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Extended and Sequential Delivery of Protein from Injectable Thermoresponsive Hydrogels

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Abstract

Thermoresponsive hydrogels are attractive for their injectability and retention in tissue sites where they may serve as a mechanical support and as a scaffold to guide tissue remodeling. Our objective in this report was to develop a thermoresponsive, biodegradable hydrogel system that would be capable of protein release from two distinct reservoirs – one where protein was attached to the hydrogel backbone, and one where protein was loaded into biodegradable microparticles mixed into the network. Thermoresponsive hydrogels consisting of N-isopropylacrylamide (NIPAAm), 2-hydroxyethyl methacrylate (HEMA), and biodegradable methacrylate polylactide (MAPLA) were synthesized along with modified copolymers incorporating 1 mol% protein-reactive methacryloxy N,hydroxysuccinimide (MANHS), hydrophilic acrylic acid (AAc), or both. In vitro, bovine serum albumin (BSA) release was studied from hydrogels, poly(lactide-co-glycolide) microparticles, or microparticles mixed into the hydrogels. The synthesized copolymers were able to gel below 37°C and release protein in excess of 3 months. The presence of MANHS and AAc in the copolymers was associated with higher loaded protein retention during thermal transition (45% vs 22%) and faster release (2 months), respectively. Microspheres entrapped in the hydrogel released protein in a delayed fashion relative to microspheres in saline. The combination of a protein-reactive hydrogel mixed with protein-loaded microspheres demonstrated a sequential release of specific BSA populations. Overall, the described drug delivery system combines the advantages of injectability, degradability, extended release, and sequential release which may be useful in tissue engineering applications.

Keywords

thermoresponsive; injectable; sequential protein delivery; hydrogel; NIPAAm

1. Introduction

The unique versatility of injectable biomaterials has led to their use in many biomedical applications including tissue engineering and drug delivery.[1,2] One primary advantage of this class of materials is that material injection may avoid the morbidity associated with the large incisions required for implantation of a pre-formed material.[3] N-isopropylacrylamide (NIPAAm) has been incorporated into many biomaterials due to its favorable sol-gel behavior.[4–10] Below its lower critical solution temperature (LCST) of 32°C, an aqueous

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solution of polyNIPAAm is liquid and can be injected through a small diameter needle. Upon reaching body temperature the polymer solution undergoes a sol-gel transition, thus forming a hydrogel in situ. Important functional properties such as mechanical strength, biodegradability, and cellular responsiveness have been imparted to NIPAAm-based injectable materials by copolymerization with specific monomers and peptides.[6,8–12] These materials have been developed for many potential applications including cartilage, bone, spinal cord and cardiac repair, as well as for drug delivery. [4,10,13–17]

Growth factor delivery has become an important tool in tissue engineering.[18] The delivery method is particularly important given that bolus solution injection leads to rapid removal of the growth factor from the injection site and extended periods of bioactivity over the tissue remodeling period in situ are desirable.[19] Hydrogels and microparticles are two delivery vehicles that have been widely studied for controlled drug release.[20,21] Combining both systems is a promising method under recent investigation to gain greater control over protein delivery rates. For example, it has been shown that putting drug-loaded microparticulates inside a hydrogel network can influence the rate of drug delivery from the microparticulates.[22–26] Additionally, combining microparticles of different compositions, where each particle type is loaded with a different growth factor, in the same hydrogel network is one way to allow for delivery of multiple drugs at rates independent of each other.[27] A variation on this theme, wherein one growth factor is loaded into microparticles that are inside the gel while another growth factor is dispersed in the gel phase, provides another means of controlling delivery rates.[27,28] The value in delivering multiple versus individual growth factors on tissue response has been demonstrated in angiogenesis and bone growth.[29,30] Further, independently controlling the rate at which each protein is delivered may allow for sequential delivery of factors, which has also been shown to be beneficial in some settings.[31–33] In angiogenesis new blood vessels must sprout and then mature with appropriate cellular components. This process may be achieved by sequential delivery of biomolecules such as vascular endothelial growth factor followed by either platelet-derived growth factor or sphingosine 1-phosphate.[19,31,33] Simultaneous delivery of these protein combinations does not elicit the same level of regeneration.

The objective of this report was to develop a thermoresponsive, biodegradable hydrogel system that would be capable of protein release from two distinct reservoirs – one where protein was attached to the hydrogel backbone, and one where protein was loaded into biodegradable polyester microparticles mixed into the hydrogel. A recently developed biodegradable and injectable NIPAAm-based biomaterial[8] was modified to allow direct protein conjugation. Poly(lactide-co-glycolide) microparticles generated by double emulsion processing were utilized to form the microparticulate protein carriers. The model protein bovine serum albumin (BSA) was delivered for extended periods from the modified thermoresponsive hydrogels, and distinct populations of labeled BSA were delivered from modified hydrogels embedded with microparticles to demonstrate sequential protein release. In addition, the effects of modifying the hydrogel to enable controlled release were examined in terms of thermoresponsive behavior.

2. Methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. NIPAAm was purified by recrystallization from hexane and vacuum-dried. HEMA was purified by vacuum distillation. Lactide was purified by recrystallization from ethyl acetate. Benzoyl peroxide (BPO), polylactide-co-glycolide (PLGA), sodium methoxide ($NaOCH_3$), poly(vinyl alcohol) (PVA), acrylic acid (AAc), N-hydroxysuccinimide (NHS) and methacryloyl chloride were used as received. Iodinated bovine serum albumin (^{125}I-BSA)

(Perkin-Elmer) and fluorescein isothiocyanate or Texas Red labeled BSA (FITC-BSA and TexR-BSA, Molecular Probes) were used as received.

2.2 Material synthesis

2.2.1 Synthesis of methacryloxy N-hydroxysuccinimide (MANHS)—MANHS was synthesized by dropping 20 g methacryloyl chloride into a solution of NHS (20 g) and triethylamine (22 g) in 350 mL dichloromethane under stirring at 0°C followed by reaction overnight at room temperature. After a filtration process to remove the precipitate, the solution was washed with water 3 times and the organic phase collected by centrifugation and dried over anhydrous $MgSO_4$. Dichloromethane solvent was removed by rotary evaporation and the solid product was purified by flash chromatography to obtain a white solid, with a yield of 85%. Synthesis was verified by ^1H NMR spectrum (in $CDCl_3$): CH_2=(6.43ppm, 1H, s), CH_2=(5.90ppm, 1H,s), -$NCOCH_2CH_2CON$-(2.88pm, 4H,s), -CH_3(2.08ppm, 3H,s).

