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A Fast One-Step Acrylate Functionalization of Hyaluronic Acid via Williamson Ether Synthesis

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Materials and Methods

Materials

Sodium hyaluronate (HA, 220 kDa, cosmetic grade) was purchased from Bloomage Freda Biopharm Co. Ltd. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), Phosphate buffer saline (PBS), disodium phosphate (Na_2HPO_4), triethylamine (TEA), hyaluronidase (Hyase type II, 1000 units/mg), alamarBlue reagent, dimethylformamide (DMF), deuterium oxide (D_2O, 99.9 atom % D), 1-Ethyl-3-[3- (dimethylamino)propyl]carbodiimide (EDCI), hydrochloric acid (HCl), sodium hydroxide (NaOH), phosphoric acid (H_3PO_4), dithiothreitol (DTT), 5,5- dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent), L-cysteine hydrochloride and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck, Germany. 2-bromoethyl acrylate (BEA) was purchased from Fisher Scientific, USA. 3,3'- dithiobis(propanoic dihydrazide) (DTPH) was purchased from Frontier Specialty Chemicals, USA. Live/Dead viability cytotoxicity kit was purchased from BioScience, Cambridge. Dialysis tubing (MW cut off 8 kDa) was purchased from Spectrum Lab, Ireland. Thiol-modified hyaluronic acid (HA-SH, 18 and 33 % SD) was purchased from Blafar Ltd, Ireland. Normal human dermal fibroblasts (NHDF) were purchased from ATCC, USA. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were purchased from Invitrogen, USA. Full cell culture media for NHDF cells was prepared using 10% FBS and 1% P/S in DMEM. All chemicals were used as delivered unless noted.

Instruments

^{1}H-NMR data was obtained at room temperature (298 K) on a Varian VnmrS 400 MHz spectrometer to confirm compound structures in solution and to calculate degrees of substitution of acrylate groups on the HA backbone. D_2O was used as the NMR solvent. Chemical shifts are reported in ppm and referenced to D_2O (1H: 4.79 ppm). Rheological

assessments were carried out at room temperature (25 ºC) on TA instruments HR-2 rheometer, equipped with 8 mm and 20 mm steel parallel plates and visible light source (OmniCure S1000, Lumen Dynamics Group Inc.). A spectrophotometer (SpectraMax M3 Molecular Devices) was used for alamarBlue cell viability test and Ellman's assay. Leica DM2500 fluorescence microscope was used to view cell staining.

Synthesis of Acrylate Modified Hyaluronic Acid (HA-A-BEA).

HA (1.00 g, 2.50 mmol) was completely dissolved in ultrapure water (50 mL) at 45 °C in a 250 mL round bottom flask. DMF (50 mL) was added to the reaction mixture as a co-solvent. Once the DMF was completely immiscible, TEA (12.20 mL, 87.50 mmol) was added dropwise to the reaction mixture whilst stirring. BEA (6.93 mL, 57.50 mmol) was then added dropwise, and the reaction mixture was left to react for 12 h. The reaction solution was then dialyzed against in dilute HCl (pH 3.5) for 2 days with 3 water changes per day and then pure deionized water for 2 further days with 3 water changes per day. The purified solution was then flash-frozen and lyophilized to afford a white foam (HA-A-BEA). The structure was confirmed by ^{1}H-NMR spectroscopy and the presence of acrylate group vinyl proton signals between 6.00 – 6.60 ppm (Figure S1).

^{1}H-NMR Calculation of Acrylate Substitution Degree (A-SD) Using Figure S1.

The acrylate degree of substitution can be directly taken from the ^{1}H-NMR spectrum (Figure S1) using the following equation.

Equation S1. $\text{A-SD \%*} = (A^1 + A^2 + A^3) / 3 \times 100$

*The integration of B (HA methyl group at 2.01 ppm) must be adjusted to 3.00 prior to calculation.

Preparation of HA-A-BEA Hydrogels.

