Tuning Hydrogel Properties to Promote the Assembly of Salivary Gland Spheroids in 3D

Tugba Ozdemir,[1$] Eric W. Fowler,[1$] Shuang Liu,[1] Daniel A. Harrington,[2] Robert L. Witt,[3, 4] Mary C Farach-Carson,[2, 3, 5] Swati Pradhan-Bhatt,[3, 4, 6]* Xinqiao Jia[1, 3, 6]*

[1]Department of Materials Science and Engineering, University of Delaware, 210 South College Ave., Newark, DE 19716, USA; [2]Department of BioSciences, Rice University, 6100 Main St., Houston, TX 77005, USA; [3]Department of Biological Sciences, University of Delaware, 210 South College Ave., Newark, DE, 19716, USA; [4]Helen F. Graham Cancer Center and Research Institute, Christiana Care Health Systems, 4701 Ogletown Stanton Rd., Newark, DE, 19713, USA; [5]Department of Bioengineering, Rice University, 6100 Main St., Houston, TX 77005, USA [6]Department of Biomedical Engineering, University of Delaware, 210 South College Ave., Newark, DE 19716, USA

Number of pages: 7; Number of figures: 3; Number of tables: 2

Supporting Information

Synthesis of mono-2-(acryloyloxy)ethyl succinate (AES). Succinic anhydride (1.05 mmol) was dissolved in 200 mL of anhydrous dichloroethylene. Following dissolution, 2-hydroxyethyl acrylate (HEA, 1.0 mmol) was added. After the vessel was heated to 65 °C and purged with nitrogen, 1-methylimidiazole (0.06 mmol) was injected. After 18 h, the reaction mixture was concentrated under reduced pressure to 25 mL. The solution was washed with deionized water, HCl (1.0 M in H_2O), and saturated NaCl. A trace amount of hydroquinone was added to inhibit polymerization and then the solution was dried over $MgSO_4$ to yield AES (Yield: 80%).

Synthesis of HA-AES. Low molecular weight HA (5 kDa, 500 mg, 1.25 mmol) was dissolved into 30 mL of deionized water and the solution was shaken over Dowex-100 (6.25 mmol) resin for 12 h. The acidic solution was neutralized with 0.2 N tetrabutylammonium hydroxide to pH 7.3–7.6 and lyophilized to produce a dry powder (HA-TBA). HA–TBA and DMAP (0.094 mmol.) were dissolved in 10 mL of anhydrous DMSO under nitrogen. AES (6.25 mmol.) was separately dissolved in 5 mL of anhydrous DMSO and added to the reaction mixture. The flask was heated to 50 °C, Boc_2O (1.88 mmol.) was added, and the reaction was allowed to proceed for 24 h. The product was precipitated into 50/50 (v/v) hexanes/ethanol then dissolved into deionized water as a slurry with Dowex-50 (6.25 mmol) resin for 12 h. The product was then lyophilized and dissolved at 10 wt% in 2.0 M NaCl and precipitated into ethanol three times. The precipitant was dialyzed for 48 hours in 0.1 M NaCl followed by 24 h in deionized waster. Following lyophilization, the product was isolated at a 45% yield while 50% acrylate modification was determined by ^{1}H NMR.

