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Sustained release formulation of erythropoietin using hyaluronic acid hydrogels crosslinked by Michael addition

Sei Kwang Hahn [a,*], Eun Ju Oh [a], Hajime Miyamoto [b], Tsuyoshi Shimobouji [b]

[a] *Department of Advanced Materials Science and Engineering, Pohang University of Science and Technology (POSTECH), San 31, Hyoja-dong, Nam-gu, Pohang 790-784, Republic of Korea*

[b] *Preclinical Research Department, Roche group, Chugai Pharmaceutical Co., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan*

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Abstract

A novel sustained release formulation of erythropoietin (EPO) was successfully developed using hyaluronic acid (HA) hydrogels crosslinked by Michael addition. Adipic acid dihydrazide grafted HA (HA-ADH) was prepared and then modified into methacrylated HA (HA-MA). ^{1}H NMR analysis showed that the degrees of HA-ADH and HA-MA modification were 69 and 29 mol%, respectively. Using the specific crosslinkers of dithiothreitol (DTT) and peptide linker, EPO was loaded during HA-MA hydrogel preparation by Michael addition chemistry between thiol and methacrylate groups. The amount of EPO recovered from both hydrogels after degradation with hyaluronidase SD (HAse SD) was about 90%. The crosslinking reaction with peptide linker (GCYKNRDCG) was faster than that with DTT. The gelation time was about 30 min for peptide linker and 180 min for DTT. In vitro release test of EPO from HA-MA hydrogel at 37 °C showed that EPO was released rapidly for 2 days and then slowly up to 7 days from HA-MA hydrogels. The released EPO appeared to be intact from the analysis with RP-HPLC. According to in vivo release test of EPO from HA-MA hydrogels crosslinked with the peptide linker in Sprague–Dawley (SD) rats, elevated plasma concentration of EPO was maintained up to 7 days. There was no adverse effect during and after the in vivo tests.

Keywords: Sustained release; Erythropoietin; Hyaluronic acid; Michael addition; Hydrogel

1. Introduction

Controlled release formulation of protein drugs has been of substantial therapeutic interest with the rapidly increasing protein drug market. For the first time, Genentech and Alkermes developed Nutropin Depot® as a sustained release formulation of human growth hormone using poly(lactic glycolic acid) (PLGA) microparticles (Jones et al., 1997). However, these delivery systems were reported to have complexities such as protein denaturation and inflammation associated with the degradation of PLGA (Elbert et al., 2001). The potential benefits of hydrogels for protein drug delivery are numerous compared to PLGA microparticles. The denaturation of protein should be greatly reduced due to the absence of organic solvent contact during the PLGA formulation and hydrophobic interaction with PLGA after the formulation (Fu et al., 2000). Additionally, hydrophilic materials and hydrogels are relatively non-inflammatory (Chowdhury and Hubbell, 1996). In order to take advantage of using hydrogel for protein drug delivery, the hydrophilic precursors must be crosslinked in a buffer at near neutral pH and should be crosslinked via reactions which do not modify proteins.

As a hydrophilic precursor, hyaluronic acid (HA) has excellent physicochemical properties, such as biodegradability, biocompatibility, viscoelasticity, and non-immunogenecity (Laurent, 1998). HA is a natural linear polysaccharide composed of alternating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with β(1 → 4) interglycosidic linkage (Laurent, 1998). This biopolymer has distinctive biological functions with a molecular weight range from 1000 to 10,000,000 Da (Laurent, 1998). HA acts to control tissue hydration and is present in hydrated networks with collagen fibers in the extra-cellular matrix. It also constitutes the backbone of cartilage proteoglycan. HA plays pivotal roles in wound healing, and in promoting cell motility and differentiation during development (Laurent, 1998). Because of unique physicochem-

* Corresponding author. Tel.: +82 54 279 2159; fax: +82 54 279 2399.
E-mail address: skhanb@postech.ac.kr (S.K. Hahn).

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ical properties and various biological functions, HA and modified HA have been widely used for arthritis treatment (Balazs and Denlinger, 1993), ophthalmic surgery (Balazs, 1983), drug delivery (Kim et al., 2005; Hahn et al., 2004a; Vercruysse and Prestwich, 1998), and tissue engineering (Ohri et al., 2004; West et al., 1985). A number of strategies for chemical modification of HA mainly through carboxyl and hydroxyl groups have been developed including esterification of HA (Campoccia et al., 1998), chemical modification of HA with carbodiimide (Kuo et al., 1991), and crosslinking of HA using divinyl sulfone (Balazs and Leshchiner, 1986) or glycidyl ether (Balazs and Leshchiner, 1987). However, the modification of HA has generally been carried out in harsh reaction conditions making difficult inclusion of sensitive molecules or living cells during the HA hydrogel preparation (Luo et al., 2000). Recently, several works on the protein delivery from crosslinked HA hydrogels have been reported (Hahn et al., 2004b; Leach and Schmidt, 2005), but there are few hydrogel synthesis approaches that account for possible deleterious effects of crosslinking reaction in the presence of bioactive protein and peptide drugs.