2.2.2 Synthesis of methacrylate polylactide (MAPLA)—MAPLA was synthesized as reported previously.[8] Polylactide-monomethyl ether (HOPLA-OCH_3) was synthesized by ring opening polymerization by dissolving lactide in dichloromethane to which $NaOCH_3$ initiator in methanol was added. After 2 h of reaction at 0°C, the polymer solution was rinsed with 0.1 M HCl and deionized water, the organic phase was dried with $MgSO_4$ and removed by rotary evaporation. MAPLA was formed by dropping methacryloyl chloride in an HOPLA-OCH_3 solution in dichloromethane containing triethylamine, followed by reaction overnight at 0°C. Following reaction, precipitates were removed, the organic phase was dried and removed by rotary evaporation, leaving raw MAPLA product. MAPLA was purified by flash chromatography. NMR confirmed the synthesis of MAPLA with an average of 2.8 PLA units per MAPLA monomer.

2.2.3 Synthesis of poly(NIPAAm-co-HEMA-co-MAPLA)—Poly(NIPAAm-*co*-HEMA-*co*-MAPLA) copolymers with or without MANHS were synthesized by free radical polymerization (Figure 1). Monomers, NIPAAm (6 g, 0.053 mol), HEMA, and MAPLA at a molar ratio of 80/10/10 were dissolved in 200 mL of 1,4-dioxane containing 160 mg BPO. The polymerization was carried out at 70°C for 24 h under argon atmosphere. The copolymer was precipitated in hexane and further purified by precipitation from THF into diethyl ether and vacuum-dried. For inclusion of MANHS the molar feed ratio for NIPAAm, HEMA, MAPLA, and MANHS was 80/9/10/1, respectively. An additional copolymer was synthesized that incorporated 1 mol% AAc as a means to increase the degradation rate of the copolymer. The molar feed ratio for this copolymer was 80/8/10/1/1 NIPAAm/HEMA/MAPLA/MANHS/AAc.

2.2.4 Synthesis of PLGA microspheres—PLGA microspheres with a lactide:glycolide ratio of 75:25 (Mw 66–107 kD) were synthesized using a water-in-oil-in-water (W/O/W) double emulsion technique.[24] FITC-BSA or ^{125}I-BSA was mixed with unlabeled BSA at a ratio of 1:10 and dissolved in 0.02x phosphate buffered saline (PBS) to make a final total BSA concentration of 1 mg/mL. Initially, 200 mg of PLGA was dissolved in 4 mL dichloromethane. After all PLGA was dissolved, 200 μL of BSA solution was added to the dissolved PLGA and vortexed for 1 min. This primary emulsion was immediately added to 60 mL of 2% PVA that was stirring at 1000 rpm, to generate the W/O/W emulsion. After 5 min, the stirring speed was reduced to 600 rpm and an additional 80 mL of 1% PVA solution was added. After stirring for 6 h to allow dichloromethane evaporation, the microspheres were collected by centrifugation, rinsed with DI water and freeze-dried.

2.3 Copolymer characterization

Copolymer molecular weight was determined by gel permeation chromatography (Waters Breeze System). The copolymers were dissolved in tetrahydrofuran at 1 mg/mL and elution time was used to determine molecular weight by comparison to a poly(methyl methacrylate) standard. LCST values were determined by measuring the optical density of the material at 500 nm wavelength over a temperature range from 2 to 24°C. The LCST of the 80/9/10/1 copolymer without protein, with low protein content (BSA at 0.2 wt% of polymer), high protein content (20 wt%), and with microspheres (10 wt%) was determined (n=4 each group). The temperature at which the absorbance reached half of its maximum value was taken as the LCST, and was determined using a custom Matlab (MathWorks) program.

Hydrogel degradation was studied by quantifying the mass loss of samples at 37°C in PBS over time. PBS was changed frequently to maintain a constant pH throughout degradation. Samples (n=3 each) were collected at specific time points and lyophilized. The mass remaining in each sample was compared against the initial dry mass to determine percent mass loss. Hydrogel and microsphere morphology was viewed with scanning electron microscopy.

The change in mechanical properties of the hydrogels during sol-gel transition was characterized on a TA instrument rheometer (AR2000). Solutions of the 80/9/10/1 copolymer(16.7 wt % in PBS), with or without the inclusion of PLGA microparticles (10 wt %), were placed between two parallel plates and a temperature sweep from 5 to 35 °C (heating rate of 4 °C/min) was applied. The shear storage modulus G′ and the loss modulus G″ were recorded as a function of temperature at a fixed strain of 2% and a frequency of 1 Hz.

2.4 In vitro protein release studies

2.4.1 Protein release from hydrogels—Copolymer was dissolved in PBS at 4°C at a concentration of 16.7 wt% and loaded with protein at a concentration of 100 μg/mL. For the 80/9/10/1 copolymer, protein was present in the PBS to be available to react with MANHS groups as the polymer dissolved (Figure 2A). For the 80/10/10 copolymer, protein was added to the solution after dissolving polymer. Gelation occurred by injecting 0.5 mL polymer solution into 1 mL of 37°C PBS. Samples (n≥ 4 for each gel type) were maintained in a water bath at 37°C and releasate was collected and replaced with fresh PBS at desired time points. ^{125}I-BSA was used for these release studies and the amount of protein released was determined by a gamma counter (Auto Gamma II, Perkin Elmer).

2.4.2 Stability of protein linkage to hydrogels—To determine the stability of the protein-polymer linkage, hydrogels with and without MANHS were loaded with ^{125}I-BSA and thermally cycled. Hydrogel solutions (n=4) were gelled at 37 °C for 3 h, released protein was collected, and gels were redissolved at 16.7 wt% at 4 °C. This process was repeated five times and released protein was measured after each cycle.

