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Supporting Information

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A Generalizable Strategy for the 3D Bioprinting of Hydrogels from Nonviscous Photo-crosslinkable Inks

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A generalizable strategy for the 3D bioprinting of hydrogels from non-viscous photocrosslinkable inks

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Materials synthesis

Sodium hyaluronate (HA, 75kDa) was purchased from Lifecore (Chaska, MN). Gelatin (type A, cat. G2500) and poly (ethylene glycol) diacrylate (PEGDA, 6kDa, cat. 701963) were purchased from Sigma. Unless otherwise stated, all other chemicals were purchased from Sigma. Synthesized materials were dissolved in deuteroxide to acquire ^{1}H-NMR spectra at 360 MHz (Bruker).

Methacrylated HA (MeHA). HA was converted to MeHA by esterification with methacrylic anhydride (MA). 3 eq MA was added dropwise to aqueous 1 wt% HA solution on ice, adjusting pH to 8 for 6-8 hours. After reacting overnight at 4 ^{o}C, another 3 eq MA was added to the reaction, followed by neutralization to pH ~7-7.5. Modification of MeHA was confirmed with ^{1}H-NMR as ~26% (Figure S10A).

Norbornene-functionalized HA (NorHA). Before NorHA synthesis, HA was first converted to its tetrabutylammonium salt (HA-TBA) to make it soluble in dimethyl sulfoxide (DMSO). HA-TBA was synthesized through adding Dowex 50W proton exchange resin to an aqueous 2 wt% HA solution (3g of resin per 1 g of HA) for a two-hour exchange reaction. The resin was removed by filtration and the filtrate was neutralized to pH ~7.02-7.05 with TBA-OH (6 mL of TBA-OH per 1 g of HA). After freezing at -80 ^{o}C and lyophilization for 4 days, HA-TBA was dissolved in DMSO together with 5-norbornene-2-carboxylic acid (Nor-COOH; a mixture of endo and exo isomers) and 4-dimethylaminopyridine (DMAP). Approximately 20 mL DMSO per 0.1g HA-TBA was used, and reagents were dissolved at a ratio of 3 eq Nor-COOH and 1.5 eq DMAP per 1 eq HA-TBA disaccharide repeat unit. Di-

tert-butyl dicarbonate (BOC2O) was then added (0.4 eq) and the reaction solution heated to 45 °C. The reaction was allowed to proceed for 24 hours, followed by quenching with water, dialysis against water, precipitation against acetone (with 1 g NaCl added per 100 mL NorHA solution), further dialysis, and lyophilization to yield a purified product. Modification of NorHA was confirmed with 1H-NMR spectra as ~22% (Figure S10C).

Gelatin methacryloyl (GelMA). Gelatin methacrylate was prepared through the conjugation of methacrylic anhydride to gelatin as described previously [1]. Gelatin was dissolved in phosphate buffered saline (PBS) at 60 °C. Methacrylic anhydride was added dropwise, and the mixture was stirred for three hours at 60 °C. The reaction mixture was subsequently dialyzed against water at 40 °C for one week. GelMA was then frozen with liquid nitrogen and stored at -80 °C until lyophilization and use. Modification of GelMA was confirmed with ^{1}H-NMR as ~50% (Figure S10B).

Lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP). LAP was synthesized according to published methods [2]. Dimethyl phenylphosphate and 2,4,6-trimethylbenzoyl chloride were combined at an equimolar ratio and stirred for 18 hours at room temperature under an inert atmosphere. A four-fold excess of lithium bromide in 2-butanone was then added and the mixture was heated to 50 °C. A solid precipitate was formed, and the mixture was cooled to room temperature. The precipitate was recovered by filtration and washed with 2-butanone to remove unreacted lithium bromide. Excess solvent was removed by vacuum. The dry product was stored at -20 °C until use.

Peptide synthesis. Peptides used to fluorescently label MeHA (GCKK-fluorescein, GCKK-rhodamine) and to enable MMP-cleavable crosslinking of NorHA gels (GCNS-GGRM ↓ SMPV-SNCG) were synthesized with thiols (cysteine residues), which added to methacrylates in MeHA via Michael addition reactions or to norbornene residues in NorHA via light-initiated thiol-ene chemistry. The italics in the MMP-cleavable sequence indicate the portion of the sequence susceptible to enzymatic cleavage, and the arrow indicates the

cleavage site [3]. Peptides were synthesized using solid-phase peptide synthesis (PS3 automated peptide synthesizer, Protein Technologies, Inc.) off of a glycinol 2-chlorotrityl resin (Novabiochem), using standard FMOC-chemistry. Sythesized peptides were cleaved, deprotected, dissolved in water, lyophilized, and stored at -20 °C until use.

Fluorescent labeling. MeHA was combined in a triethanolamine buffer (pH 10) with a fluorescent peptide (either GCKK-fluorescein or GCKK-rhodamine), such that the molar ratio MeHA:fluor-peptide was approximately 1:2 (moles of full-length MeHA molecule:moles fluorescent peptide). The reaction proceeded at room temperature for 2 hours, after which the solution was neutralized and then purified by dialysis and lyophilization to yield the final product.

Bioprinter and bioprinting process

The adapted 3D bioprinter was a commercially available machine that was modified for extrusion of materials from syringes. A customized printerhead unit with a holder for commercial syringes was incorporated in the printer and was connected via a series gears to the stepper motor used to drive filament extrusion [4]. To apply the in-situ-crosslinking approach introduced in this study, the core modification was the light-permeable needle, which can be generalized to other 3D bioprinters without changing the main hardware framework.

Here, normal straight stainless steel and PTFE dispensing needles (McMaster-Carr) were used as basic needles. As an extended light-permeable part, a glass capillary (Kimble Chase) or silicone tubing (Scientific Commodities) 30 mm in length was inserted onto these needles over the needle tip and were fixed against movement with superglue or a helical cover, respectively. To reduce the extrusion resistence, the glass capillary was perfused with a glass water repellent (Rain-X) for 10 min as hydrophobic surface modification. The disposable silicone tubing was used without modification.

