Macromolecular Research, Vol. 14, No. 1, pp 87-93 (2006)

Preparation of Thermo-Responsive and Injectable Hydrogels Based on Hyaluronic Acid and Poly(*N*-isopropylacrylamide) and Their Drug Release Behaviors

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Received October 24, 2005; Revised November 29, 2005

Abstract: Copolymers composed of hyaluronic acid (HA) and poly(*N*-isopropylacrylamide) (PNIPAAm) were prepared to create temperature-sensitive injectable gels for use in controlled drug delivery applications. Semi-telechelic PNIPAAm, with amino groups at the end of each main chain, was synthesized by radical polymerization using 2-aminoethanethiol hydrochloride (AESH) as the chain transfer agent, and was then grafted onto the carboxyl groups of HA using carbodiimide chemistry. The result of the thermo-optical analysis revealed that the phase transition of the PNIPAAm-grafted HA solution occurred at around 30~33 °C. As the graft yield of PNIPAAm onto the HA backbone increased, the HA-*g*-PNIPAAm copolymer solution exhibited sharper phase transition. The short chain PNIPAAm-grafted HA (M_w=6,100) showed a narrower temperature range for optical turbidity changes than the long chain PNIPAAm-grafted HA (M_w=13,100). PNIPAAm-grafted HA exhibited an increase in viscosity above 35 °C, thus allowing the gels to maintain their shape for 24 h after *in vivo* administration. From the *in vitro* riboflavin release study, the HA-*g*-PNIPAAm gel showed a more sustained release behavior when the grafting yield of PNIPAAm onto the HA backbone was increased. In addition, BSA released from the PNIPAAm-*g*-HA gels showed a maximum concentration in the blood 12 h after being injected into the dorsal surface of a rabbit, followed by a sustained release profile after 60 h.

Keywords: hyaluronic acid, poly(*N*-isopropylacrylamide), injectable gel, drug delivery.

Introduction

Hydrogels have been extensively studied in the last decades for application in drug delivery and tissue engineering.[1-3] Particularly, in recent years there is a growing interest with *in situ* gel-forming systems as candidates for injectable drug and cell delivery.[4-11] Various methods have been developed for preparing self-gelling hydrogels that are cross-linked by non-permanent bonds based on physical interactions between the polymer chains. Such systems can be administered by injection as liquid formulation and gellify *in situ*. The following mechanisms may be involved in the *in situ* gel formation: gelation in response to temperature or pH change, ionic cross-linking, solvent exchange or crystallization, and thickening upon removal of the injection shear.[4,7] Gel formation through chemical cross-linking can also occur when UV light is used as a trigger.[4,12-14]

In this study, we focused on the thermo-responsive injectable hydrogels that can be formulated at room temperature and form a gel at body temperature. Poly(*N*-isopropylacrylamide) (PNIPAAm), the most popular thermo-sensitive, water-soluble polymer, exhibits a lower critical solution temperature (LCST) of almost 32 °C. PNIPAAm chains hydrate to form an expanded structure at temperatures lower than 32 °C, but undergo a sharp phase transition at higher temperatures to form inter- and intra-chain associations, resulting in precipitation.[15-23]

Note that an injectable gel should solidify rapidly after injecting into the body, otherwise, the polymer solution may dissipate into the surrounding tissues or organs.[4] Comb-type grafted materials were introduced to improve the kinetic temperature sensitivity; thus rapid phase transition occurs as

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a result of the free mobility of the temperature sensitive polymer at the chain end.[21,22] Our previous studies revealed that the PNIPAAm comb-type grafted alginate hydrogels showed a rapid swelling and shrinking behavior.[24-26]

Hyaluronic acid (HA) consists of 2-acetamide-2-deoxy-α-D-glucose and β-D-glucuronic acid residues linked by alternate (1-3) and (1-4) glycoside bonds.[4] HA, a component of the glycosaminoglycans (GAGs) in the extracellular matrix (ECMs), is biocompatible, biodegradable, and performs important biological functions such as stabilizing and organizing the ECM, regulating cell adhesion and mobility, and mediating cell proliferation and differentiation.[8-10,27,28] Recent biomedical applications of HA included scaffolds for wound healing and tissue engineering, as well as ophthalmic surgery, arthritis treatment, and components for implant materials.[8-10,27,28]

The objective of this study is to prepare the comb-type PNIPAAm-grafted HA copolymers and to then investigate the feasibility of temperature-sensitive injectable drug carriers. In the present study, we synthesized semi-telechelic PNIPAAm with amino groups at the end of the main chains by radical polymerization and then prepared PNIPAAm-grafted HA copolymers using carbodiimide. The phase transition and viscosity changes of PNIPAAm-grafted HA copolymer solution was investigated depending on the molecular weight and graft yield of PNIPAAm chain in response to temperature changes. In addition, the release behavior of drug from the PNIPAAm-grafted HA hydrogel was evaluated *in vitro* and *in vivo*.