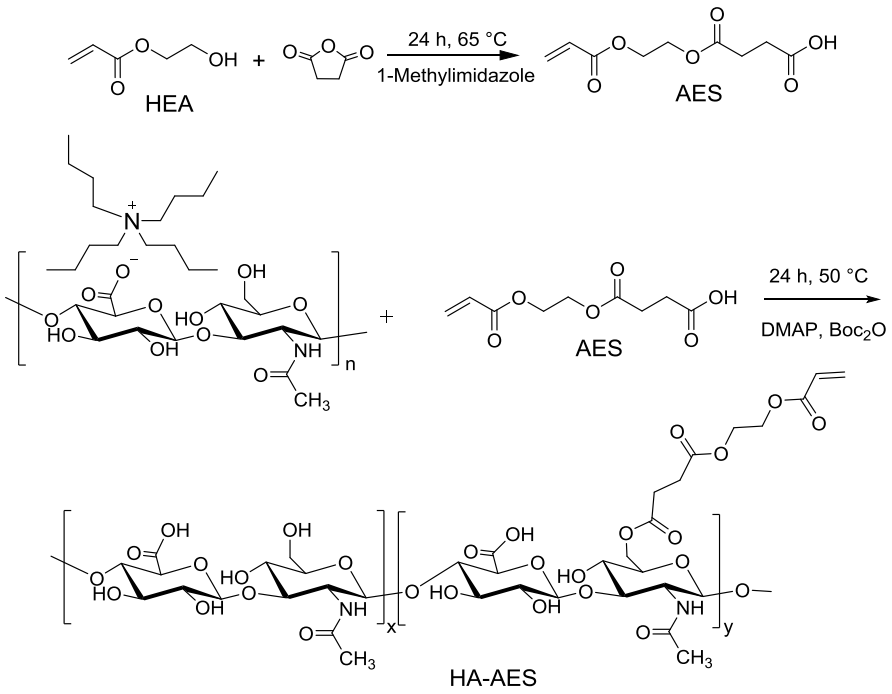
Identification of cell surface CD44. 2D hS/PC cultures were lifted from the culture plate by 15-min incubation with TrypLE™ Express, followed by neutralization with trypsin soybean inhibitor and cell culture media. Cells (1,000,000 cells/mL) were then incubated with either IgG control or APC-conjugated anti-CD44 (0.1 µg/µL) at room temperature in the dark. Fifteen min later, the fluorescently labeled cells were analyzed by flow cytometry (FACSAria II, BD Biosciences) counting 1,000 events/case. All measurements were performed in triplicate and data was analyzed using FlowJo Software (Ashland, OR). Briefly, the characteristic hS/PC populations were gated based on their forward scatter (FSC-A) and side scatter (SSC-A) dot plots, and the CD44-specific fluorescence intensities of individual cells were then plotted as a histogram against the null IgG labeled cells. To quantify cellular expression of CD44 in 3D, the cell/gel constructs were treated with HAase (10 kU/mL) for 4 h at 37 °C and cells were further dissociated using TrypLE™ Express for 15 min. After achieving a single-cell suspension, the flow cytometry protocol described above was used to quantify the CD44 levels.

Table S1. Primary antibodies used for immunofluorescence.

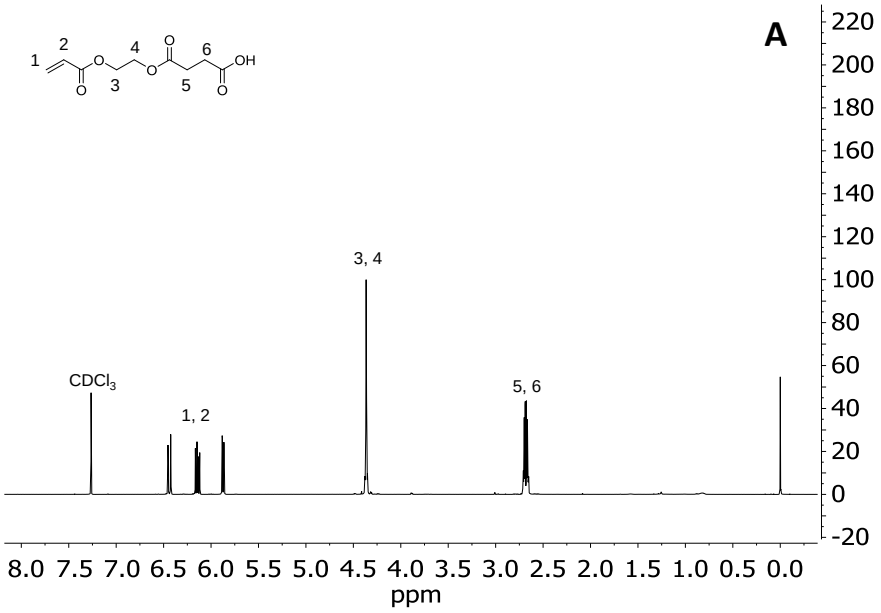
Target protein	Vendor	Item #	Host Species	Clone	Dilution
β-Catenin	Santa Cruz BioTechnology	SC-7963	mouse	Monoclonal (E5)	1:100
Caspase-3 (activated)	Abcam	Ab13847	rabbit	Polyclonal	1:250
Laminin-1	Novus	NB-300-144	rabbit	Polyclonal	1:50
Occludin	Thermo Fisher	33-1500	mouse	Monoclonal (OC-3F10)	1:100
Cytokeratin 5 (K5)	Biolegend	PRB160-P	rabbit	Polyclonal	1:1000
Cytokeratin 14 (K14)	Abcam	Ab49747	mouse	Monoclonal (CKB1)	1:200
CD44	Thermo Fisher	MA4400	rat	Monoclonal (Hermes-1)	1:100
Collagen IV	Abcam	Ab6586	rabbit	Polyclonal	1:100