For good control of the filament deposition, the speeds of filament generation (v_{ink}) and nozzle movement (v_{noz}) were matched. v_{ink} was determined by extrusion flux and capillary size and could be measured as Figure S5 showed, while v_{noz} was an input parameter specified by the g-code printing commands. Normally, a velocity of ~0.5 mm/s was used for printing filaments of medium size (~500 μm). The printing process can be altered through variations of these parameters.

For printing of tubes on a rotating rod, v_{ink} and v_{noz} were determined by rod diameter (D), filament diameter (d) and angular velocity of rotor (ω) as indicated in Figure 2B. Two rods of 5 mm and 1.5 mm in diameter were used to coil filaments of ~500 μm and ~200 μm in diameter, respectively. After deposition, the tube was treated with added initiator and UV-irradiation (1~2min) for post-stabalization, followed by immersion in PBS.

Co-axial printing

A normal co-axial needle (Figure S3B, core: 23G, shell: 18G) was used to print core-shell and heterogenerous filaments. Core-shell filaments were generated when both channels were opened, while heterogenerous filaments were generated when flows from the two channels were alternately switched on and off. The ratio of the two inks in the core/shell or along the filament could be tuned by extrusion flux or switch time, respectively. Basically, extrusion flux of 0.1-0.4 mL/hr, switch time of 3-10 s, and silicone tubing of 30 mm in length were used.

A co-axial needle with a 10 mm longer core (Figure S3C, core: 24G, shell: 18G) was used to print hollow filaments. Silicone tubing was applied such that its internal diameter matched the outer diameter of the shell needle, and the tubing was cut so its outlet was even with the outlet of the core needle. Prior to the addition of tubing, the extended part of the core needle was immersed in water repellent (Rain-X) for 10 min to reduce extrusion resistence (Figure S3D). Because of the core needle in the middle, a bifurcated light guide was connected to the lamp for equal light irradiation in order to maintain homogenerous crosslinking (Figure S1D). Typically, extrusion flux of 0.1-0.2 mL/hr was used.

Rheological characterization

Rheological measurements were performed at 25 °C on an AR2000 rheometer (TA Instruments) using a cone-plate geometry (20 mm diameter, 59 min 42 s cone angle, and 27 μm gap). To measure the initial viscosity of the formulations, continuous flow tests were conducted with shear rates from 0.1-500 s^{-1}. To measure the response of rheological properties to photopolymerization, in situ polymerization was performed with UV or visible light for different formulations for 5-10 min via a light-curing stage during oscillatory time sweeps at a frequency 1 Hz and a strain 0.5%.

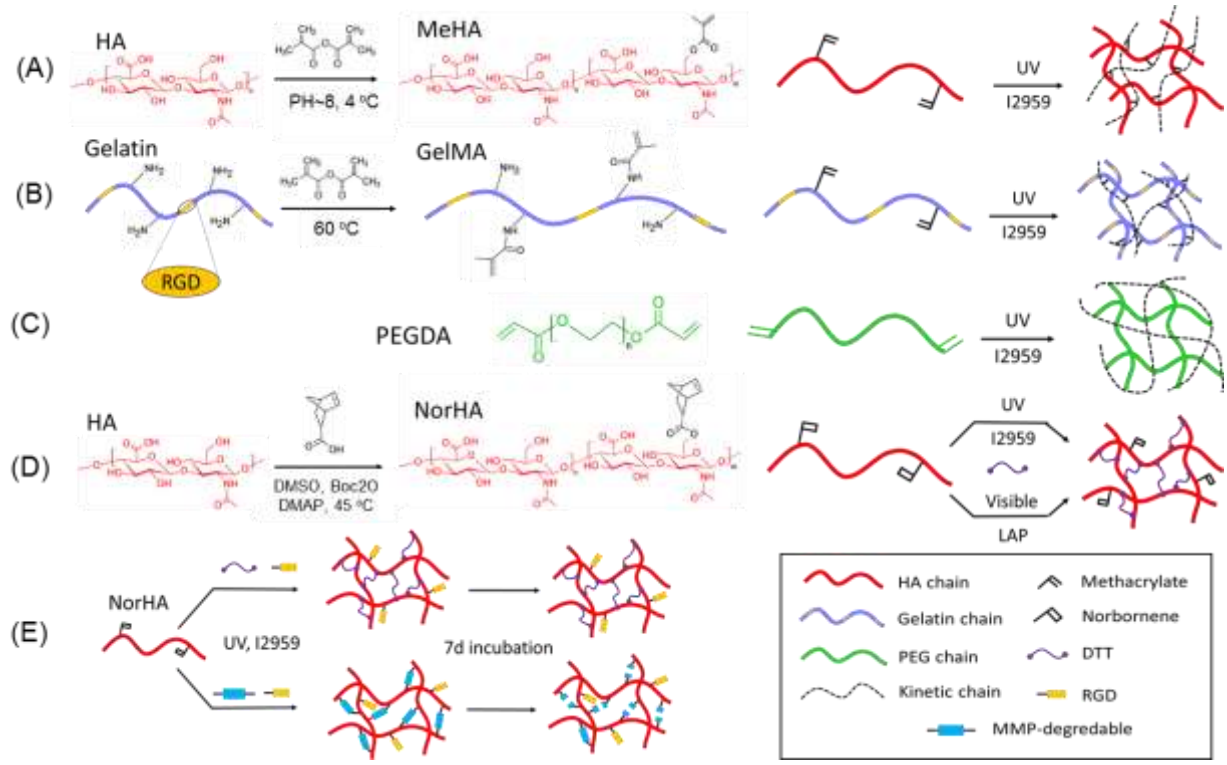


Figure S1. Chemical structures and crosslinking of various bioink formulations. (A) MeHA, where methacrylates were added to HA at pH ~8 on ice for 6-8 hours; (B) GelMA, where reactive groups were added to gelatin at 60°C for 3 hours; (C) PEGDA, where commercial product was used; and (D) NorHA, where norbornenes were added to HA via HA tetrabutylammonium salt (HA-TBA) through reaction in DMSO in the presence of Boc2O for 2 hours. (A-C) and (D) used radical chain-growth polymerizations and thiol-ene crosslinking, respectively. (D) For NorHA crosslinking, both UV light with I2959 initiator and visible light with LAP initiator were used. (E) NorHA formulation with non-degradable (DTT) and degradable (MMP-degradable) crosslinkers and with RGD modification.

