Electronic Supplementary Information (ESI)

A Facile One-pot Synthesis of Acrylated Hyaluronic Acid

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Materials and Methods

Materials: Hyaluronic acid (220 kDa, food grade) was purchased from Stanford Chemistry Ltd. HA-SH (R&D grade) was purchased from Vornia Ltd. 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (photo initiator 2959, I2959), acryloyl chloride (97%), $CDCl_3$ (99.8 atom % D) and D_2O (99.9 atom % D) were purchased from Sigma-Aldrich and used as received. Glycidol (96%), triethylamine (TEA, 99%), glycidyl methacrylate (97%) were purchased from Sigma-Aldrich and dried with 4A molecular sieve. Dichloromethane (DCM, 99.9%), N,N-dimethylformamide (DMF, 99.9%) were purchased from Fisher and used as received. Dialysis tubing (MWCO = 14 kDa) and silica gel were purchased from Sigma.

Instruments: ^{1}H-NMR spectra were collected on a Varian 400 MHz NMR spectrometer. Rheological assessments were conducted on an HR-2 Rheometer equipped with an 8 mm steel parallel-plate geometry and a UV lamp. The inherent viscosity was tested by a Lauda Viscocool 6 viscometer.

3T3 were purchased from Sigma-Aldrich and normal human keratinocytes (NHK) were purchased from Lonza.

Synthesis of acrylated hyaluronic acid (HA-A) via one-pot approach:

Glycidol (250 mmol, 18.5 g) and TEA (275 mmol, 27.9 g) were dissolved in anhydrous DCM (100 mL). Acryloyl chloride (250 mmol, 22.6 g) was added to the stirring solution dropwise and precipitation was formed. After addition of acryloyl chloride, the mixture was warmed up to RT and stirred for 2 h. 0.5 g of the mixture was taken and purified by column chromatography (solvent eluent, ethyl acetate : hexane = 1:5) to achieve the pure intermediate and ^{1}H-NMR was used to verify the structure of intermediate glycidyl acrylate. Afterwards, HA (5.0 mmol, 2.0 g) solution (in 100 mL DI water) was added to the flask. TEA (250 mmol, 25.3 g) was dissolved in DMF (100 mL) and added to the flask. The mixture was stirred at RT for 5 days and the solution was dialysis against DI water for 5 days with the outer solution changed three times per day. Afterwards, the solution was freeze-dried to achieve the HA-A as faint yellow cotton-shaped foam. The product was stored at -20 °C and protected by argon before use.

Synthesis of methacrylated hyaluronic acid (HA-MA)[1]:

HA (5.0 mmol, 2.0 g) was dissolved in DI water (100 mL). Glycidyl methacrylate (250 mmol, 35.5 g) and TEA (250 mmol, 25.3 g) were dissolved in DMF (100 mL). The water phase and organic phase was mixed and stirred at RT for 3 days and the solution was dialysis against DI water for 5

days with the outer solution changed three times per day. Afterwards, the solution was freeze-dried to achieve the HA-MA as white cotton-shaped foam and the product was stored at -20 °C and protected by argon before use.

Hydrogel fabrication:

Chemically crosslinked hydrogel:

HA-A was dissolved in 1X PBS (2%, 4%, and 6% w/v). HA-SH was dissolved in 1X PBS (2% w/v). The hydrogel was formed by mixing the two solutions at 1:1 volume ratio.

UV crosslinked hydrogel:

Photo initiator 2959 was dissolved in DI water with a concentration of 0.5% (w/v). HA-A was dissolved in the prepared solution (2%, 4%, and 6% w/v). The hydrogel was crosslinked by 5 min of UV irradiation (365 nm).

Rheological property analysis

Rheological properties of hydrogel were measured on an HR-2 Rheometer equipped with an 8 mm steel parallel-plate geometry and a UV lamp. For time sweep assessment, 100 μL hydrogel was formed *in situ* with a gap of 800 μm. Constant frequency (1 Hz) and strain (1%) were utilized. Gelling point was defined as the point of the storage modulus (G') increased rapidly. For UV crosslinked hydrogel, the UV irritation was on from the second min for a duration of 5 min. The strain sweep test was performed right after the time sweep assessment from 0.1-1,000 % with a constant frequency of 1 Hz. The critical strain value was defined as the cross-point of the storage modulus and loss modulus.

Hydrogel degradation

The degradation of the hydrogel was determined by measuring the weight loss of the hydrogel at particular time points after incubation in 1X PBS (with or without hyaluronidase) in a shaker at 37 °C and 100 rpm.

Degradation in 1X PBS

200 μL of each hydrogel was fabricated by the method mentioned above and placed into a pre-weighed 20 mL glass vial. 5 mL of 1X PBS was added to the glass vial and then the vial was kept at 37 °C in a shaker. The weight of the hydrogel was recorded at the particular time points. Each group has three replicates. The degradation data was collected after the hydrogel reached equilibration (a maximum weight was achieved).