Experimental

Materials. *N*-isopropylacrylamide (NIPAAm) (Aldrich Chemicals, Milwaukee, WI, USA) was purified by recrystallization from *n*-hexane/toluene (Duksan Pure Chemicals, Seoul, Korea). 2-Aminoethanethiol hydrochloride (AESH) was purchased from Aldrich Chemicals. *N,N'*-Azobisisobutyronitrile (AIBN) (Aldrich Chemicals) was recrystallized from methanol (Duksan Pure Chemicals). HA, in a sodium form ($M_w = 1.7 \times 10^6$), was obtained from Pacific Chemical Co., Ltd. (Ansan, Korea). *N,N*-dimethylformamide (DMF) (Duksan Pure Chemicals) was purified by distillation. 1-Ethyl-(3-3-dimethylaminopropyl), carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma Chemicals (St. Louis, MO, USA). The sodium hydroxide, tetrahydrofuran (THF), and *n*-hexane (Duksan Pure Chemicals) were used without any further purification. The water used in the experiments was first treated using a reverse osmosis system (Sambo Glove, Ansan, Korea), and further purified using a Milli-Q Plus system (Millipore, Billerica, MA, USA). Phosphate buffered saline (PBS) was purchased from Gibco BRL (Rockville, MD, USA). FITC-labeled BSA (fluorescein isothiocyanate conjugate bovine serum albumin, 7-12 mols FITC per mol albumin) and riboflavin were purchased from Sigma Chemicals.

Animals. Male New Zealand white rabbits (weighing 1.65-3.5 kg) were obtained from the Korea Laboratory of Animal Development (Seoul, Korea) and Marshall Farms (New York, NY, USA). The animals were housed in a light-controlled room kept at 22 ± 1 °C and a humidity of 55 ± 10% with food (Samyang Company, Seoul, Korea).

Synthesis of Semi-Telechelic PNIPAAm. Telechelic PNIPAAm, having an incorporated amino group at the chain end, was synthesized by radical polymerization using AESH as a chain transfer agent, and AIBN as an initiator. The synthesis and characterization was performed using similar procedures and compositions to those described in previous work.[24-26,29] The chain length of the PNIPAAm-NH_2 was controlled by varying the feed molar ratio of initiator to NIPAAm monomer. Briefly, NIPAAm (88.37 mmol) was dissolved in DMF (20 mL) with AESH (3.68 mmol). Dried nitrogen was bubbled into the solution for 30 min prior to polymerization, which was initiated by AIBN (long chain: 1.22 mmol, short chain: 7.06 mmol). Polymerization was carried out at 70 °C for 6 h under a vacuum. The reactant was precipitated into an excess of diethyl ether and dried in a vacuum at room temperature. The dried polymer was purified by repeatedly precipitating and dissolving it in hot water, after which the purified product was freeze dried.

The molecular weight distribution of the amino semi-telechelic PNIPAAm was determined by gel permeation chromatography (GPC, Waters Mode 510 HPLC pump, Milford, MA, USA) using the Millennium software program. A nonaqueous potentiometric titration method was used to quantitatively determine the incorporation of terminal amino groups of PNIPAAm-NH_2.[21,26] Semi-telechelic PNIPAAm-NH_2 (0.2 g) was dissolved in 20 mL acetic acid, and titrated with 0.1 M perchloric acid-acetic acid standard solution using crystal violet as an indicator.

Preparation of PNIPAAm-Grafted Hyaluronic Acid.

Table I. Composition and Graft Yield of PNIPAAm-Grafted HA Copolymer

Sample	Feed Weight Ratio(wt%)		Molecular Weight of PNIPAAm-NH_2[a]	Graft yield (wt%)[b]
	PNIPAAm	HA		
L37	30	70	13,100	42.8
L55	50	50	13,110	56.7
L73	70	30	13,110	67.0
S37	30	70	6,100	51.8
S55	50	50	6,100	63.5
S73	70	30	6,100	78.9

[a]Determined by GPC measurement.
[b]Graft yield (%)$=(W_2 - W_1)/W_1 \times 100$ (W_1: weight of HA before grafting, W_2: total weight after grafting PNIPAAm-NH_2 onto the HA main chain).

