Electronic Supplementary Information

Supramolecular Hydrogels Encapsulating Bioengineered Mesenchymal Stem Cells for Ischemic Therapy

Byung Woo Hwang,[a] Young-Eun Kim,[b] Mungu Kim,[c] Seulgi Han,[a] Seoyeon Bok,[b] Kyeng Min Park,[d] Annadka Shrinidhi,[d] Ki Su Kim,[c,e] G-One Ahn,[b] and Sei Kwang Hahn,[a,c,*]

[a] Department of Materials Science and Engineering, [b] School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-ro, Pohang 37673, Korea.

[c] PHI BIOMED Co., 175 Yeoksam-ro, Gangnam-gu, Seoul 06247, Korea.

[d] Center for Self-assembly and Complexity (CSC), Institute for Basic Science (IBS), Pohang 37673, Republic of Korea.

[e] Department of Organic Materials Science and Engineering, College of Engineering, Pusan National University, 2 Busandaehak-ro 63 beon-gil, Gumjeong-gu, Busan, 46241, Korea.

*** CORRESPONDING AUTHOR FOOTNOTE**

Tel.: +82 54 279 2159; Fax: +82 54 279 2399; E-mail : skhanb@postech.ac.kr (S. K. Hahn)

EXPERIMENTAL SECTION

Materials. Sodium hyaluronate, sodium salt of hyaluronic acid (HA), with a molecular weight (MW) of 100 kDa and HA with a MW of 210 kDa was purchased from Lifecore (Chaska, MN). Phosphate buffered saline (PBS) tablet, N -hydroxysulfosuccinimide (sulfo-NHS), cysteamine hydrochloride, hydroxybenzotriazole (HOBt), succinic anhydride, tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 1,6-diaminohexane (DAH), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), carbazole, hyaluronidase from *Streptomyces hyalurolyticus* were purchased from Sigma (St. Louis, MO). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Snake-skin-pleated dialysis tube was obtained from Thermo Scientific (Rockford, IL). Mouse embryonic fibroblast (NIH 3T3) cell lines were obtained from American Type Culture Collection (ATCC). Human-derived umbilical cord MSCs were supplied from Genexine Co. (Seoul, Korea) after genetic modification to express angiogenic factors such as HGF and VEGF. Low-glucose Dulbecco's modified Eagle's medium (LDMEM), fetal bovine serum (FBS). Hank's balanced salts solution (HBSS) was obtained from WelGENE (Daegu, Korea). 6-week-old Balb/c female mice were purchased from Coatech (Pyeongteak, Korea). All animal procedures conformed to the guidelines of POSTECH.

Synthesis of mono-amino CB[6]. *Mono*-allyloxy CB[6] was synthesized as previously reported elsewhere.[1] One hundred mg of allyloxy CB[6] was dissolved in 5 mL of DI water. Then, 50 mg of TCEP and 50 mg of cysteamine hydrochloride was dissolved in 5 mL of DI water, and reacted for 15 min. After mixing them together, we purged the mixed solution with Ar gas for 15 min. The solution was reacted at 80°C in oil bath for 3 h, adding 72 mg of $K_2S_2O_8$. The fully reacted solution was washed with methanol twice. Then, the precipitated product was dried for a day, and analysed by ^{1}H NMR and MALDI-TOF MS (Fig. S1).

Synthesis of amino CB[6]-HA (mCB[6]-HA) and preparation of mCB[6]/DAH-HA hydrogel. Fifty mg of HA (100 kDa) was dissolved in 10 mL of DI water. Then, we added 100 mg of EDC and 75 mg of HOBt, then 70 mg of mCB[6] sequentially. The solution was reacted at pH 5.0 overnight. The product was dialyzed for 3 days and then lyophilized. We synthesized and characterized a counterpart of DAH-HA with 45% substitution rate as previously reported elsewhere.[2] Then, we mixed a solution of mCB[6]-HA (50 μL, 3.0 wt/v%) in PBS with a solution of DAH-HA (50μL, 2.0 wt/v%), vortexing them to form mCB[6]/DAH-HA hydrogels.

***In vitro* cell viability assay of the mCB[6]-HA and the DAH-HA solutions.** DAH-HA and mCB[6]-HA were dissolved at a concentration of 6 wt/v% in cell culture media (DMEM, 10% FBS, 1× antibiotics). NIH 3T3 cells were seeded into a 96-well cell culture plate at an initial density of 10^3 cells per well (n = 5) and further incubated in the hydrogel precursor solutions at different concentrations (6, 3, 1.5, 0.75 wt/v%) in culture media at 37°C for 3 days. The proliferation of NIH 3T3 cells was assessed by MTT assay in 3 days.

