Supporting Information

Robust Biopolymeric Supramolecular "Host−Guest Macromer" Hydrogels Reinforced by in Situ Formed Multivalent Nanoclusters for Cartilage Regeneration

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Experimental section

Preparation of HGM hydrogels and MeHA hydrogels

Functionalized hyaluronic acids (AD_xHA or MeHA) were firstly dissolved in PBS (pH=7.4) and then mixed with the photoinitiator I2959 (final concentration: 0.05 wt%). For AD_xHA, the host monomer mono-Ac-βCD (the molar ratio between βCD and AD was always kept as 1:1) was then added into the solution before it was loaded in the homemade molds. After 8 minutes of UV irradiation (7 mW/cm^2), disk like hydrogel objects were obtained.

In vitro chondrogenesis of hMSCs

hMSCs (purchased from Lonza) were photoencapsulated (10 million cells/mL) in hydrogels (each hydrogel was fabricated from 50 μL precursor solution, loaded with 100 ng TGF-β1), which were cultured in chondrogenic media (DMEM, 1% (vol/vol) ITS+ Premix, 50 μg/mL L-proline, 0.1 μM dexamethasone, 0.9 mM sodium pyruvate, 50 μg/mL ascorbate, and antibiotics) and changed three times per week.

BSA release study

BSA was mixed with the pre-gelation solution of HGM or MeHA hydrogels for hydrogel preparation as described above (50 μg BSA for each hydrogel). The as-prepared hydrogels were then immersed in 1 mL PBS. 50 μL PBS from each sample was collected for quantification of the released BSA proteins from the hydrogels. A BCA protein quantification kit was used to quantify the BSA concentration in the medium.

Construct analysis

For gene expression analysis, samples were homogenized in TRIzol reagent and RNA

was extracted. After the extracted RNA was reverse-transcribed into cDNA, PCR was performed (primers listed in Table S1). The relative gene expression was calculated using the ΔΔCT method, and relative expression was calculated using the expression $2^{\Delta\Delta Ct}$, normalized to GAPDH. For quantification of GAG content, samples were digested in proteinase-K and assessed for GAG content with dimethylmethylene blue. All data are presented as mean ± SD. Statistica (Statsoft) was used to perform statistical analysis using two-way ANOVA with Tukey's honestly significant difference post hoc test of the means used to make comparisons between groups (n = 4 samples per group), with experimental group as independent factors.

Animal study

Hydrogel constructs were made as aforementioned and then sliced to suitable size before implanted in osteochondral defects (d=1mm) in the knees of Sprague-Dawley rats (male, age 8 weeks). All animal procedures were approved and guided by the Institutional Animal Care and Use Committee at the Chinese University of Hong Kong.

Synthesis and characterization

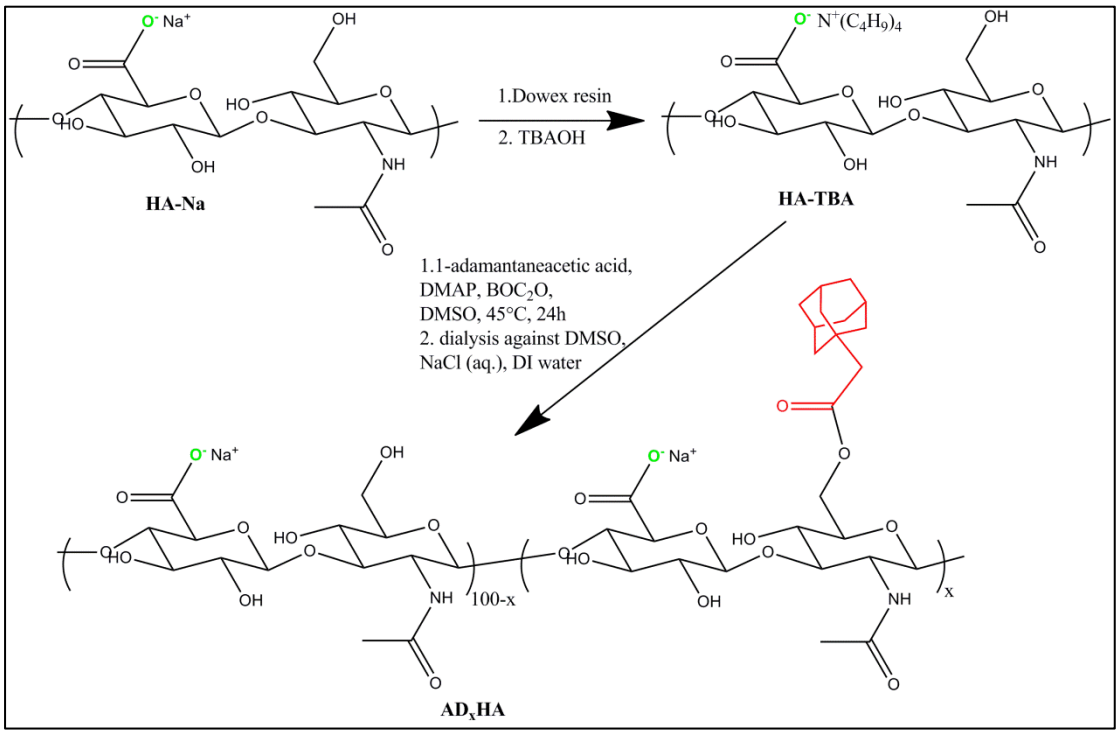

Figure S1. Synthesis of guest polymer AD_xHA.