Table S2. Flow cytometry analysis of CD44 expression by hS/PCs grown on 2D and in 3D DRC18 gels. The characteristic hS/PC population was first gated using FlowJo software based on their sizes. Within the characteristic population, the mean geometric fluorescence intensity of fluorescently labeled CD44 molecules was tabulated.

Sample	Mean Geometric Intensity ±STD
IgG 2D	333 ± 91
CD44 2D	6,351 ±1,051
IgG 3D	280 ± 23
CD44 3D	9,583 ± 1,237



Scheme S1: Synthetic pathways for the preparation of HA-AES.



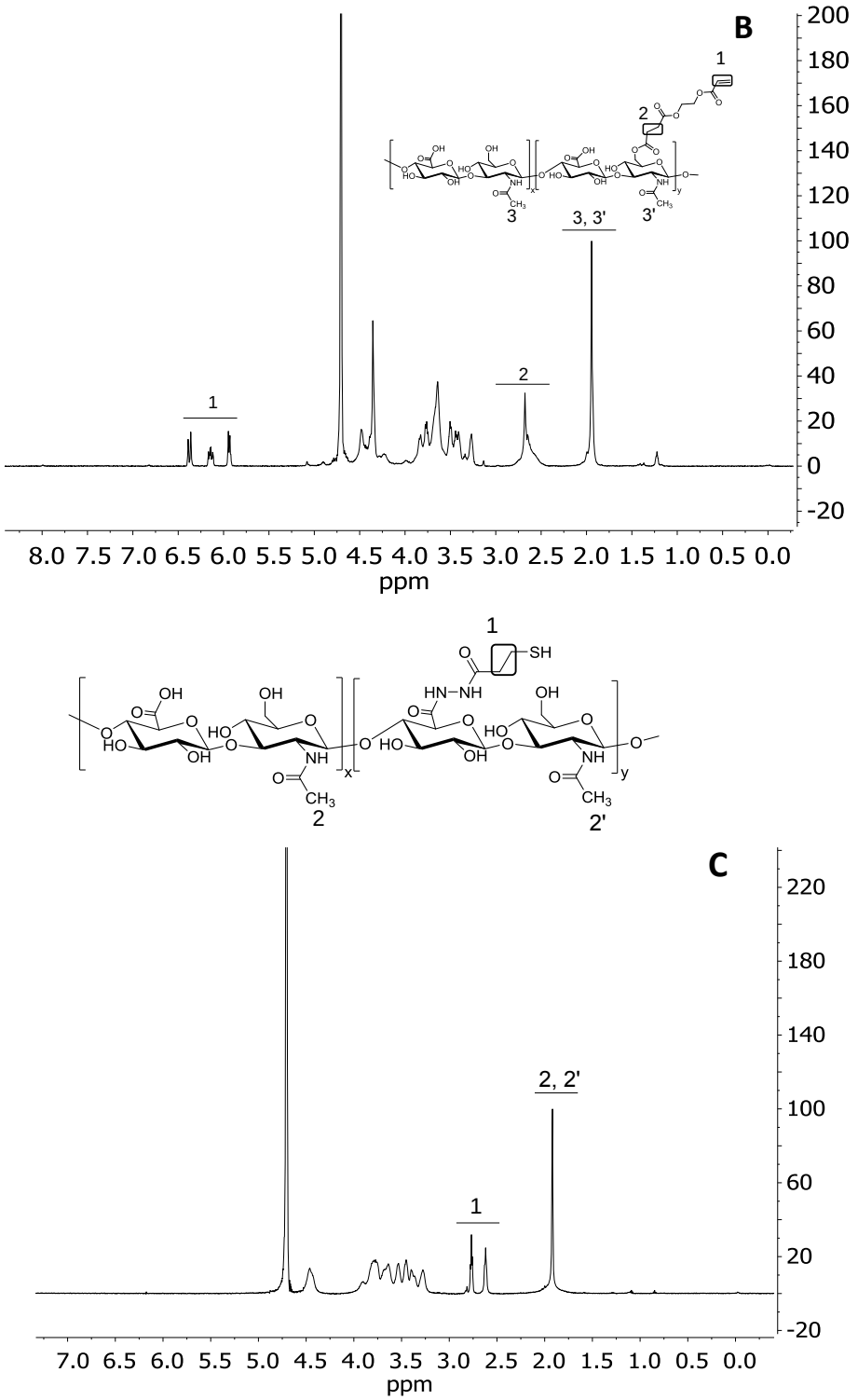


Figure S1. ^{1}H NMR spectra of AES (A, in $CDCl_3$), HA-AES (B, in D_2O) and HA-SH (C, in D_2O)

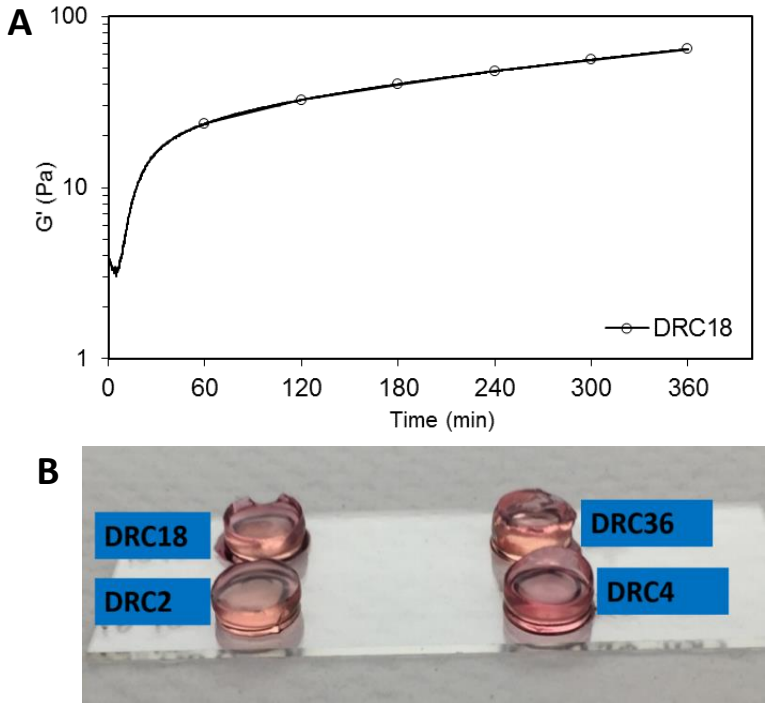


Figure S2. Hydrogel stiffening (A) and construct stability (B). (A) Elastic modulus (G') for DRC18 gels as a function of time. Data shown are from 0 to 6 h post mixing. (B) Representative images of cell-laden DRC gels with various thiol/acrylate molar ratios, designated as DRC2, DRC4, DRC18, DRC36, after 28 days of culture.

