Cell Microencapsulation Within Engineered Hyaluronan Elastin-Like Protein (HELP) Hydrogels

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Three-dimensional cell encapsulation has rendered itself a staple in the tissue engineering field. Using recombinantly engineered, biopolymer-based hydrogels to encapsulate cells is especially promising due to the enhanced control and tunability it affords. Here, we describe in detail the synthesis of our hyaluronan (i.e., hyaluronic acid) and elastin-like protein (HELP) hydrogel system. In addition to validating the efficacy of our synthetic process, we also demonstrate the modularity of the HELP system. Finally, we show that cells can be encapsulated within HELP gels over a range of stiffnesses, exhibit strong viability, and respond to stiffness cues. © 2023 Wiley Periodicals LLC.

Basic Protocol 1: Elastin-like protein modification with hydrazine
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Keywords: cell encapsulation • dynamic covalent chemistry • elastin-like protein • hyaluronan • hydrogel

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INTRODUCTION

The encapsulation of various cell types in 3D hydrogels, whether it be in the form of individual cells, cell clusters (Du & Yarema, 2014), or organoids (Hunt et al., 2021), has transformed the field of tissue engineering and regenerative medicine and portends further advancements in cell-based transplantation therapies and disease modeling. Reproducibility challenges that often accompany 3D hydrogels isolated from natural sources, such as decellularized matrices (Hughes et al., 2010), have prompted the development of fully synthetic 3D hydrogel networks. Encapsulating cells in these hydrated,

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3D-crosslinked polymer networks provides a mechanism to study how the 3D microenvironment can influence cell morphology and behavior, while maintaining tight control over the experimental parameters, as is characteristic of 2D *in vitro* culture (Khetan & Burdick, 2009). The biomechanical properties of 3D hydrogels more closely recapitulate the natural extracellular matrix (ECM) compared to tissue culture polystyrene. Additionally, 3D hydrogels can be tuned to match parameters such as stiffness and diffusivity and permit cell–cell and cell–ECM interactions that are more characteristic of native tissue (Geckil et al., 2010). Applications of 3D cell encapsulation range from cell delivery for *in vivo* transplantation and localization (Youngblood et al., 2018) to developing models for *in vitro* drug screening (Li & Kumacheva, 2018). Moving forward, an ideal hydrogel system would allow for reproducible mimics of the native ECM, which includes both extracellular proteins and polysaccharides (Frantz et al., 2010). Toward this goal, here we provide detailed protocols for a fully recombinant, reproducible 3D hydrogel developed by our team that includes both protein and polysaccharide components for encapsulation of cells.

Recombinant materials possess desirable traits of both natural and synthetic polymers (Werkmeister & Ramshaw, 2012). They integrate the inherent biocompatibility of natural polymers with the enhanced control and reproducibility of synthetic polymers. Protein engineering constitutes a major portion within the domain of recombinant materials. This is due largely in part to the ability to refine properties inherent to naturally occurring proteins, such as self-assembly in response to certain stimuli (e.g., temperature, ion concentration), susceptibility to degradation, and ability to achieve high biocompatibility and low immunogenicity (Schloss et al., 2016). Elastin-like proteins (ELPs) are recombinant proteins modeled after the naturally occurring protein elastin that is present in connective tissue. ELPs are modular, and therefore amino acid sequences from other ECM proteins can be engineered into the sequence (Straley & Heilshorn, 2008). Here, we have incorporated an extended cell-adhesive, fibronectin-derived sequence that includes the RGD integrin-binding ligand. However, this strategy can be replicated with other ECM proteins such as laminin and collagen (Lin & Liu, 2016). Although recombinant materials have focused primarily on protein engineering, the utilization of recombinant hyaluronic acid (HA) has also been used at great length in biomaterials (Liu et al., 2011). Although HA is often selected for its biocompatibility and biodegradability, it possesses other advantageous characteristics, such as its ability to directly signal to cells through interactions with their surface receptors (Garantziotis & Savani, 2019). When recombinant proteins and recombinant HA are combined, signaling can be elicited from both components to exert more precise control over cell behavior. Thus, designing a hydrogel system comprising both ELP and recombinant HA allows for a more versatile and realistic ECM mimic.

