24}.

The LCST property of poly(NIPAAm) copolymers makes them attractive candidate materials for injectable drug release systems. Such systems could avoid surgical implantation, thus simplifying drug administration. Drugs or cells can be suspended in the polymer solution and then, upon injection, the polymer solution will gel due to an increase in temperature from room temperature to body temperature. The suspended drugs will then diffuse out of the gel. In a physiological environment, the polymer will undergo hydrolysis, which is expected to lead to an increase in the LCST with time. When the LCST increases above body temperature, the polymer becomes soluble again, diffuses away and is cleared²⁰.

In our previous work, we have reported the synthesis and characterization of poly(N-isopropylacrylamide-co-dimethyl- γ -butyrolactone acrylate) (poly(NDB)) and poly(N-isopropylacrylamide-co-dimethyl- γ -butyrolactone acrylate-co-acrylic acid), (poly(NIPAAm-co-DBA-co-AAc), P(NDBA)), as hydrolysis-dependent, thermosensitive polymer for an injectable degradable system²⁵. It has been confirmed that the degradation mechanism employed under physiological conditions is hydrolysis of the ester group in the side chain ring structure. Thus, there is no low molecular weight byproduct after degradation, eliminating toxic effects from low molecular weight chemicals. This copolymer would be useful for injectable, in situ-gelling controlled drug delivery systems. With the incorporation of acrylic acid, the initial LCST increases and the hydrolysis rate is increased. The materials can be designed so that the LCST can reach above body temperature within two weeks. The P(NDB) and P(NDBA) copolymers have been synthesized in dioxane which resulted in copolymers with molecular weight of more than 100kDa. For copolymers as temporary drug delivery carriers, it has been shown that the polymers will not be cleared by kidney when molecular weight is above 40kDa. For this purpose, tetrahydrofuran (THF) has been selected as the polymerization solvent to obtain low molecular weight polymers. The synthesized copolymer has been characterized by GPC, NMR, acid titration, DSC, cloud point, % mass loss, and swelling ratio. The cytotoxicity and biocompatibility have also been investigated.

Materials and Methods

Materials

All materials were reagent grade and obtained from Aldrich unless otherwise noted. NIPAAm was dissolved in hexanes (10g in 100mL at 40°C) and then re-crystallized at room temperature. (R)-(+)- α -Acryloyloxy- β,β -dimethyl- γ -butyrolactone acrylate (DBA) was used as received. Acrylic acid was purified by vacuum distillation. 2, 2'-Azobisisobutyronitrile (AIBN) was dissolved in methanol (1g/20mL) at room temperature and re-crystallized at -20°C. Anhydrous 1,4-Dioxane was used as the polymerization solvent and was treated by molecular sieve to remove the dissolved water before use. HPLC grade Tetrahydrofuran (THF) was used as the mobile phase for Static Light Scattering (MiniDawn, Wyatt Technology Corporation)/Gel Permeation Chromatography (GPC) (Shimadzu Corporation). Phosphate Buffered Solution (PBS) at pH 7.4 was used as the solvent for multi-cell Differential Scanning Calorimetry (DSC) (Calorimetry Science Corporation). 3T3 fibroblast cells (ATCC, USA) were used in cytotoxicity tests. Cell culture media was made of Dulbecco's Modified Eagles' Medium (DMEM) with 4.5g/L Glucose, without L-glutamine (BioWhittaker), 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products), 2% L-glutamine (200 μ M, 29.2mg/ml) (Mediatech Cellgro), 1% Penicillin-streptomycin (10,000IU/ml and 10,000 μ g/ml) (Mediatech Cellgro, US). EDTA trypsin was used to detach cells from flasks, while PBS was used to wash cells. Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation) was used to test the cytotoxicity. 24-well Transwell® plate (6.5mm diameter inserts, 3.0 μ m pore size, polycarbonate membrane sterile, polystyrene plates) was used for in vitro degradation at 37°C/5% CO₂ to obtain test samples (Corning, USA). 96-well tissue culture plate (Sarsted, USA) was used for absorbance reading for cytotoxicity.