HA and PNIPAAm-NH_2 were dissolved in deionized water with 0.5 wt% concentration at room temperature. EDC and NHS were added to the solution to form amide bonds between the carboxyl groups of hyaluronic acid and the amino groups of PNIPAAm-NH_2. The solution had various weight ratios of the two polymers, as shown in Table I, and a HA/EDC/NHS molar ratio of 2 : 2 : 1 with a reference to the carboxyl group of hyaluronic acid. The mixed solution was continuously stirred overnight at room temperature. After precipitation in a THF-hexane solution (4:1), the precipitant was purified by Soxhlet extraction with methanol, dialyzed for three days in deionized water, and then freeze-dried. Fourier transform infrared (FTIR, Nicolet model Magna IR 550, Madison, WI, USA) spectroscopy was used to confirm the grafting of amino-terminated semi-telechelic PNIPAAm onto HA. The graft yield, based on the weight change, was calculated using the following equation: [Graft yield(wt%) $=[(W_2 - W_1)/W_1] \times 100]$; where W_1 and W_2 are the weight of HA before grafting and the total weight after grafting PNIPAAm-NH_2 onto the HA main chain, respectively, as shown in our previous studies.[29]

Thermo-Optical Analysis (TOA) of PNIPAAm-Grafted HA Copolymer Solution. TOA provides a simple, rapid, and reliable experimental method to determine cloud point curves of binary polymer/solvent systems.[30] A thermo-optical analyzer consists of a polarizing microscope (Nikon Optiphot-Pol, Kawasaki, Japan), a heating-cooling stage (Mettler Toledo FP82HT, Greifensee, Switzerland), a photodiode (Mettler FP 82, Greifensee, Switzerland), and a microprocessor (Mettler FP 90, Greifensee, Switzerland).

The sealed sample tube containing the solution was placed and centered in the microscope heating/cooling stage. A high scan rate of heating or cooling (5 °C/min) was first used to find the approximate range for the cloud point of the sample. The sample was then repeatedly heated or cooled over a temperature range near the cloud point with a low scan rate (0.5 °C/min), while the intensity of the light was monitored. The photodiode quantitatively detected the light intensity penetrating the sample tube as a function of temperature. Each measurement was then repeated at least three times to assure reproducibility.

Viscosity Measurement. The viscosity of the PNIPAAm-grafted HA solution was determined for a specific temperature range (25~45 °C) using a digital Brookfield Viscometer, model RTV (Brookfield engineering laboratories, Stoughton, MA, USA). Appropriate spindles with six different speeds (2.5, 5, 10, 20, 50 and 100 rev/min) were used to obtain the correct dial readings, which were taken at 20 min intervals, to allow time for the solution to stabilize. Three readings were taken for each sample, and the mean dial reading was corrected using factors supplied by the instrument manufacturer. Viscosity measurements were performed with a HA-*g*-PNIPAAm copolymer/PBS solution of 5 wt% concentration.

***In vitro* Riboflavin Release Study.** To evaluate the release of drug from the injectable PNIPAAm-grafted HA hydrogel, a measuring system was designed. The copolymer was dissolved in PBS (pH 7.4) with a concentration of 5 wt%. Two milliliters of prepared solution with riboflavin (0.1 mg/mL) was poured into test tubes (diameter = 10 mm). The test tubes were kept at 37 °C for gelation and then a PBS release medium solution of 10 mL was added to the test tubes. Aliquots (3 mL) were withdrawn from the release medium to evaluate the released content of the drug and replaced by an equal volume at each time point. The riboflavin released was detected using UV-vis spectroscopy at 375 nm and the amount was determined by reference to a riboflavin standard curve.

***In vivo* BSA Release Study.** PNIPAAm-grafted HA (S73) solution was prepared at a concentration of 5 wt% in PBS and sterilized by exposure to ethylene oxide gas. FITC-labeled BSA (0.12 mg) was added to 1.2 mL of the sterilized PNIPAAm-grafted HA injectable solution, which was subcutaneously injected on the dorsal surface of a rabbit. Three to four milliliters of blood was collected from an ear vein of the rabbit at a specified time after injection. The blood plasma was isolated by centrifuging at 3,000 rpm for 5 min to assay FITC-labeled BSA concentration using a spectrofluorometer. The steady-state fluorescence spectrum was obtained with a Spex-Fluorolog 3.2.2 (Horiba Jobin Yvon, Longjumeau, France) spectrofluorometer at room temperature.