Both ELP and HA can be modified in such a way that they form hydrogels that are held together by dynamic covalent crosslinks (DCCs; Wang et al., 2017). DCCs are characterized by their ability to rapidly and reversibly form and break under equilibrium control. This is in contrast to many chemical systems that form through traditional covalent bonds that are irreversible (Rowan et al., 2002). This crosslinking scheme is advantageous for a multitude of reasons. In our case, it eliminates the need for potentially cytotoxic crosslinkers and does not require external stimuli (such as ionic concentrations or changes in pH) that are outside the boundaries of physiologic conditions (Wang et al., 2017). These gels can exhibit shear-thinning and self-healing properties and undergo stress relaxation in a manner similar to the native ECM (Lou et al., 2018). Gels that are based on DCCs can be tailored to be injectable, making them promising candidates for the delivery of drugs and other therapeutics (Suhar et al., 2022). The structural domain of ELP contains repeats of the elastin consensus repeat sequence VPGXG, where X represents a guest residue. At this position, lysine residues have been engineered into the

Table 1 Summary of Recommended Percent Modification of HA-Alkyne and HA-BZA and Final Concentration of HA-BZA and ELP-HYD Based on Desired Gel Stiffness[a]

Desired gel stiffness, Pa	HA-alkyne modification, %	HA-BZA modification, %	Final HA-BZA concentration, % w/v	Final ELP-HYD concentration, % w/v	Final RGD concentration, mM
300	12	6	1	1	1
800	12	12	1	1	1
1500	30	30	1	1	1
3000	30	30	1	2[b]	1

BZA, benzaldehyde; ELP-HYD, elastin-like protein–hydrazine; HA, hyaluronic acid.
[a]With this hydrogel system, the final concentration of the integrin-binding domain can be maintained while the gel stiffness is altered.
[b]1% (w/v) ELP-RGD-HYD and 1% ELP-RDG-HYD.

sequence (Straley & Heilshorn, 2008). These lysine residues are chemically modified with hydrazine moieties that can form DCCs with benzaldehyde moieties that have been conjugated to the backbone of HA. Collectively, we refer to this HA- and ELP-based viscoelastic hydrogel system as HELP. In this HELP system, the mechanical properties can be controlled by modulating the weight percent of the polymers and/or by controlling the degree of functionalization of HA. Independently, the concentration of integrin-binding ligands can be controlled by changing the amino acid sequence of the ELP (Table 1) to introduce a scrambled, non-cell-adhesive domain.

The expression and purification of ELP has previously been reported in detail (LeSavage et al., 2018). In summary, engineered plasmids are transformed into the BL21(DE3) strain of *Escherichia coli*, and protein expression is induced using β-isopropyl thiogalactoside under control of the T7-lac promoter. Since the elastin-like domain is hydrophobic, inverse phase transition cycling can be implemented for purification by alternating centrifugation steps at temperatures below and above the lower critical solution temperature of ELP (4°C and 37°C, respectively). Should an additional level of purification be desired, the ELP was also designed to contain a histidine tag to facilitate resin purification. The resulting ELP solution is dialyzed at 4°C, lyophilized, and then stored in this fibrous state (Straley & Heilshorn, 2008). The procedures outlined in the upcoming methods are written with the consideration that this lyophilized ELP fiber is a starting material. Thus, we begin by detailing the functionalization of the lysine residues present on the elastin-like domain of the protein with hydrazine groups to produce an ELP-hydrazine (ELP-HYD) product (Fig. 1A; Basic Protocol 1).The degree of functionalization is quantitatively confirmed using nuclear magnetic resonance (NMR; Basic Protocol 2).

We also describe the procedure for the chemical modification of HA to HA-benzaldehyde (HA-BZA; Basic Protocol 3). This modification is carried out in a two-step process, where we first modify HA with propargylamine to produce HA-alkyne (Fig. 1B). This is followed by the copper-mediated click chemistry cycloaddition of azido-benzaldehyde (azido-BZA) to HA to yield HA-BZA (Fig. 1B). Different percent modifications of HA-alkyne and HA-BZA can be achieved by altering the equivalents of propargylamine and azido-BZA, respectively. Modulating the degree of functionalization of either HA-alkyne or HA-BZA will tune the resulting gel mechanical properties for the desired application of the material (Table 1). The degree of functionalization can be quantitatively confirmed using NMR (Basic Protocol 4).

Mixing ELP-HYD with HA-BZA forms a hydrogel crosslinked together by dynamic covalent hydrazone bonds (Fig. 1C). Along with altering the degree of HA modification, hydrogel stiffness can be controlled by changing ELP concentration (Table 1), while keeping the concentration of integrin ligands constant. For cell microencapsulation, cells are premixed with ELP-HYD and added to molds containing HA-BZA to form a 3D