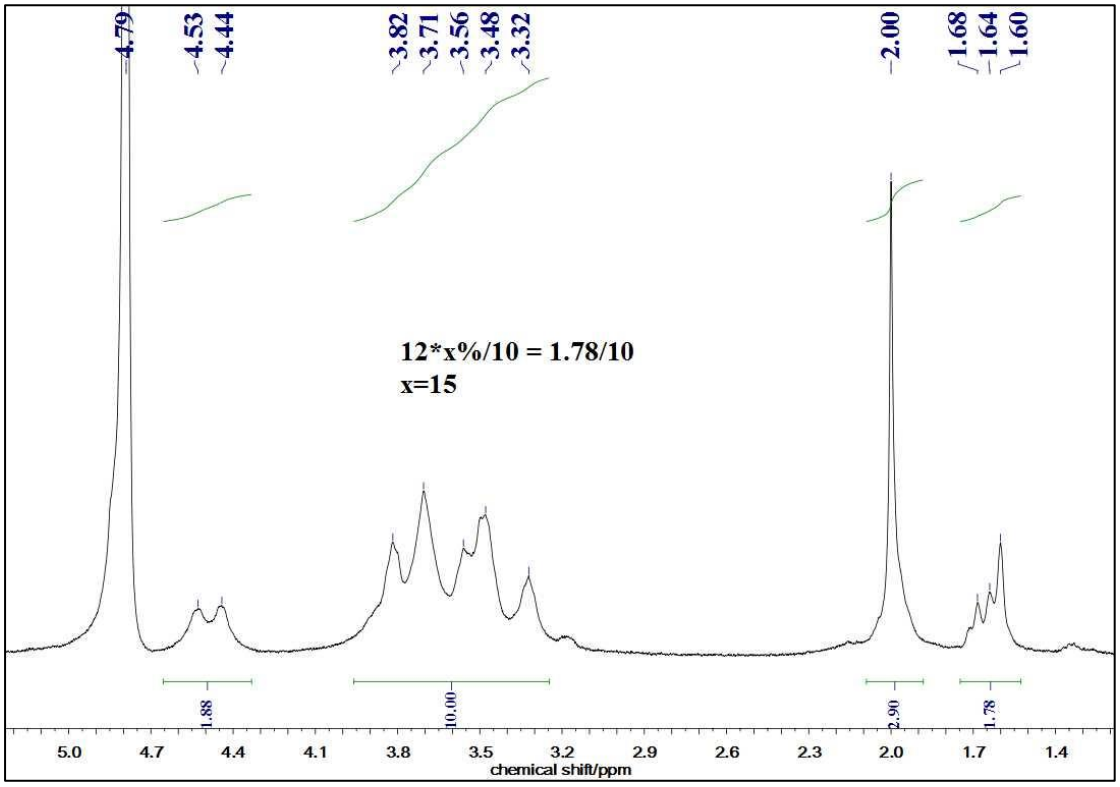

Figure S2. ^{1}H NMR (D_2O) of Ad_xHA, x=15.

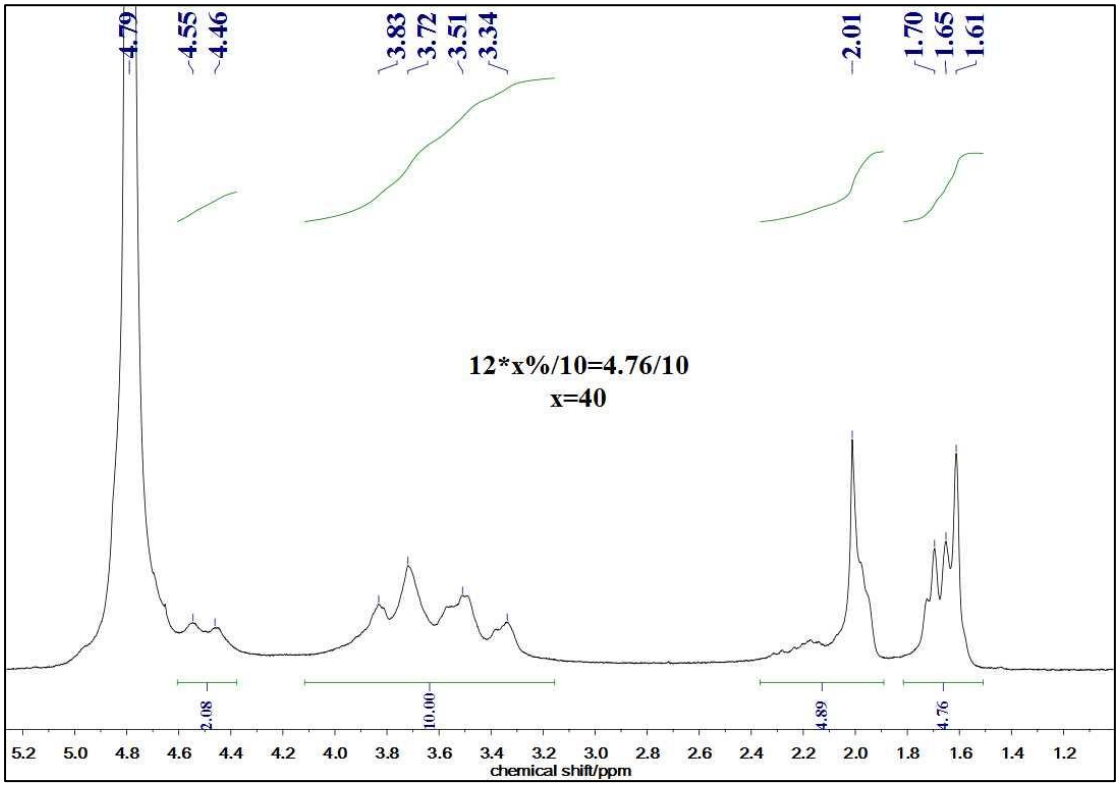

Figure S3. ^{1}H NMR (D_2O) of Ad_xHA, x=40.