2.4.3 Protein release from microspheres—For studies involving PLGA microspheres, microspheres were either free in PBS or were contained within the hydrogel network (Figure 2B). For inclusion inside the gel, microspheres were added to aqueous polymer solutions and mixed for 30 min until homogeneous, at which point the solution was heated to form the gel. For these studies ^{125}I-BSA was encapsulated in the microspheres and release quantified by a gamma counter (n=4 for each group).

2.4.4 Conformational stability of released protein—The conformation of the released protein was assessed by circular dichroism (CD) spectroscopy as previously

reported.[34,35] FITC-BSA released from hydrogels without MANHS was collected after 3 h of gelation and concentrated using a centrifuge filter (Millipore) with a 50 kD molecular weight cutoff to remove interfering soluble polymer chains. FITC-BSA encapsulated in PLGA microparticles was collected using a method described previously.[34] Specifically, protein-loaded PLGA microparticles (75 mg) were dissolved in 1 mL dichloromethane to which 4 mL of deionized water was added. The solution was sonicated for 90 min at 25 °C and centrifuged at 2000 x g for 10 min. The aqueous phase was decanted and the BSA was concentrated in a centrifuge filter. All protein solutions were measured using a Jasco J-810 spectropolarimeter, and compared against stock FITC-BSA.

2.4.5 Dual protein release from microspheres inside hydrogel—Release of two distinct protein populations, FITC-BSA and TexR-BSA, was studied. TexR-BSA was allowed to react with the 80/9/10/1 copolymer in PBS to form a protein-polymer conjugate followed by addition of microspheres encapsulating FITC-BSA to the polymer solution. After mixing until microspheres were homogeneous, the solution (n=4) was heated to 37°C for gelation to occur – Figure 2C. Protein concentration in the releasate from the hydrogel was measured by a fluorescence microplate reader (SpectraMax M2, Molecular Devices) with excitation/emission wavelengths of 495/520 nm and 594/615 nm for FITC-BSA and TexR-BSA, respectively. In all experiments measured levels of radioactivity or fluorescence were compared against standard curves to determine concentration.

2.5 Statistics

Protein release rates during specific phases in the release profile were compared between polymer types using unpaired t-tests. Where three or more groups were being compared, one-way ANOVA was used. To determine differences between hydrogel compositions during thermal cycling two-way ANOVA was applied. Tukey's post hoc test was used in conjunction with each ANOVA. Statistical significance was defined as $p<0.05$.

3. Results

3.1 Characterization of thermoresponsive copolymers

Thermoresponsive hydrogels were successfully synthesized with yields >75%. The 80/10/10 copolymer had a M_n of 22K with a polydispersity index (PDI) of 1.6. The M_n and PDI of the 80/9/10/1 copolymer was 26K and 1.5, respectively. All copolymers were able to form bulk gels upon heating to 37°C. Representative thermal transition profiles are shown in Figure 3 with quantification of the LCST values in Table I. Although altering the LCST as much as 5°C, the presence of protein or PLGA microspheres did not interfere with the polymer's ability to form a gel below body temperature. The presence of microparticles increased the turbidity of the polymer solution leading to a smaller relative change in optical density upon heating, but the final gel appeared similar to those without microparticles. Likewise, the addition of microparticles did not have an effect on the mechanical properties of the hydrogel above or below the LCST. The maximum of the shear storage and loss moduli for the hydrogel without microparticles was 10290 Pa and 21940 Pa, respectively. Similarly, the maxima of these same parameters for the hydrogel with microparticles was 10720 Pa and 22620 Pa, respectively – Figure 4. The degradation of the copolymer, which occurs as hydrolysable MAPLA side chains are cleaved leaving a more hydrophilic polymer chain that becomes soluble at 37°C, is complete after approximately 4 to 6 months. The inclusion of 1 mol% MANHS increased the degradation rate of the copolymer as shown in Figure 5. However, the degradation profile remained similar to the 80/10/10 copolymer wherein mass loss was slow in the first few months of gel formation followed by a rapid loss of mass as MAPLA side chains were cleaved to increase hydrophilicity and allow solubilization at 37°C.[8]

3.2 Protein release from hydrogels

The BSA release profile for 80/10/10 (no MANHS, protein mixed), 80/9/10/1 (1% MANHS, protein reactive), and 80/8/10/1/1 (1% MANHS and 1% AAc, protein reactive) copolymers are shown together in Figure 6. For ease of comparison, release profiles were separated into distinct phases and rates were quantified using previously reported methods – Table II.[27] The loading efficiency was here defined as the amount of protein remaining after the 3 h necessary for the complete stabilization of hydrogel size and water content.[8] Protein release was relatively quick during the burst release phase from 3 to 24 h. Following the burst there was a period of continued high diffusion rates (phase I, days 1 to 7) followed by slower release (phase II, weeks 1 to 6). Hydrogel degradation became more prominent as time progressed leading to higher release rates in phase III (week 6 until 98% of protein was released).

The loading efficiency increased from 22.0 ± 2.1% in the 80/10/10 hydrogel up to 44.6 ± 6.9% when 1 mol% of MANHS was present ($p<0.05$). The 80/9/10/1 hydrogel showed significantly higher release rates in phases I, II, and III compared to the 80/10/10 hydrogel ($p<0.05$ each phase) and demonstrated a more linear release profile. The presence of hydrophilic AAc sped polymer degradation and displayed near zero order release kinetics such that the release rates in phases I, II, and III were not significantly different from each other ($p>0.05$), although in all phases they were higher than the 80/10/10 copolymer ($p<0.05$ each phase). In hydrogels without AAc, BSA continued to be released for over 3 months in vitro, whereas the duration of release was shortened to 2 months in the copolymer with hydrophilic AAc. After the burst phase the copolymer without MANHS released protein at a slow rate until polymer degradation became more substantial after 60 days.

The influence of MANHS on protein retention within the hydrogel is demonstrated in Figure 7. More protein remained after each thermal cycle in hydrogels with MANHS compared to those without ($p<0.05$). The percent of protein lost from the preceding thermal cycle is presented in Table III. Hydrogels without MANHS lost a significantly greater percentage of their remaining protein at each subsequent cycle compared to hydrogels with the MANHS linker ($p<0.05$).