The objective of this study was to develop a novel sustained release formulation of protein drugs using HA hydrogels crosslinked by Michael addition in the presence of proteins to be loaded. According to the preliminary simulation study with RP-HPLC, EPO appeared not to be denatured by dithiothreitol (DTT) and peptide-linker, which was thought to be used for the preparation of HA hydrogels. Thus, adipic acid dihydrazide grafted HA (HA-ADH) was prepared and then modified into methacrylated HA (HA-MA). Without free thiols on the surface, erythropoietin (EPO) was used as a model protein for its sustained release test from HA-MA hydrogels. EPO is used for the treatment of patients not only with anemia associated with chronic renal failure but also with many types of anemia caused by diseases which interfere with RBC production, such as cancer, rheumatoid arthritis, HIV infection, ulcerative colitis, sickle cell anemia, and so on (Marchetti and Barosi, 2004; Ferraiolo et al., 1992). EPO was loaded during HA-MA hydrogel preparation by Michael addition between thiol of the specific crosslinker and methacrylate groups of HA-MA. In vitro release test of EPO was carried out and the released EPO was analyzed with RP-HPLC. This selectively crosslinked HA-MA hydrogel was assessed for the sustained release of EPO in Sprague–Dawley rats.

2. Materials and methods

2.1. Materials

EPO was supplied from Chugai Pharmaceutical Co. (Tokyo, Japan) and HA from Denkikagaku Kogyo Co. (Tokyo, Japan). Peptide linker of GCYKNRDCG was purchased from Qiagen (Tokyo, Japan) and hyaluronidase SD (HAse SD) from Seikagaku Co. (Tokyo, Japan). Irgacure 2959 were purchased from Ciba specialty chemicals (Basel, Swiss) and 1,8-bis-maleimidotriethyleneglycol ($BM(EG)_3$) from Pierce (Rockford, IL). Methacrylic anhydride (MA), polyethylene glycol diacrylate (PEG-DA), and triethanolamine (TEA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Dithiothreitol (DTT), iodoacetate (IA), DMSO, ethanol, acetonitrile, and trifluoroacetic acid were purchased from Wako Pure Chemicals Industries (Osaka, Japan). All the chemicals were used without further purification.

2.2. Simulation of EPO denaturation during HA hydrogel preparation

The effect of various crosslinkers for HA hydrogel preparation on the protein denaturation during the crosslinking reaction was assessed as follows. As described in Section 2.4, the concentration of HA (MW = 200,000) solution was assumed to be approximately 4 wt% containing EPO of 800 μg/mL and the concentration of crosslinkers to be 30 mol% of HA repeating units. On the basis of this crosslinking reaction condition, EPO was mixed at 37 °C for 4 h with HA-MA, PEG-DA, Irgacure 2959 (photosensitizer in 20% ethanol), $BM(EG)_3$ and DTT, and then analyzed with RP-HPLC after eight times dilution. RP-HPLC was carried out using Waters 600S with 717 auto-sampler and 486 UV detector. The injection volume was 100 μL and the concentration of standard EPO was 100 μg/mL. As a mobile phase, Solution A (water/acetonitrile/trifluoroacetic acid = 400/100/1) and Solution B (water/acetonitrile/trifluoroacetic acid = 100/400/1) were used with the gradient from A/B of 65/35 to 0/100. The column used was C4 (diameter of 5 μm, 4.6 mm × 250 mm) and the flow rate was 1 mL/min.

2.3. HA-MA preparation

HA-ADH (MW of HA = 200,000) was prepared as described elsewhere (Luo et al., 2000) and then modified into HA-MA as follows. HA-ADH (100 mg) was dissolved in water (4.5 mL) for 2 h. Phosphate buffer (0.5 mL, pH 8, 100 mM) was added to the HA-ADH solution to adjust the reaction pH at 8 (PB of 10 mM). Twenty time molar excess of methacrylic anhydride (MA, MW = 154.17, 616.7 mg) to ADH content in HA-ADH was added to the HA-ADH solution (2%, w/v, pH 8). After reaction for 4 h, 1 day, or 3 days, HA-ADH-MA was recovered by ethanol precipitation, washed with ethanol three times, and dried at room temperature. The recovered HA-MA was analyzed with ^{1}H NMR (JNM-ECA500, Nihondensi Co., Japan). Three replicates were carried out for HA-MA preparation.

