**Electronic Supplementary Information**

# Hyaluronic acid decorated pH- and temperature-induced injectable bioconjugates for sustained delivery of bioactive factors and highly efficient wound regeneration

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## Materials and methods

### Materials

Dibutyltin dilaurate (DBTL, 95%), 1-(2-Hydroxyethyl)piperazine (HP, 98%), 4-hydroxybutyl acrylate (HBA, 90%), poly(ethylene glycol) chips (mPEG, $M_n$=2,050 g/mol), 1,6-diisocyanatohexane (HDI, ≥99%), succinic anhydride (≥99%), triethylamine, 4-(dimethylamino)pyridine (≥99%), *N,N'*-dicyclohexylcarbodiimide (99%), *N*-hydroxysuccinimide (98%), ethylenediamine (≥99.5%), methylthiazolyldiphenyl-tetrazolium bromide (MTT, ≥97.5%), and fluorescein diacetate, were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium hyaluronate was purchased from Lifecore Biomedical (Chaska, MN, USA). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium, trypsin-EDTA solution, penicillin-streptomycin solution (10,000 U/mL), and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Human embryonic 293T kidney cells were bought from Korean Cell Line Bank (Seoul, Korea).

### Synthesis of HA-PAEU bioconjugates

Temperature and pH-sensitive HA-PAEU bioconjugates were synthesized by subsequent fusion of both synthetic copolymer and polysaccharides to obtain the bioconjugates, as shown in Fig. 1A. For fusion synthesis, PAEU copolymer was synthesized through polycondensation polymerization. Subsequently, the chain-end of copolymer was terminated to amine using a two-step process to obtain PAEU-$NH_2$. Thereafter, PAEU-$NH_2$ was coupled to HA through amide bond formation.

#### *Synthesis of PAEU-$NH_2$*

The PAEU-$NH_2$ was prepared via a four-step process, as shown Fig. S1. PAEU copolymer was

synthesized using our previously reported synthesis procedure.[1] Carboxylated PAEU (PAEU-COOH) was synthesized by the ring-opening reaction between hydroxyl group of PAEU and succinic anhydride. In brief, PAEU (1 g, 0.22 mmol) was dissolved in chloroform. DMAP and succinic anhydride were then added and stirred for 12 h. The crude mixture was diluted with dichloromethane and precipitated using excess ether, filtered, and dried under vacuum.

PAEU-COOH (0.9 g, 0.19 mmol), DCC (0.12 g, 0.58 mmol) and DMAP (0.024 g, 0.58 mmol) were dissolved in 10 mL chloroform and stirred for 6 h. Thereafter, excess ethylenediamine in DMF was added and stirred further for 6 h. The reaction mixture was diluted with water and transferred to dialysis membrane tubes, dialyzed against excess deionized water, lyophilized, and freeze-dried to obtain PAEU-$NH_2$.

***Synthesis of HA-PAEU bioconjugates***

HA (1 g, 0.015 mmol) was dissolved in de-ionized water at 5 mg/mL concentration. Then, PAEU-$NH_2$ solutions prepared at pH 6.6 was added to HA solution. Acid-amine coupling reaction was initiated by the addition EDC and NHS followed by continuously stirring for two days. The reaction mixture was then dialyzed under acidic pH (pH 6.5) for 24 h and subsequently replaced with excess deionized for two days. The purified solution was lyophilized to obtain HA-PAEU bioconjugate.

**Characterization**

***$^1$H NMR.*** Molecular structures and compositions of synthesized copolymer and conjugate were determined using $^1$H NMR spectra (Varian Unity Inova 500NB, Varian Inc., USA).

***FT-IR.*** FT-IR spectrum of copolymers and conjugates was recorded using an attenuated total reflection (ATR) IFS 66/S FT-IR spectrometer.

***GPC.*** Polydispersity index ($Đ$) and molecular weight ($M_n$) of synthesized PAEU copolymers and commercial PEG were measured using an Agilent 1100 system with THF as an eluent at a flow rate of 1.0 mL/min.