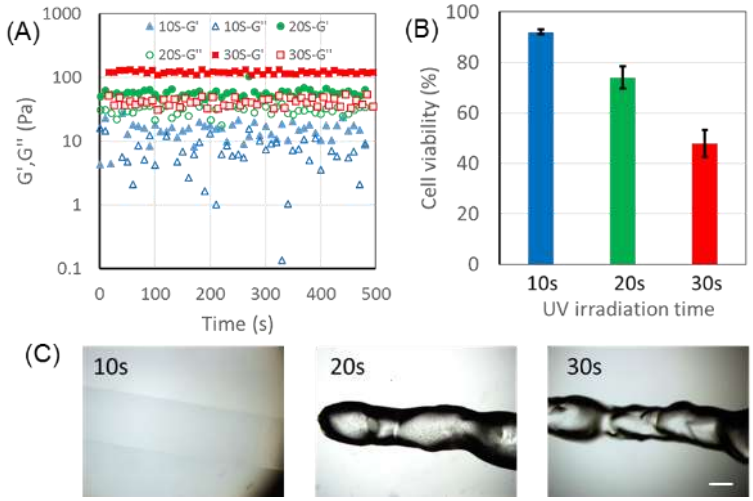


Figure S2. Illustration of pre-crosslinking approach. (A) Time sweeps of storage modulus (G') and loss modulus (G'') for 5 wt% MeHA treated with pre-UV-irradiation for 10, 20, and 30 seconds. (B) Quantified cell viability and (C) microscopy images of printed filaments using 5 wt% MeHA with pre-UV-irradiation. UV intensity of 10mW/cm^2 was used. Scale bar is 500 μm.

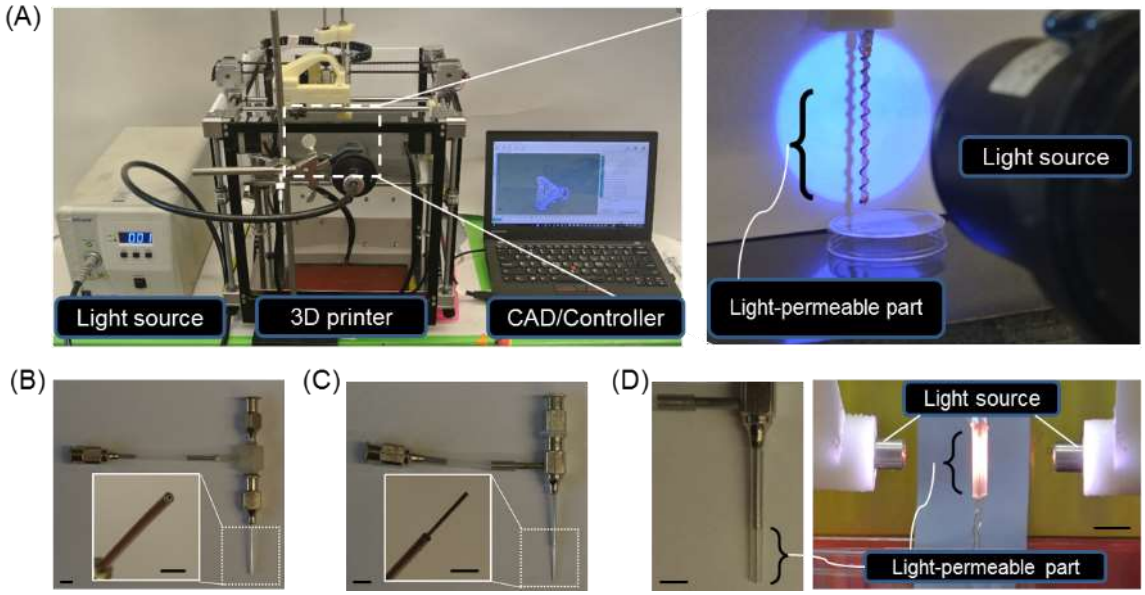


Figure S3. Set-up of printer and nozzle systems. (A) Set-up of 3D bioprinter with curing light unit and CAD/controller (left), where the light was introduced to the light-permeable capillary of the nozzle (right). (B) Co-axial nozzle with the same length for core and shell needles. (C) Co-axial nozzle with 10 mm longer core needle. (D) Set-up for fabricating hollow filaments using nozzle in (C), where the light-permeable capillary was matched to cover the longer part of the core needle, leaving a shell space between the core needle and light-permeable capillary. A bifurcated light guide was used to shine light on both sides of the photopermeable capillary for homogeneous irradiation. Scale bars are 5 mm.