2.4. HA-MA hydrogel synthesis

HA-MA (b3, 44 mg) was dissolved for 1 h in PBS (760 μL, pH 7.4, 10 mM) containing 16 μL/mL of TEA (hereafter PBS/TEA). Addition of TEA changed the pH of PBS to ca. 9.5 as measured with a pH-meter. Then, 200 μL of EPO (5 mg/mL in PBS) was added to the solution and mixed slowly for 30 min. As a specific crosslinker, 40 μL of DTT (MW = 154, 92.6 mg/mL) or 40 μL of GCYKNRDCG (MW = 1015.2, 610 mg/mL) in PBS/TEA was added to the above solution and mixed slowly. After mixing for 10 min, 200 μL of each solution was added to 1 mL syringe. The final precursor solution was mixed with 20 passages in the syringe and then incubated at 37 °C to complete

the crosslinking reaction for HA-MA hydrogel preparation. The molar ratio of MA to dithiol was 1:1 in both cases. Three replicates were carried out for HA-MA hydrogel preparation.

2.5. In vitro release test of EPO

The amount of EPO incorporated in HA-MA hydrogel (200 μL) was determined by RP-HPLC analysis after degradation with HAse SD (0.5 unit in 300 μL). The hydrogel was incubated for complete degradation at 37 °C for 5 h. Then, the degraded solution (500 μL) was diluted with 1.5 mL of phosphate buffer saline (PBS, 10 mM, pH 7.4). In case of 100% recovery, EPO concentration was expected to be 100 μg/mL. In vitro release tests of EPO from HA-MA hydrogels were carried out at 37 °C in PBS (1.75 mL, pH 7.4, 10 mM) using a shaking incubator. Iodoacetic acid (10 μL, 223.2 mg/mL) was added to the HA-MA hydrogel in the vial for the prevention of EPO denaturation by the remaining free thiols during in vitro release test. The released amount of EPO was quantified from the peak area of RP-HPLC. In order to study the swelling of HA-MA hydrogels, the weight of swollen hydrogel samples during the in vitro release test was measured with a microbalance (Mettler, Columbus, OH) after removal of the surface water and presented as a percentage to the original weight. Three replicates were carried out for each sample.

2.6. In vivo release test of EPO using SD rats

HA-MA (b3, 44 mg) was dissolved for 2 h in 760 μL of PBS/TEA. Then, 200 μL of EPO (1 mg/mL in PBS) was added to the solution and mixed slowly for 30 min. As a specific crosslinker, 40 μL of GCYKNRDCG (MW = 1015.2, 610 mg/mL) in PBS/TEA was added to the above solution and mixed slowly for 10 min. After that, 180 μL of the precursor solution was filled into 1 mL syringe, mixed with 20 passages in the syringe, and then incubated at 37 °C for 1 h to complete the crosslinking reaction for HA-MA hydrogel preparation. Each hydrogel containing 36 μg of EPO was implanted in the back of SD rats. The average body weight of SD rats was 360.4 ± 7.1 g. As a control, aqueous EPO with a same dosage of 100 (μg EPO)/(kg SD rats) was also injected. The degradation of HA hydrogels implanted in rats was investigated in 3 months.

2.7. Statistical analysis

The statistical analysis was carried out using a Sigma Plot Software (Aspire Software International, Leesburg, VA). All data are presented as means ± S.E.M.

3. Results and discussion

3.1. HA-MA preparation

HA hydrogel was considered as a suitable drug carrier encapsulating peptide and protein drugs within its three-dimensional network (Hahn et al., 2004b; Elbert et al., 2001). Among various analytical methods to examine the protein denaturation during HA hydrogel preparation, RP-HPLC appeared to reflect the denaturation of protein drugs in a most sensitive and qualitative manner. Even slight changes in protein conformation can result in changes in reversed-phase elution, since RP-HPLC is very sensitive to the "hydrophobic foot" of a protein (Carr, 2002). As well known, RP-HPLC is sensitive to protein modifications, such as deamidation or oxidation, and widely used for protein purification (Carr, 2002; Fahrner et al., 1999). Hermentin et al. (2002) reported EPO batch release assays with RP-HPLC. RP-HPLC method was also reported to be useful for quality control of rhEPO in final drug preparations (Wilczynska et al., 2005). According to the preliminary simulation study with RP-HPLC, EPO was denatured after incubation at 37 °C for 4 h with PEG-DA, Irgacure 2959 (photosensitizer), and $BM(EG)_3$