***SEM:*** Microstructures of hydrogels were using a Scanning Electron Miscroscope (JSM-7600F, JEOL, Tokyo, Japan) at an accelerating voltage of 20 kV. For analysis, hydrogels were coated with platinum via sputter coating.

***TEM.*** Morphology of HA-PAEU bioconjugates has been observed using a Transmission Electron Microscope (TEM, JEM-2100F, JEOL, USA). At different concentrations (1 mg/mL and 2mg/mL), 20 µL of HA-PAEU conjugate solutions were dropped on to a copper grid. Thereafter, the solution loaded copper grid was stained using uranyl acetate solution (10 µL, 2% w/w) and dried before visualization.

***Zeta potential.*** The zeta potential of copolymers, proteins and copolymer-protein complexes, was investigated using the Zeta-potential analyzer (Malvern Instruments Inc., Massachusetts, USA). For zeta potential measurement, the copolymers and proteins were stabilized at room temperature for 20 min, and then the pH was adjusted to pH 6.0. To prepare copolymer-protein complex, the hGH or lysozyme was mixed to the copolymer solutions at pH 6.0 and then the pH was adjusted to 7.4. Thereafter, the protein solutions or copolymer-protein complexes were incubated for 20 min at room temperature, and then measured the zeta potential.

**Sol-gel phase transition**

Phase transition patterns of copolymer and conjugate solutions were examined using the vial tilting test *in vitro*. Briefly, 20 wt.% copolymers were dispersed in PBS. Thereafter, the pH of the solution was adjusted to desired value using 5N NaOH and 5N HCl and kept at 0 °C for 6 h. Vials containing copolymer sols were transferred to thermo-sensitive water bath and the temperature was gradually raised from 10 °C to 70 °C. At specific points, vials were examined and gelation status was determined by tilting vials.

***In vitro* cytotoxicity**

Cytotoxicities of copolymers, conjugates, and hydrogel extracts collected at different time points were examined using MTT assay. Human embryonic kidney 293T cells were seeded into a 96-well plate ($1 \times 10^4$ cells per well) and incubated with DMEM containing 10% FBS and 1% penicillin-streptomycin at 37 ℃ in a humidified incubator containing 5% $CO_2$ for 24 h. These cells were then incubated with cell culture medium containing different concentrations of copolymers, conjugates, or hydrogel extracts collected at different points for 48 h. Thereafter, the medium was removed and cells were washed with PBS, and then 100 μL of fresh medium containing MTT (5 μL, 5 mg/mL) was added and incubated further for 3 h. The media were removed and insoluble purple formazan crystals were dissolved using DMSO. To examine cell viability, absorbance at 490 nm was measured using a microplate reader. Cell viability measured for wells containing only free medium served as control.

***In vivo* gelation and biodegradation**

All live animal experiments were performed in compliance with relevant laws and the institutional guidelines of Sungkyunkwan University. Institutional committees of Sungkyunkwan University approved all animal experiments.

Sprague-Dawley (SD) rats (6 weeks old, Hanlim Experimental Animal Laboratory, Seoul, Korea) were used to examine gelation and biodegradability of copolymers and bioconjugates. Solutions of copolymers and conjugates were prepared at ~pH 6.8 at 0 °C. Then, 300 μL of each sample solutions was transferred to a 26G needle and subcutaneously injected to the dorsal region of each rats. After injection, rats were euthanized at pre-determined time points and the shapes of gels were photographed. Remaining hydrogel weight at different time points was collected, freeze-dried and weighed. The extent of degradation was calculated using mass loss method.

***In vivo* 3D cell culture**

For *in vivo* 3D cell culture examination, 100 μL of cultured 293T cells ($5\times10^6$ cells/mL) were mixed with 20 wt.% of copolymer or conjugate sol (1 mL). These cells were then mixed with polymer sols by gentle vortex. Thereafter, 300 μL of 293T cell mixed copolymers was administrated subcutaneously into the back of SD rat using 26G hypodermic needles. After 48 h, gels were harvested and washed thoroughly using PBS and FDA stained. Thereafter, hydrogels were cross-sectioned and fluorescence images were obtained under a laser scanning confocal fluorescence microscope (Zeiss LSM 510, Carl Zeiss MicroImaging GmbH, and Germany). For fluorescence measurement, stained hydrogel sections were excited at 490 nm and images were taken at a depth of 200 μm to verify the 3D distribution and viability of 293T cells.