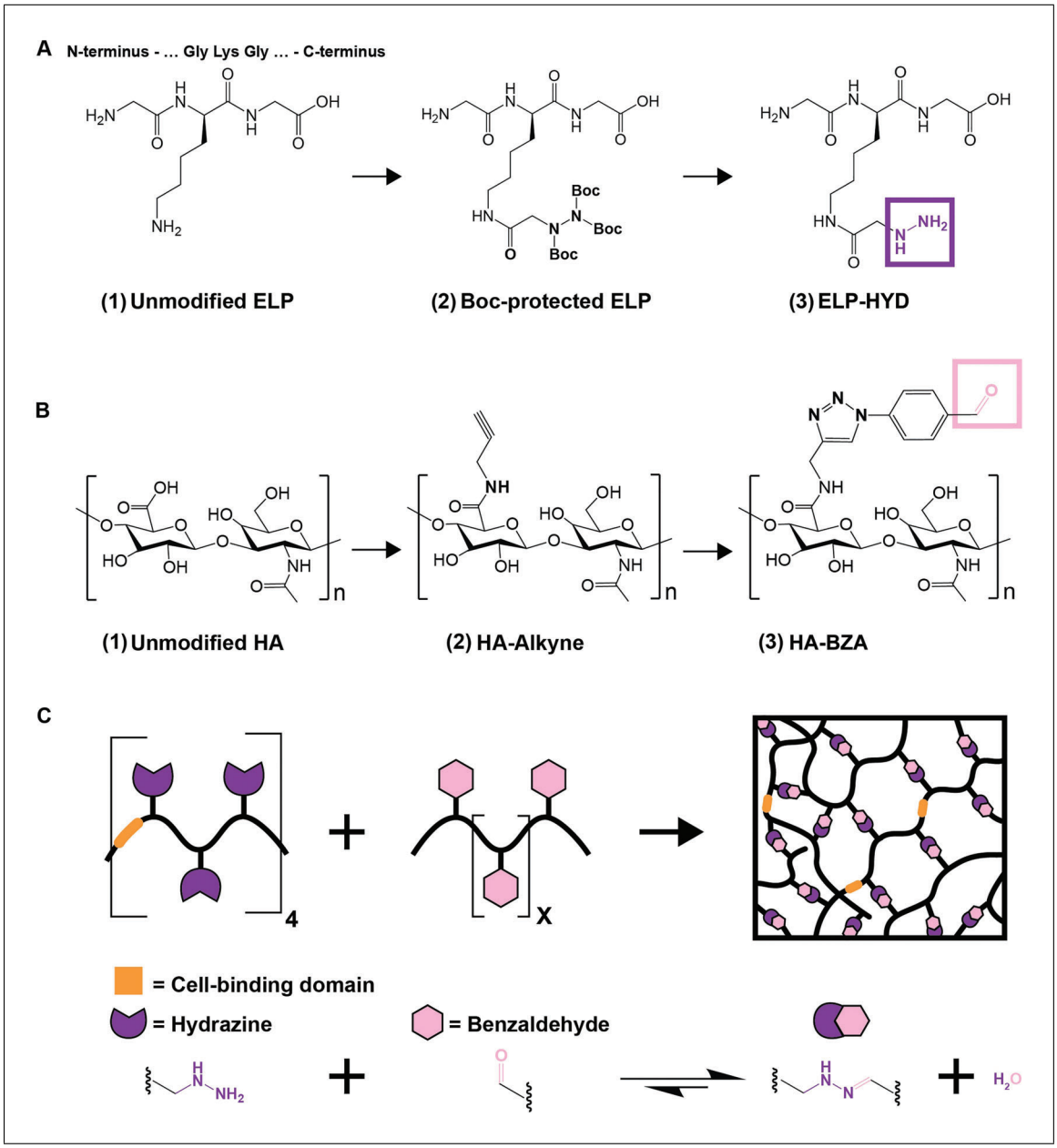

Figure 1 Overview of chemical modifications and crosslinking scheme for the hyaluronan elastin-like protein (HELP) system. (**A**) Lysine groups on unmodified ELP (1) are functionalized through a hexafluorophosphate azabenzotriazole tetramethyl uronium–mediated amidation, adding a Boc-protected hydrazinoacetic acid to the protein (2), which is then deprotected using trifluoroacetic acid:dichloromethane to yield a hydrazine (ELP-HYD) moiety (3). (**B**) A fraction of the carboxylic acid moieties on unmodified hyaluronic acid (HA) (1) are first converted to alkyne groups using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide chemistry (2). Then, copper click chemistry is used to conjugate a benzaldehyde (HA-BZA) moiety to the alkyne (3). (**C**) Hydrazine moieties on the ELP form reversible hydrazone dynamic covalent crosslinks with the benzaldehyde moieties on the HA, creating a polymer network.

culture (Basic Protocol 5). We further demonstrate that, in addition to being a hydrogel system with tunable mechanical properties, HELP gels are cyto-compatible and exhibit high viability of encapsulated cells. Not only do these encapsulated cells survive, but they also display expected differences in morphologies based on the given stiffness of the gel in which they are encapsulated. This modularity of the HELP system makes it well suited for studies of cell interactions with their microenvironment.