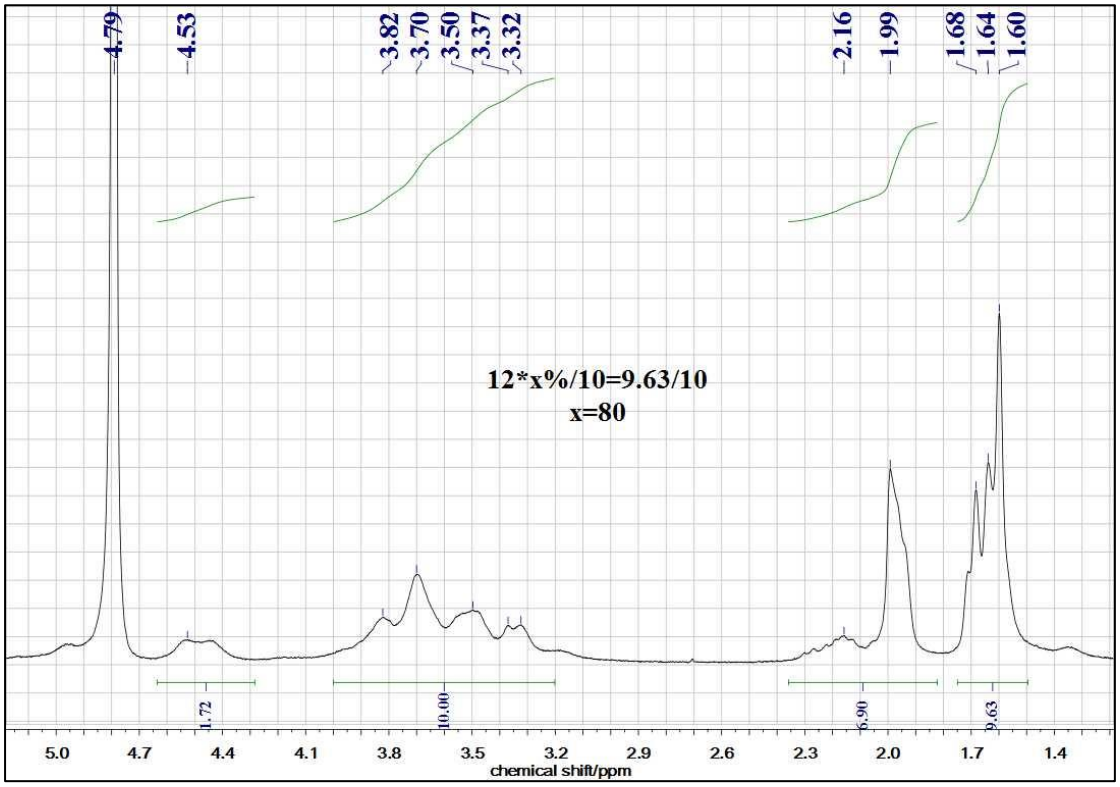

Figure S4. ^{1}H NMR (D_2O) of Ad_xHA, x=80.

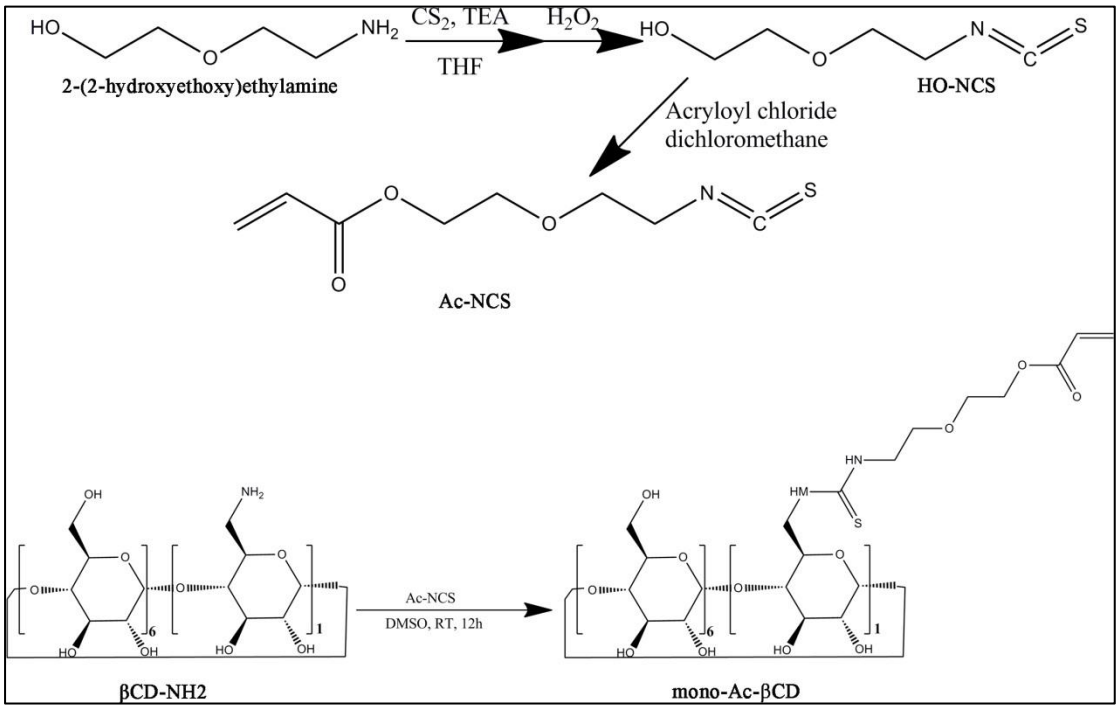

Figure S5. Synthesis of mono-Ac-βCD.