Synthesis of Poly(NIPAAm-co-DBA-co-AAc)

Poly(NIPAAm-co-DBA-co-AAc) was synthesized in anhydrous tetrahydrofuran (THF) at 65°C for 16 hours by radical polymerization. The feed ratio of DBA was 7 mol. % and the feed ratio of acrylic acid was at 2 mol% (Table 1). AIBN (7×10^{-3} mol AIBN/mol monomer) was used as the initiator for all reactions. The reaction was bubbled with Nitrogen (N₂) for 15 min. before adding the initiator and then maintained under a nitrogen environment throughout the reaction to reduce oxygen. After polymerization, the solution was filtered and the solvent was evaporated. Then the copolymer was dissolved in acetone and collected by precipitation in 14- to 15-fold-excess of diethyl ether. Finally, the copolymer was vacuum-dried for 24 hours. The synthesized polymer was dialyzed against water to remove unreacted NIPAAm and DBA monomer.

Molecular Weight and Polydispersity Determination

The molecular weight and the polydispersity of the synthesized copolymers were determined by gel permeation chromatography (GPC) (Shimadzu VP) in conjunction with static light scattering, using tetrahydrofuran (THF) as the mobile phase. The molecular weight for each copolymer was approximated from light scattering data (Wyatt MiniDawn). The sample was prepared by dissolving the copolymer in THF with a concentration of 5 mg/ml. The flow rate is 1ml/min.

Chemical Composition Determination

The DBA and AAc contents were determined by acid titration in conjunction with ¹H NMR quantification before hydrolysis. Titration was conducted manually for each copolymer using standard sodium hydroxide volumetric solution with the concentration of 0.01N. The

polymer was dissolved in distilled water with the concentration of 0.15g/10mL. At a minimum, measurements were made in triplicate.

Hydrolysis

The time-dependent hydrolysis was evaluated at both 37°C and 70°C. Solutions of the copolymer were prepared in 0.1M PBS at 5 wt. % in vials and then placed in 37°C and 70°C water bath, respectively. The pH of the polymer solution was adjusted to 7.4 daily to maintain a nearly constant pH. Daily changes in pH were less than 0.5 pH units. All solutions were maintained at either 37°C or 70°C. DSC were conducted after hydrolysis for each polymer. The polymers were dialyzed against water and lyophilized before DSC tests.

Differential Scanning Calorimetry (DSC)

The lower critical solution temperature (LCST) of the synthesized copolymers was evaluated by differential scanning calorimetry (DSC). Scans were taken on 5 wt. % solutions in 0.1 M PBS (pH 7.4) at 1°C/min from 0°C to 80°C. A minimum of triplicates was performed for each copolymer before, after and at various time points during hydrolysis.

Cloud Point Test

A cloud point test was conducted for materials before and during hydrolysis at 37°C. 1 wt.% polymer solution was used to take the measurement at 500nm using UV spectroscopy from 5°C to 55°C. The rate of temperature increase was 0.25°C/min using a water circulating system. The cloud point temperature is determined at which the transmittance reach 50%. The results are reported as the mean±standard deviation.

Swelling Ratio and Percent Mass Loss

25 wt.% solutions of NIPAAm-based copolymers in PBS (pH 7.4, 0.1M) were prepared and the pH was adjusted to 7.4 with 1N NaOH. The 0.4ml polymer solution was placed in a pre-weighed vial. The samples were placed at 37°C. After gelation and equilibrium, 4ml pre-warmed PBS (pH 7.4, 0.1M) solution was added. At 4 hours, 8 hours, 1 day, 4 day and 8 day, etc., the excess water was removed and the vial/polymer was weighed. Then, the samples were lyophilized and the vial/dried polymer was weighed. The swelling ratio was defined as $100(W1-W0)/W0$, where $W0$ is the weight of the original dried polymer, $W1$ is the weight of swollen polymer. The percent mass remaining was defined as $100 \times W2/W0$, where $W2$ is the weight of the dried polymer after degradation.

Cytotoxicity Test

An indirect/extraction method was used to investigate the cytotoxicity of the synthesized copolymer. The materials were sterilized by Eto (Ethylene Oxide) sterilization and dissolved in cell culture media without serum at 25 wt.%. The pH of the polymer solution was adjusted to 7.4, and 100μL of the polymer solution was placed into the inserts of a 24-well Transwell® plate. The inserts and polymer solution were incubated at 37°C/5% CO₂ for 1–2 hour until the materials had completely gelled. Then, 0.6 ml of cell culture media was added to the bottom of the Transwell® plate, which was then placed at 37°C incubator. The cell culture media with empty insert on top was used as control. At designated time points, samples of cell culture media at the bottom of the plates that had been exposed to the gels were collected for testing of degradation products. In order to determine the effect of the degrading polymer on cells, 2000 3T3 A31 cells in a total of 100μL media were seeded at the bottom of 96-well plates for 2–3 hours, and then the media was exchanged with media exposed to the degrading polymer in the Transwell® plates for different time periods. The 96-well plates were then placed at 37°C/5% CO₂. Following 48 hour incubation, 20μL of Promega's CellTiter 96 Aqueous One Solution Cell Proliferation Assay solution was added

into each well of the 96-well plate, and the plate was incubated at 37°C/5% CO₂ for 2–3 hours. The absorbance of each well was measured and the cell number was calculated according to the standard curve of cell number determined at the same time. The cell number from control group was normalized to 100%. The results were expressed as relative cell number (compared with control samples) and the error was expressed as the standard deviation of 6 replicates.