As a general note, to prevent condensation, allow any refrigerated or frozen reagents to come to room temperature before opening. Before use, rinse any glassware to be used as reaction vessels with acetone three times (disposing waste in an appropriate container), and then dry with nitrogen gas. Then, wash with soap and water, and rinse with ultrapure water. In our laboratory, the Millipore Milli-Q Advantage A10 Water Purification System was used to produce ultrapure water with a resistivity of 18.2 MΩ•cm at 25°C. In general, try to use a flask or reaction vessel that can hold approximately double the necessary solvent volume. When degassing/purging steps are required, use nitrogen gas. Insert a needle to function as an exit line before starting nitrogen flow. In the laboratory, these reactions have been run on starting amounts ranging from 50 mg to upward of 1 g with consistent results.

ELASTIN-LIKE PROTEIN MODIFICATION WITH HYDRAZINE

BASIC PROTOCOL 1

This protocol details the modification of ELP with hydrazine reactive groups so that the ELP can crosslink with modified HA. It is written for 300 mg ELP; therefore, scale appropriately. Make sure to use nitrogen-flushed syringes to withdraw dimethyl sulfoxide (DMSO) and dimethylformamide (DMF). DMF is not compatible with pipette-gun serological pipettes, so do not use these for transferring. Only use glass Pasteur pipettes to transfer dichloromethane (DCM), trifluoroacetic acid (TFA), and ether. This protocol is divided into two parts. In part 1, the ELP is modified with Boc-protected hydrazinoacetic acid. In part 2, the Boc protective groups are removed to yield the final ELP-HYD product.

Materials

ELP (prepared as described in LeSavage et al., 2018)
DMSO, anhydrous (e.g., Sigma-Aldrich, cat. no. 276855)
DMF, anhydrous (e.g., Sigma-Aldrich, cat. no. 227056)
Tri-Boc hydrazinoacetic acid (tri-Boc; e.g., Sigma-Aldrich, cat. no. 68972)
Hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU; e.g., Sigma-Aldrich, cat. no. 445460)
4-methylmorpholine (e.g., Millipore Sigma, cat. no. M56557)
Diethyl ether (e.g., Fisher Scientific, cat. no. E138-1)
DCM (e.g., Millipore Sigma, cat. no. DX0835-3)
TFA (e.g., Millipore Sigma, cat. no. T6508)
Tri-isopropylsilane (e.g., Sigma-Aldrich, cat. no. 233781)

50-ml round-bottom flask (e.g., Fisher Scientific, cat. no. CG150689) with stopper
Magnetic stir plate and stir bars
10-ml vial
Glass pipettes
Centrifuge, refrigerated
Spatula
Nitrogen source
22-gauge needles
Dialysis tubing, 10-kD molecular weight cut-off (MWCO; e.g., Spectrum Labs, cat. no. 132576)
0.22-μm syringe filter units (e.g., Millipore Sigma, cat. no. SLGPR33RB)
Vacuum filter units (e.g., Millipore Sigma, cat. no. SCGP00525)
50-ml polytetrafluoroethylene (PTFE) centrifuge tubes, ether grade (e.g., Thermo Fisher Scientific, cat. no. 79013)
Lyophilizer

Part 1: ELP modification with Boc-protected hydrazinoacetic acid

1. Dissolve 300 mg ELP (MW: 37914 Da, 14-amine; 0.00791 mmol ELP, 0.111 mmol amines) in 4.1 ml anhydrous DMSO in a 50-ml round-bottom flask (∼10 min). Once dissolved, add 4.1 ml anhydrous DMF. Keep ELP solution stirring using a magnetic stir bar and cover with stopper.

2. In a separate 10-ml vial, dissolve 90.83 mg tri-Boc (0.232 mmol, 2.1 equivalents per amine, 390.43 g/mol) in 4.1 ml anhydrous DMF. Once tri-Boc has completely dissolved, add 84.24 mg HATU (0.221 mmol, 2.0 equivalents per amine, 380.23 g/mol). Immediately after, add 60.89 µl 4-methylmorpholine (0.553 mmol, 5.0 equivalents per amine, 101.15 g/mol, 0.92 g/ml). Stir reaction for 10 min at room temperature.

3. Add tri-Boc and HATU reaction dropwise with a glass pipette to the ELP solution. Cap round-bottom flask with no ventilation. Keep reaction stirring for ∼20 to 24 hr at room temperature.

   It is normal for the solution to turn a yellow color at this point.

4. Precipitate Boc-protected ELP product in ice-cold diethyl ether. Let product incubate in ether on ice for ∼10 min to fully precipitate.