3.3 Protein release from PLGA microspheres

BSA-loaded PLGA microspheres were synthesized with an average diameter of 34 ± 30 μm and could be easily incorporated into the hydrogel networks – Figure 8. Microspheres free in PBS demonstrated a substantial early-stage protein release – Figure 9 and Table IV. The burst release, which included all release during the first 24 h, for microspheres in PBS was 17.4 ± 5.4% compared to 6.6 ± 1.0% when microspheres were entrapped in hydrogel ($p<0.05$). During the remainder of the first week (phase I) release rates were 16.9 ± 2.5% and 6.6 ± 1.2%, respectively ($p<0.05$). The release rate of BSA from microspheres inside the hydrogel was higher than the PBS counterparts only during phase III as polymer degradation became more substantial.

CD spectroscopy of protein encapsulated in PLGA microparticles and protein released from the hydrogels is shown in Figure 10. The spectra of both BSA released from hydrogels and BSA encapsulated in microparticles are similar to native FITC-BSA. Each has minima near 208 and 222 nm and increased ellipticity below 200 nm, characteristic of the largely alpha-helical BSA.[35]

3.4 Sequential protein release from microspheres embedded within the hydrogel

The release of two differently labeled BSA proteins (FITC-BSA and TexR-BSA) from the same hydrogel samples using the MANHS-containing polymer with microspheres together

showed sequential protein release – Figure 11. TexR-BSA, which was in the gel phase, showed a large release initially, whereas during the same early time points FITC-BSA release from entrapped PLGA microspheres was relatively slow. Specifically, in the first 3 wk 54.2 ± 3.1% of TexR-BSA was released compared to 17.9 ± 5.4% of FITC-BSA. Over the next 10 wk TexR-BSA release averaged 1.6%/wk whereas FITC-BSA release gradually accelerated from an average of 1.4%/wk up to a high of 3.7%/wk.

4. Discussion

Of the many applications of injectable materials in tissue engineering, one of recent focus in the literature is that of material injection into the myocardium following ischemic injury.[36] An appropriate biomaterial, upon injection into the heart wall, may act as a bulking agent to mechanically support the weakened myocardium or as a focal point for tissue remodeling and angiogenesis. These benefits have been shown using a number of injectable synthetic and biological materials. It has been reported that intramyocardial injection of a NIPAAm-based material with similar design features as the one presented in the current report was able to halt deleterious cardiac remodeling following ischemic injury.[14] Use of the current injectable material in this same application would also be feasible. Another treatment for cardiac disease of clinical interest has been therapeutic angiogenesis, which aims to increase collateral blood flow and attenuate ischemic damage by delivering pro-angiogenic proteins to ischemic myocardium. Of the clinical trials that have investigated cardiac therapeutic angiogenesis, the longest and most substantial benefit was seen when basic fibroblast growth factor was delivered to the heart in sustained release capsules compared to direct intracoronary infusion.[37,38] Studies using animal models have also confirmed the potential for improved cardiac function when pro-angiogenic factors are released in a controlled fashion in the heart wall.[31,39,40] In this report we have sought to combine these two beneficial effects by developing an injectable, biodegradable material capable of serving as a bulking agent with controlled release capabilities.

The developed thermoresponsive polymer system was amenable to molecular design changes that resulted in predictable effects on protein delivery. For example, addition of just 1 mol% MANHS consistently improved the loading efficiency of this system and allowed more protein to be delivered over an extended duration in a more linear fashion. Additionally, inclusion of 1 mol% AAc predictably increased the degradation rate of the polymer allowing the protein to be delivered over a shorter duration and in a near zero order fashion. These features may allow for a more specific release profile to be engineered depending on the hypothesized drug delivery needs of the target biological environment.

The addition of microspheres to the system provided another means to control the timing of protein delivery and allow for multi-factor release in a sequential manner. PLGA was chosen for microsphere synthesis because of a hypothesized affinity of the hydrophobic PLGA to associate with the hydrophobic MAPLA and NIPAAm residues of the copolymer. This interaction appeared to exist as the microparticles were not excluded from the hydrogel during phase transition. Likewise, the fact that the mechanical properties were not altered by the inclusion of microparticles in the gel network may be a manifestation of a favorable interaction between these two moieties. Release profiles from protein-loaded microspheres suggested that only about 60% of the loaded protein was released. However, measurements of the sample tubes at the end of the experiments demonstrated that 100% of protein was released, but the remaining 40% of the protein was adsorbed onto the inner surface of the polypropylene tubes, making it unavailable for measurement in the collected fluid samples.

It was apparent that the initial release of protein was delayed when the microspheres were entrapped in the hydrogel versus being free in PBS. While releasing some protein in the first

few weeks, acceleration of the delivery rate did not occur until after 6 wk. The extent of the delay may be related to the degradation rate of PLGA used, which would constitute another control point in this delivery system. For example, PLGA with lower molecular weight or higher PLA content would lead to faster degradation and potentially quicker release. Additionally, the W/O/W fabrication technique used in this study produces a dense inner structure and porous surface on the microparticles, which also plays a role in the release profile. Particle morphology is influenced by many different processing parameters and has been shown to influence drug delivery rates.[41–43] In particular, surface porosity will lead to a larger burst release than would be seen from a nonporous surface. The high polymer volume fraction of the dense internal structure provides a more tortuous diffusional path that would favor a more extended release compared to hollow particles, which lose all encapsulated drug once the outer shell is breached. Thus, processing of the microparticles may provide yet another control mechanism for drug release.

It is clear that the sequential nature of the current drug delivery system does not follow a strict on-off mechanism wherein all of one protein is delivered before all of another. Indeed, in both the hydrogel and the microparticle system there is continued release throughout the duration of the study. However, a strict sequential delivery may not be necessary to see desired biological effects. Previous studies have shown a beneficial biological outcome when growth factors are delivered in a sequential manner similar to the one shown in the current report.[31,32,44] For example, Ruvinov et al. demonstrated different rates of IGF1 and HGF release from an alginate gel.[44] Similar to the current report, the growth factors were both continuously released from the gel but with one releasing faster than the other. Nevertheless, the sequential nature was adequate to elicit an improved biological response. One advantage of the current system is that the release profile of each of the two components – hydrogel and microsphere – could be independently manipulated, allowing each drug to be released in a desired profile to meet the perceived biological need.