Chemically Crosslinked HA-A-BEA Hydrogel (HA-A-CX)

HA-A-CX hydrogels were prepared by dissolving HA-A-BEA in 0.2 M Na_2HPO_4 (1 and 2 % w/v). HA-SH was dissolved in 0.2 M Na_2HPO_4 (1 and 2 % w/v). HA-A-BEA of 18 and 27 % SD and HA-SH of 18 and 33 % SD were used for the formation of HA-A-CX hydrogels. The details regarding the hydrogel formulations are outlined in Table 1. The hydrogels were formed by mixing the HA-A-BEA and HA-SH solutions at 1:1 volume ratio at room temperature (RT, 25 °C).

Photo Crosslinked HA-A-BEA Hydrogel (HA-A-VL)

Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), was dissolved in DI water at a concentration of 0.5% (w/v). HA-A-BEA (SD 18%) was dissolved in the prepared solution (1 and 2 % w/v). The polymer was exposed to visible light (405 nm) for 1 min at RT.

Rheological assessment of HA-A-BEA hydrogels

Rheological assessments were carried out at RT on a TA instrument HR-2 rheometer, equipped with an 8 mm (time-sweep) and 20 mm (compression) steel parallel plate. For time sweep tests, the HA-A-CX hydrogels were pre-mixed and applied (100 µL) to the bottom plate with the plate gap set to 1500 µm and the test started immediately. The HA-A-VL gels were prepared by adding 100 µL of pregel solution to the bottom plate with the plate gap set to 1500 µm. The samples were equilibrated for 60 s and then cured by visible light for 60 s, the time-sweep data was collected for a further 2 mins. All time sweep tests were carried out at a frequency of 1.0 Hz and a strain of 1%. For further rheological studies, 200 uL hydrogels were prepared in 1.5 mL Eppendorf caps using previously stated formulations. For the compression studies, the hydrogel samples were placed on the center of compression plate of the tester and compressed at a rate of 1% strain /s. Frequency sweep measurements were carried out over a range of

frequencies from 0.1 to 10 Hz, at 1% strain. Each rheological property test was carried out in triplicate.

Swelling and Degradation Studies of HA-A-BEA Hydrogels

The swelling and degradation profile of HA-A-CX and HA-A-VL hydrogels was determined by measurement of hydrogel weight after incubation in 37 ºC / 100 rpm shaker. 200 uL hydrogels were prepared as previously stated. Degradation medium solutions: 5 mL of 1X PBS buffer and 5 mL of 100 U/mL hyaluronidase in 1X PBS buffer. 200 μL gels were added to 7 mL glass vials. 5 mL of medium was added to each vial and the vials were added to the shaker. The degradation/swelling profile was then measured by removing the medium completely at the time points and weighing the vials containing the gels. Each experiment was conducted in triplicate. The quantity of degradation/swelling was calculated using the following equation:

Equation S2. Percentage of initial hydrogel mass: $W_t/W_0 \times 100$.