Degradation in hyaluronidase

200 μL of each hydrogel was fabricated by the method mentioned above and placed into a pre-weighed 20 mL glass vial. 5 mL of 1X PBS containing 10 U/mL or 100 U/mL of hyaluronidase was added to the glass vial and then the vial was kept at 37 °C in a shaker. The weight of the hydrogel was recorded at the particular time point. Each group has three replicates. The degradation data was collected after the hydrogel reached equilibration (a maximum weight was achieved).

Light transmission determination of HA-A solutions:

HA-A solutions with the concentrations of 2%, 4%, and 6% (w/v) were prepared in DI H_2O. The light transmission was collected on a plate reader with DI H_2O as the reference.

Cytotoxicity test of HA-A polymer solution by AlamarBlue assay:

3T3 and NHK are used in the in vitro cytotoxicity test. For both cell lines, 8,0000 cell/mL were seeded into 96-well plates and cultured overnight to support cell attachment under standard cell culture conditions (37 ^{o}C and 5% CO2). Polymer was then dissolved in full cell media in a series of concentrations (0, 250, 500, 1000, 2000, 4000, 6000, 8000, 10000, 15000, 20000 μg/mL) and placed into 96-well plates (n = 4). Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher), containing fetal bovine serum (FBS, 10% v/v, Gibco) and penicillin/streptomycin (P/S, 1% v/v, Thermo Fisher) was prepared as full cell media for 3T3 culture. KGM-2 Keratinocyte Growth media from Lonza was used as full cell media for NHK culture. AlamarBlue assay was used to test cell viability according to the protocol after 24 h incubation. The reduction of alamarBlue reagent was tested by a plate reader.

Cell viability in HA-A/HA-SH hydrogels:

The 3T3 or NHK encapsulated HA-A/HA-SH hydrogels were prepared as follows,

Cell suspension, 4% HA-A, and 2 % HA-SH solutions were mixed together and dropped on taflon tape to obtain hydrogels with 30 uL per gel and a final cell concentration of 1×10^6 /mL. The cell seeded hydrogel systems were incubated in 24-well plates under standard cell culture conditions. 1 mL full cell media were added into each well. Cell media was changed every three days. AlamarBlue assay was performed at day 1, 3 and 7. Live/dead staining was performed on day 1. 2D cultured cells with the same number in each gel was used as positive control group. For each well, 500 uL of 2 mM of calcein AM and 1 mM of EthD-1 mixture in Hanks' buffer were added. Fluorescent images were obtained from an inverted fluorescence microscope (Olympus IX81).

Calculation of conjugation degree based on Figure S3 and S4 (CD):

$$CD_{HA\text{-}A} = \frac{\frac{a}{c}}{\frac{3}{3}} \times 100\% = \frac{0.25}{\frac{3}{3}} \times 100\% = 25\% \quad \text{Equation S1}$$

$$CD_{HA\text{-}MA} = \frac{\frac{a}{c}}{\frac{3}{3}} \times 100\% = \frac{0.20}{\frac{3}{3}} \times 100\% = 20\% \quad \text{Equation S2}$$

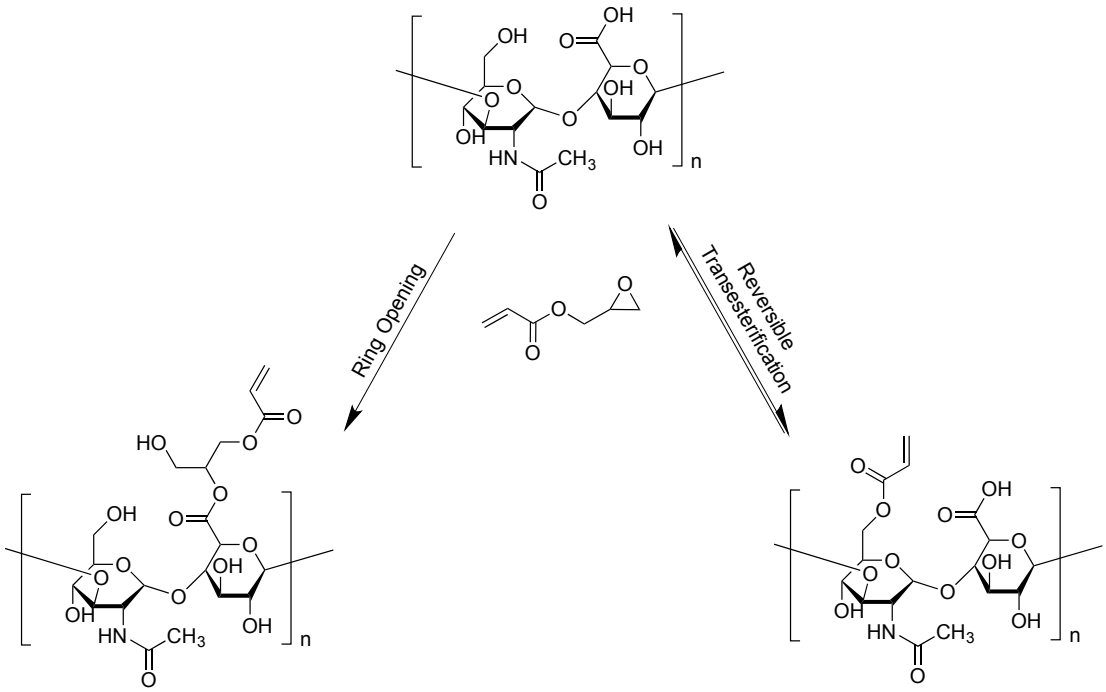

Figure S1. The competition mechanism between transesterification and ring-opening reaction.