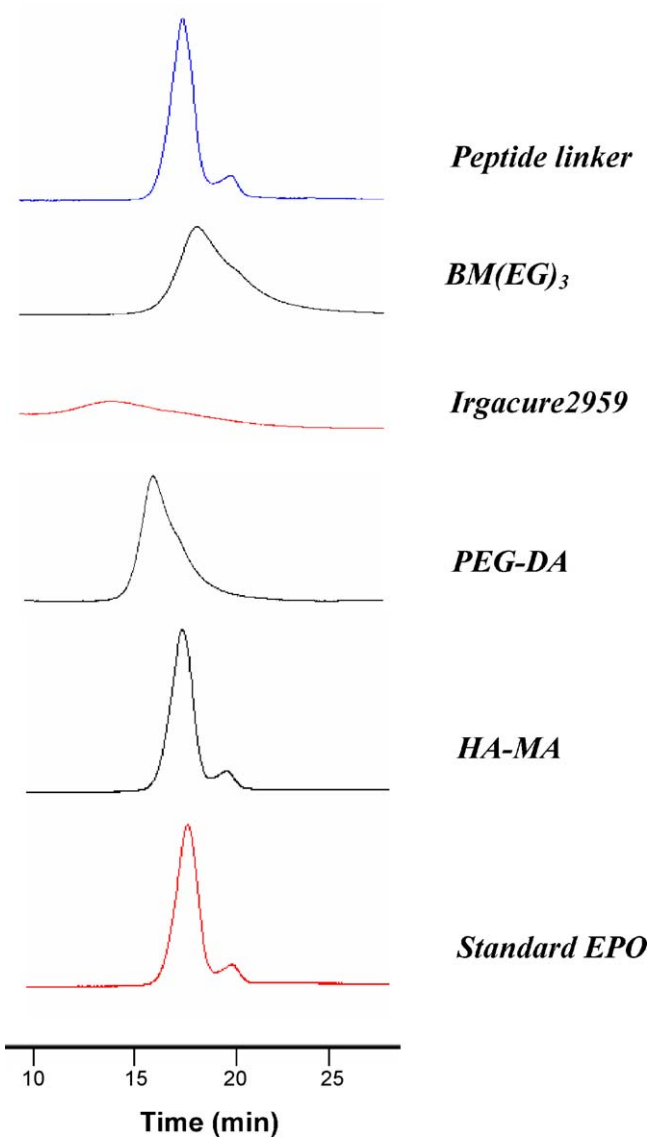

Fig. 1. RP-HPLC analysis for the simulation of EPO denaturation during HA hydrogel preparation. EPO was incubated at 37 °C for 4 h with methacrylated HA (HA-MA), polyethylene diacrylate (PEG-DA), photosensitizer of Irgacure 2959, 1,8-bis-maleimidotriethyleneglycol ($BM(EG)_3$), and peptide linker (GCYKNRDCG). The final concentration of EPO was 100 μg/mL after eight times dilution for RP-HPLC analysis.