***In Vivo* Gelation and Degradation**

25 wt.% solutions of the selected copolymer was prepared by dissolving in 0.1M PBS after being sterilized. The pH was adjusted to 7.4 with 1N NaOH. The polymer solution was placed at 4°C for 48 hours. Upon dissolution, 0.4 ml aliquots were loaded into sterile, 1 ml luer-lock syringes, which were then capped with 18G needles. 0.4ml PBS solution was used as the control sample. All material handling prior to injection was performed in a biosafety hood under sterile conditions. All loaded syringes were kept at 5°C until ready to inject to prevent polymer from precipitating. Male Sprague Dawley rats (~200 g) were used for the *in vivo* 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 polymer solution (25 wt%) were administered subcutaneously. PBS solution was used as control. For the degradation study, animals with implant were sacrificed at time points of 1, 3, 7, 10 days post-implantation. Upon sacrifice, the implants (n=2) were surgically exposed by carefully dissecting the tissue around the implant. Each implant was then photographed and carefully removed.

***In Vivo* Biocompatibility**

For *in vivo* biocompatibility study, the materials preparation and implant procedure are the same as described in *In vivo* Gelation and Degradation study. Upon sacrifice, the tissue around the implant was harvested and preserved in formalin solution. All tissue was sent to the Medical College of Georgia for sectioning and staining. 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 100x total magnification were taken of tissue cross-sections from each time period. The number of dark staining cells in H&E sections, which were assumed to be leukocytes and fibroblasts, were 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 12µm, and fibroblasts nuclei are similarly sized. A cell area of 50 µm², which corresponds to a cell diameter of around 8 µm, will be 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 selected material and control samples were reported as the mean cell count ± standard deviation.

Statistics

LCST data for each polymer are reported as the average peak temperature for three DSC runs per sample, the error is reported as standard deviation. The DBA content is reported as the mean ± standard deviation for 3 samples. The cytotoxicity data are reported as relative

cell number $\pm$ standard deviation for 6 samples. The student t-test was conducted on the cytotoxicity test data and $p < 0.05$ is considered statistically significant.

Results

The synthesis scheme of polymerization is shown in Figure 1. Table 1 shows the chemical composition, molecular weight, and the polydispersity. The result shows that by using THF as the polymerization solvent, the molecular weight dropped to below 30kDa compared with the molecular weight of the copolymer synthesized in dioxane in our previous investigation.

The degradation property of this copolymer was characterized by time-dependent LCST, cloud point and percentage mass loss. Figure 2 shows the time-dependent LCST property under two degradation conditions: 37°C and 70°C both of pH 7.4. At 70°C, it took about 4 days for LCST to reach above 37°C and after 10 days, the LCST reached a plateau of about 47.7°C indicating that hydrolysis is complete; while at 37°C, after about 20 days, the LCST increased to be approximately 35°C indicating a slower degradation rate under physiological conditions. At both conditions, the polymer showed a time-dependent LCST property. The cloud point results (Figure 3) show that at 0, 7, 12, and 20 day degradation time, the cloud point temperature increased from 16°C, to 24, 30 and 37°C respectively. The cloud point test also shows the time-dependent hydrolysis property. The mass degradation result (Figure 4) shows that there is a trend of gradual mass loss over hydrolysis time and after 20 day's degradation, there is no gel mass left. These degradation results show that this material has both the time-dependent LCST and time-dependent mass loss properties.