The thermoresponsive hydrogels reported were able to deliver protein, with or without protein-reactive moieties in the backbone, for a period in excess of 3 months. This contrasts with many hydrogel systems which, because of their high water content, are associated with quick molecular diffusion.[45] Previous studies have shown that the equilibrium water content of the 80/10/10 hydrogel is near 45%.[8] This relatively low water content was associated with a more collapsed gel structure with smaller pore sizes and thus likely lower aqueous convection rates for protein release. In conjunction with this, the large size of the model protein BSA (MW 66 kD) may inhibit its quick release from the gel. This size effect has been shown in polyNIPAAm hydrogel systems previously.[46,47]

The presence of MANHS in the 80/9/10/1 hydrogels consistently improved loading efficiency compared to 80/10/10 hydrogels – an effect attributed to the putative linkage of the protein to the polymer backbone. The occurrence of this linkage is also supported by the protein loss data during hydrogel thermal cycling. Presumably protein that is not bound to the hydrogel will be lost more quickly as the gel solubilizes and reforms, with the collapse of the gel pushing unbound protein out. Accordingly, hydrogels undergoing thermal cycling retained protein to a greater extent when MANHS was present compared to those without the linker. Because more protein remains in the gel after formation there is subsequently more protein to be released over time, and it appears to be released in a more linear fashion.

Protein bioactivity is a primary concern for any drug delivery system. Synthesis of the PLGA microparticles involved a W/O/W emulsion technique that includes the possibility of protein denaturation by various mechanisms.[48] CD spectroscopy confirmed that BSA encapsulated in microparticles maintained its native confirmation following synthesis, as compared to the changes in ellipticity that would occur following denaturation.[35] Likewise,

BSA released after 3 h from 80/10/10 hydrogels did not exhibit a conformational change. One potential concern associated with the protein-reactive hydrogel approach would be the effect that polymer-protein conjugation could have on protein bioactivity. Covalent attachment of polymers, specifically poly(ethylene glycol), to protein therapeutics is an established method being used clinically to improve protein half-life in vivo for better pharmacokinetics.[49] However, it has been shown that uncontrolled conjugation can lead to a decrease in activity of the therapeutic if the polymer chain blocks protein active sites or influences protein conformation.[50] Each protein that would be conjugated to this polymer would require a bioactivity assessment to define the impact of conjugation. Should conjugation prove detrimental, a more careful linking strategy could be pursued by different conjugation chemistries, analogous to the approaches used for pharmaceutical pegylation.

5. Conclusion

Injectable, biodegradable polymers with thermoresponsive properties were synthesized and characterized as a drug delivery system. These hydrogels were able to release model protein BSA in a controlled manner extending 3 months. Modification of the polymer backbone, specifically inclusion of a protein-reactive MANHS group, led to higher protein loading in the gel during thermal transition. Further, addition of hydrophilic AAc to the polymer increased the drug delivery rate as predicted. The presence of both MANHS and AAc led to a more linear protein release profile than from hydrogels without these monomers. Protein loaded microspheres entrapped in the hydrogel network during gel formation released protein in a delayed fashion relative to microspheres in saline. Neither protein conjugation nor microsphere presence interrupted the ability of the copolymer to transition from sol to gel when warmed to body temperature, thus maintaining the injectability of this system. Combining both systems provided a sequential release of two distinct protein populations where the majority of protein associated with the gel phase was released before protein loaded into microparticles. Thus, this composite material combines the advantages of injectability, degradability, extended release, and sequential release into a single delivery system.

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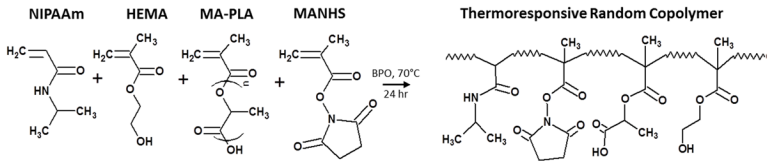

Figure 1.
Synthesis scheme for a thermally responsive copolymer containing protein-reactive MANHS.

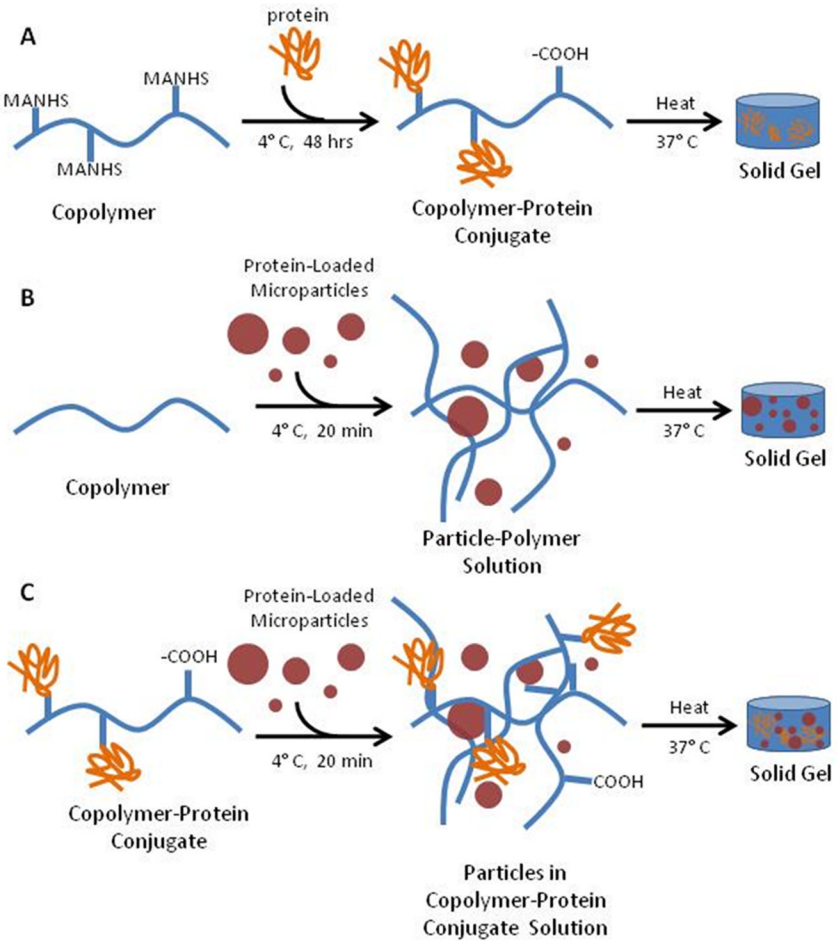

Figure 2.