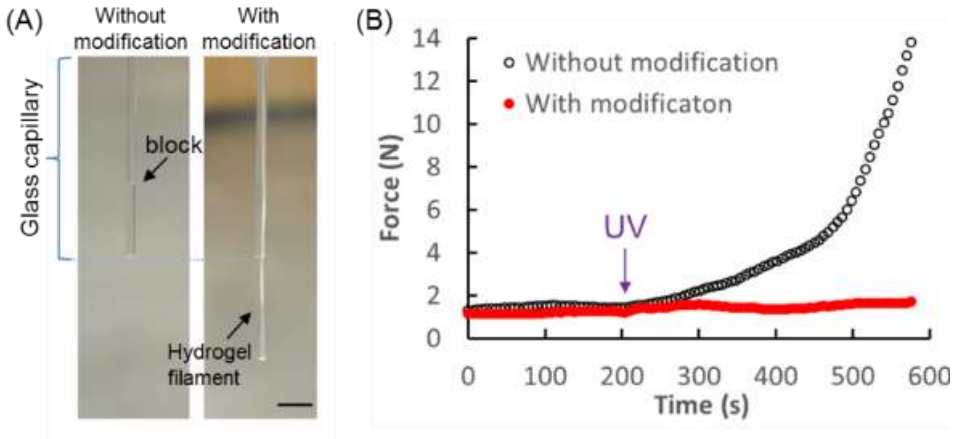


Figure S4. Demonstration of glass capillary for printing. (A) Extrusion status and (B) driving force for printing 5 wt% MeHA using a glass capillary with or without hydrophobic modification. (A) The bioink was finally blocked in an unmodified capillary, while a hydrogel filament was generated with modified capillary. (B) The time point when UV irradiation (10mW/cm^2) was applied was indicated as arrow shown. Scale bar is 5 mm.

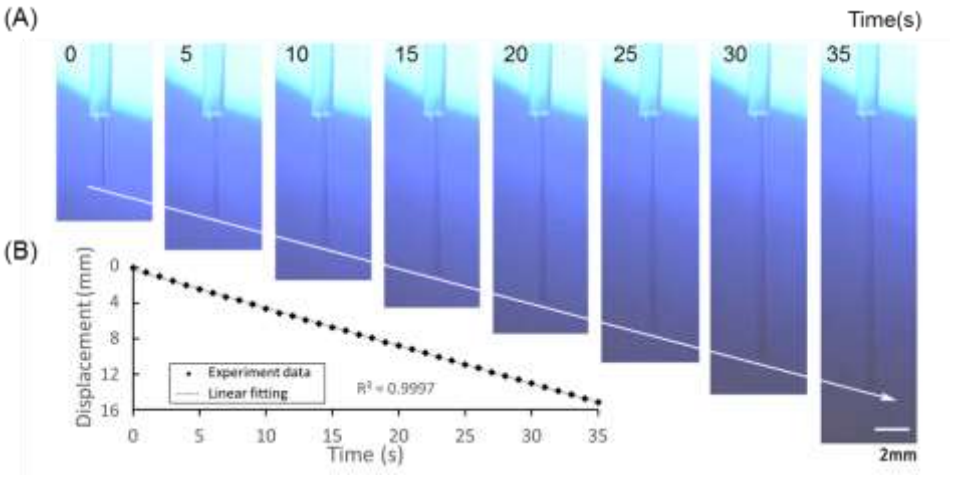


Figure S5. Demonstration of consistent filament generation under 10 mW/cm^2 UV irradiation. (A) Images of 5 wt% MeHA filaments during printing process indicate the continuous generation of filaments with time. (B) Relationship of filament displacement and printing time with linear fitting, indicating a stable filament printing speed. Medium silicone tubing and extrusion flux of 0.2 mL/hr was used. Scale bar is 2 mm.

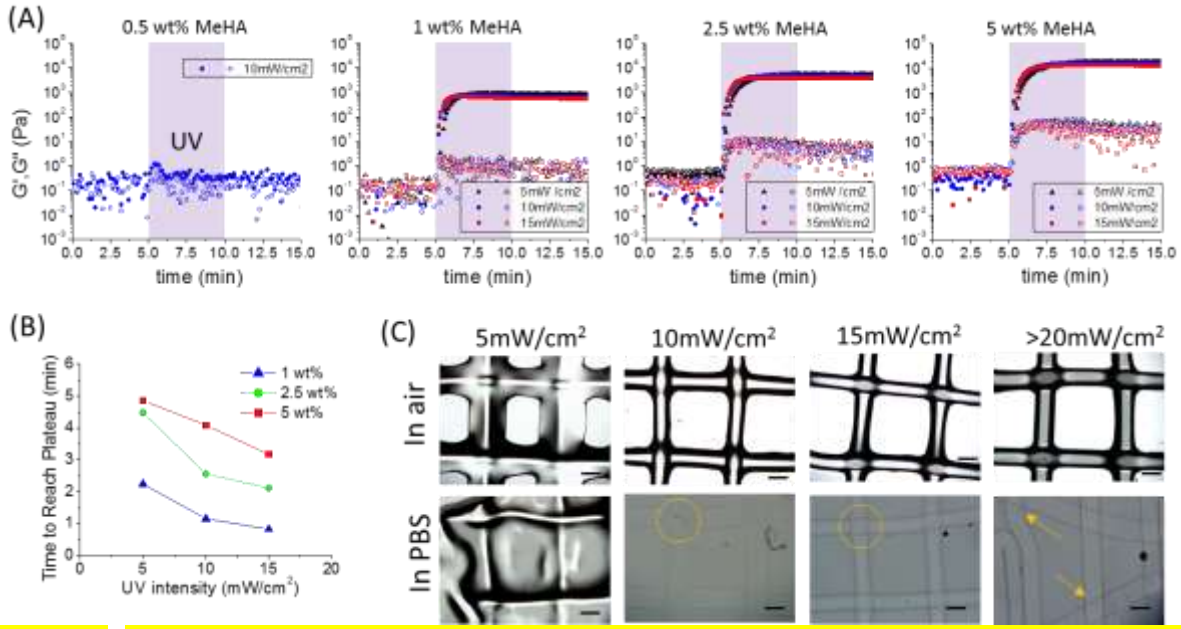


Figure S6. (A) Rheological profiles during the polymerization of MeHA bioinks with varied concentrations and UV light intensities. The low MeHA concentration of 0.5 wt% did not undergo gelation. (B) Quantification of time to reach a plateau storage modulus (G') for various MeHA concentrations and UV light intensities. (C) Lattice structures were printed under different UV intensities with 1 wt% MeHA and visualized before and after being immersed in PBS. Filaments with 5 mW/cm^2 were not polymerized well; however, co-adhesion was observed as indicated (circle) with 10 and 15 mW/cm^2, but did not occur (arrow) with more than 20 mW/cm^2. Scale bars are 500 μm.