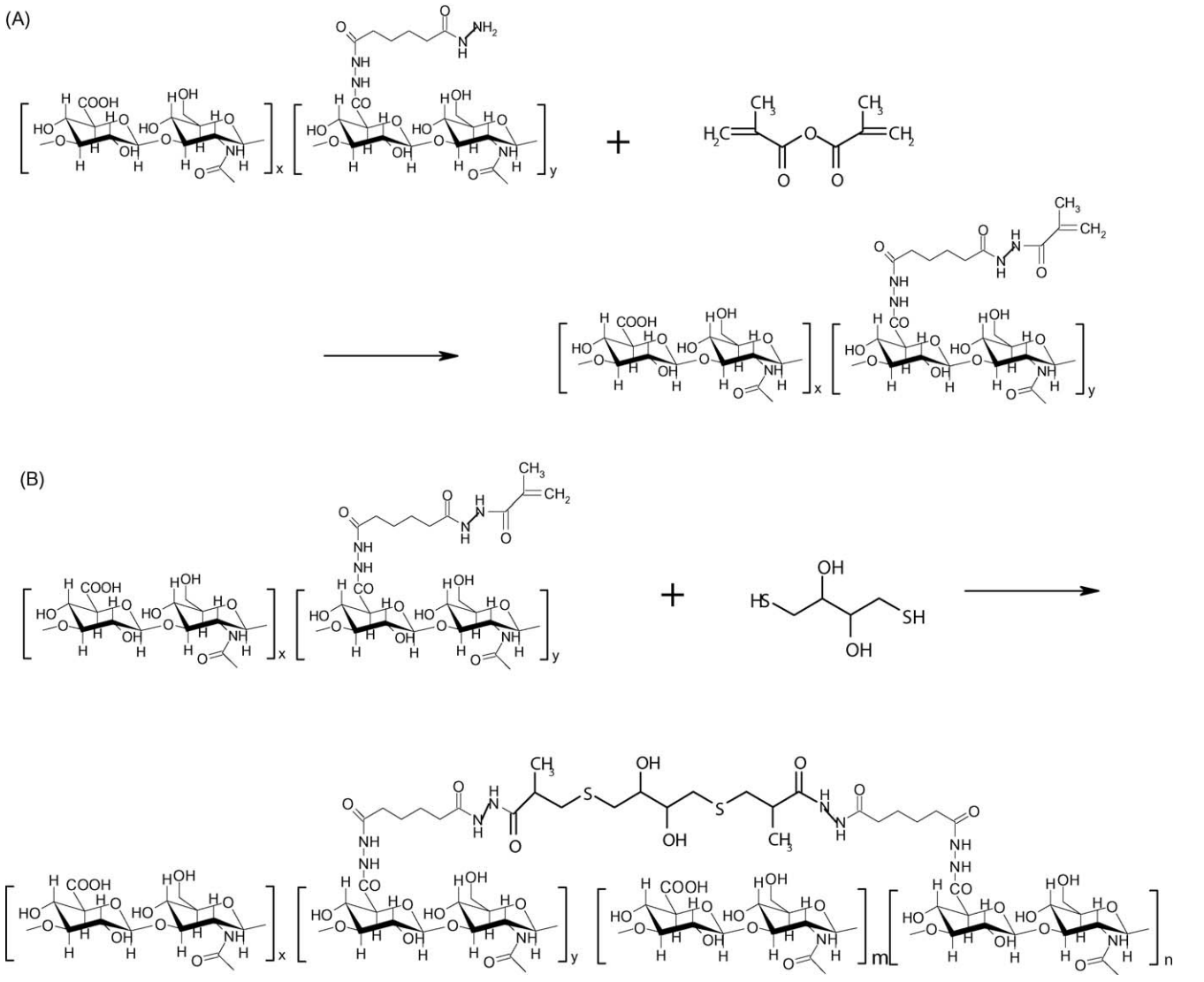

Fig. 2. Schematic representations of: (A) HA-MA preparation by grafting methacrylic anhydride onto HA-ADH and (B) HA-MA hydrogel preparation by crosslinking with dithiothreitol (DTT).

(Fig. 1). Interestingly, PEG-DA appeared to cause EPO denaturation likely due to the high reactivity of acryloyl group toward amine of EPO. It has been reported previously that acrylates react considerably faster than other unsaturated esters such as methacrylates, crotonates, cynnamates, and cyclopropancarbonylates (Lutolf et al., 2001). In addition, photosensitizer appeared to cause EPO denaturation during the photocrosslinking reaction (Fig. 1). To the contrary, EPO remained intact after incubation with HA-MA, DTT, and peptide linker (GCYKNRDCG). From the results, we thought HA-MA may be selectively crosslinked with DTT and peptide linker by Michael addition between thiol and methacrylate groups (Fig. 2).

On the basis of the simulation studies, HA-ADH was prepared and then modified into HA-MA with methacrylic anhydride (MA) (Fig. 2A). The peak assignments of HA-ADH in ^{1}H NMR spectra (Fig. 3) and the degree of HA-ADH modification were determined as described elsewhere (Pouyani and Prestwich, 1994). The methyl resonance (δ = 1.85–1.95 ppm) of acetamido moiety of the *N*-acetyl-D-glucosamine residue was used as an internal standard. The degrees of HA-ADH and HA-MA modification were determined by the peak areas of methylenes of ADH at δ = 1.7 and 2.4 ppm, and those of methacrylate groups between δ = 5.6 and 5.9 ppm, respectively (Fig. 3). As described in Table 1, the degree of HA-MA modification increased up to 30% with increasing ADH content on HA backbone. In case of direct grafting of MA to the carboxyl group of HA, the degree of HA-MA modfication was only 4% even after reaction for 3 days. The reaction time of 4 h appeared to be enough for the chemi-

Table 1
Degree of chemical modification (mol%) of HA-MA prepared by grafting methacrylic anhydride to HA-ADH according to ^{1}H NMR analysis

	HA-MA 0	HA-MA 1	HA-MA 2	HA-MA 3
ADH content (%)	0	26	46	69
MA content (%)	4	12	17	29