NMR spectrums

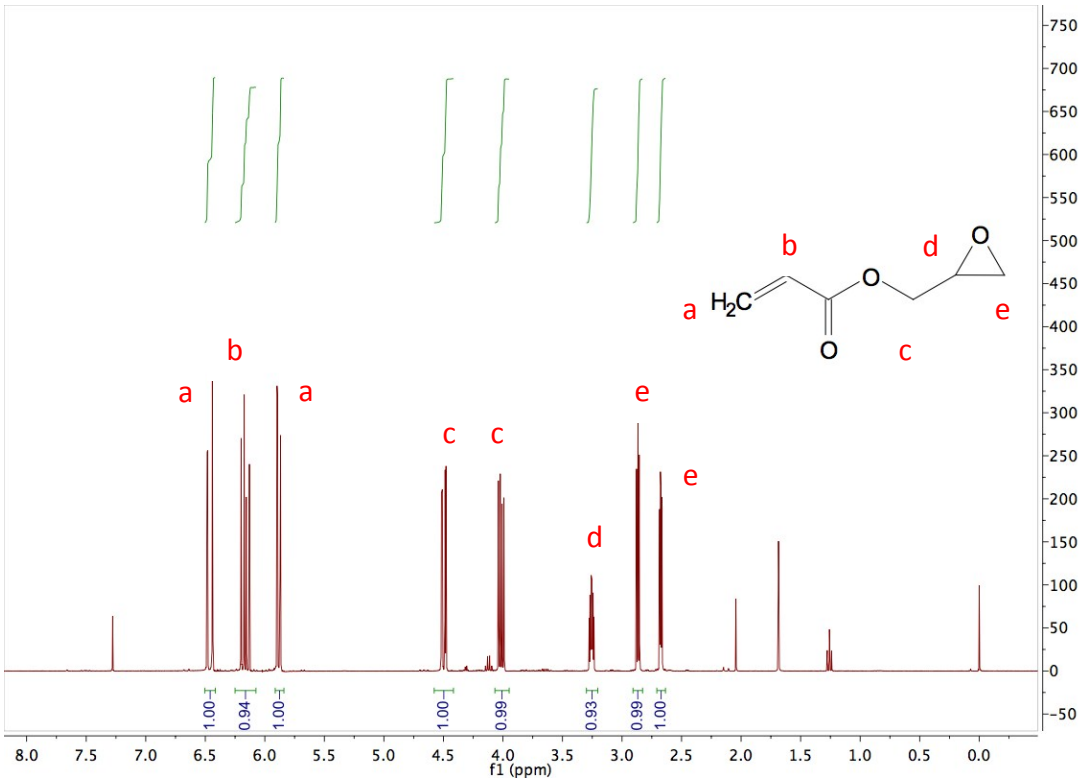

Figure S2. ^{1}H-NMR spectrum of Glycidyl acrylate

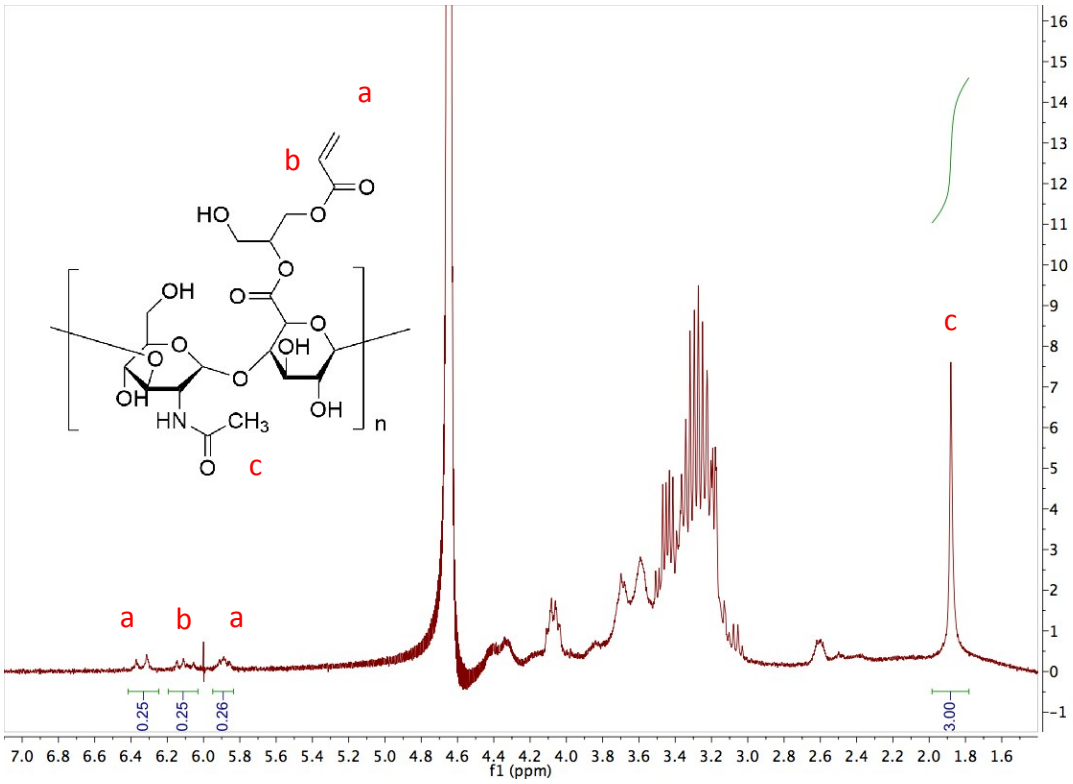

Figure S3. ^{1}H-NMR spectrum of HA-A

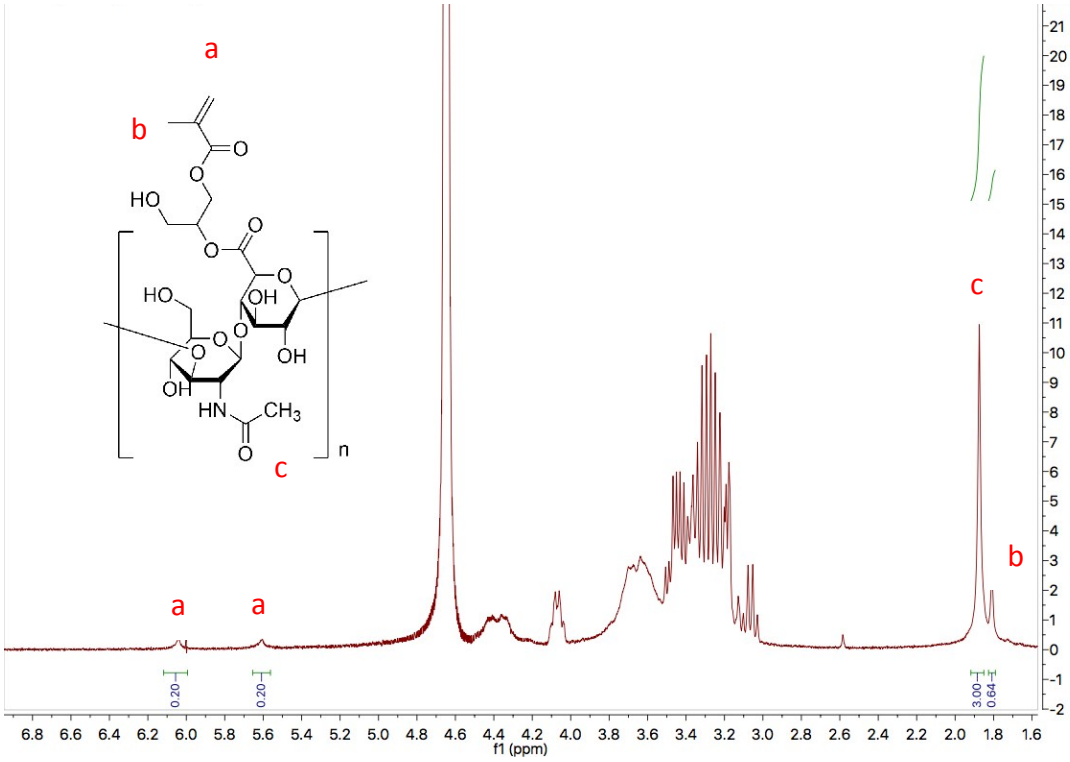

Figure S4. ^{1}H-NMR spectrum of HA-MA

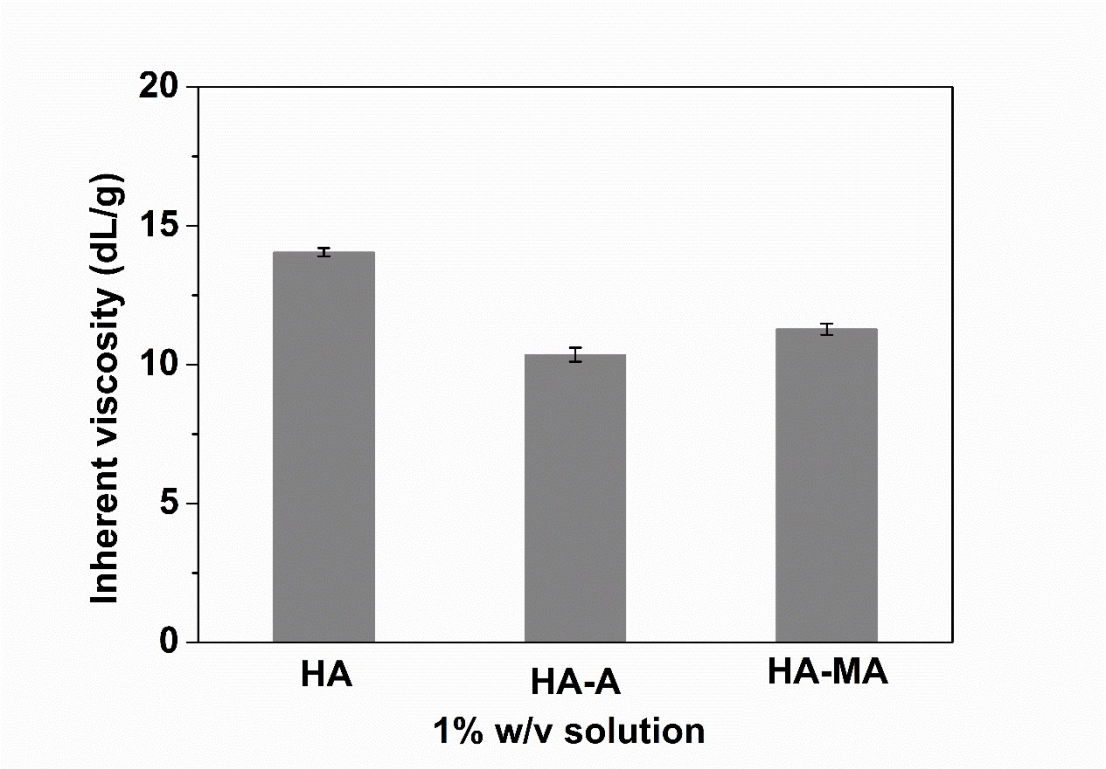

Figure S5. Inherent viscosity of HA, HA-A, and HA-MA solution with the concentration of 1% w/v. Each test was conducted in triplicate.