Approaches to protein loading in thermally responsive hydrogels. A) Protein is mixed with reactive copolymer to form protein-polymer conjugates in solution before hydrogel formation. B) Protein-loaded microspheres are mixed with copolymer solution before heating. C) Combination of (A) and (B) where one protein population is reacted with the copolymer in solution to form protein-polymer conjugates followed by mixing with microparticles loaded with a second protein population prior to thermally induced gel formation.

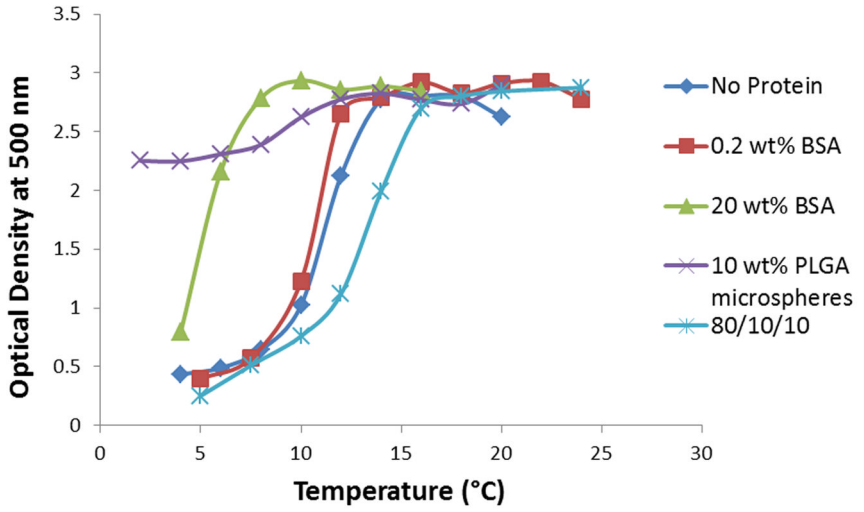

Figure 3.
Representative optical absorption curves of hydrogel solutions with various protein and microparticle additives as solutions are heated.

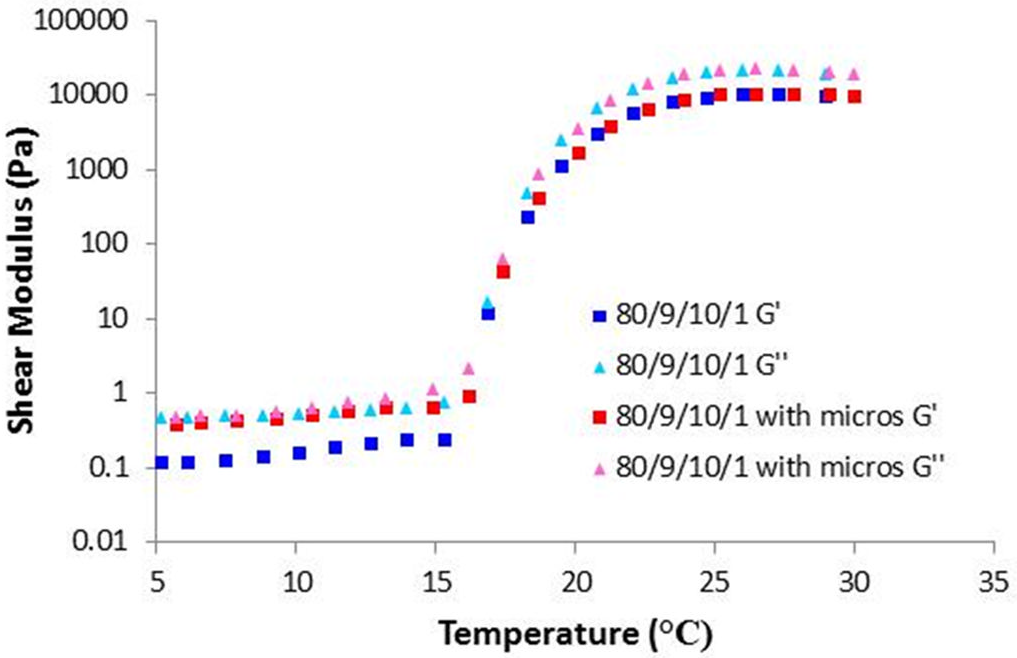

Figure 4.

Shear mechanical properties during heating of the 80/9/10/1 hydrogel with or without the inclusion of microparticles in the gel network. The presence of microparticles did not alter the mechanical properties of the hydrogel.

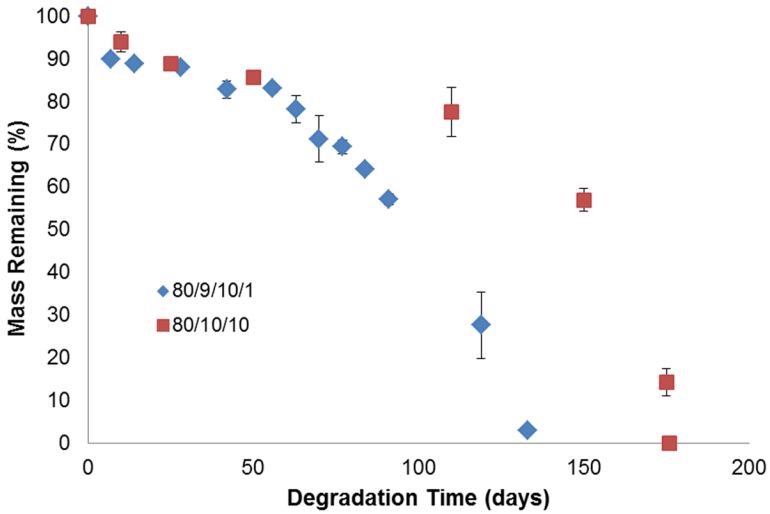

Figure 5.
Mass loss curves for hydrogels with and without the MANHS linker. Incorporation of 1 mol % MANHS increased the polymer degradation rate. Data for 80/10/10 hydrogel reproduced from ref 8.