The gelation and degradation process is also illustrated in Figure 5. Figure 5a shows that this polymer is soluble in aqueous solution below LCST (PBS, 4°C). After incubation in water bath of 37°C for 1 day, it forms a complete gel at the bottom of the vial. The inverted vial shows that the gel is a solid mass at the bottom (Figure 5b). After incubation in water bath of 37°C for 10 days, the polymer has been hydrolyzed and becomes viscoelastic material which follows down along the wall when the vial is inverted (Figure 5c). 12 days later, the polymer has become soluble again seen as turbid liquid (Figure 5d). This process shows that during hydrolysis, the polymer becomes more and more hydrophilic due to the hydrolysis of the side ester group in DBA structure. Finally, the polymer becomes soluble again when LCST is above body temperature. The swelling test was also conducted and the result shows that the polymer hydrogel swells while it degrades (Figure 6). For the first day of degradation, the swelling ratio stays around the same as day 0 and then starts to increase after that. After 8 days, the swelling ratio reached about 8 folds and then, the material starts disintegrating and the material no longer remains as a whole piece of solid mass. This indicates that the polymer hydrogel absorbs more and more water while it degrades and at the same time becomes weaker and weaker until it breaks into small pieces.

The indirect/extraction method was used for cytotoxicity test of the low molecular weight copolymers (Table 1). The indirect method showed that during hydrolysis, this copolymer has 93.1%, 89.0% and 90.7% relative cell numbers on day 1, 4 and 10 compared with the control sample which indicated only a small decrease in cell number although the p-value from the t-test on these three days are 0.022, 0.031, 0.030 respectively, which implies a significant decrease on cell number. (Figure 7).

The *in vivo* gelation and degradation test were conducted and similar results were found (data not shown) as in our previous study using high molecular weight polymer²⁶. It was found that the polymer gel was observed 1 day after subcutaneous injection on rat back. After 4 days, the polymer gel had a moderate swelling and more noticeable swelling was observed after 7 days which represented the maximum swelling and are in good agreement

with *in vitro* study. After 14 days, total dissolution of polymer was observed and no damage to adjacent tissues observed at this point. These *in vivo* gelation and degradation results are in good agreement with our *in vitro* studies.

Figure 8 shows the H&E staining of the dermal tissue sections surrounding the implant (or implant vacancy) after 1 and 3 days. The nuclei of leukocytes and fibroblasts stain as dark purple and are thus easily differentiated from the surrounding acellular connective tissue. It indicates an increased wound healing response. Figure 9 shows the Mason's Trichrome staining of the dermal tissue sections 1 and 7 days post implant. Mason's trichrome stains collagen in blue and it indicates the increased collagen deposition after 7 days as part of the wound healing response and the collagen seems to be more oriented.

Discussion

As shown in Figure 1, there are three monomers of this synthesized copolymer. NIPAAm is the monomer which contributes to the thermosensitivity of the copolymer. DBA makes the copolymer degradable due to the ester group in its structure. Before degradation, the LCST decreases because of the hydrophobicity of this monomer. After degradation, this copolymer becomes more hydrophilic and the LCST increases. AAc is employed to finely tune the initial LCST to be between room temperature and body temperature.

As seen in Table 1, the obtained copolymer has a weight average molecular weight of below 30kDa. For biomedical applications, low molecular weight polymer of less than 40kDa has been reported as being sufficiently small to be cleared by the kidney^{27–29}. There are several factors affecting the clearance of polymers by kidney, such as molecular weight of the polymer, the shape of the polymer and the charge of the polymer, etc. There is no single factor dominating the clearance. Usually, a polymer with low molecular weight of below 40kDa and linear shape would make the clearance easier^{27–29}. Thus, the synthesized copolymer with a low molecular weight of 27kDa should be cleared by kidney.

The degradation mechanism of this copolymer has been illustrated in our previous investigation²⁵. Basically, while the material degrades, due to the hydrolysis of the ester side group on DBA monomer, the copolymer becomes more and more hydrophilic resulting in the increase in LCST. After about two weeks of degradation time under physiological conditions, the polymer is hydrophilic enough and becomes soluble again. In Figure 2, the LCST reached about 35°C after 20 days under physiological conditions. While in Figure 5, the polymer becomes soluble again after 12 days under physiological conditions. The discrepancy between these two observations is because during the hydrolysis, the polymer becomes more heterogeneous and on day 12, most of the polymer chains already have a LCST of above 37°C. But there is a portion of polymer chains which has a LCST below 37°C and the concentration of it is not enough to form gel. This is also why the polymer solution is turbid as observed in Figure 5d. In Figure 2, the LCST was determined as the peak value of the DSC diagram. While the peak value is still below 37°C as observed in Figure 2, the whole peak has become wider and wider and the majority of the copolymer already have a LCST above body temperature. In the cloud point test (Figure 3), as compared with day 0 and day 7, the % transmission curve becomes wider on 12 and 20 day. This is due to the heterogeneity of the copolymer during hydrolysis. Also, there is a plateau on day 20. This is because within that temperature range, some of the polymer chains in the solution start precipitating down to the bottom of the vial while other polymer chains precipitating out of solution making % transmission staying the same. When the temperature increases further, the transmission decreases again. The % mass loss results indicates that while the polymer degrades, the polymer gels become more and more hydrophilic (Figure

4). Once the LCST of some of the polymer chains is above body temperature, it becomes soluble again. Thus, the polymer gels lose its mass while it degrades.