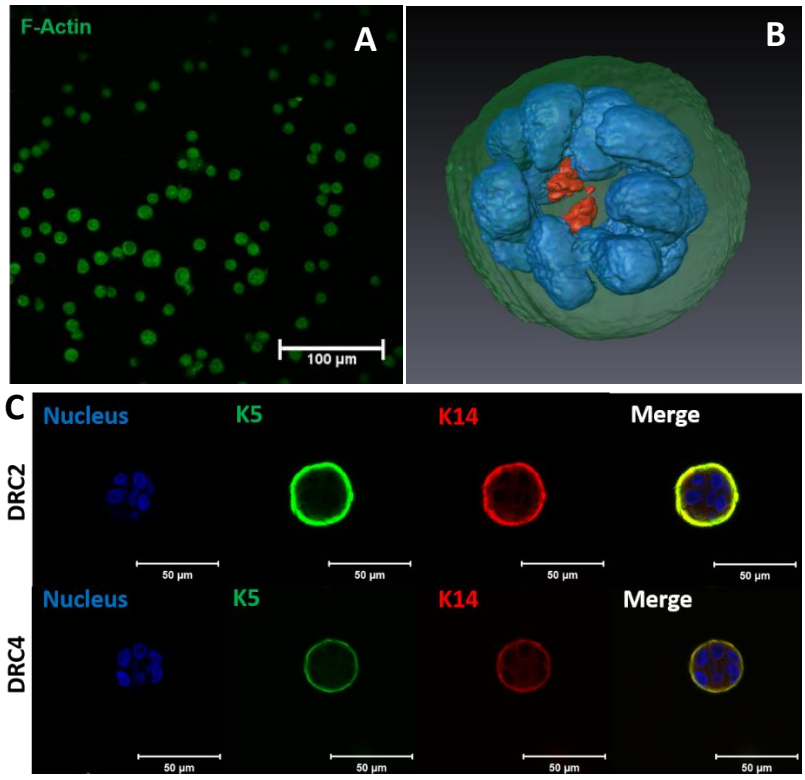


Figure S3. Characterization of hS/PC cultures by fluorescent staining and confocal imaging. (A) Confocal image of hS/PCs grown in HA-SH/HA-AM gels for 28 days. This type of HA gels is conducive to the assembly of tumoroids,[1-2] but does not promote the formation of organized spheroids from dispersed hS/PCs after 28 days of culture. F-actin was stained green. (B). 3D reconstructed confocal image of hS/PC spheroids in DRC18 gels showing the presence of PI-positive dead (red) cells in the core of the hS/PC spheroid. SYTO13 positive live (green) cells comprise most of the spheroid. DAPI positive cell nuclei of live cells are seen in blue. Magnification: 25×. (C) Expression of progenitor markers (K5, green; K14, red) by hS/PCs grown in DRC2 and DRC4 for 28 days. K5/K14 were stained green and red. Cell nuclei was counter stained blue.

References

1. Xu, X.; Gurski, L. A.; Zhang, C.; Harrington, D. A.; Farach-Carson, M. C.; Jia, X., Recreating the tumor microenvironment in a bilayer, hyaluronic acid hydrogel construct for the growth of prostate cancer spheroids. *Biomaterials* **2012,** *33* (35), 9049-60.
2. Xu, X.; Sabanayagam, C. R.; Harrington, D. A.; Farach-Carson, M. C.; Jia, X., A hydrogel-based tumor model for the evaluation of nanoparticle-based cancer therapeutics. *Biomaterials* **2014,** *35* (10), 3319-30.