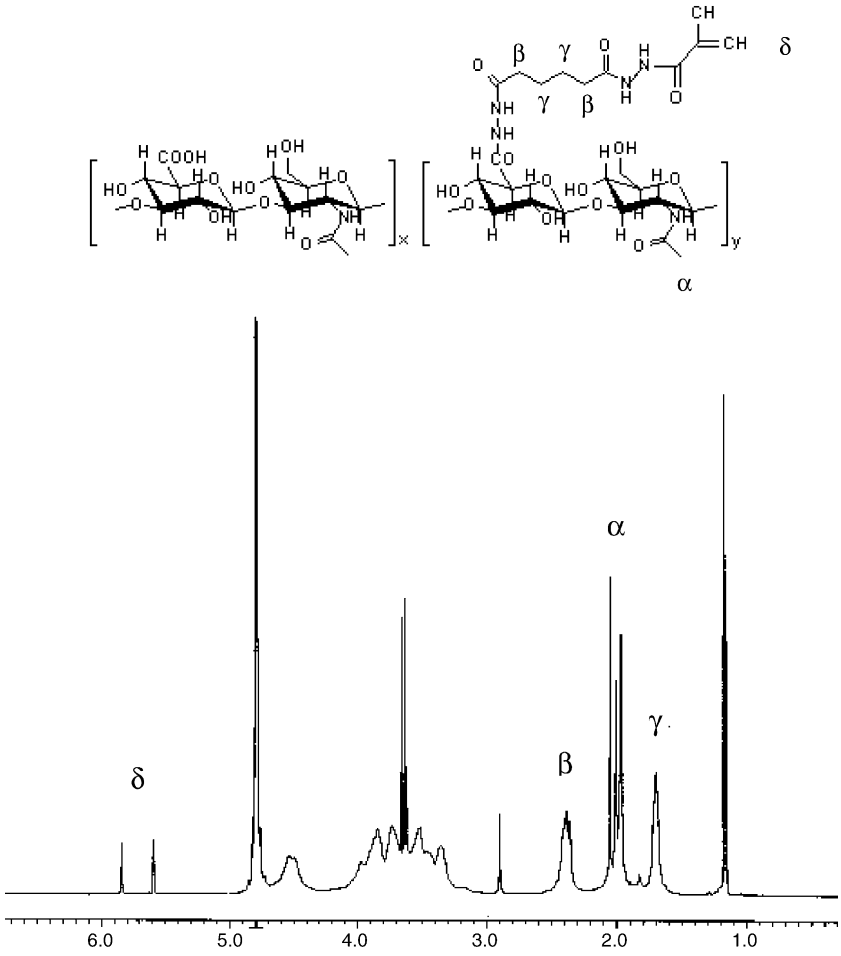

Fig. 3. ^{1}H NMR spectra of methacrylated HA (HA-MA) synthesized by grafting methacrylic anhydride onto adipic acid dihydrazide grafted HA (HA-ADH).

cal modification of HA-ADH with MA. The degree of HA-MA modification did not increase at a value of ca. 30% regardless of different reaction times of 4, 24, and 72 h.

3.2. HA-MA hydrogel synthesis

Fig. 2B shows the schematic representation of HA-MA hydrogel preparation using DTT. The crosslinking reaction with peptide linker (GCYKNRDCG) was faster than that by DTT. The gelation time was about 30 min for peptide linker and 180 min for DTT. It has been reported that positive charges close to the thiol groups accelerated Michael addition reaction considerably, while the opposite was shown by nearby groups of negative charge (Lutolf et al., 2001). Accordingly, lysine (K) and arginine (R) was thought to contribute for fast reaction of free thiols to HA-MA. Peptide linker appeared to be a good specific crosslinker to synthesize HA-MA hydrogel in a short reaction time. DTT was also thought to be useful because it is stable and cheap without immunogenecity issues. In both cases, the ratio of methacrylate groups versus dithiols of 1:1 showed the fastest Michael addition reaction as reported on the previous studies (Shu et al., 2004). After synthesis of HA-MA hydrogel with dithiols, the remaining reactive thiol groups were terminated with iodoacetate (IA) for EPO stability. As well known, IA has relatively higher reactivity toward sulfhydryl than amine group (Hermanson, 1996).