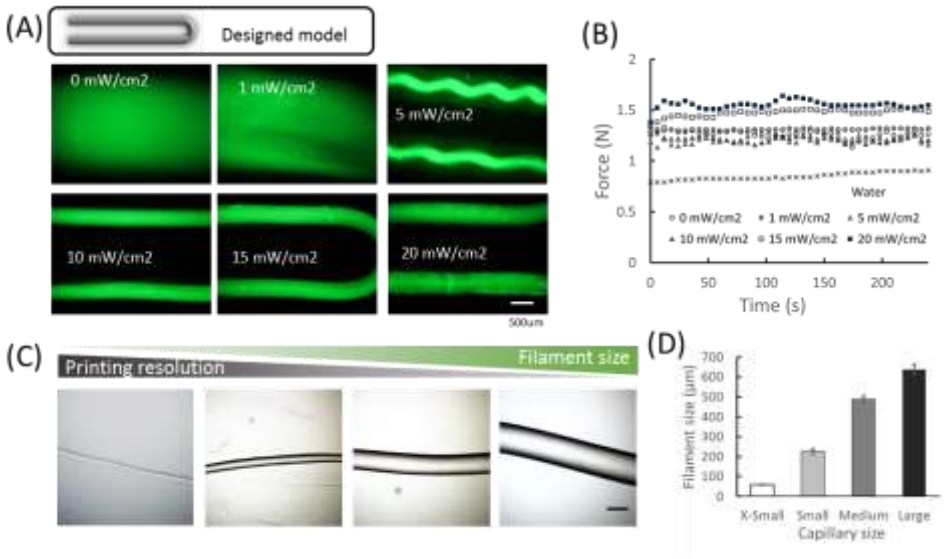


Figure S7. (A) Fluorescence images of printed filaments and (B) driving force consistency with varied UV intensity. (C) Microscopy images and (D) quantified diameter of printed filaments using a photopermeable capillary of varied size (with UV intensity of 10 mW/cm^2). 5 wt% MeHA was used. Scale bars are 500 μm.

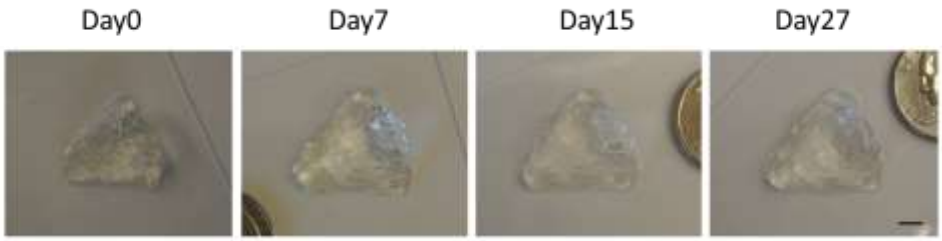


Figure S8. Images of printed nose at different time points of incubation in PBS at room temperature after fabrication. 5 wt% MeHA was used. Scale bar is 5 mm.

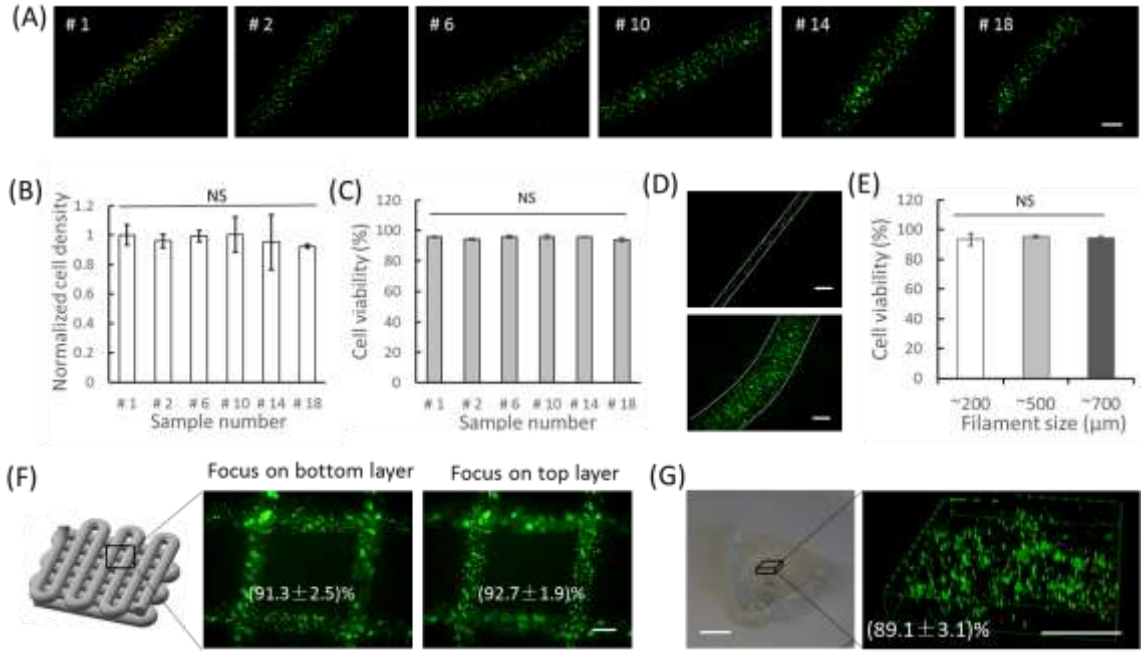


Figure S9. Cell viability study at day 0. (A) Live/dead stained images of cell-laden filaments printed at different time points within 40 min (equal time interval for samples). (B) Normalized cell density and (C) quantified cell viability for different samples based on (A). (D) Live/dead stained images of smaller and larger cell-laden filaments. (E) Quantified cell viability for filaments with three different sizes. Cell viability was also measured for printed (F) lattice structures and (G) a macro nose structure. (A-E) used 5 wt% MeHA, (F-G) used 2.5 wt% MeHA, and a UV intensity of 10mW/cm^2 was used. Scale bars are 500 μm.