**hGH and lysozyme release from hydrogels *in vivo***

To evaluate the capability of copolymer hydrogels in controlling the therapeutic protein release, hGH and lysozyme were chosen as anionic and cationic proteins, respectively. Rats were randomly divided into six groups ($n$ = 3 rats per group) for checking the *in vivo* release. The groups are (i) 300 μL of hGH-loaded conjugate solution (20 wt% HA-PAEU conjugate, 5 mg/mL hGH), (ii) 300 μL of hGH-loaded copolymer solution (20 wt% PAEU copolymer, 5 mg/mL hGH), (iii) 300 μL of hGH solution (5 mg/mL), (iv) 300 μL of lysozyme-loaded conjugate solution (20 wt% HA-PAEU conjugate, 5 mg/mL hGH), (v) 300 μL of lysozyme-loaded copolymer solution (20 wt% PAEU copolymer, 5 mg/mL hGH), and (vi) 300 μL of lysozyme solution (5 mg/mL). Therapeutic protein-loaded or free proteins were administered subcutaneously into the back of SD rats and blood samples were collected from tail veins. The concentration of hGH and lysozyme in the serum was analyzed using an immunoenzymatic assay kit (hGH-EASIA, DIAsource ImmunoAssays, Belgium) and Enzyme-Linked Immunosorbent Assay (ELISA kit, Cusabio, China), respectively.

**Wound healing**

To develop cutaneous wounds rats were anesthetized first and the hair was shaved for surgical excision. Linear open wounds (1 cm) were created using surgical knife and the rats were randomly divided into two groups ($n$ = 3 rats per group). Thereafter, hGH-loaded PAEU and HA-PAEU hydrogel were injected and the extent wound contraction was rate was photographed at different time intervals (0, 1, 3, 5 and 8 days).

The dynamic healing process was examined using histological analysis. After the treatment, skin tissues were recovered and fixed using 10% neutral buffered formalin. The fixed

tissues were embedded in paraffin and cut into 4 μm thickness slices. Thereafter, tissue sections were stained using hematoxylin & eosin (H&E). Masson's trichrome (MT) staining was also done to examine collagen deposition. Finally, stained sections were visualized under a microscope.

1. M. S. Gil, J. Cho, T. Thambi, V. H. Giang Phan, I. Kwon and D. S. Lee, *Journal of Controlled Release*, 2017, **267**, 119-132.