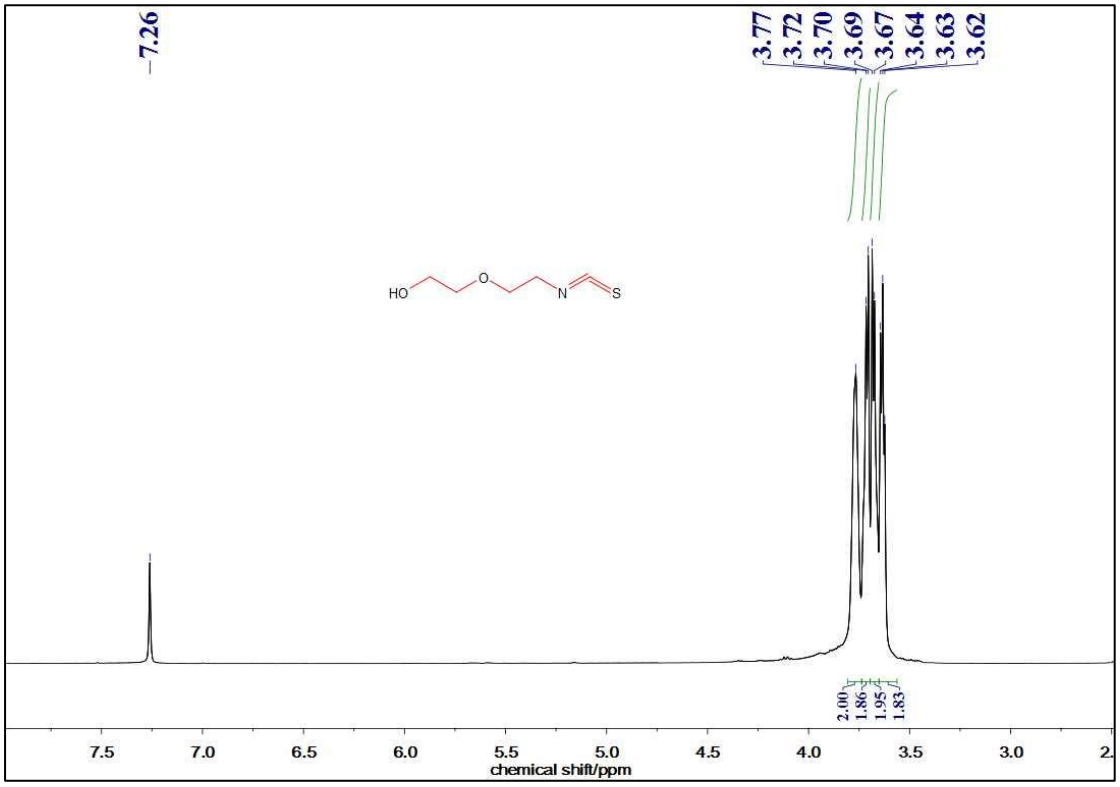

Figure S6. ^{1}H NMR ($CDCl_3$) of HO-NCS.

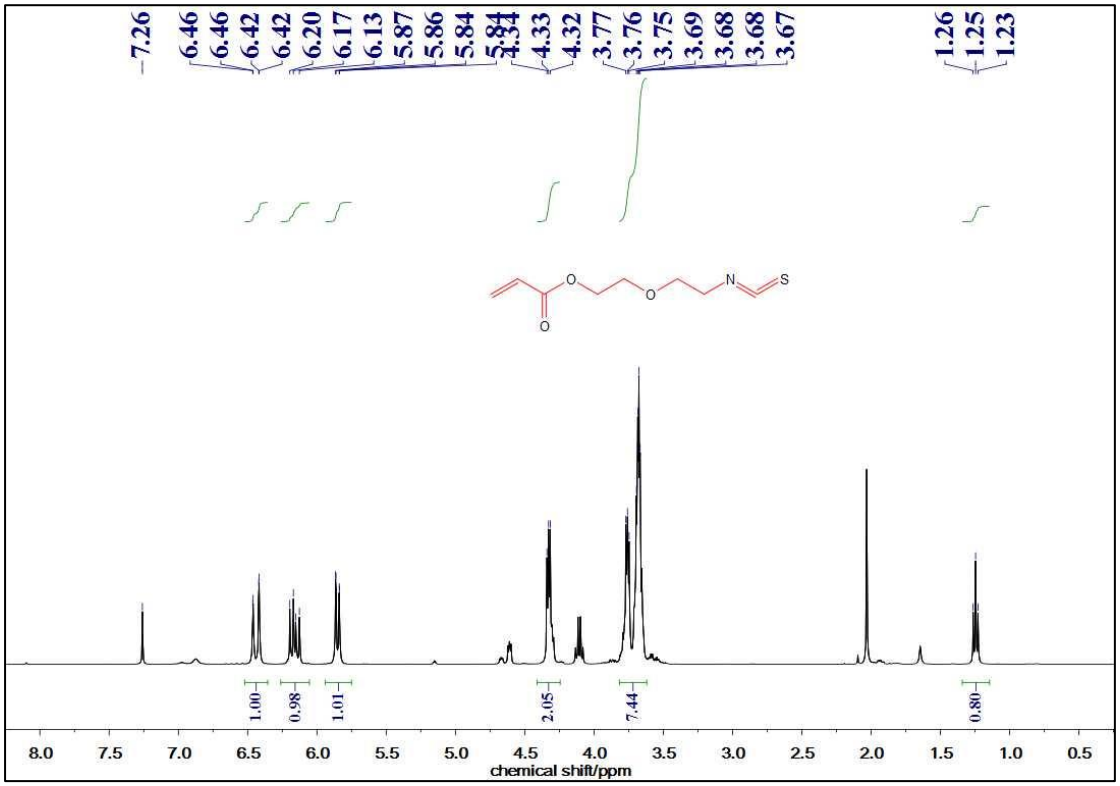

Figure S7. ^{1}H NMR ($CDCl_3$) of Ac-NCS.