Results and Discussion

Preparation of Telechelic PNIPAAm-NH_2. In previous studies, we synthesized the amino-terminated PNIPAAm for use in the preparation of the comb-type graft hydrogel with polysaccharides containing the carboxyl group as a pendent group.[25,26] To covalently graft the PNIPAAm on the carboxyl group in HA, telechelic PNIPAAm-NH_2 was synthesized by radical polymerization using AESH as a chain transfer agent. The procedure for synthesis of a PNIPAAm-grafted HA copolymer is shown in Figure 1. To confirm the preparation of telechelic PNIPAAm-NH_2, the existence of NH_2 groups at the end of the chain and the molecular weight of PNIPAAm were investigated using FTIR and GPC, respectively, as shown in our previous paper.[25] The molecular weight distribution of the telechelic PNIPAAm-NH_2 determined by GPC showed that the weight-average molecular weights (M_w) were 13,100 and 6,100 in the long and short chains, respectively. The efficiency of PNIPAAm possessing the amino end groups was 91%, which was determined by comparing the molecular weight of PNIPAAm measured from GPC with the amino-group content obtained from a titration assay (data not shown).

Preparation and Characterization of HA and Comb-Type Graft Hydrogels. To prepare the comb-type graft

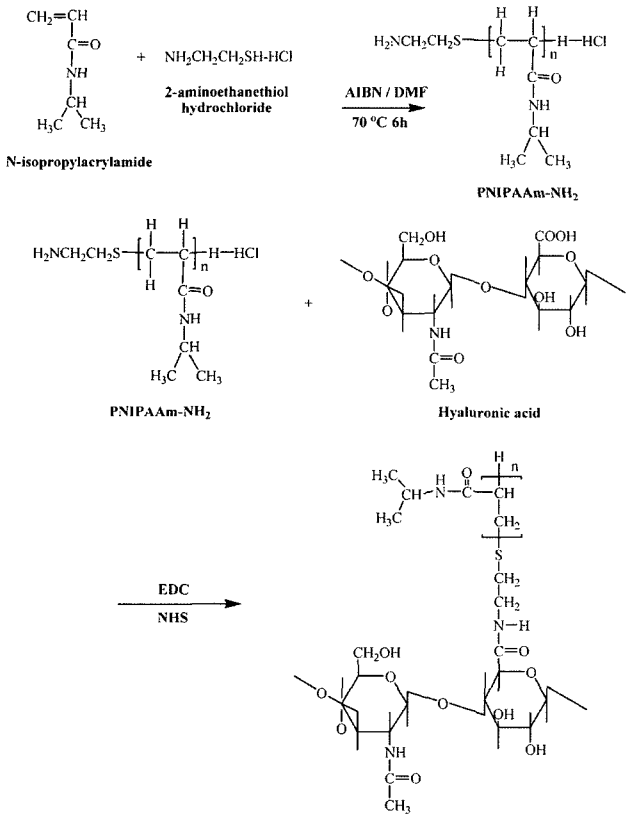

Figure 1. Synthetic scheme of amine-terminated telechelic PNIPAAm and PNIPAAm-grafted HA copolymer.

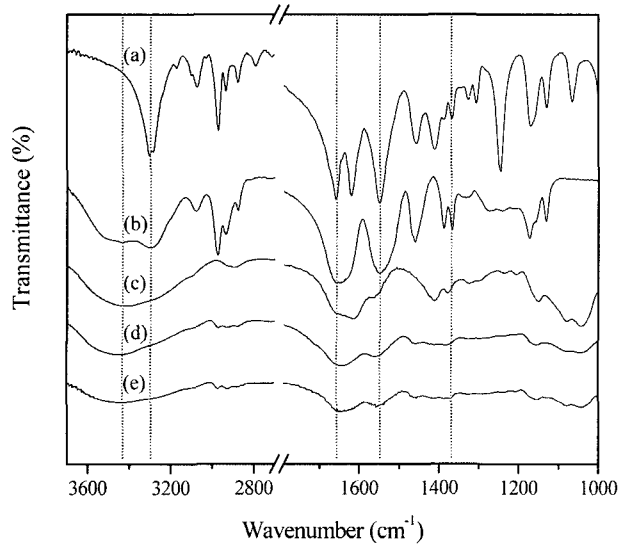

Figure 2. FTIR spectra for (a) NIPAAm monomer, (b) telechelic PNIPAAm-NH_2, (c) HA, (d) long chain PNIPAAm-grafted copolymer (M_w of PNIPAAm-NH_2: 13,100), and (e) short chain PNIPAAm-grafted copolymer (M_w of PNIPAAm-NH_2: 6,100).