***In vitro* degradation test with and without hyaluronidase.** Two different mCB[6]-HA derivatives (7.1% and 21.5% of carboxylate on HA substituted by mCB[6]) were prepared as hydrogel precursor solutions (50 μL, 3.0 wt/v% in PBS). After mixing mCB[6]-HA with DAH-HA (50 μL, 2.0 wt/v% in PBS), two types of hydrogels (7.1% mCB[6]/DAH-HA and 21.5% mCB[6]/DAH-HA) were prepared in a cylinder shape. Then, they were placed in 1.9 mL hyaluronidase solution (100 units/mL in PBS). The hydrogel degradation proceeded at 37 °C for 13 days was measured every 3 days (n = 6). The concentrations of the degraded HA were determined by carbazole assay. As control experiments, the same experiments were performed in PBS except using hyaluronidase.

Effect of the supramolecular hydrogel on in vitro transgene expression of VEGF-A and HGF. We assessed the effect of supramolecular hydrogel on transgene expression of eMSC that VEGF-A or HGF is genetically modified as previously reported[1]. eMSCs were cultivated in a 24-well cell culture plate for control group. For formation of the eMSCs containing hydrogel, mCB[6]-HA was dissolved in PBS (50 μL, 3 wt/v%), and HGF expressing MSCs (eMSCs/HGF) and/or VEGF-A expressing (eMSCs/VEGF-A) (total number of cells: 1×10^5) were added. DAH-HA precursor solution was prepared to dissolve in PBS (50 μL, 2 wt/v%), and mixed together to give hydrogel formation. eMSCs/VEGF-A and eMSCs/HGF were cultivated on polyester (PET) membrane transwell inserts (mesh size = 8 μm, Corning) in a 24-well cell culture plate. Cell culture supernatants were collected on days 1, 2, 4, 7, and 15 days after the culture medium was replaced one day prior to collection. The amount of VEGF-A and HGF produced by eMSC were determined by human VEGF ELISA kit (Sunbio, Korea) and human HGF ELISA kit (R&D Systems, Minnesota, MN, USA), according to the manufacturer's instructions.

Mouse ischemic hind limb model and treatments. To give rise to hind-limb ischemia, 6-8 weeks old Balb/c mice were anesthetized by isoflurane on a 37°C temperature pad. The area of incision site was shaved before incision. The femoral artery was ligated at the proximal point and its branch, denoted by black arrows in Figure 1b, then we closed the overlying skin. One day after arterial ligation, mice were randomly distributed to four experimental groups, then injected 100μL of the materials as follows; media only (G1), eMSC/HGF within media (G2), within Matrixen (G3), and eMSC/HGF within CB[6]/DAH-HA (G4) for first experiment, and untransfected MSC (G1), eMSC/VEGF-A (G2), eMSC/HGF (G3), and eMSC/HGF+VEGF-A (G4) within mCB[6]/DAH-HA for second experiment.

Laser Doppler imaging analysis. We used a laser Doppler perfusion imager (LDI; Perimed, North Royalton, Ohio) for *in vivo* physiological evaluation of perfusion enhancement. We monitored the blood perfusion of mice by scanning of surface of hind limbs on predetermined days; day 0, 2, 10 and 24, for first experiment and 0, 3, 7, 14, and 28 for second experiment. We analysed the digital color-coded images to evaluate the blood flow in the ischemic region, and we calculated mean values of perfusion.

Statistical Analysis. We expressed the quantitative data as mean ±SD. Statistical analysis was calculated with the t-test using Microsoft Excel.