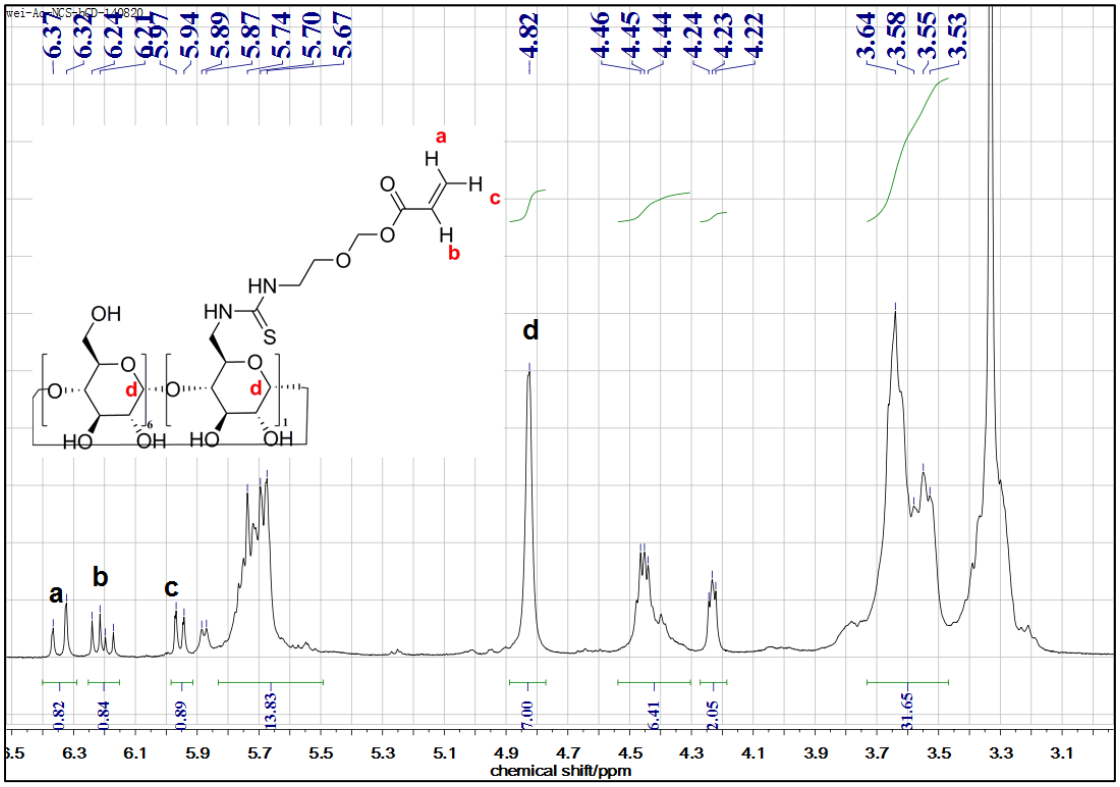

Figure S8. ^{1}H NMR (DMSO-*d6*) of mono-Ac-*β*CD.

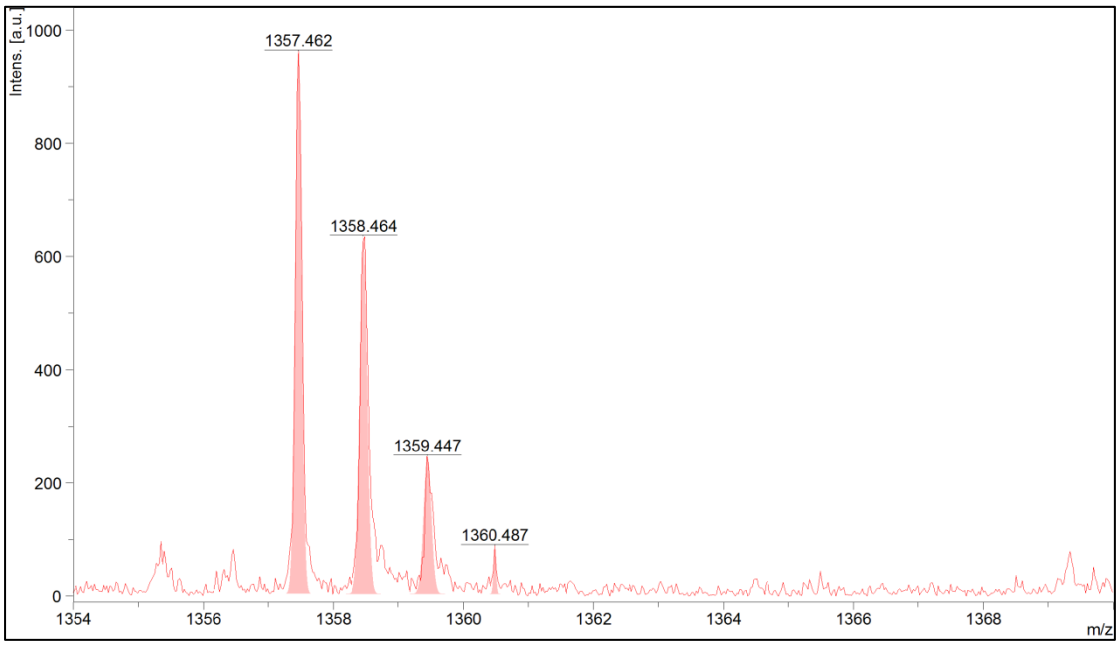

Figure S9. MALDI-TOF MS of mono-Ac-*β*CD.

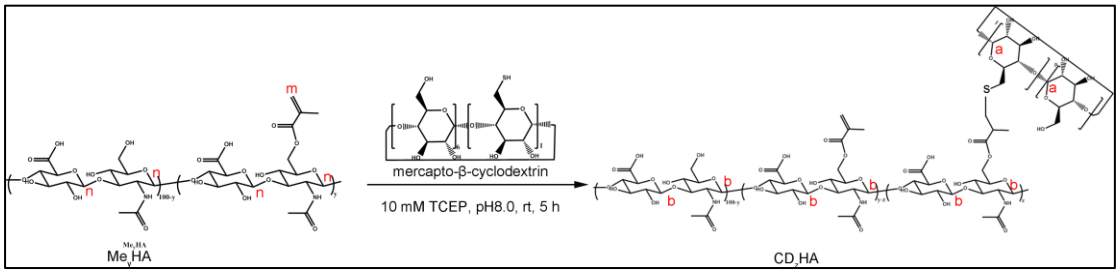

Figure S10. Synthesis of host polymer $CD_{15}HA$.