   At ∼30 min before this step, begin cooling the centrifuge to 4°C. In addition, add the relevant volumes of diethyl ether to ether-grade centrifuge tubes. This should be ∼30 ml diethyl ether for every 5 to 10 ml ELP solution. Prechill the diethyl ether by fully submerging the tubes in ice. It is imperative that PTFE ether-safe tubes, such as those noted in the materials list, are used for this step. They are necessary for not only the ether but also the additional solvents that are added before the second ether crash.

5. Centrifuge tubes containing Boc-protected ELP and ether 25 min at 18,000 × *g*, 4°C. Decant supernatant to collect the pelleted product.

6. Use a spatula to cut and collect an ∼10-mg sample of Boc-protected ELP for NMR. To evaporate excess ether, allow both collected sample and large pellet to dry for ∼3 hr under nitrogen flow. Use two 22-gauge needles—one for nitrogen flow and the other to vent. Freeze 10-mg Boc-protected sample at −80°C.

   This step functions as a potential pause point. The pellet can be left to dry with nitrogen flow overnight.

Part 2: deprotection of Boc-protected hydrazinoacetic acid

7. In a 50-ml round-bottom flask or scintillation vial, redissolve entire Boc-protected ELP pellet in 8.2 ml of a 50:50 DCM:TFA solution with 5% (v/v) tri-isopropylsilane. Once the ELP has fully dissolved (∼20 min), start a 4-hr timer. Ventilate with a 22-gauge syringe needle.

   To avoid any unnecessary injuries or accidents during the above ELP deprotection step, it is imperative to handle TFA with care, as it is corrosive. Appropriate personal protective equipment should be worn, and glass pipettes and measuring utensils should be used when handling TFA; this applies to DCM as well. It is recommended to first add the required amount of DCM to a glass graduated cylinder. Fill to the final volume with TFA using a glass pipette. It is normal to see a slight fume when mixing these components; however, ensure there is proper ventilation within the chemical fume hood.

8. Using the same volume and ratio as in step 4, precipitate resulting product in diethyl ether. Centrifuge 25 min at 18,000 × *g*, 4°C. Discard supernatant.

9. Evaporate remaining diethyl ether from the sample overnight in a fume hood.

10. Dissolve remaining ELP sample in ∼30 ml ice-cold ultrapure water (1% [w/v] ELP-HYD). Leave ELP-HYD overnight with agitation at 4°C to fully dissolve.

11. Use 10,000-MWCO tubing to dialyze the ELP-HYD solution against 4 L ultrapure water for 3 days at 4°C with stirring. Change water twice per day for a total of five switches. Make sure ultrapure water has been prechilled to 4°C ahead of time.

    Cover the bucket with aluminum foil to prevent debris from falling into it. If 10,000-MWCO tubing is not available, any dialysis tubing within the range of 1000 to 10,000 MWCO should be sufficient. As an extra precaution to prevent losing material at this step, we recommend wrapping rubber bands around the dialysis clips.

12. In a sterile biosafety cabinet, run ELP-HYD solution through a 0.22-µm filter into a 50-ml tube to remove precipitates. Freeze solution overnight at −80°C, and then lyophilize sample for 72 hr. Store final product as a sterile powder at −20°C in desiccant.

    The ELP-HYD solution should no longer be cloudy after filtration.

NUCLEAR MAGNETIC RESONANCE QUANTIFICATION OF ELASTIN-LIKE PROTEIN MODIFICATION WITH HYDRAZINE

BASIC PROTOCOL 2

The degree of modification of ELP with hydrazine is quantitatively confirmed using NMR. NMR characterization of the unmodified and modified ELP is carried out using the same general procedure. All samples are analyzed via H^1 NMR 600 MHz in DMSO. We use a 600-MHz NMR spectrometer (Varion Inova console) and MNOVA 14.0 for all spectra analysis and peak quantification. The degree of modification is characterized by comparing specific peaks unique to the unmodified protein and the modified protein. Quantification of each protein variant (unmodified ELP, ELP with Boc-protected hydrazine, and ELP-HYD) is also described.

Materials

10-mg Boc-protected ELP sample (see Basic Protocol 1)
10-mg Boc-removed ELP sample (see Basic Protocol 1)
Deuterated DMSO (e.g., Sigma-Aldrich, cat. no. 570672-10)

1.7-ml microcentrifuge tube
NMR tubes (e.g., Sigma-Aldrich, cat. no. Z565229)
H^1 NMR spectrometer (e.g., Varion Inova)
Computer running analysis software (e.g., MNOVA 14.0)

1. Retrieve sample of ELP to be characterized, and bring to room temperature.
2. Dissolve ELP sample in deuterated DMSO at a concentration of 10 mg/ml in a 1.7-ml microcentrifuge tube, and allow to dissolve for 1 hr.
3. Transfer 700 µl into an NMR tube that is compatible with the NMR spectrometer being used.
4. Analyze sample via H^1 NMR 600 MHz in DMSO.
5. Use MNOVA 14.0 for all spectra analysis and peak quantification.