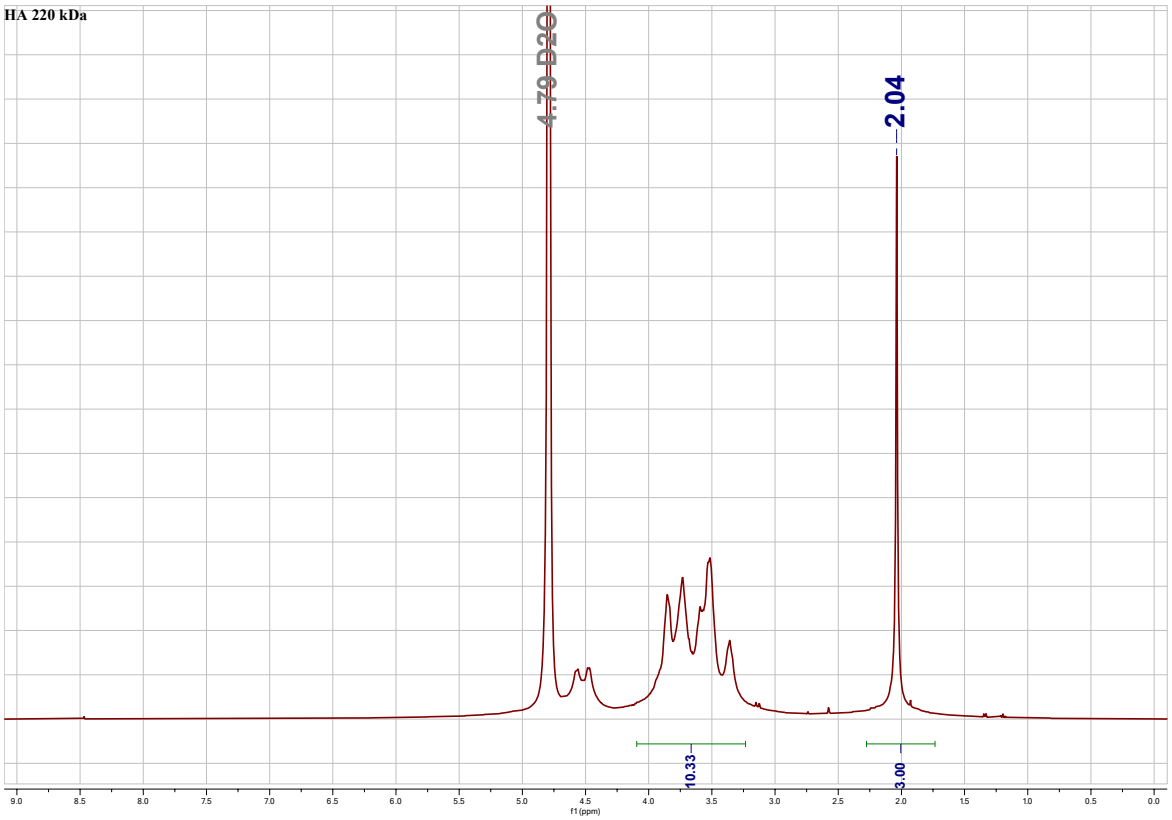
Where W_t is the weight of the hydrogel at the scheduled time point and W_0 is the initial weight of the hydrogel.

Cytotoxicity Studies of HA-A-BEA Biopolymers

A direct in vitro cytotoxicity test was performed according to ISO 10993-5:2009. All solutions for cell viability tests were prepared using DMEM buffer and filtered for sterilization using a 0.22 μm pore size filter. For the blank control groups, cells were cultured in DMEM buffer without treatment. Cytotoxicity of HA-A-BEA was carried out using NHDF cell lines. 7.0×103 cells/well were seeded into a 96-well plate and cultured overnight in full cell media (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1%

penicillin) under standard cell culture conditions (37 °C, 5% CO_2). The cell media was then changed to a series of HA-A-BEA (from 0.25 to 5 mg/mL). The cell viability assay was performed after 24 and 72 h following co-culture with alamarBlue for 4 h. Cell viability of the HA-A-BEA was calculated based on the untreated cell viability data (blank). LIVE/DEAD kit (calcein/ethidium) staining was utilised to confirm the living status of the cells. After 24 h and 72 h, the culture medium was removed and replaced with the LIVE/DEAD stain. After 30 minutes of incubation at 25 °C, the stain was washed away from the well plates with PBS. The images were captured using a fluorescence microscope. Absorbance values (n = 3) are reported as the "Relative Cell Viability" in which 100 % equals the control absorbance. All samples were prepared in triplicate.

(A)