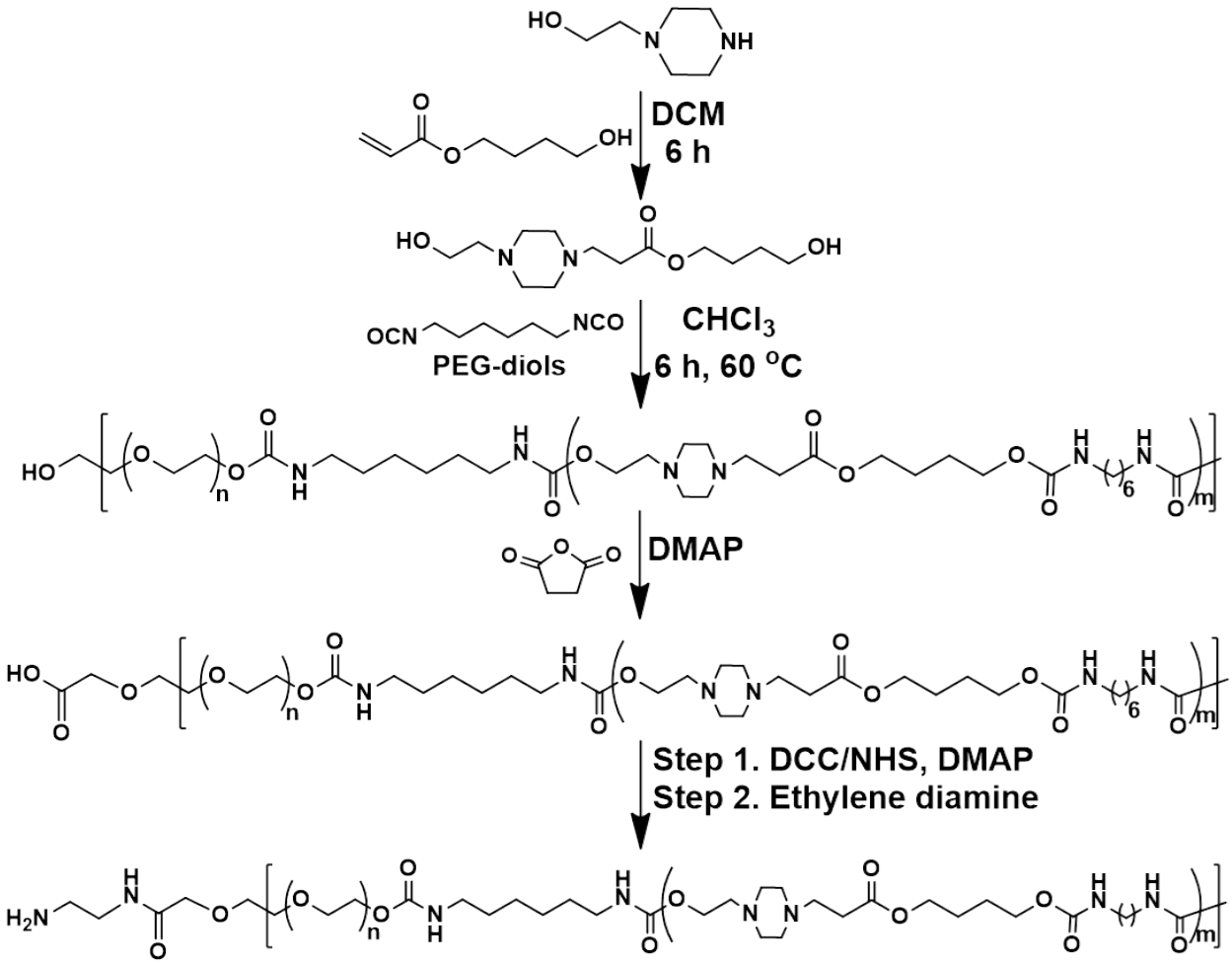


**Fig. S1.** Synthesis of PAEU-$NH_2$.

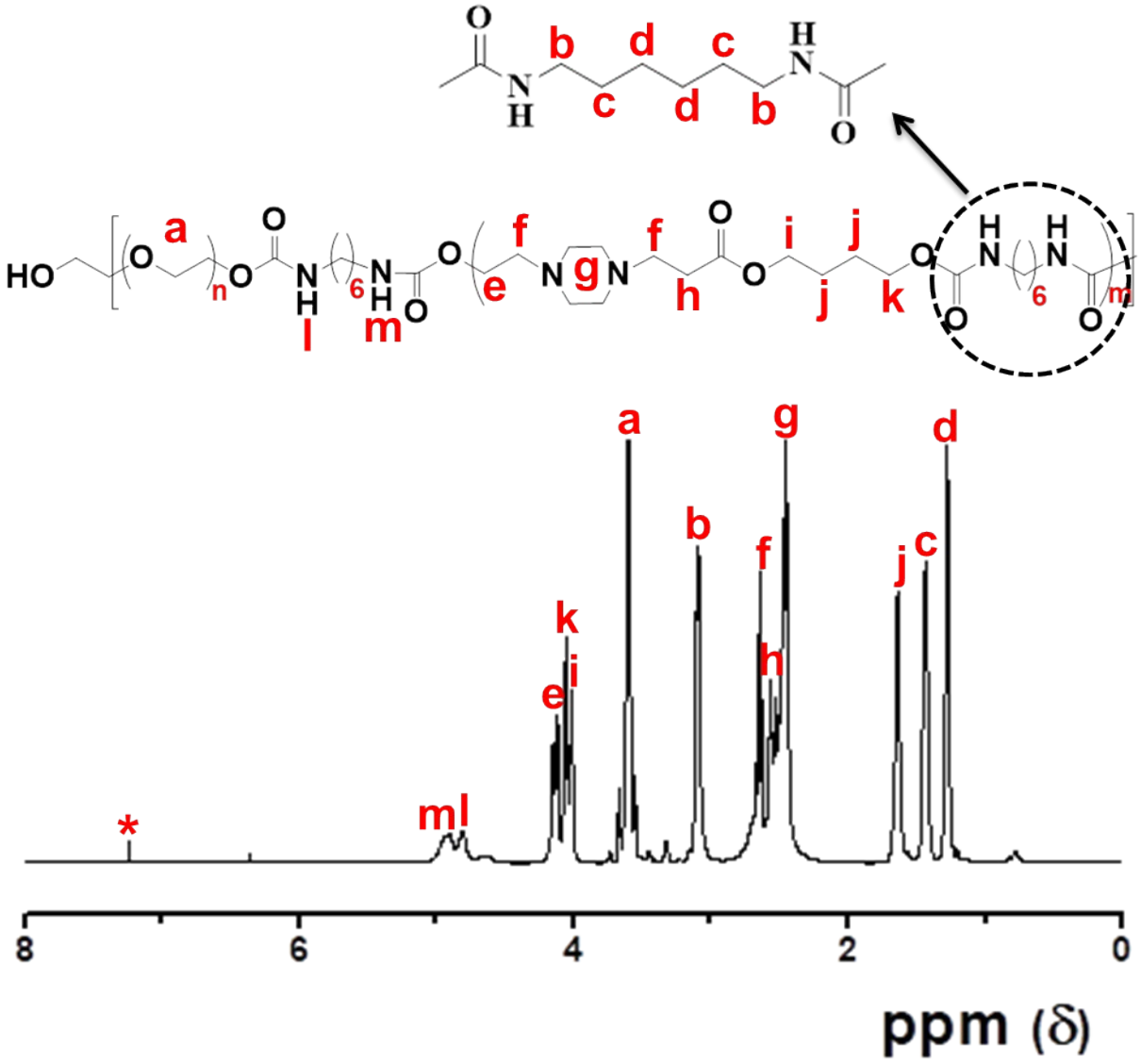


**Fig. S2.