Methacrylate hyaluronic acid ($Me_{37}HA$) and host polymer $CD_{15}HA$

Methacrylated HA was synthesized according to the previously reported procedure.[1] Briefly, a 20-fold excess of methacrylic anhydride was added to 1% (wt/vol) HA solution in deionized water, pH of the solution was kept around 8, adjusting by 5M NaOH (aq.). After stirring at 5°C for 24h, the product was purified via dialysis against DI water (molecular weight cutoff 6-8 kDa), and then freeze dried. Methacrylation degree of HA was calculated by ^{1}H NMR integration ratio between protons denoted as ***m*** and ***n*** in Figure S10-S11 (y~37%).

$Me_{37}HA$ (50 mg) and mercapto-*β*-cyclodextrin (65 mg) were mixed in 4 mL phosphate buffer (pH 8.0) containing 10 mM tris(2-carboxyethyl)phosphine (TCEP) at room temperature. The mixture was kept on gentle vortex for 5 h, and then dialyzed against water (cutoff Mw. 7000 Da) for one week. After lyophilization, the product was characterized by ^{1}H NMR and the modification degree was determined by the integration ratio between protons denoted as ***a*** and ***b*** in Figgure S10 and Figure S12 (z ~15%).

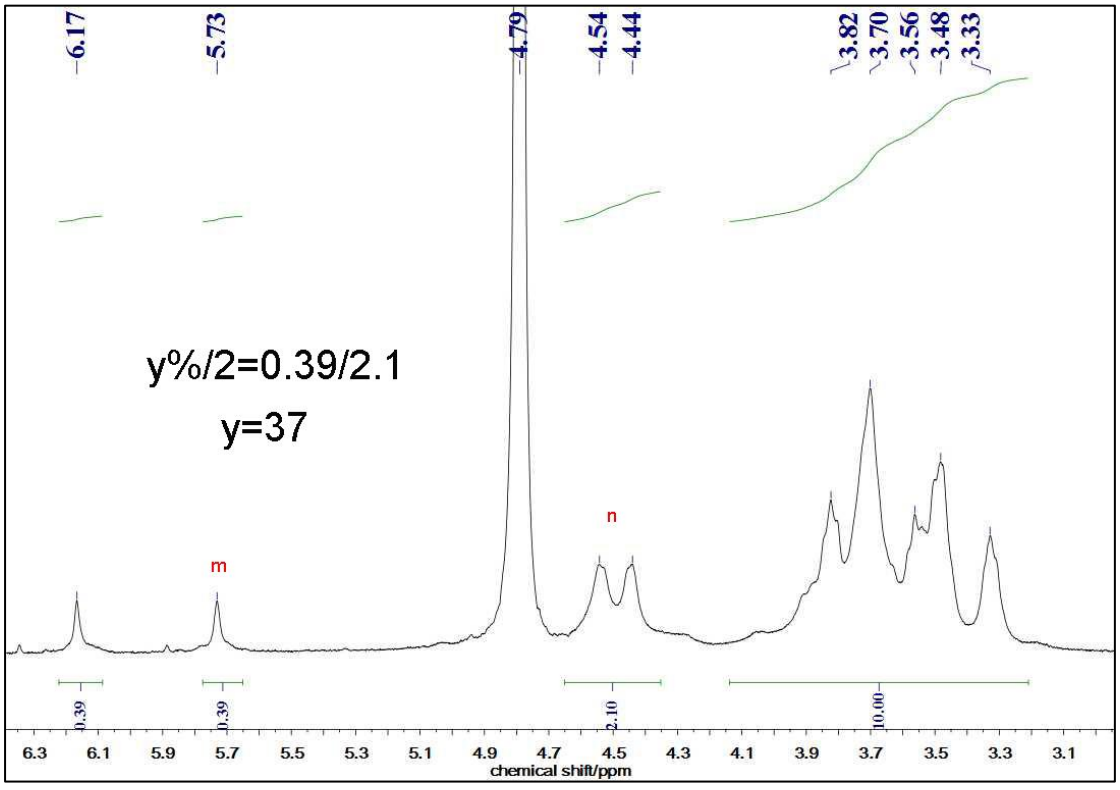

Figure S11. ^{1}H NMR (D_2O) of $Me_{37}HA$.