    The quantification of each protein variant is described as:

    Unmodified ELP is characterized by comparing the methylene group adjacent to the amine on the lysine (2 H per methylene group, peak at 2.8 ppm) to the aromatic protons of the tyrosine (4 H per tyrosine, peaks at 6.6 [2 H] and 7.1 [2 H] ppm). Specifically, the integrated areas under the peaks are compared to assess the correct proportion of amino acids in the sequence, establishing a baseline. To quantify the peaks assigned, the lysine peak was set to 28.0 to account for the 14 lysines (14 methylene groups, 2 H each) in the amino acid sequence and compared to the peaks assigned to the 4 tyrosines available (16 H total, peaks at 6.6 [8 H total] and 7.1 [8 H total] ppm).

Conjugation efficiency of the ELP with Boc-protected hydrazine is characterized by comparing the integrated spectra of the methyl groups on the Boc moieties (27 H per Boc-protected hydrazine, represented by two sharp peaks at 1.5 ppm) to the aromatic protons of tyrosine (4 H per tyrosine, peaks at 6.6 [2 H] and 7.1 [2 H] ppm). To quantify the conjugation efficiency, we set the peak at 6.6 ppm to 8.0, as this value represents the aromatic protons at this position, and then quantified the area of the peaks at 1.5 ppm. The protons at 1.5 ppm were compared to the theoretical maximum value of 378 H of the total Boc-protected hydrazines (14 possible hydrazines, each with 27 H from the Boc groups) to determine the conjugation efficiency.

Finally, the ELP with the deprotected hydrazine is characterized by observing the suppression of the methyl groups on the Boc-protected hydrazine (27 H per Boc-protected hydrazine, represented by two sharp peaks at 1.5 ppm) in comparison to the aromatic protons of tyrosine (4 H per tyrosine, peaks at 6.6 [2 H] and 7.1 [2 H] ppm). The two distinct peaks at 1.5 ppm will no longer be present, and the spectrum can be qualitatively compared to the unmodified ELP spectrum. If deprotection was incomplete, the two peaks at 1.5 ppm will still be present, and the number of remaining Boc groups can be quantified.

BASIC PROTOCOL 3

HYALURONIC ACID–BENZALDEHYDE SYNTHESIS

Basic Protocol 3 delineates the synthesis of HA-BZA, the second component of the HELP system, which forms crosslinks with the ELP-HYD from Basic Protocol 1. HA-BZA synthesis consists of two distinct parts: (1) HA modification with alkyne and (2) HA modification with azido-BZA. Take HA out ahead of time to bring to room temperature before opening.

Materials

Sodium hyaluronate, research grade, HA100K (HA; e.g., Lifecore Biomedical, cat. no. HA100K-5)
MES buffer (see recipe)
Propargylamine (e.g., Sigma-Aldrich, cat. no. P50900)
1 M NaOH (e.g., Sigma-Aldrich, cat. no. S8045)
N-hydroxysuccinimide (NHS; e.g., Thermo Fisher, cat. no. 24500)
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; e.g., Thermo Fisher, cat. no. 22980)
Beta-cyclodextrin (e.g., Sigma-Aldrich, cat. no. C4767)
10× isotonic phosphate-buffered saline (PBS; see recipe)
Copper sulfate, 5-hydrate (e.g., JT Baker, cat. no. 1841-01)
Sodium l-ascorbate (e.g., Sigma-Aldrich, cat. no. A7631)
Azido-BZA (e.g., Santa Cruz Biotechnology, cat. no. 24173-36-2)
DMSO, extra dry (e.g., Acros Organics, cat. no. 61097-1000)
50 mM ethylenediamine tetraacetic acid (EDTA), disodium salt dihydrate, pH 7.0 (e.g., Fisher Scientific, cat. no. O2793-500)

100-ml round-bottom flask (e.g., Fisher Scientific, cat. no. 50-153-1122) and stopper
Magnetic stir plate and football-shaped stir bars
pH strips
Dialysis tubing, 10-kD MWCO (e.g., Spectrum Labs, cat. no. 132576)
0.22-μm syringe filter units (e.g., Millipore Sigma, cat. no. SLGPR33RB)
Vacuum filter units (e.g., Millipore Sigma, cat. no. SCGP00525)
Lyophilizer
Nitrogen source
22-gauge, 3-in. hypodermic needles (e.g., Fisher Scientific, cat. no. 14-817-101)
18-gauge, 6-in. deflected point septum penetration needles (e.g., Cadence Science, cat. no. 89234-220)