** $^{1}$H NMR spectra of PAEU-$NH_2$.

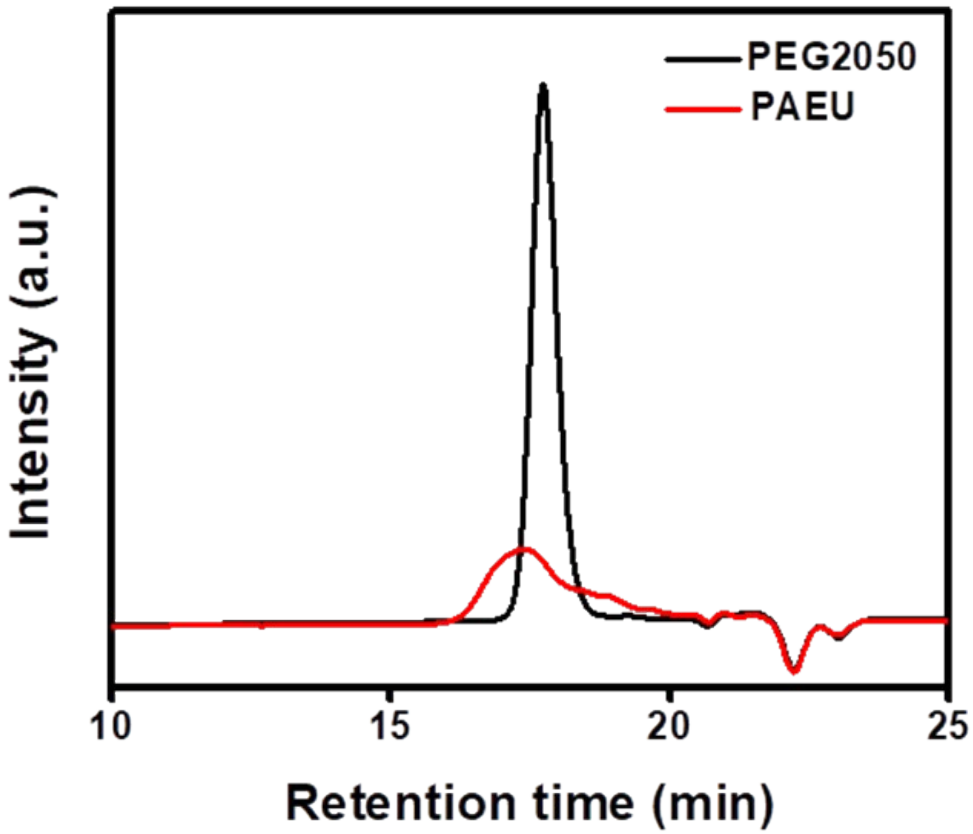


**Fig. S3.** GPC trace of PAEU copolymers.

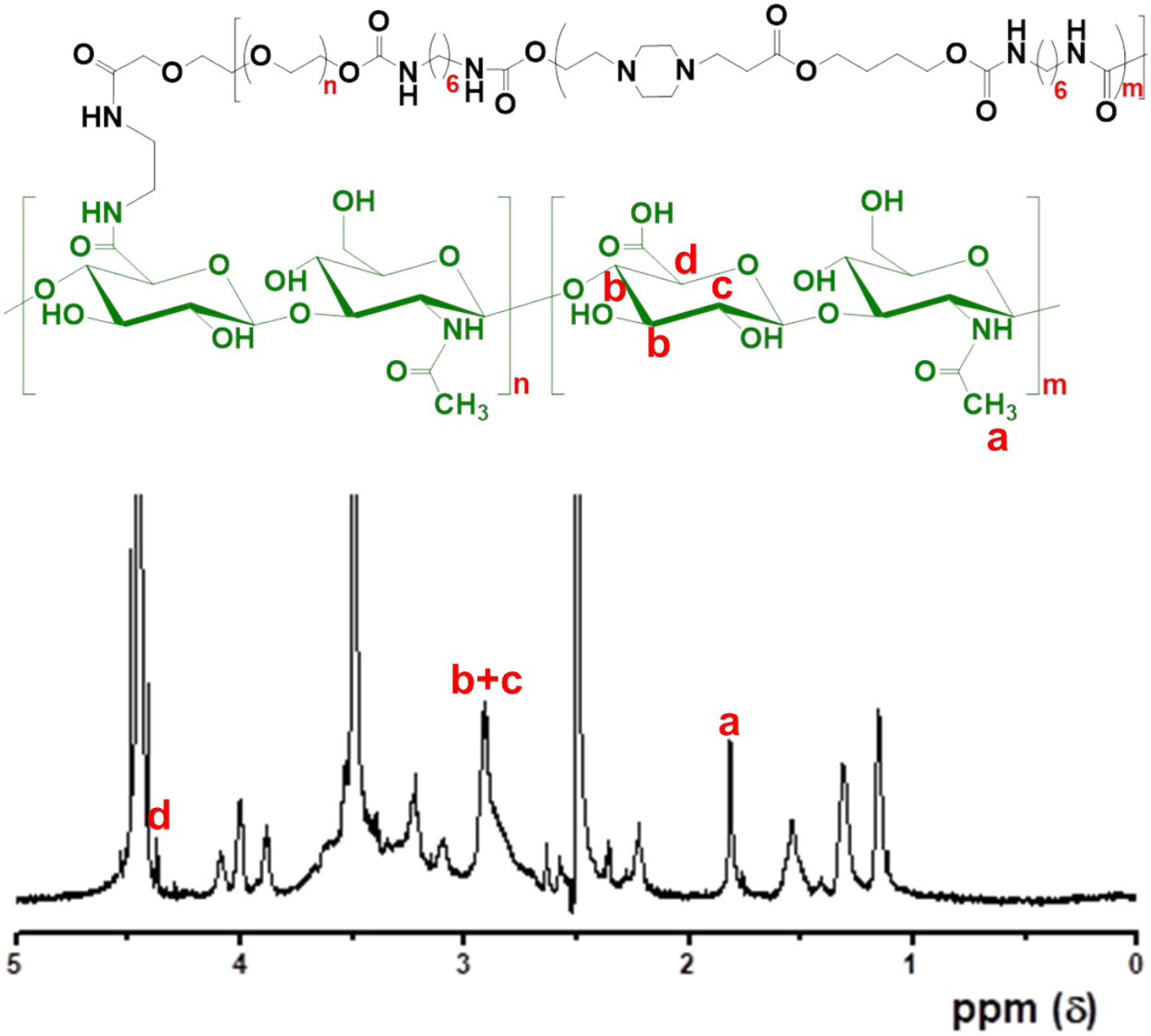


