Supplementary Material (ESI) for Soft Matter

Supplementary Information

Molecular design of hyaluronic acid hydrogel networks for long-term controlled delivery of human growth hormone

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Materials and Methods

Materials. Sodium hyaluronate, the sodium salt of hyaluronic acid (HA), with a molecular weight (MW) of 100 kDa was purchased from Shiseido Co. (Tokyo, Japan) and HA with a MW of 230 kDa was purchased from Lifecore Co. (Chaska, NM). Human growth hormone (hGH) was kindly provided by LG life Sciences Co. (Daejeon, Korea). Phosphate buffered saline tablet (PBS) was purchased from Invitrogen (Carlsbad, CA) and Bradford protein assay reagent from Thermo scientific (Rockford, IL). Dowex 50WX8-40 ion-exchange resin, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), 2-aminoethylmethacrylate hydrochloride (AEMA), N,N-diisopropyl ethylamine (DIPEA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), trifluoroacetic acid (TFA), and hyaluronidase from *Streptomyces hyalurolyticus* were obtained from Sigma-Aldrich (St. Louis, MO). Cystamine dihydrochloride and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 1-Hydroxybenzotriazole (HOBT) was purchased from Daejung Chemical (Siheung, Korea) and tetra-n-butylammonium hydroxide (TBA-OH) was obtained from Alfa Aesar (Ward Hill, MA). Dimethyl sulfoxide (DMSO) was obtained from Junsei Chemical Co. (Tokyo, Japan) and acetonitrile from J. T. Baker (Phillipsburg, NJ). The hGH ELISA kit was purchased from Roche Diagnostics (Basel, Swiss). All reagents were used without further purification.

Synthesis and characterization of HA-AEMA. Aminoethyl methacrylated HA (HA-AEMA) was synthesized and characterized as reported elsewhere [S1]. Ion exchange resin of Dowex 50WX-8-400 (25 g) was washed with 500 mL of water and filtered to remove the supernatant three times. Then, 1.5 molar excess of TBA-OH (48.9 mL) was added to the Dowex resin and mixed for 30 min. HA (2 g, MW = 230 kDa) was dissolved in water (5 mg/mL), which was poured into the Dowex-TBA resin. After mixing for 3 h, the supernatant was filtered through 0.45 μm filter and lyophilized for 3 days. The resulting HA-TBA was dissolved in DMSO. Then, BOP, 2-AEMA and N,N-DIPEA were added to the solution and mixed for a day. Finally, the reaction product was dialyzed against a large excess amount of water and lyophilized for three days. The obtained HA-AEMA was characterized by ^{1}H NMR (NMR, DPX300, Bruker, Germany).

Synthesis and characterization of thiolated HA. Thiolated HA (HA-SH) was synthesized and characterized as described elsewhere [S2]. HA (MW = 100 kDa) was dissolved in water (5 mg/mL) and conjugated with cystamine dihydrochloride at pH 4.8 for 6 h after activation of the carboxyl group of HA with EDC and HOBT. The resulting product was poured into the pre-washed dialysis membrane tube (MWCO of 7 kDa) and dialyzed against a large excess amount of water. Then, TCEP was added to the solution to reduce the disulfide bond of cystamine grafted HA at pH 7. After stirring for 3 h, the pH of the solution was dropped to 3.5 by the addition of 0.1 N HCl. Finally, HA-SH was precipitated in the excess ethanol, re-dissolved in water, and freeze-dried for 3 days. The degree of thiol modification was determined by ^{1}H NMR.

Synthesis and characterization of HA hydrogels. HA-AEMA and HA-SH were dissolved in PBS at a concentration of 4 wt%, respectively. HA hydrogel was formed by simple mixing of HA-AEMA and HA-SH solutions. The sol-gel transition was determined by a flow test utilizing the test tube inversion method. For the determination of crosslinking density, HA hydrogel fabricated in the 1 mL syringe was degraded with hyaluronidase (100 U/mL) and the remaining unreacted thiol group and methacryloyl group were analyzed by ^{1}H NMR. In addition, the gelation process and the mechanical property of HA hydrogels were characterized by the rheological analysis at 25°C with a TA ARES rheometer having a parallel-plate geometry (25 mm diameter). The gelation process was monitored by measuring storage (G') and loss (G'') moduli of the HA hydrogel precursor solution (2 wt%) at a constant frequency of 50 rad s^{-1} and a fixed strain amplitude (10 %) with increasing time. Then, HA hydrogel prepared with 4 wt% HA precursor solution was cured for 3 h, and the storage (G') and loss (G'') moduli were analyzed by frequency sweep rheology.

***In vitro* release test of hGH.** HA-AEMA and HA-SH were dissolved in PBS at a concentration of 4 wt%, respectively. HA hydrogels encapsulating hGH was prepared by simple mixing of HA-AEMA and HA-SH solutions (each 100 μL) with hGH solution (15 mg/mL, 50 μL) at a final hGH concentration of 3 mg/mL in a syringe. After incubation for 10 min, the HA hydrogel was put into 20 mL of fresh PBS in a vial. The amount of released hGH at predetermined time intervals was measured by Bradford protein assay and ELISA.

Characterization of *in vitro* released hGH. *In vitro* released hGH was characterized by reverse phased - high performance liquid chromatography (RP-HPLC) and circular dichroism (CD). RP-HPLC analysis was performed using the following systems: Waters 717 plus autosampler, Waters 1525 binary HPLC pump, Waters 2487 dual absorbance detector, Waters symmetry 300^{TM} C_4 column. As a mobile phase, 0.05 M Tris (pH 7.5)/n-propanol (71/29) was used at a flow rate of 0.5 ml/min. The detection wavelength was 280 nm. CD spectra for hGH in PBS (pH 7.4) were obtained with a UV spectrophotometer (JASCO J-715) at 25°C over the wavelength range of 200 ~ 250 nm under a nitrogen atmosphere. A quartz cuvette with a path length of 2 mm was used. Raw data were acquired at 0.2 mm intervals with a response time of 1 s. Each spectrum was subtracted by the spectrum of PBS and the residual ellipticity was calculated as an average of three scans.

***In vivo* release test of hGH in SD rats.** As a preliminary study, *in situ* HA hydrogel formation was confirmed by anatomization 10 min after mixing and subcutaneous (*sc*) injection of HA-AEMA and HA-SH solutions (each 200 μL) at a concentration of 4 wt% to female SD rats. After that, female SD rats at a mean body weight of 300 g were divided into three groups (five animals in each group). Group 1 was treated by *sc* injection of PBS as a control. Group 2 was treated by *sc* injection of the precursor solution of HA-AEMA (200 μL), HA-SH (200 μL), and hGH (15 mg/mL, 100 μL). Group 3 was treated by *sc* surgical implantation of hGH loaded HA bulk hydrogels (500 μL) prepared as described above. In both cases, the injected dose of hGH was 5 mg/kg-body weight.

References

S1. K. Motokawa, S. K. Hahn, T. Nakamura, H. Miyamoto and T. Shimoboji, *J. Biomed. Mater. Res.*, 2006, **78A**, 459.
S2. H. S. Lee, S. H. Choi and T. G. Park, *Macromolecules,* 2006**, 39**, 23.