20-ml glass scintillation vials
U-100 insulin syringes (e.g., BD, cat. no. 329461)
Aluminum foil

Part 1: HA modification with alkyne

Steps 1 through 6 are written for 200 mg of 100 kDa HA. Make sure to scale measurements appropriately. The steps are written to achieve 12% HA-alkyne modification for a softer gel (see Table 1). This involves using 0.8 equivalents of propargylamine, which represents a ratio of 8 propargylamine groups to 10 carboxylic acid groups on the HA. For 20% or 30% HA-alkyne modification, three or six equivalents, respectively of propargylamine are used.

1. In a 100-ml round-bottom flask, dissolve 200 mg HA (sodium salt) in 20 ml MES buffer to 1% (w/v). Do this in a fume hood, and use a football-shaped stir bar, covering with a stopper. Ensure all HA aggregates are dissolved (∼45 to 60 min) before proceeding.

   In case any HA gets stuck to the sides of the flask, add the MES buffer after the HA solid. While HA is dissolving, it is recommended that propargylamine, NHS, and EDC be taken out to bring them to room temperature.

2. Add 25.5 µl propargylamine (0.8 equivalents to the HA dimer unit) to the reaction mixture.

3. Immediately after adding propargylamine, use pH strips to assess and adjust the pH to 6 with 1 M NaOH.

   For the 0.8 equivalent reaction, adding 1 ml of 1 M NaOH should bring the pH to 6. It is likely that the pH of the 6 equivalent reaction will already be around 6.

4. Add 45.9 mg NHS (0.8 equivalents to HA dimer unit) dissolved in ∼400 µl MES buffer to the reaction flask, and ∼1 min later, add 76.4 mg EDC (0.8 equivalents to HA dimer unit) also dissolved in ∼400 µl MES buffer to the reaction mixture. Prepare solutions in MES buffer immediately before adding to the vial using a pipette.

   Note that the recommended amounts in this step are for the 0.8 equivalent reaction (12% HA modification). For the 30% HA modification, use 6 equivalents of NHS/EDC:HA dimer unit.

5. Cover reaction vessel with a rubber stopper, and stir for 24 hr at room temperature.

6. Dialyze mixture against 4 L ultrapure water in the same manner as done for ELP-HYD (10,000-MWCO dialysis tubing for 3 days at 4°C). Change dialysis water twice per day. Once dialysis has been completed, filter HA-alkyne solution using a 0.22-µm filter unit to remove any precipitates, and freeze samples overnight. Lyophilize for 72 hr, and store lyophilized polymer in desiccant at −20°C.

   For the filtration step, larger vacuum filter units with a funnel capacity of ∼150 to 250 ml work well.

Part 2: HA modification with azido-BZA

Steps 7 through 16 are written for 100 mg HA-alkyne. Make sure to scale measurements as desired. Before beginning, measure out an ∼10-mg sample (>7.5 mg) of HA-alkyne for NMR.

7. Prepare 1 mg/ml beta-cyclodextrin in 10× isotonic PBS. Then dissolve 100 mg HA-alkyne in 8 ml beta-cyclodextrin-supplemented 10× isotonic PBS (∼1 hr). Once the HA-alkyne has fully dissolved, degas vial using nitrogen for 30 min. Use one long 22-gauge needle as the input and another shorter needle as the output for air to escape.

This 1-hr period of wait time provides a good opportunity to prepare the solutions in step 8. If space and resources permit, all components can be degassed concurrently. The solutions should be visibly bubbling.

8. In 20-ml glass scintillation vials, prepare 2.4 mM copper sulfate in ultrapure water (249.68 g/mol; 6 mg/10 ml) and 45.2 mM sodium ascorbate (stock) in ultrapure water (198.11 g/mol; 89.5 mg/10 ml). Degas solutions for 30 min using nitrogen.

   Small, 20-ml glass scintillation vials work well for this step. Make sure the vials have a top that a needle can penetrate. Both solutions should dissolve quickly, but vortexing can expedite the process. Due to the relatively large size of the copper sulfate crystals, it is often easier to prepare a 10× solution of 24 mM (60 mg/10 ml) copper sulfate and then dilute to the final 2.4 mM concentration. The 30-min wait for the degas step also provides an opportunity to determine how much azido-BZA to add in subsequent steps.

9. Prepare stock solution of azido-BZA in anhydrous DMSO with the appropriate amount for the desired modification and final gel stiffness (see Table 1).