**Fig. S4.** $^{1}$H NMR spectra of HA-PAEU conjugates.

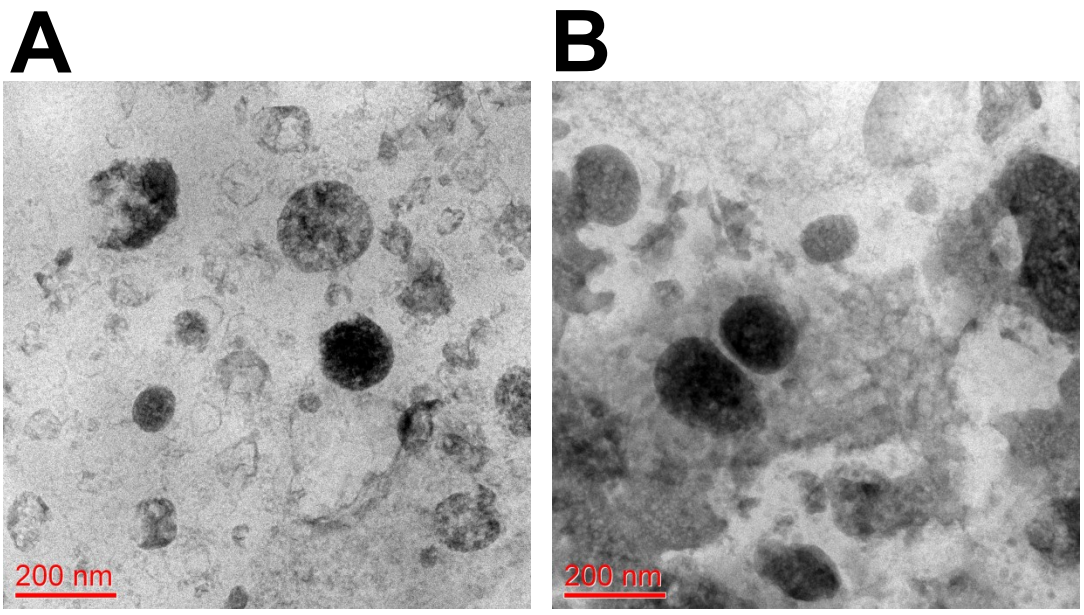


**Fig. S5.** TEM image of HA-PAEU conjugates at (A) 1 mg/mL and (B) 2 mg/mL concentrations. With increasing concentration of HA-PAEU conjugates induced the aggregation that eventually facilitates the gelation.