copolymers with free and mobile chain ends, the telechelic PNIPAAm-NH_2 was grafted on the carboxyl groups of HA. FTIR spectroscopy measurements were carried out to confirm the synthesis of telechelic PNIPAAm-NH_2 and PNIPAAm-grafted HA copolymer. Figure 2 shows the FTIR spectra for (a) NIPAAm monomer, (b) PNIPAAm-NH_2, (c) HA, (d) long chain PNIPAAm-grafted HA (M_w of PNIPAAm-NH_2 = 13,100), and (e) short chain PNIPAAm-grafted HA copolymer (M_w of PNIPAAm-NH_2 = 6,100). The telechelic PNIPAAm homopolymer shows characteristic peaks at 1654, 1542, and 1375 cm^{-1}, which can be attributed to the characteristic peaks of amide I, amide II, and a methyl group in $CH(CH_3)_2$, respectively (Figure 2(b)). Characteristic peaks of NIPAAm monomer at 1617 cm^{-1} (C=C) and 1410 cm^{-1} (CH_2=) disappeared. Also, two bands at 3435 and 3300 cm^{-1} for the primary amine (NH_2) were observed. Characteristic peaks of HA appeared at 1615 and 1410 cm^{-1} for the asymmetric COO- stretching vibration and the symmetric COO- stretching vibration, respectively (Figure 2(c)). The graft of PNIPAAm-NH_2 on HA was confirmed by the appearance of the characteristic peaks of PNIPAAm at 1654, 1542, and 1375 cm^{-1}, and by the shift of a carboxyl peak of HA at 1615 cm^{-1} caused by the formation of amide linkages (Figure 2 (d) and (e)).

Table I shows the feed compositions of NIPAAm:HA and graft yields of HA-*g*-PNIPAAm copolymers. PNIPAAm-grafted HA copolymers with different graft yields and molecular weights of PNIPAAm were synthesized. The graft yield of PNIPAAm onto HA backbones gradually increased with increasing feed weight ratio of NIPAAm to HA. In addition, the short chain PNIPAAm-NH_2 (M_w=6,100) showed higher graft yield than long chain PNIPAAm-NH_2 (M_w= 13,100) at the same feed ratios. This may be due to an increase in steric hindrance with increasing PNIPAAm chain length.

Thermo-Responsive Properties of PNIPAAm-Grafted HA Copolymer. Figure 3 shows the turbidity change of HA-*g*-PNIPAAm copolymer solution as a function of temperature. The turbidity measurement of the injectable gel was performed with a solution of 5 wt% concentration. The reduction of photodiode transmittance observed upon heating rapidly occurred at 30~33 °C. The gels composed of HA and PNIPAAm underwent a volume phase transition in water at around the LCST of PNIPAAm (32 °C), because PNIPAAm chains hydrate to form expanded structures in water when the solution temperature is below the LCST, but become compact structures by dehydration when heated above the LCST. The cloud point, which means the phase separation temperature, was affected on the concentration of copolymer and the PNIPAAm content in the copolymer.[15] In this study, the concentration of the gel was fixed at 5 wt% by considerations of gel viscosity. As the graft yield of PNIPAAm onto HA backbones increased, HA-*g*-PNIPAAm copolymer solutions exhibited a sharper phase transition (Figure 3(a)). Compared with a long chain PNIPAAm-grafted