In order to determine the amount of EPO encapsulated in HA-MA hydrogels without denaturation, HA-MA hydrogels were synthesized and then degraded with hyaluronidase (HAse) SD. HAse SD purified from the culture broth of *Streptococcus dysgalactiae* catalyzes the eliminative cleavage of *N*-acetalhexosaminide linkages of HA. The HAse solution of 1 unit/mL (0.5 mL in PBS) degraded 200 μg HA hydrogel in 3 h completely. Although it might be difficult to compare directly, bovine HAse of 500 unit/mL was reported to degrade HA-ADH hydrogels in 20 h formed by crosslinking of ca. 65% ADH-functionalized HA with polyethylene glycol bis(succinimidyl propionate) (Bulpitt and Aeschlimann, 1999). The recovery of EPO from HA-MA hydrogel after degradation with HAse SD was ca. 90%. The 10% loss of EPO might be ascribed to the denaturation of EPO during the crosslinking reaction by Michael addition.

3.3. In vitro release test of EPO from HA-MA hydrogel

Fig. 4 shows in vitro release of EPO from HA-MA hydrogel crosslinked with DTT. The EPO release profile was dependent on the crosslinking reaction time and temperature. EPO was

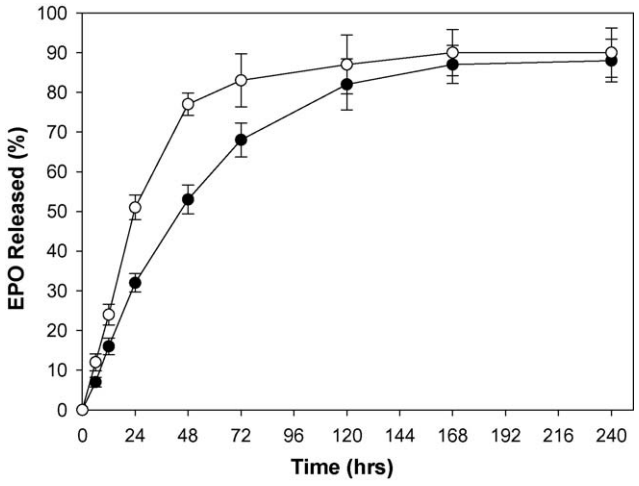

Fig. 4. In vitro release of EPO from two different HA-MA hydrogels prepared by the incubation of precursor solutions at 25 °C for 3 h (◯) and at 37 °C for 5 h (●). The 100% release of EPO corresponds to 100 μg/mL. Three replicates were carried out for each sample.

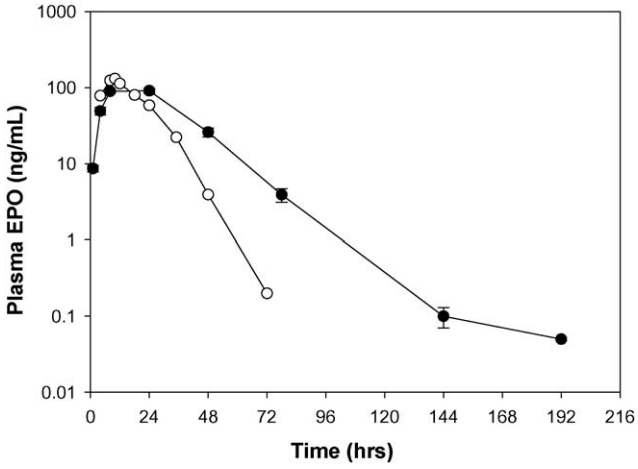

Fig. 6. Plasma concentration of EPO released from HA-MA hydrogels in comparison with that of aqueous EPO formulation. HA-MA hydrogel was prepared by Michael addition between methacrylate group of HA-MA and thiol group of specific crosslinker of peptide linker. Three replicates were carried out for the in vivo tests.

released rapidly for 2–4 days and then slowly released up to 7 days from HA-MA hydrogels. In vitro release of EPO from HA-MA hydrogels crosslinked with peptide-linker showed same release pattern. Considering the recovery of EPO by HAse degradation of 90%, almost all the EPO was thought to be released from HA-MA hydrogels. Fig. 5 shows the swelling of HA-MA hydrogels during the in vitro release test. With longer incubation for HA-MA hydrogel preparation, more highly crosslinked HA-MA hydrogel was prepared rendering longer sustained release of EPO (Figs. 4 and 5). The released EPO appeared to be intact according to RP-HPLC analysis.