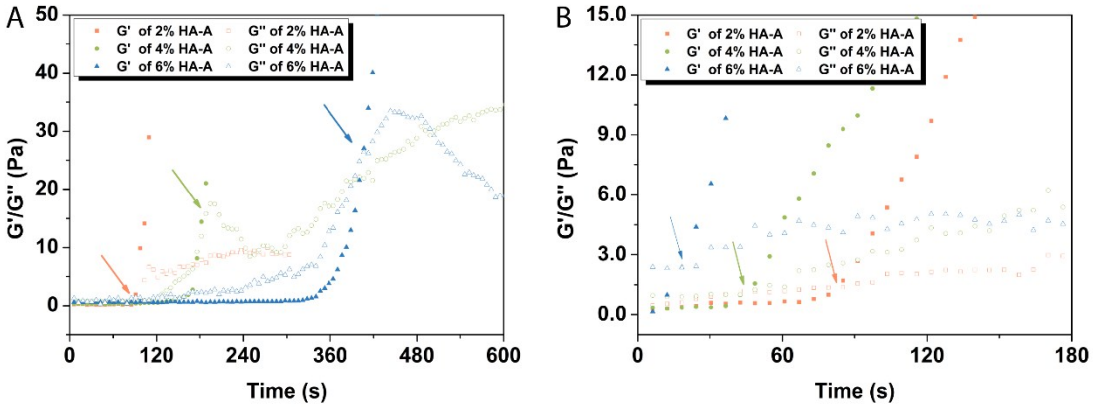

Figure S6. Zoom in area of rheological assessments of HA-A hydrogels to show the gelling point. (A) Time sweep assessments of HA-A (2%, 4%, and 6% w/v) in the presence of 0.5% w/v of I2959 with a frequency of 1 Hz and a strain of 1%. UV irradiation was started from 1 min. The arrows indicate the gelling points. (B) Time sweep assessments of HA-A (2%, 4%, and 6% w/v) with HA-SH (2% w/v) with a frequency of 1 Hz and a strain of 1%. The arrows indicate the gelling points.

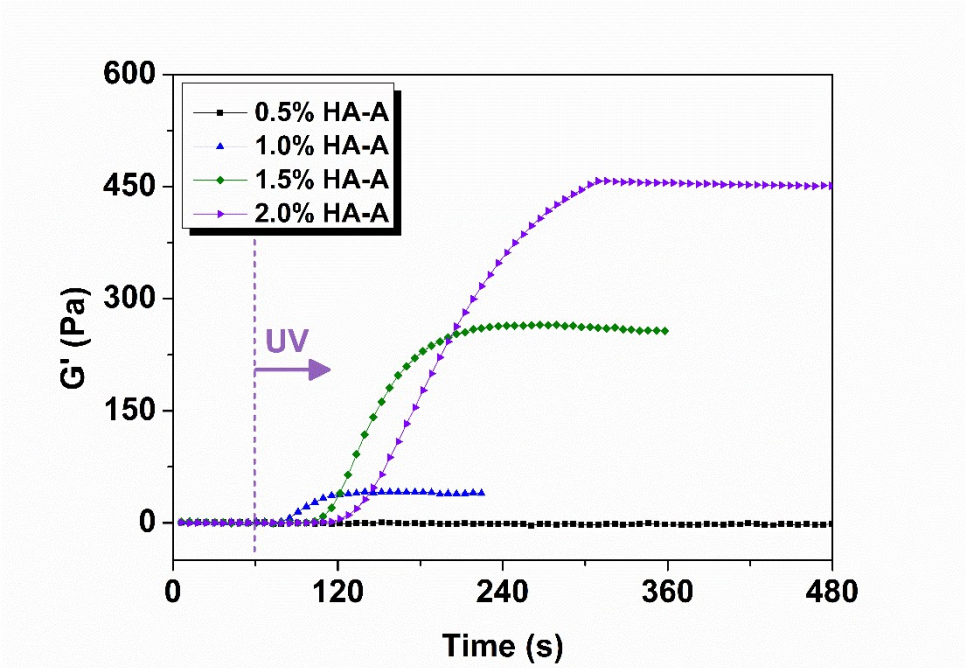

Figure S7. Time sweep assessments of HA-A (0.5%, 1%, 1.5%, and 2% w/v) in the presence of I2959 (0.5% w/v) with a frequency of 1 Hz and a strain of 1%. UV irradiation was started from 1 min and lasted until a maximum storage modulus was achieved.