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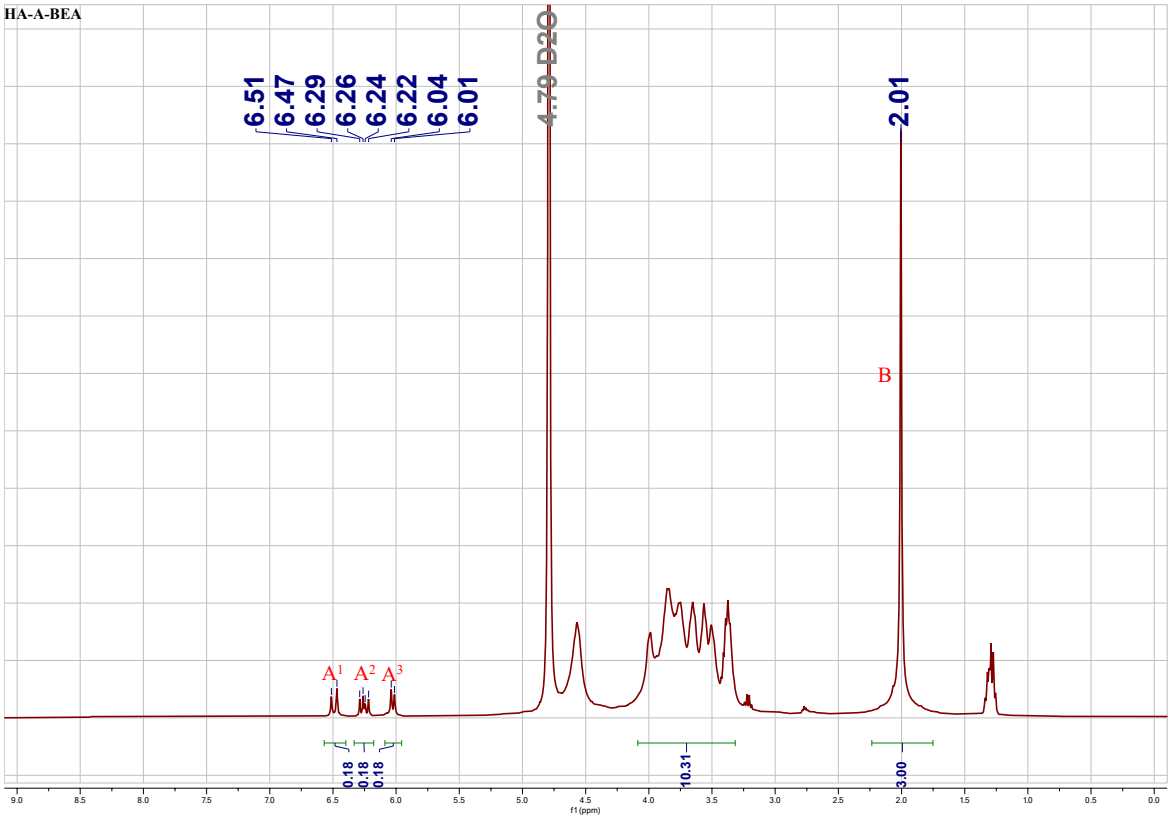

Figure S1 ^{1}H-NMR spectrum for (A) HA raw material and (B) HA-A-BEA (18% SD) in D_2O.

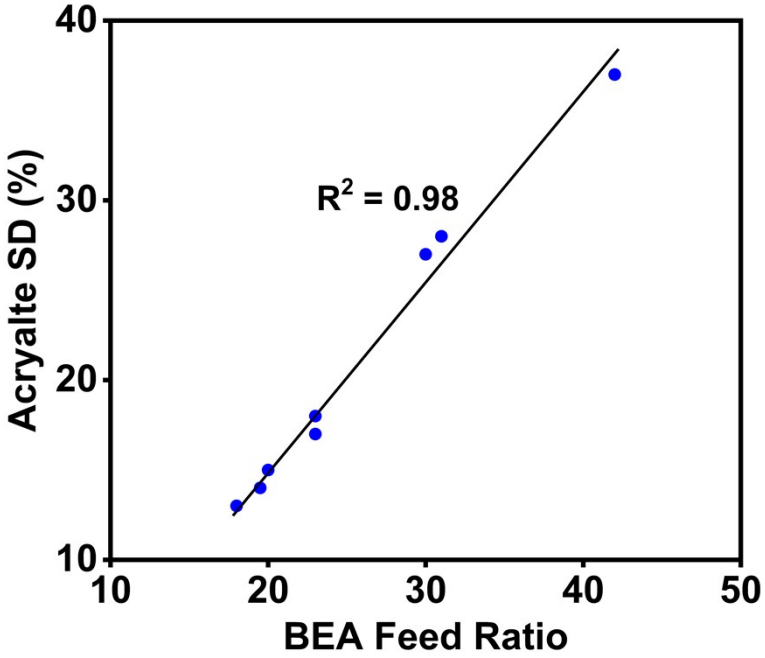

Figure S2 Relationship between BEA molar feed ratio and acrylate SD % in HA-A-BEA (A-SD).