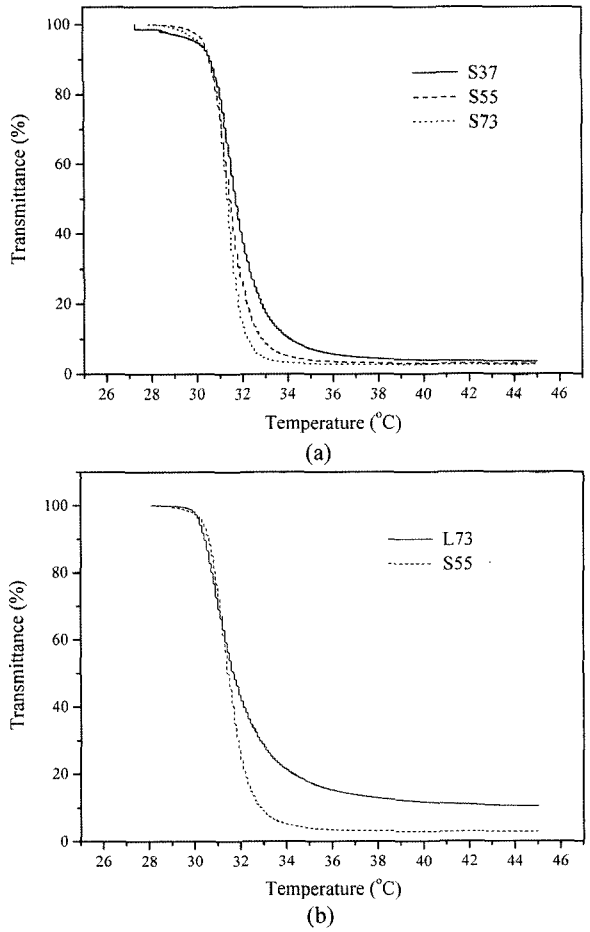

Figure 3. Thermo-optical analysis (TOA) of PNIPAAm-grafted HA copolymer; (a) PNIPAAm-grafted HA with different graft yields and (b) PNIPAAm-grafted HA with different PNIPAAm chain length and similar graft yield (L73: M_w of PNIPAAm= 13,100, graft yield=67.0%; S55: M_w of PNIPAAm=6,100, graft yield=63.5%). Heating rate was 0.5 °C/min and the concentration of copolymer was 5 wt%.

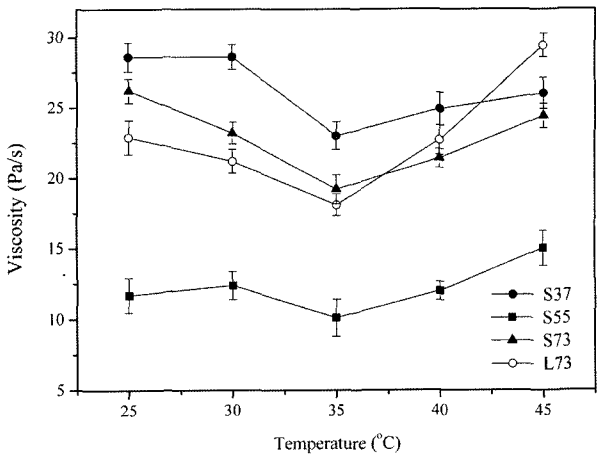

Figure 4. Viscosity change of PNIPAAm-grafted HA copolymer solution (5 wt% concentration) depending on temperature.

HA (M_w=13,100) [L37, graft yield=67.0%], the short chain PNIPAAm-grafted HA (M_w=6,100) [S55, graft yield=63.5%] showed a narrower temperature range for optical turbidity changes, because the mobility of the PNIPAAm chain was restricted to the main chain of HA (Figure 3(b)).

Viscosity Change HA-*g*-PNIPAAm Copolymer Depending on Temperature. The viscosity of the injectable gel at room temperature must be considered for its effect on the needle gauge required. If the viscosity is too high, the solution cannot be injected. All PNIPAAm-grafted HA copolymer solutions (5 wt%) were injected through a 21-gauge needle at room temperature.

Figure 4 shows the viscosity changes of PNIPAAm-grafted HA solutions at various temperatures. All PNIPAAm-grafted HA samples exhibited a slight decrease in viscosity with increasing temperatures below 35 °C. This result may be due to the viscous effects of the HA solution with increasing temperatures. However, the viscosity of HA-*g*-PNIPAAm