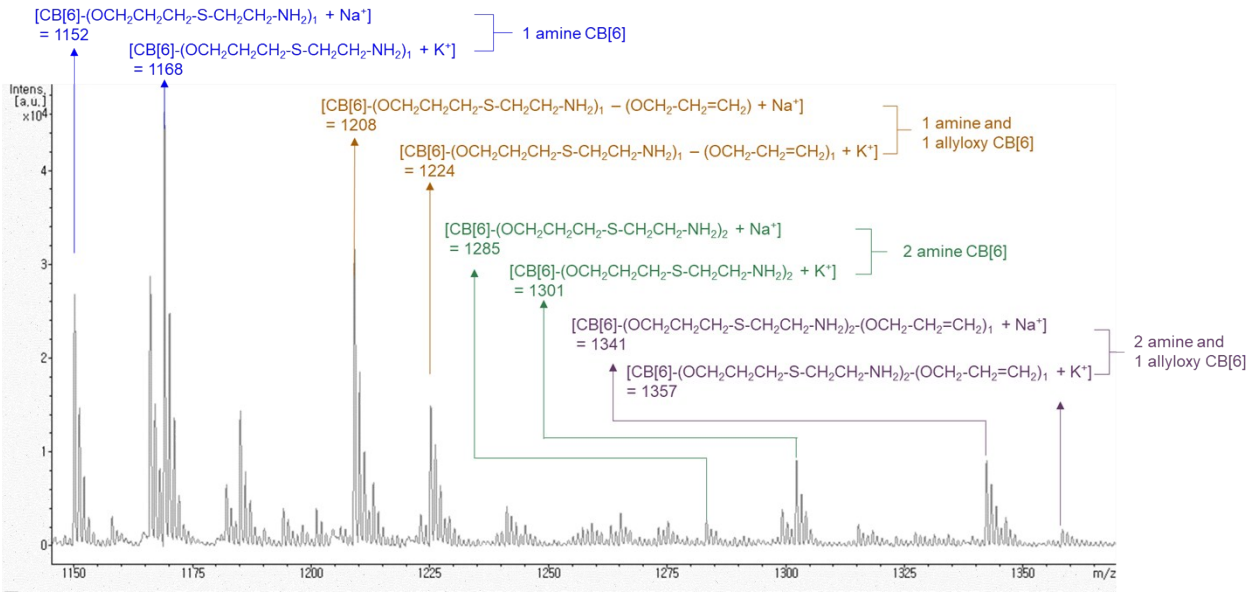

Fig. S1. MALDI-TOF-MS spectrum of amino CB[6] and its complexes with Na^+ and K^+.

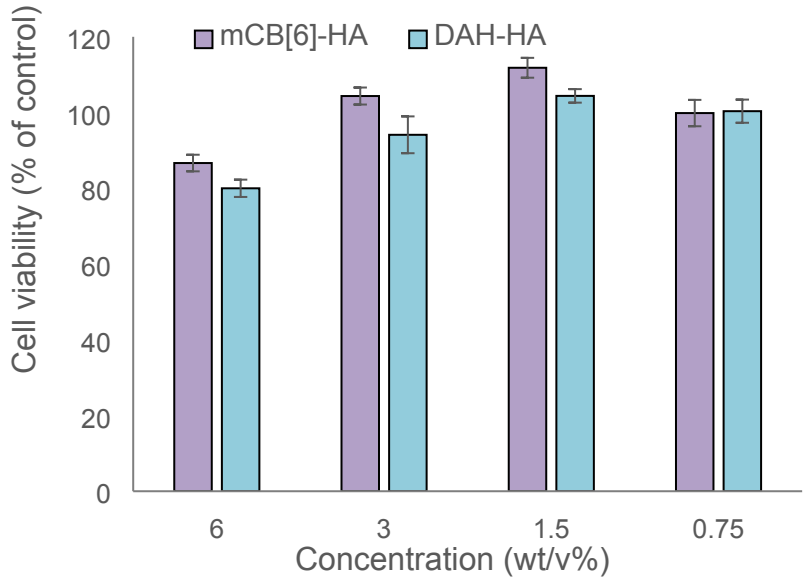

Fig. S2. NIH 3T3 Cell viability after incubation with the hydrogel precursor solutions (mCB[6]-HA, DAH-HA), assessed by MTT assay (mean ± SD, $n = 5$).

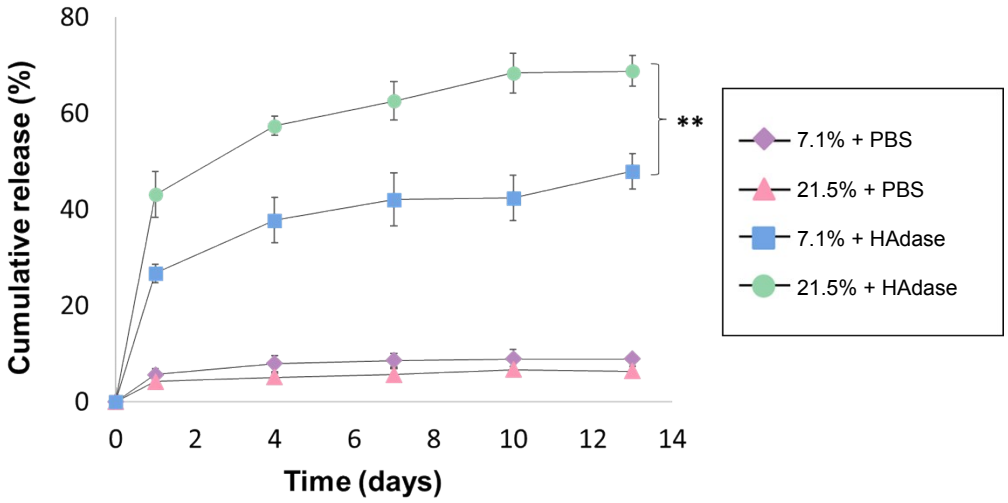

Fig. S3. *In vitro* degradation test for 7.1% mCB[6]/DAH-HA hydrogels and 21.5% mCB[6]/DAH-HA hydrogels in PBS and the hyaluronidase solution (HAdase) for 13 days (mean ± SD, n = 5, **P = 0.00001).

REFERENCES

1. J. Yeom, S. J. Kim, H. Jung, H. Namkoong, J. Yang, B. W. Hwang, K. Oh, K. Kim, Y. C. Sung and S. K. Hahn, *Adv. Healthc. Mater.*, 2015, **4**, 237–244.
2. S. Karmakar, S. Roy Choudhury, N. L. Banik and S. K. Ray, *J. Cancer Sci. Ther.*, 2010, **2**, 107–113.