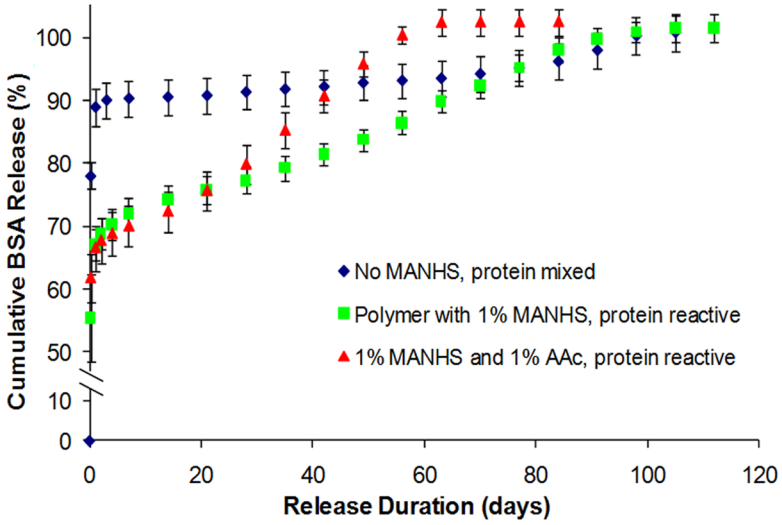

Figure 6.

In vitro BSA release from hydrogels with different compositions. Polymers with protein-reactive MANHS showed higher loading efficiency and more constant release rates than copolymers without. Addition of hydrophilic AAc increased the rate of protein delivery.

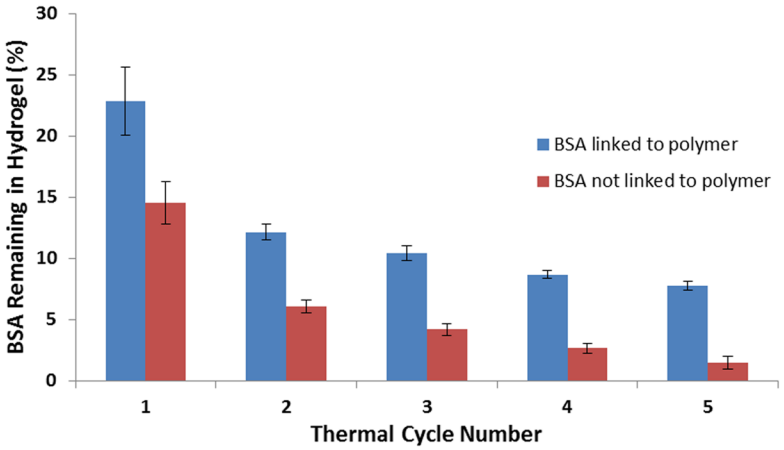

Figure 7.
Protein remaining in hydrogels during repeated thermal cycling. Hydrogels incorporating the MANHS linker retained more protein after each cycle than hydrogels without linker.

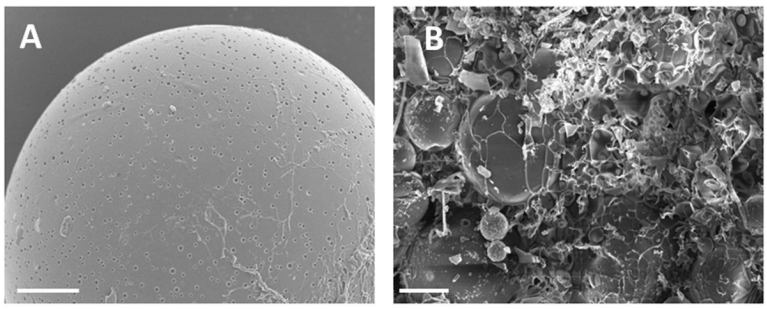

Figure 8.
Scanning electron micrographs showing dehydrated structure of A) PLGA microparticle (scale bar = 5 μm) and B) PLGA microparticles embedded in the hydrogel polymer network (scale bar = 20 μm).

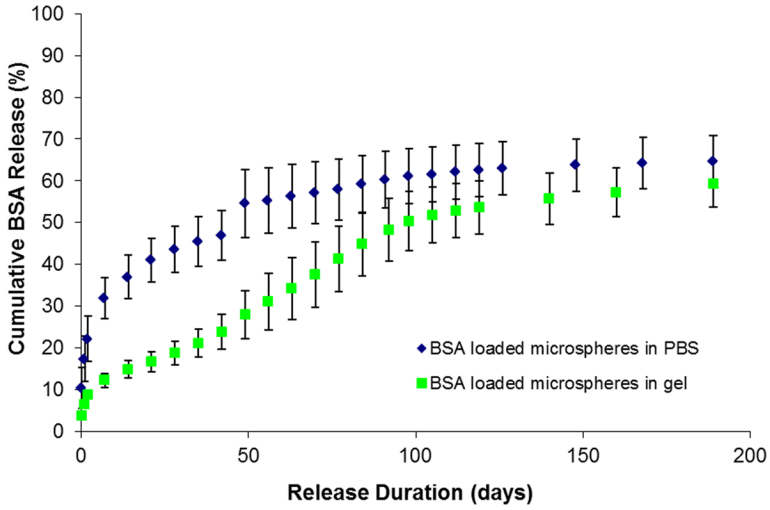

Figure 9.
BSA release from microspheres in PBS and mixed into hydrogel network. Protein release was delayed when microspheres were entrapped in the hydrogel.