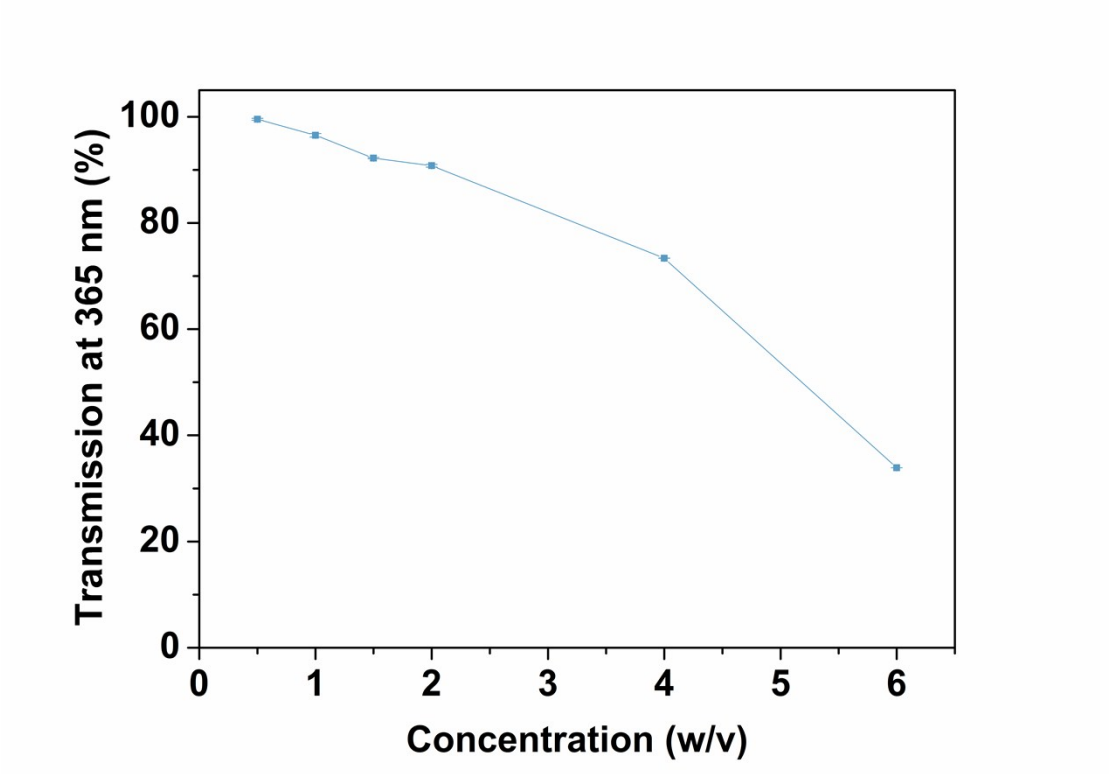

Figure S8 Light transmissions of HA-A solutions at 365 nm.