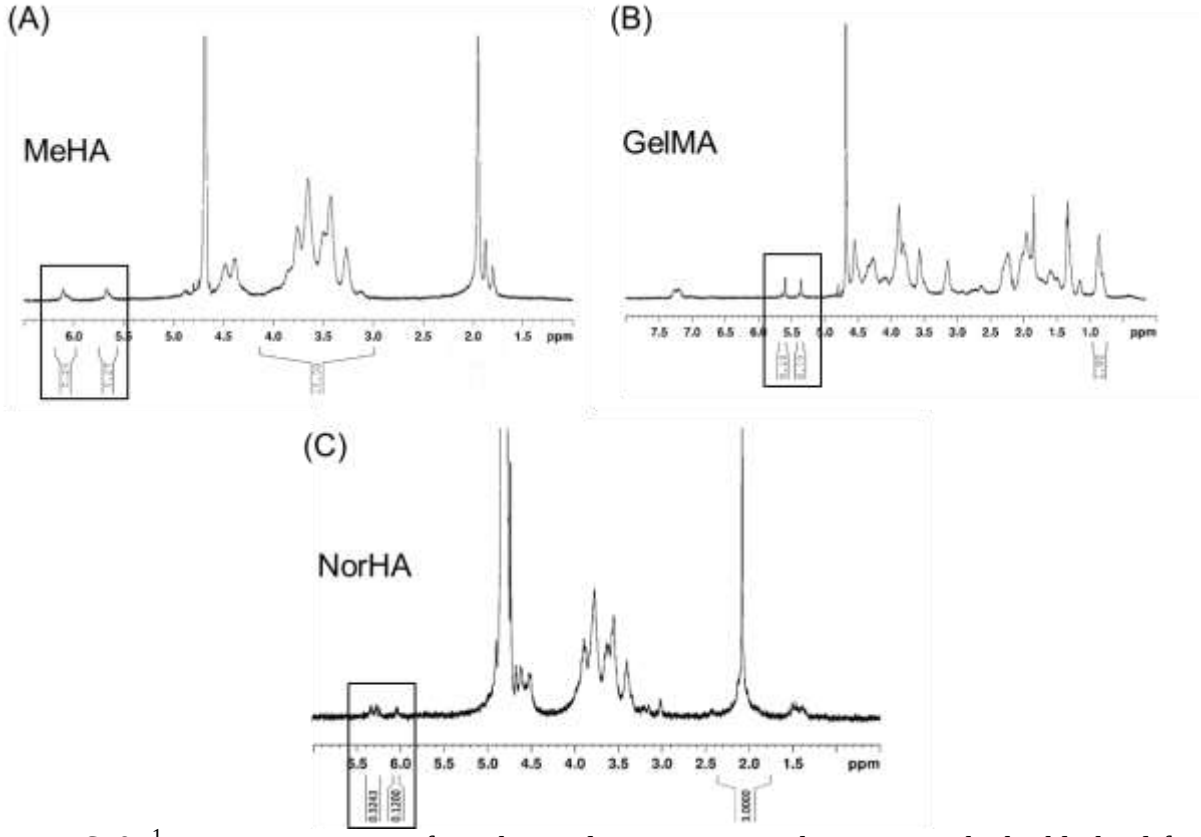


Figure S10. ^{1}H-NMR spectrum of synthesized macromers with proton peaks highlighted for determining (A) methacrylate modification in MeHA, (B) methacrylamide modification in GelMA, and (C) norbornene modification in NorHA.

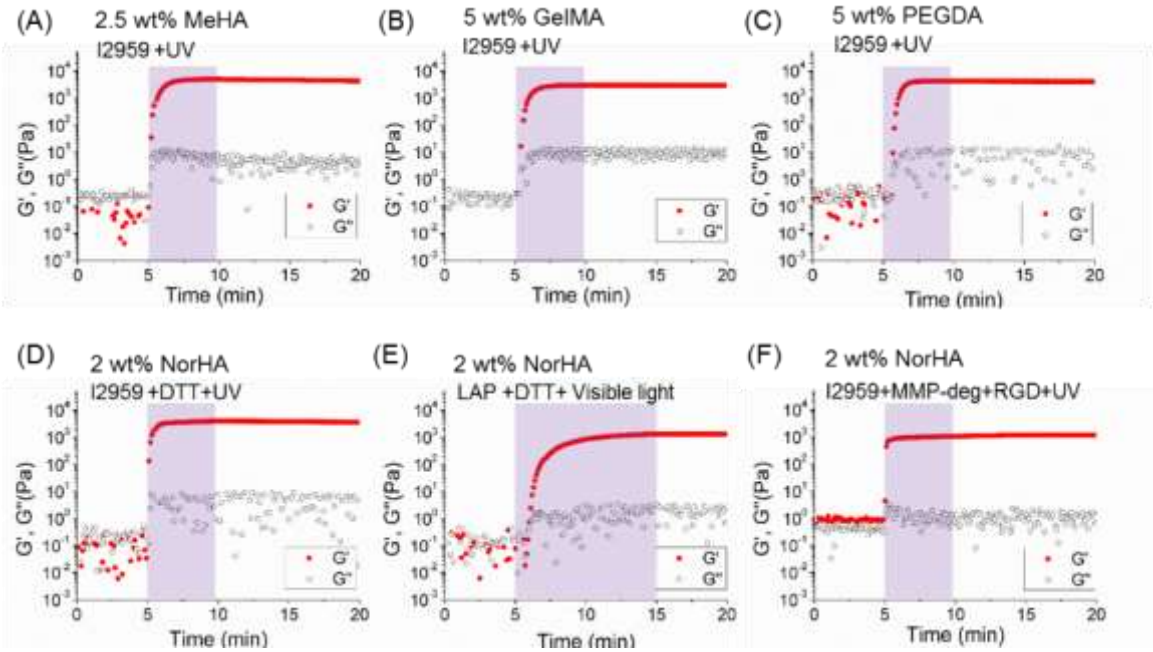


Figure S11. Rheological profiles during the polymerization of bioinks with UV or visible light. (A-D, F) used 0.05% I2959 and 10 mW/cm^2 UV light. (E) used 0.05% LAP and 15 mW/cm^2 visible light. (D-E) used X_{DTT}=0.6. (F) used $X_{MMP\text{-}deg}$=0.6. Shaded area indicated the light irradiation duration. Details about the formulations are indicated in Table S1.