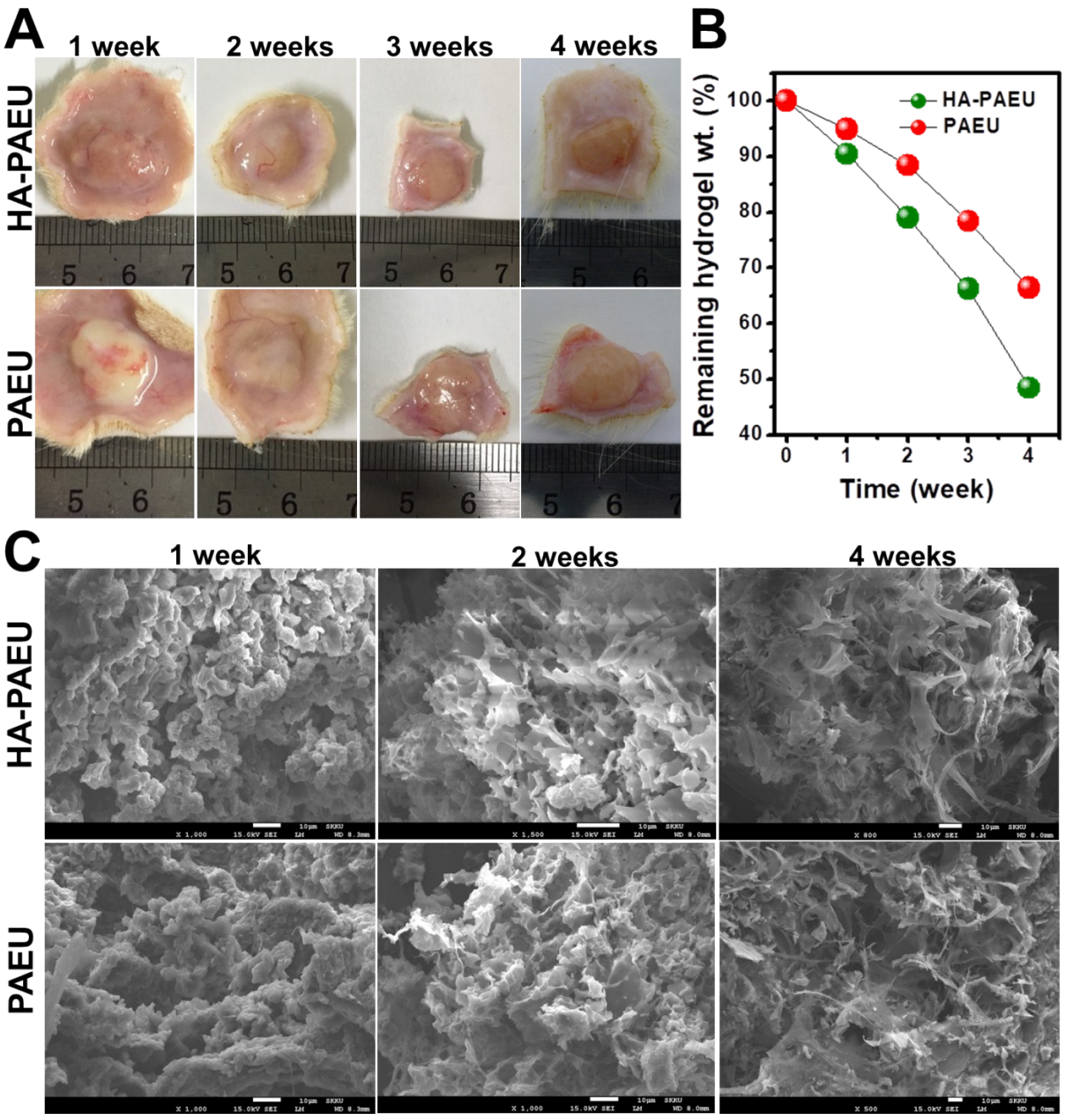


**Fig. S6.** (A) Biodegradation of hydrogels *in vivo*. (B) Hydrogel weight changes as a function of time. (C) Porosity changes of hydrogels during the degradation.