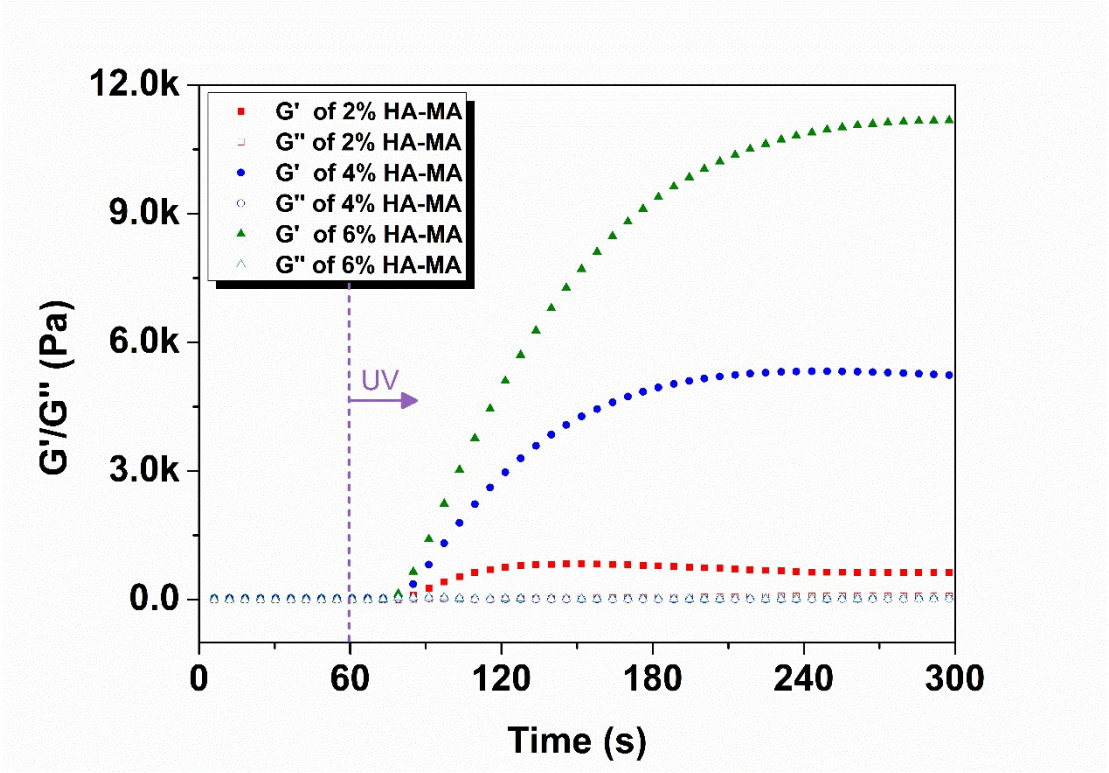

Figure S9 Time sweep assessments of HA-MA (2%, 4%, and 6% w/v) in the presence of 0.5 % w/v I2959 with a frequency of 1 Hz and a strain of 1%. UV irradiation was started from 1 min and lasted until a maximum storage modulus was achieved.

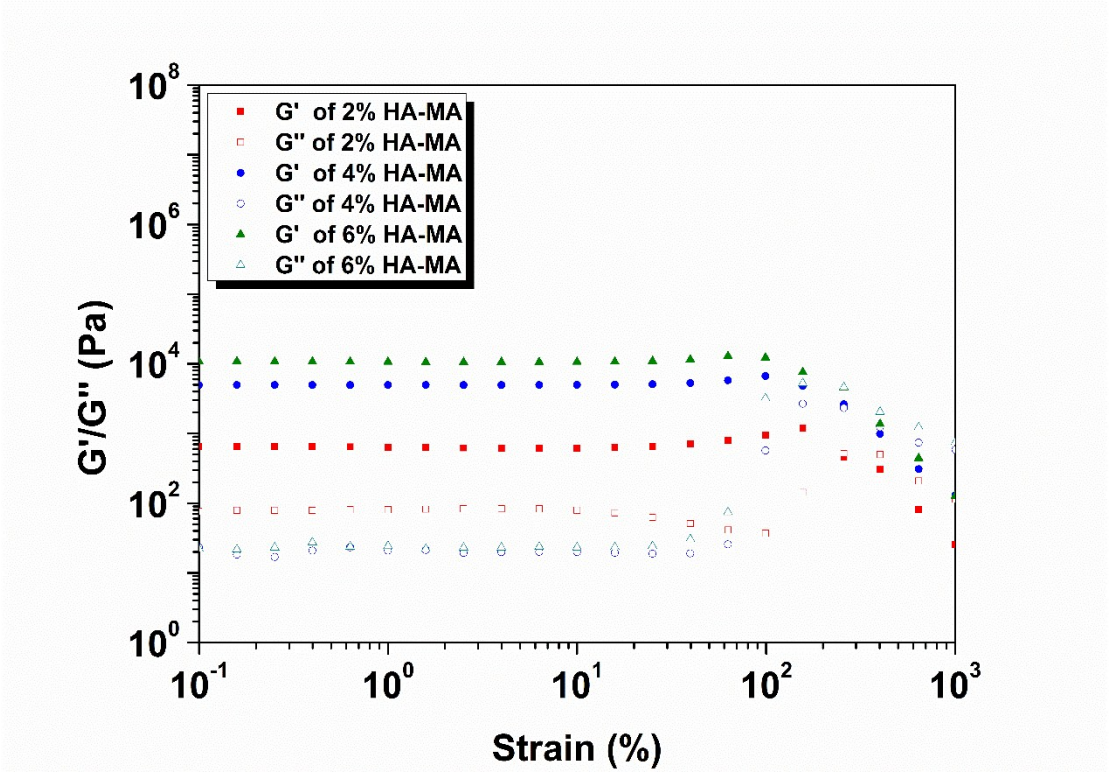

Figure S10 Strain sweep assessments (0.1-1,000%) of HA-MA hydrogels with a constant frequency of 1 Hz.