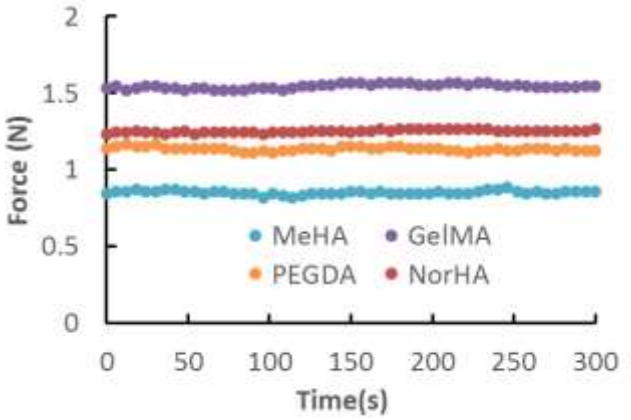


Figure S12. Driving force for printing different ink formulations: 2.5 wt% MeHA, 5 wt% GelMA, 5 wt% PEGDA and 2 wt% NorHA (DTT+I2959).

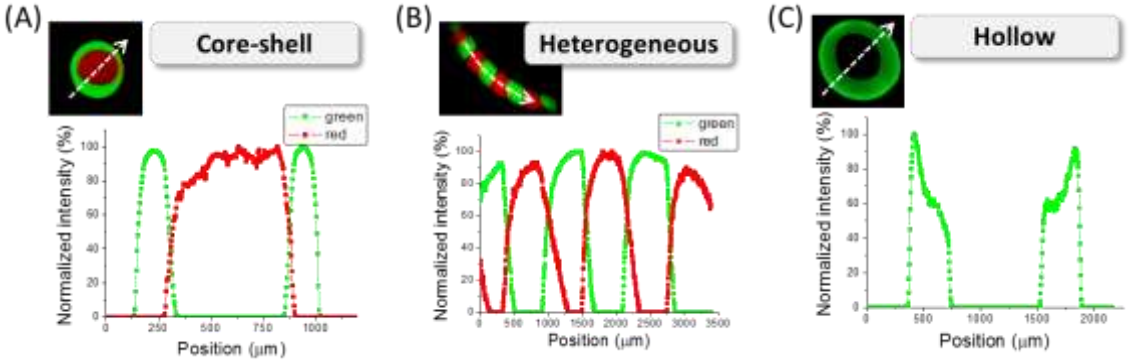
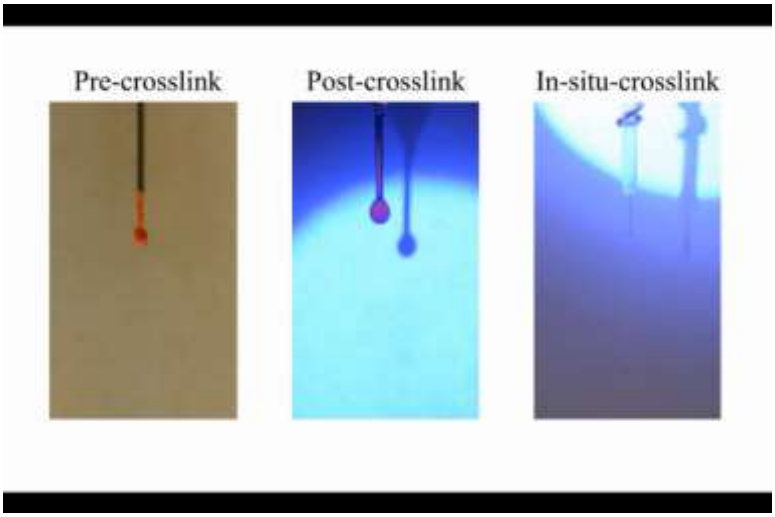


Figure S13. Fluorescence signal intensity profiles along white dot arrow for (A) core-shell, (B) heterogeneous, and (C) hollow filaments.

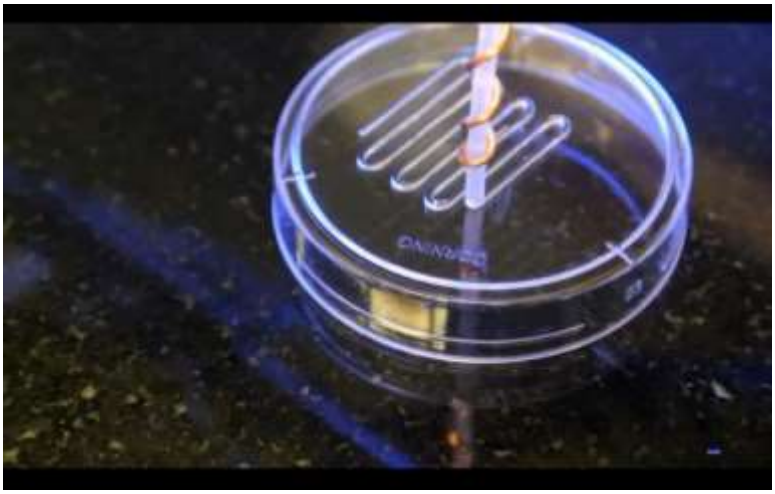
Table S1. Details of formulations and their initial viscosities.