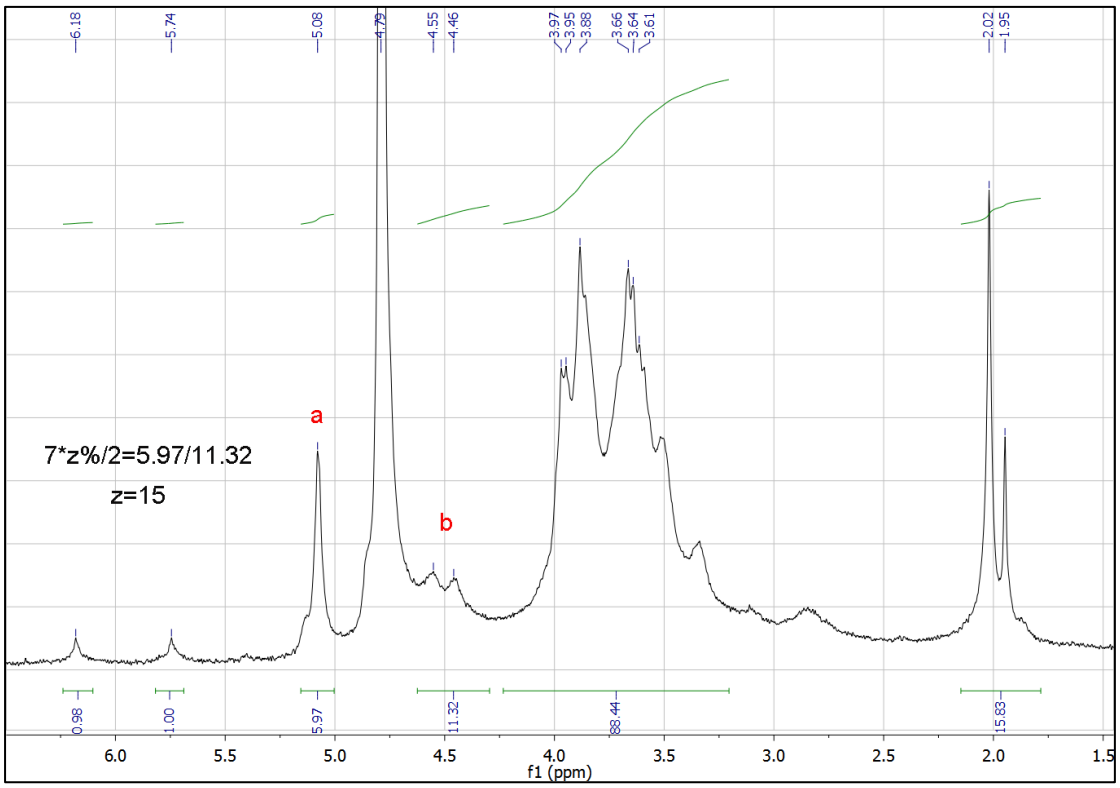

Figure S12. ^{1}H NMR (D_2O) of host-polymer $CD_{15}HA$.

Characterization of the host-guest-macromer (HGM)

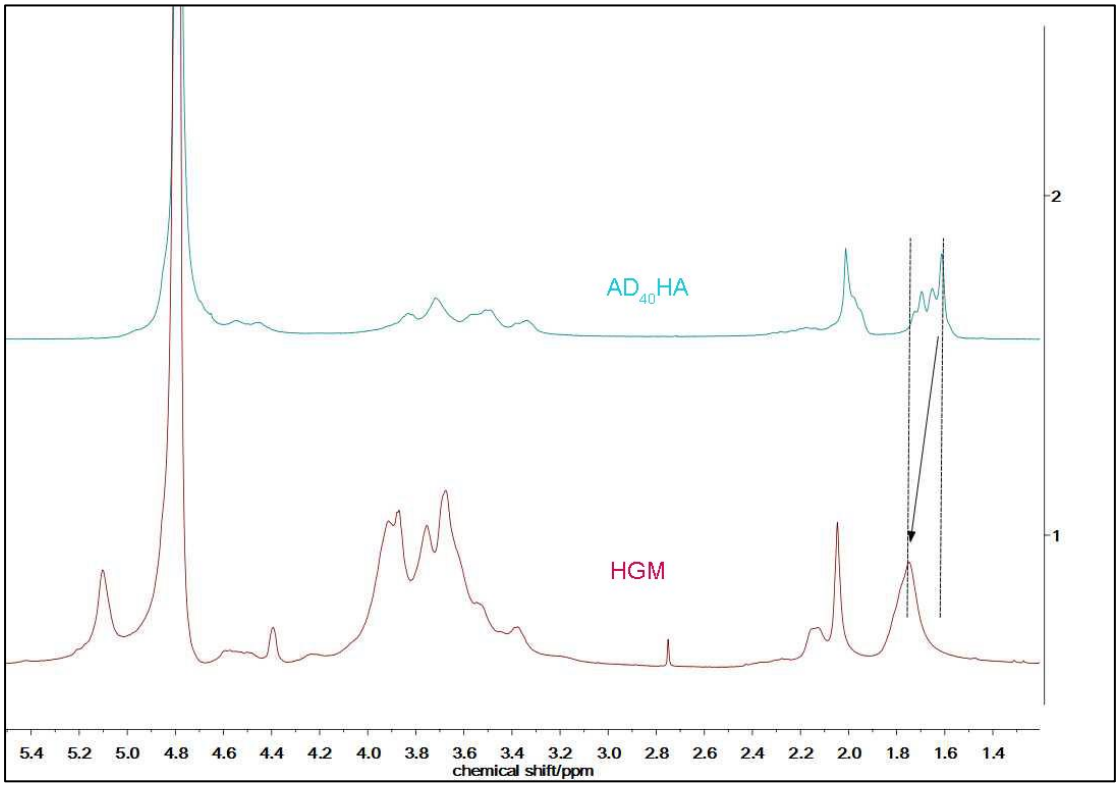

Figure S13. ^{1}H NMR (D_2O) of $AD_{40}HA$ and HGM consisting of $AD_{40}HA$ and mono-Ac-βCD. Upon HGM formation, the chemical shift of the ethyl multiplet of AD

changes significantly, and the corresponding peak is broadened.

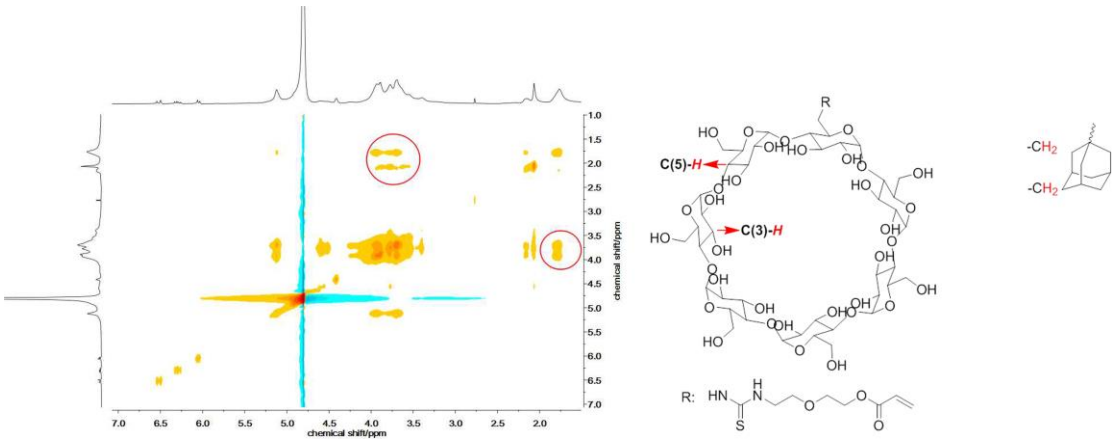

Figure S14. 2D NOESY NMR (D_2O) of HGM consisting of $AD_{40}HA$ and mono-Ac-βCD, the ADA $-CH_2$ proton peaks are found correlated with the inner protons (C(3)-H and C(5)-H) of βCD, indicating successful molecular self-assembly between $AD_{40}HA$ and mono-Ac-βCD.