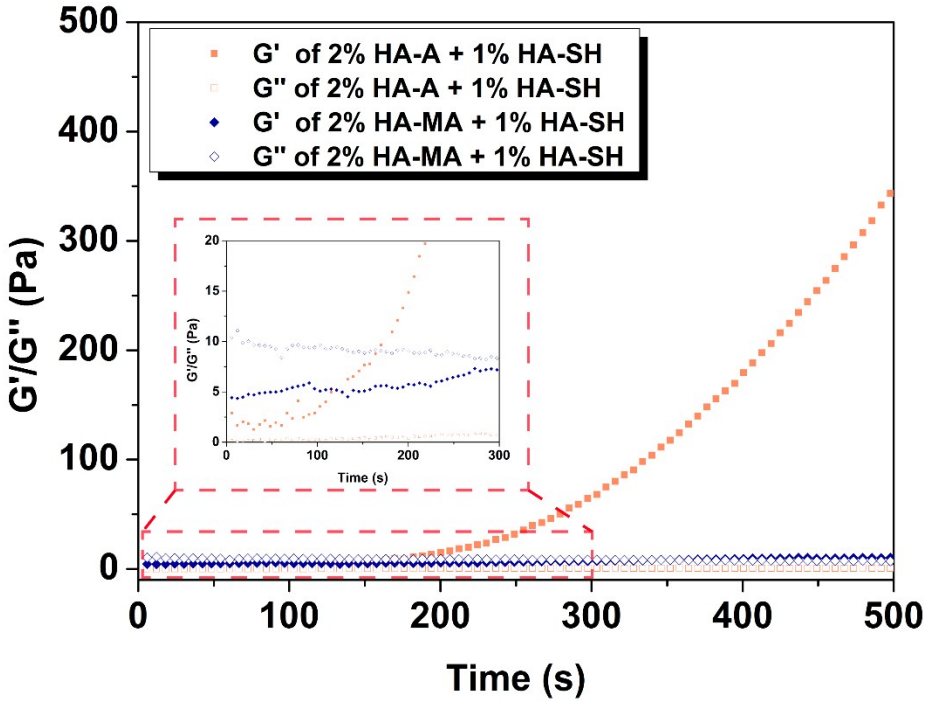

Figure S11 Real time gelation process determined by time sweep rheological assessments of HA-SH with HA-A (orange) and HA-MA (blue).

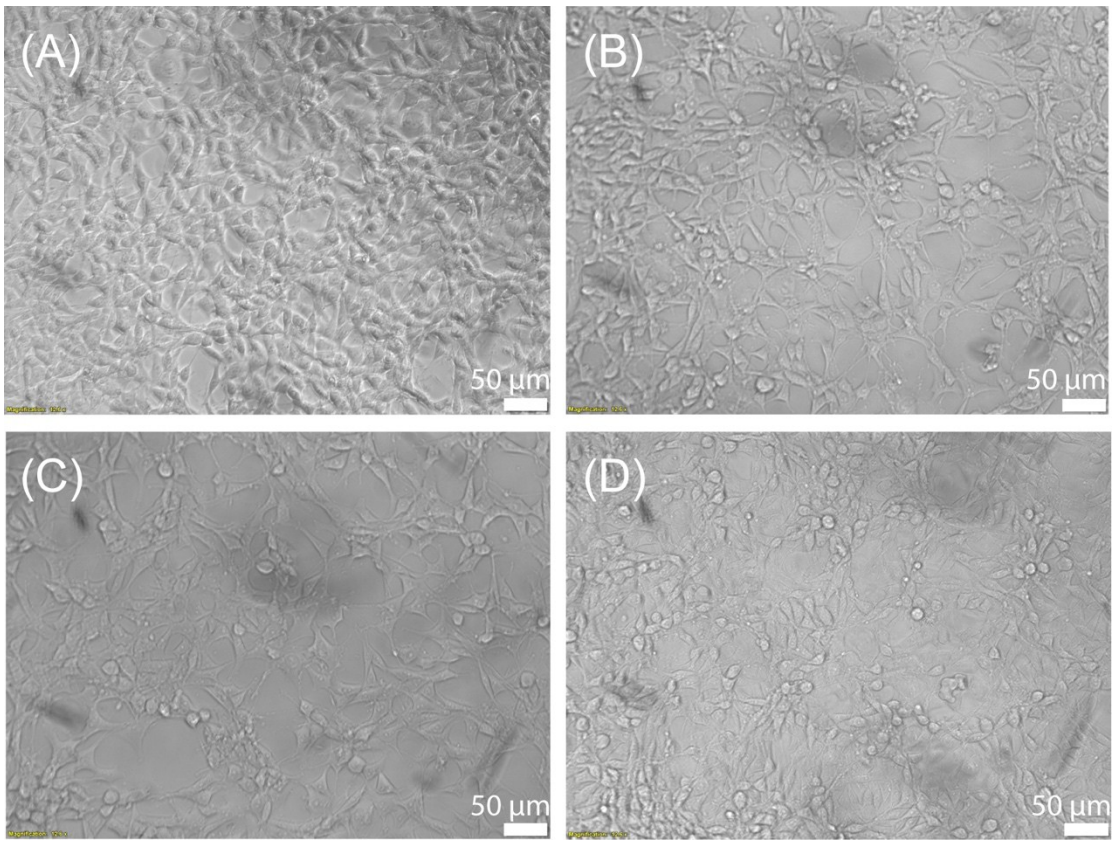

Figure S12 Morphology of 3T3 cells when co-cultured with various concentrations of HA-A display the same fibroblast shape. (A) 250 µg/mL. (B) 4,000 µg/mL. (C) 10,000 µg/mL. (D) Control group. Scale bars: 50 µm.

Reference:

1 S. A. Bencherif, A. Srinivasan, F. Horkay, J. O. Hollinger, K. Matyjaszewski and N. R. Washburn, *Biomaterials*, 2008, **29**, 1739–1749.