Formulation	Raw	Initiator	Crosslinker	RGD	Light	Initial viscosity (mPa s)
MeHA-5%	5 wt% MeHA	0.05% I2959	/	/	0-20mW/cm^2 UV	73.4±1.1
MeHA-2.5%	2.5 wt% MeHA	0.05% I2959	/	/	10mW/cm^2 UV	15.1±0.3
GelMA	5 wt% GelMA	0.05% I2959	/	/	10mW/cm^2 UV	5.1±0.7
PEGDA	5 wt% PEGDA	0.05% I2959	/	/	10mW/cm^2 UV	2.5±0.6
NorHA (DTT+I2959)	2 wt% NorHA	0.05% I2959	X_{DTT}=0.6	/	10mW/cm^2 UV	9.9±0.5
NorHA (DTT+LAP)	2 wt% NorHA	0.05% LAP	X_{DTT}=0.6	/	15mW/cm^2 visible	9.3±0.3
NorHA (DTT+I2959+RGD)	2 wt% NorHA	0.05% I2959	X_{DTT}=0.6	3mM	10mW/cm^2 UV	8.9±0.6
NorHA (MMP-deg+I2959+RGD)	2 wt% NorHA	0.05% I2959	$X_{MMP\text{-}deg}$=0.6	3mM	10mW/cm^2 UV	9.9±0.7

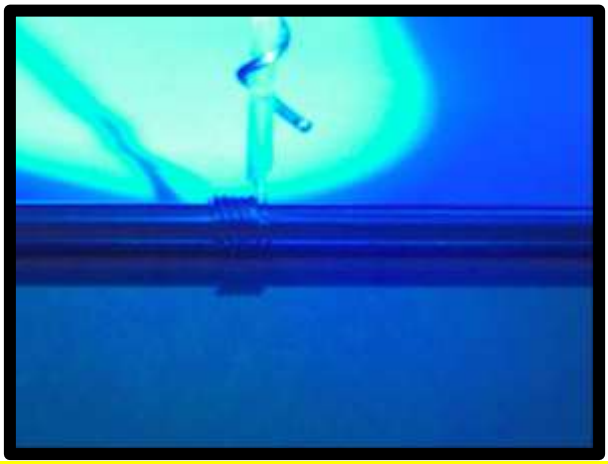
Note: The initial viscosity was obtained under shear rate of 10 s^{-1}.



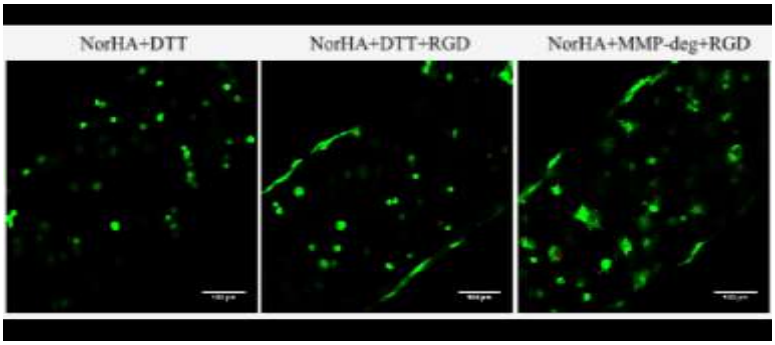
Movie S1. Injection of 5 wt% MeHA using: i) pre-crosslinking, where inks came out as an irregular filament with obvious fracture; ii) post-crosslinking, where inks came out drop-by-drop; and iii) in-situ-crosslinking, where inks formed a regular filament continuously. Pre-UV-irradiation of 30 seconds was applied in pre-crosslinking, while UV light was continuously shined under the needle or at the light-permeable capillary for post-crosslinking or in-situ-crosslinking, respectively.



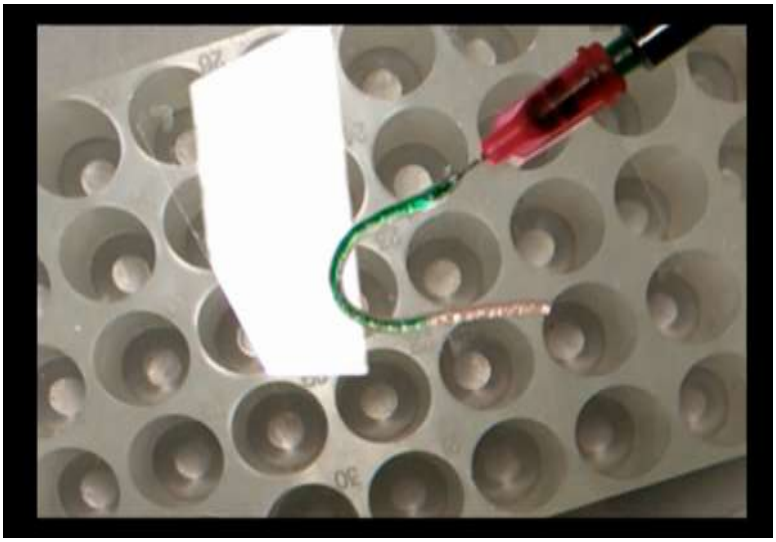
Movie S2. Snapshots of printing of line structure and a hollow tube structure. In the end of the movie, PBS was added to the hollow tube to test the structure stability. 5 wt% MeHA was used.



Movie S3. Video of filament deposition on a rotating rod for fabricating hollow tube structures. 2.5 wt% MeHA was used. Video was 12 times faster than realtime.



Movie S4. Cell fluorescence images for different formulations from bottom to top with a z-stack confocal scanning of cell-laden filaments. Cell morphology was clearly observed throughout the filament, including the inner and near-surface parts. The details of these formulations were indicated in Table S1.



Movie S5. Perfusion of a printed hollow filament, which was printed using a co-axial needle with a longer inner needle (Figure S3C-D). Green dye was perfused through the filament smoothly, which demonstrated the open channel along the filament and closed radial shell. 2.5 wt% NorHA with DTT crosslinker was used.

Supplementary References

[1] A. I. Van Den Bulcke, B. Bogdanov, N. De Rooze, E. H. Schacht, M. Cornelissen, H. Berghmans, *Biomacromolecules* 2000, 1, 31.
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