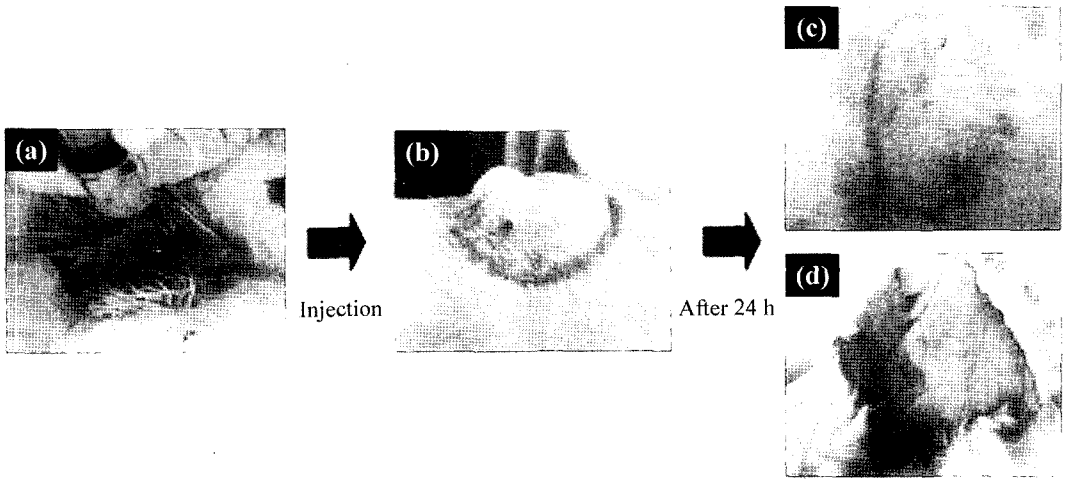

Figure 5. PNIPAAm-grafted-HA gels maintained their shapes for 24 h after injection.

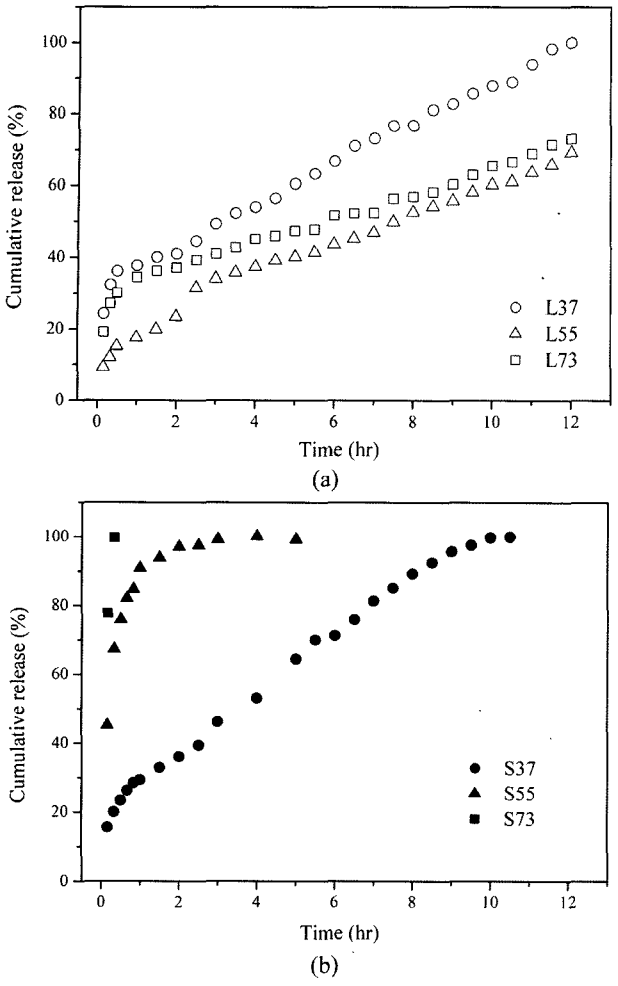

Figure 6. *In vitro* release profiles of riboflavin from PNIPAAm-grafted HA gels in PBS at 37 °C. (a) long chain PNIPAAm-grafted HA (M_w of PNIPAAm = 13,100) and (b) short chain PNIPAAm-grafted HA (M_w of PNIPAAm = 6,100).

solution increased above 35 °C, which is related to the LCST of the branched PNIPAAm chain. This can be explained by the fact that copolymers were aggregated within the temperature range of 35~45 °C. Particularly, long chain PNIPAAm-grafted HA (L73) showed a higher increase in viscosity than short chain PNIPAAm-grafted HA samples (S37, S55 and S73). To investigate *in vivo* gelation behavior, 5 wt% HA-*g*-PNIPAAm copolymer solution (S73) was subcutaneously injected into the dorsal surface of a rabbit. Although viscosity data (Figure 4) didn't change greatly with temperature, the HA-*g*-PNIPAAm gels maintained their shapes for 24 h after administration as shown in Figure 5.