The swelling behavior of HGM hydrogels

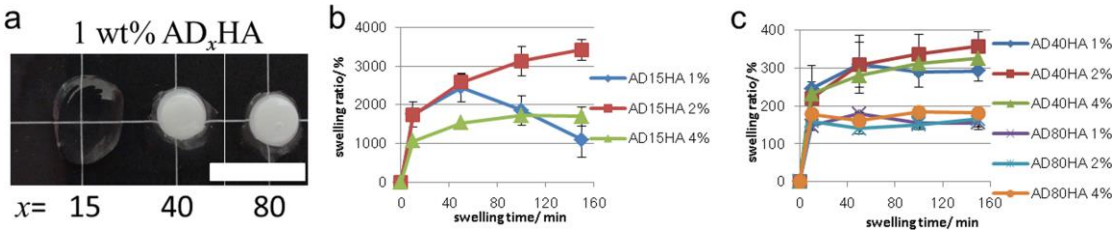

Figure S15. Swelling of HGM hydrogels prepared from AD_xHA of varied concentrations. **(a)** Freestanding HGM hydrogels (1 *wt*% AD_xHA) at equilibrium swollen state (for $AD_{15}HA$ HGM hydrogels, the equilibrium state is considered at the swelling time of 50min, as shown in panel **(b), scale bar: 1 cm**); **(b)** swelling kinetics of $AD_{15}HA$ HGM hydrogels, showing that the hydrogel made from 1 *wt*% $AD_{15}HA$ is not stable after 50min of swelling; **(c)** Identical swelling kinetics of HGM hydrogels composed of AD_xHA with the same modification degree indicates that the modification degree of AD dictates the inter-crosslink polymer chain length, thereby confirming the crosslinking function of AD in the hydrogel networks.

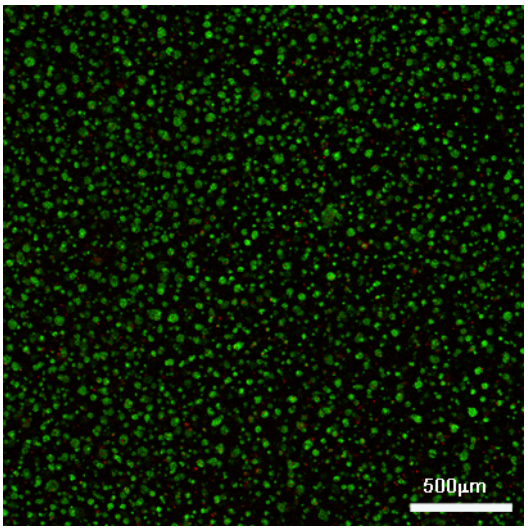

Figure S16. Viability staining of the hMSCs encapsulated in the re-molded HGM hydrogels obtained 24 hours after the remolding procedure (Green: living cells; red: dead cells).

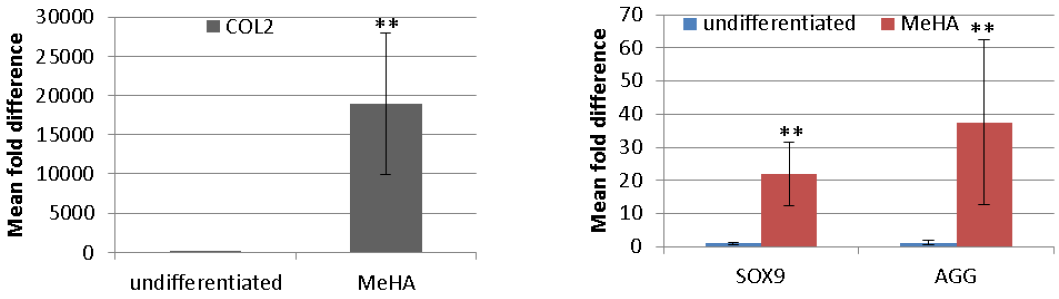

Figure S17. Gene expression level of COL2 **(a)**, SOX9 and AGG **(b)** in MeHA hydrogels after 7 days of culture (in chondrogenic media (DMEM, 1% (vol/vol) ITS+ Premix, 50 µg/mL L-proline, 0.1 µM dexamethasone, 0.9 mM sodium pyruvate, 50 µg/mL ascorbate, and antibiotics) supplemented with 10 ng/mL TGF-β1 and changed three times per week.).

Table S1. Sequences of primers and probes used for real-time PCR

Gene	**Forward primer**	**Reverse primer**	**probe**
COL2	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT	CTGCACGAAACATAC
GAPDH	AGGGCTGCTTTTAACTCTGGTAAA	GAATTTGCCATGGGTGGAAT	CCTCAACTACATGGTTTAC
SOX9	AAGCTCTGGAGACTTCTGAACGA	GCCCGTTCTTCACCGACTT	CCGGATTACAAGTACCAGC
AGG	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA	ATGGAACACGATGCCTTTCACCACGA

Reference

(1) Smeds K. A.; Grinstaff M. W.; *J. Biomed. Mater. Res.* **2001**, *54*, 115-121.