3.4. In vivo release test of EPO from HA-MA hydrogel

In vivo release test of EPO was performed from HA-MA hydrogels selectively crosslinked by Michael addition. Fig. 6 shows the plasma concentration profile of EPO released from HA-MA hydrogels in comparison with that of aqueous EPO formulation. Elevated plasma EPO concentration higher than 0.1 ng/mL, which is a critical minimum concentration for EPO efficacy, could be maintained up to 7 days for HA-MA hydrogels (Fig. 6). Concerning with the bioactivity of released EPO, preliminary study showed the elevation of plasma hematocrit level in the range of 30–60% up to a week for HA-MA hydrogels. The results demonstrated that the released EPO was biologically active stimulating hematopoiesis. The remaining peptide linker in the HA-MA hydrogel was thought to be diluted and removed quickly from the HA-MA hydrogels in the subcutaneous of SD rats without causing EPO denaturation during the in vivo release tests. HA-MA hydrogels appeared to be degraded partially in 3 months from their weight changes. There was no adverse effect during and after the in vivo tests. In case of aqueous EPO formulation, however, the plasma concentration of EPO dropped to the baseline level in 3 days. The pharmacokinetic parameters are summarized in Table 2. T_{max} was elongated from 10 h of aqueous formulation to 24 h of HA-MA hydrogels reflecting sustained release of EPO.

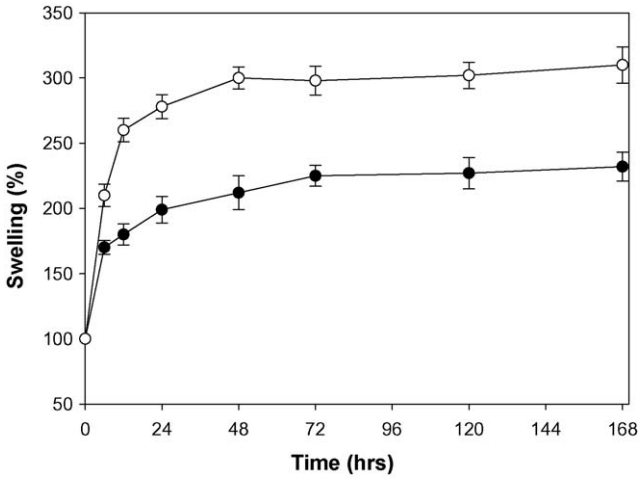

Fig. 5. Swelling of two different HA-MA hydrogels prepared by the incubation of precursor solutions at 25 °C for 3 h (◯) and at 37 °C for 5 h (●). Three replicates were carried out for each sample.

3.5. Advantages of HA-MA hydrogels

HA is a biodegradable, biocompatible, non-immunogenic, and non-inflammatory polysaccharide which can be used adequately for sustained release formulation of protein and peptide drugs (Laurent, 1998). While conventional drug delivery systems using PLGA exhibited inflammation and protein denaturation associated with the degradation of PLGA (Elbert et al., 2001), hydrophilic HA hydrogels appeared to be used as efficient protein-friendly drug carriers. Furthermore, HA hydrogels can be completely degraded by hyaluronidase in a body after releasing encapsulated protein drugs whereas PEG hydrogels may be difficult to be cleared in a body due to their

Table 2
Pharmacokinetic parameters of EPO formulations in SD rats

Formulation	Dose (μg/kg)	T_{max} (h)	C_{max} (ng/mL)	AUC (h ng/mL)	AUC/dose (h kg/mL)
Aqueous EPO	100	10	130.8 ± 4.9	2720	0.027
HA-MA hydrogel	100	24	91.1 ± 6.8	3804	0.038

high molecular weight after the crosslinking reaction. Considering all these results, HA-MA hydrogels were thought to be an excellent drug carrier for sustained release formulation of EPO. In order for longer sustained release formulation of EPO using HA-MA hydrogels, optimization study will be followed.

4. Conclusions

HA-MA hydrogel was assessed as a novel sustained release drug carrier for a model protein of EPO. HA-MA was prepared by grafting methacrylic anhydride to HA-ADH. ^{1}H NMR analysis showed that the degree of HA-ADH modification was ca. 69 mol% and that of HA-MA modification was ca. 29 mol%, respectively. HA-MA hydrogel was prepared by Michael addition chemistry between thiol and methacrylate groups using the specific crosslinkers of DTT and peptide linker (GCYKNRDCG) in the presence of EPO. The gelation time for peptide linker was about 30 min whereas that for DTT was about 180 min. The amount of EPO recovered from both hydrogels after degradation with HAse SD was ca. 90%. In vitro release test of EPO from HA-MA hydrogel at 37 °C showed that EPO was released rapidly for 2 days and then slowly up to 7 days from HA-MA hydrogels. The released EPO appeared to be intact from the analysis with RP-HPLC. According to in vivo release test of EPO from HA-MA hydrogels in SD rats, elevated plasma concentration of EPO was maintained up to 7 days. There was no adverse effect during and after the in vivo tests. Optimization study will be followed for longer sustained release of EPO.

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