***In vitro* Riboflavin Release Study.** Figure 6 shows the cumulative amount of riboflavin released from the PNIPAAm-*g*-HA gel as a function of time at 37 °C. The riboflavin released was monitored by UV-vis spectroscopy of the solution taken at set time intervals. The initial burst of the riboflavin was low because the drug formulation was

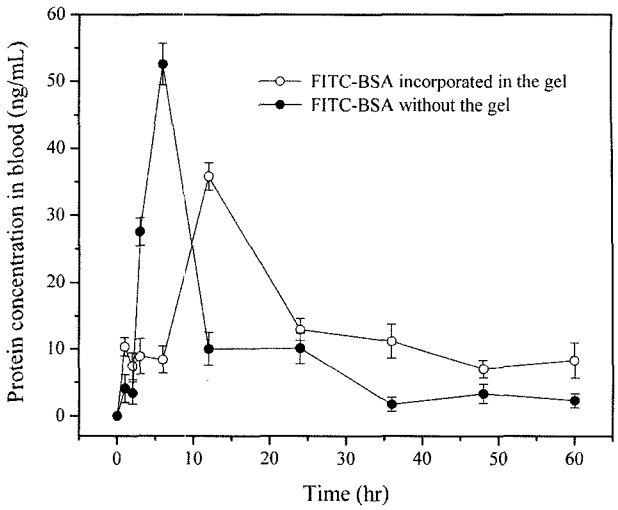

Figure 7. *In vivo* release profile of FITC-labeled BSA from PNIPAAm-grafted-HA gel (5 wt%) (S73) in rabbits.

homogeneous; the drug was not localized on the surface of the gel. As the grafting yield of PNIPAAm onto the HA backbone increased, HA-*g*-PNIPAAm gel showed more sustained release behaviors (Figure 6(a) and (b)).

When the chain length was considered in the drug release, the release profiles of long chain PNIPAAm-grafted HA gels showed a sustained release of riboflavin from the gel for 12 h (Figure 6(a)). However, short chain PNIPAAm-grafted HA gel showed much faster release kinetics than the long chain-grafted HA gel (Figure 6(b)). In particular, riboflavin was completely released from S37 and S55 within 2 h. This is probably due to the small viscosity change by low graft yield of PNIPAAm as shown in the viscosity measurements of Figure 5. For the S73 sample, which had higher graft yield (78.9%), the sustained release kinetics of riboflavin was observed. Thus, the release kinetics of drug from PNIPAAm-grafted HA gel could be controlled by adjusting the graft yield and chain length of the PNIPAAm.

***In vivo* FITC-BSA Release Study.** To investigate *in vivo* release kinetics of the drug from PNIPAAm-grafted HA gel, the concentration of FITC-labeled BSA in blood was measured after the injection of PNIPAAm-*g*-HA copolymer solution containing FITC-BSA into the dorsal surface of a rabbit. As a control, the same amount of FITC-BSA was injected without the gel.

Figure 7 shows the concentration of FITC-BSA released from the PNIPAAm-grafted HA gels (S73) and untreated FITC-BSA as a function of time. As a control, the same amount of albumin was injected without the gel. For free BSA solutions without the gel, the concentration in the blood plasma increased immediately after injection and most of the BSA was released within 6~8 h. However, the BSA released from the PNIPAAm-*g*-HA gel showed a maximum concentration after 12 h and a more sustained release profile,

and the BSA was detected in the blood for more than 60 h.

Conclusions

Temperature-sensitive injectable gels were prepared by grafting amino-terminated semi-telechelic PNIPAAm onto HA backbones. Results from thermal-optical analysis revealed that the HA-*g*-PNIPAAm copolymer solution exhibited sharper phase transition with increased graft yield of PNIPAAm onto the HA backbone. Compared with a long chain PNIPAAm-grafted HA (M_w=13,100), the short chain PNIPAAm-grafted HA (M_w=6,100) showed narrower temperature ranges for optical turbidity changes. All PNIPAAm-grafted HA samples exhibited a slight decrease in viscosity with increasing temperatures below 35 °C. This may be due to the viscous effects of HA solutions with increasing temperatures. However, above the 35 °C the viscosity of HA-*g*-PNIPAAm solution increased, which is related to the LCST of the branched PNIPAAm chain. Particularly, the long chain PNIPAAm-grafted HA showed a higher increase in viscosity than the short chain PNIPAAm-grafted HA samples. The subcutaneously injected HA-*g*-PNIPAAm solution into the dorsal surface of a rabbit formed a gel within seconds and maintained its shape for 24 h after administration. As the grafting yield of PNIPAAm onto the HA backbone increased, the riboflavin incorporated within HA-*g*-PNIPAAm gel showed a more sustained release behavior. From the *in vivo* release study results, the BSA released from PNIPAAm-*g*-HA gel showed a maximum concentration after 12 h and more sustained release profile than free BSA without gel, and the BSA was detected in blood plasma for more than 60 h. Thus, this thermo-responsive PNIPAAm-grafted HA hydrogel can be useful as an injectable drug carrier and its release behaviors can be specifically designed by controlling the graft yield and chain length of PNIPAAm.

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