In both Figures 5c and 5d, turbidity is observed in the solution. As mentioned earlier, this is because even the majority of polymer chains have a LCST of above body temperature, some of the polymer chains still have a LCST below body temperature but the concentration is so low that no gels can be formed. In the long run, these polymers will continue degrading and reach a LCST of above body temperature so that it could be cleared by kidney.

Our *in vitro* cytotoxicity test showed that this material has only minimal cytotoxic effect on 3T3 fibroblast cells which would render this material a candidate for subcutaneous injection applications. The *in vivo* gelation and degradation study showed good agreement with *in vitro* study. From H&E staining, it was observed that there is a clear incidence of leukocyte and fibroblast hyperplasia in response to the polymer implants at both 1 and 3 days after injection. Our previous study also found that this response seems to diminish with time because the cell population after 1 month closely resembles that of native tissue²⁶. Therefore, there is an acute wound healing response after implantation and the tissue return to normal after 30 days. This is also confirmed by cell counting of leukocyte and fibroblast cells in our previous study from H&E staining²⁶. Collagen staining shows an increased presence of collagen deposition around the implant site after 1 and 7 days. Our previous study shows that collagen density decreases significantly by 30 days; however, the collagen density at this time is still above that of normal tissue²⁶. These *in vivo* biocompatibility tests show that this NIPAAm-based copolymer is reasonably biocompatible with little tissue irritation remaining after an initial wound healing phase.

Conclusion

Low molecular weight, thermosensitive, injectable and bioresorbable copolymer, poly(NDBA) has been synthesized in THF with 7.00 mol.% DBA content. The degradation property of this material has been characterized by time-dependent LCST, cloud point and % mass loss. The time-dependent LCST and cloud point test show that under physiological conditions, it takes about 2 weeks for LCST to reach above body temperature. The % mass loss shows that after 20 days, there is no mass left in the gel state. The polymer swells during degradation and become disintegrated after 8 days degradation. The cytotoxicity test shows a low toxic effect to fibroblast cells during degradation of the polymer. The *in vivo* gelation and degradation study showed a good agreement with *in vitro* investigation and no damage to adjacent tissues was observed after the complete dissolution of the copolymer.

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Figure 1.
Polymerization Scheme and Degradation

Figure 2. Time-dependent LCST at different degradation temperature for poly(NIPAAm-co-DBA-AAC) with 7.00% DBA, pH=7.4. (y-error bar smaller than data point) (5 wt. %, n=3,4)

Figure 3.
Cloud Point Test for poly(NIPAAm-co-DBA-AAc) with 7.00% DBA, UV spec, 1 wt.%, 500nm (n=3)

Figure 4.
% Mass Remaining for poly(NIPAAm-co-DBA-AAc) with 7.00% DBA at 37°C (n=3)

Figure 5. Gelation and Degradation of poly(NDBA) with 7.0% DBA content (5 wt. %). a: 0 day, 4°C, solution, b: 1 day, 37°C, complete gel,, c: 10 day, 37°C, viscoelastic material, d: 12 day, 37°C, solution

Figure 6.
Swelling Ratio for poly(NIPAAm-co-DBA-AAc) with 7.00% DBA at 37°C (n=3)

Figure 7.
Indirect Cytotoxicity Test for poly(NIPAAm-co-DBA-AAc) with 7.00% DBA content during hydrolysis (n=6)

Figure 8.

H&E staining of a: 1 day; b: 3 days post implantation

Figure 9.
Tissue sections stained with Masson's Trichrome to reveal collagen density. a: Control ; b: 1 day; c: 7 day post implantation

Table 1

Feed ratio, composition, molecular weight and polydispersity

Feed Ratio (mol. %)		Composition (mol. %)				MW (g/mol x 10 ⁴)	Pd
		NIPAAm	DBA	AAc	NIPAAm	DBA	AAc
91	7	2	90.89±0.14	7.00±0.01	2.11±0.15	2.7	1.95