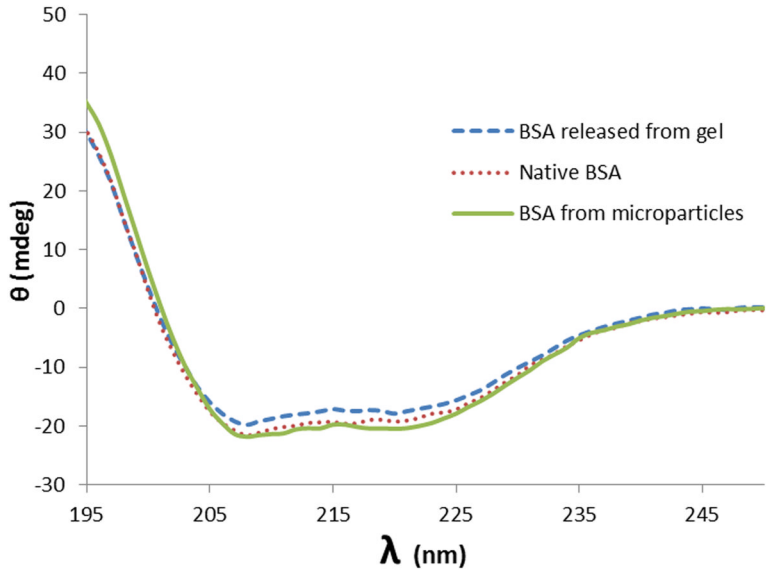

Figure 10.

CD spectroscopy of native BSA compared to BSA encapsulated in PLGA microparticles and BSA released from hydrogels without MANHS. Spectra demonstrate that protein conformation is not altered by this polymeric drug delivery system.

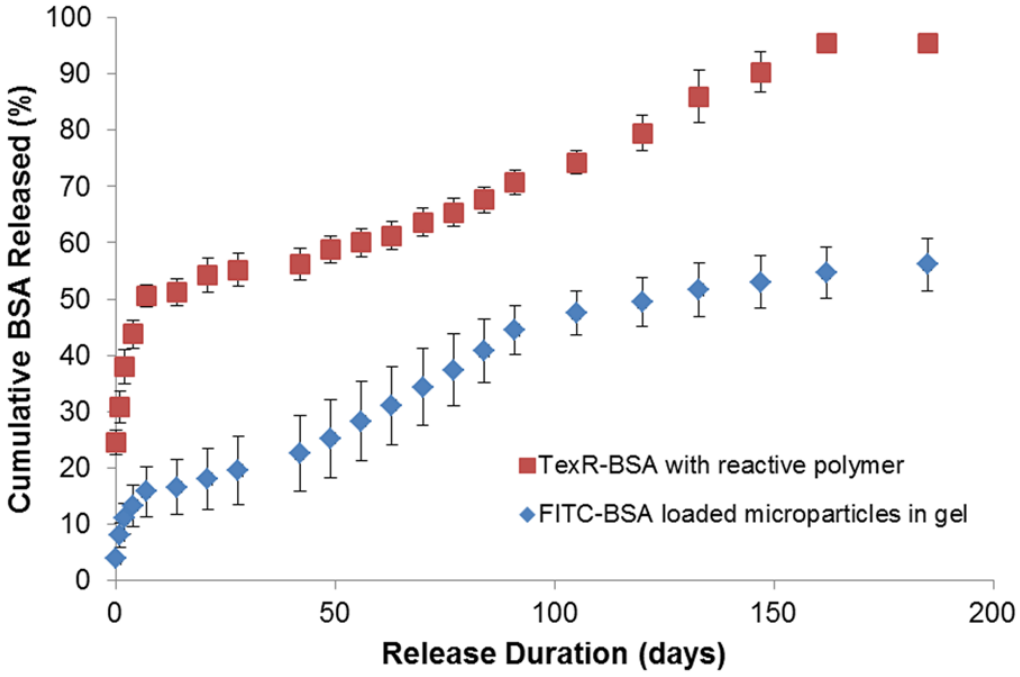

Figure 11.
Sequential release of distinct BSA protein populations. A majority of TexR-BSA was released from the reactive polymer system during the first three weeks compared to the delayed release of FITC-BSA from microspheres inside the hydrogel.

Table I

LCST of Hydrogels

Hydrogel	Additive	Transition Temp (°C ± SD)
80/9/10/1	none	11.0 ± 0.1
	0.2 wt% BSA	10.5 ± 0.1
	20 wt% BSA	5.4 ± 0.3
	10 wt% PLGA microspheres	9.2 ± 0.6
80/10/10	none	12.4 ± 0.4

Table II

In Vitro BSA Release Kinetics from Hydrogels

Hydrogel	Loading Efficiency (%)	Burst (%)	Phase I (%/wk)	Phase II (%/wk)	Phase III (%/wk)
80/10/10	22.0 ± 2.1	10.9 ± 1.6	1.61 ± 0.32	0.37 ± 0.05	0.82 ± 0.04
80/9/10/1	44.6 ± 6.9*	11.7 ± 6.3	5.81 ± 0.73*	1.88 ±	2.77 ±
80/8/10/1/	38.3 ± 3.9*	4.8 ± 1.3*	4.05 ± 1.18*	4.14 ±	4.86 ±
1				0.32*	1.33*

*Denotes p<.05 compared to 80/10/10 hydrogel during same phase

Table III

BSA Lost After Each Thermal Cycle

Cycle Number	Not Linked (%± SD)	Linked (%± SD)*
Cycle 1	85.4 ± 1.7	77.1 ± 2.8
Cycle 2	57.8 ± 5.6	46.4 ± 5.3
Cycle 3	30.9 ± 5.3	14.0 ± 1.4
Cycle 4	36.6 ± 2.7	16.7 ± 2.5
Cycle 5	46.0 ± 14.0	10.9 ± 2.8

* denotes $p<0.05$ for all cycles compared to not linked

Table IV

In Vitro BSA Release Kinetics from Microspheres

Microsphere Environment	Burst (%)	Phase I (%/wk)	Phase II (%/wk)	Phase III (%/wk)
Microspheres in PBS	17.4 ± 5.4	16.9 ± 2.5	2.99 ± 0.31	1.13 ± 0.05
Microspheres in 80/10/10 gel	6.6 ± 1.0*	6.6 ± 1.2*	2.33 ± 0.58	2.27 ± 0.18*

* Denotes $p<.05$ compared to microspheres in PBS during same phase