   As long as the appropriate equivalents are added, the exact concentration of the azido-BZA solution is not as important. We select for a volume that is easy to pipette with an azido-BZA concentration between 20 and 150 mg/ml.

   *To achieve a soft gel of ∼300 Pa (**HELP low**, 1% ELP-HYD/1% HA-BZA), use azido-BZA:alkyne stoichiometric ratios <1 (0.6 equivalent) with a 12% HA-alkyne modification. For an intermediate stiffness gel of ∼800 Pa (**HELP medium**, 1% ELP-HYD/1% HA-BZA), use the same HA-alkyne modification as for the soft gel (12%) while increasing the azido-BZA:alkyne ratio to 2 equivalents to modify all available alkynes. Although it is not directly used in this demonstration, a moderately stiffer gel can be prepared at ∼1500 Pa (1% ELP-HYD/1% HA-BZA) by using a higher azido-BZA:alkyne ratio (2 equivalents) with the 30% modified HA-alkyne. To achieve the stiffest gel iteration of ∼3000 Pa (**HELP high**), use the recommended HA equivalents for the 1500 Pa gel (2 equivalents), but increase the weight percent of ELP to ∼2%. To keep the concentration of the cell-adhesive RGD domain on the ELP consistent for the ∼3000 Pa gel, use a 50:50 mixture of ELP-RGD to the scrambled ELP-RDG.*

   Table 1 provides a summary of the recommended HA-alkyne modification, HA-BZA modification, and final concentrations of HA-BZA and ELP-HYD suggested to produce gels of a desired stiffness and a final RGD concentration of 1 mM.

10. Add 1 ml of 45.2 mM sodium ascorbate to the reaction flask using an insulin syringe.

11. Add 1 ml of 2.4 mM copper sulfate to the reaction flask using an insulin syringe. Add appropriate volume of azido-BZA solution to the reaction flask using a pipette tip. Pipette directly into the solution with effort being made to minimize exposure to the surrounding air.

12. Degas final solution with nitrogen for 10 min.

13. Secure stopper over the reaction flask. Wrap flask in aluminum foil, and stir reaction for 24 hr at room temperature.

14. After the reaction has been stirred for 24 hr, add an equal volume of 50 mM EDTA, pH 7.0, to the reaction flask, and stir at room temperature for 1 hr.

    The solution is no longer under inert gas and does not need to be covered with aluminum foil.

15. Dialyze HA-BZA solution against 4 L ultrapure water for 3 days at 4°C. Use 10,000-MWCO dialysis tubing. Change water twice per day.

16. Run dialyzed HA-BZA solution through a 0.22-μm filter into sterile 50-ml tubes. Freeze samples at −80°C overnight, and then lyophilize for 72 hr. Store HA-BZA as a sterile powder at −20°C in desiccant.

Although it is possible to filter the HA-BZA after lyophilization in small, reconstituted volumes, this is advised against due to greater amount of product loss, especially considering the high molecular weight of HA.

NUCLEAR MAGNETIC RESONANCE QUANTIFICATION OF HYALURONIC ACID–BENZALDEHYDE

BASIC PROTOCOL 4

The degree of modification of HA is quantitatively confirmed using NMR. NMR characterization of the unmodified and modified HA is carried out using the same general procedure. All samples are analyzed via H^1 NMR 600 MHz in deuterium oxide (D_2O). We use a 600 MHz NMR spectrometer (Varion Inova console) and MNOVA 14.0 for all spectra analysis and peak quantification. The degree of modification is characterized by comparing specific peaks unique to the unmodified HA and the modified HA. Quantification of each HA variant (unmodified HA, HA-alkyne, and HA-BZA) is also described.

Materials

10-mg HA-alkyne sample (see Basic Protocol 3)
10-mg HA-BZA sample (see Basic Protocol 3)
D_2O, for NMR, 99.8 atom % D (e.g., Thermo Fisher, cat. no. AC166301000)

1.7-ml microcentrifuge tube
NMR tubes (e.g., Sigma-Aldrich, cat. no. Z565229)
H^1 NMR spectrometer (e.g., Varion Inova)
Computer running analysis software (e.g., MNOVA 14.0)

1. Retrieve sample of HA to be characterized, and bring to room temperature.
2. Dissolve HA sample in D_2O at a concentration of 10 mg/ml in a 1.7-ml microcentrifuge tube, and allow to dissolve for 1 hr.
3. Transfer 700 µl into an NMR tube that is compatible with the NMR spectrometer being used.
4. Characterize sample via H^1 NMR 600 MHz in D_2O.
5. Use MNOVA 14.0 for all spectra analysis and peak quantification.

The quantification of each HA variant is described as:

Unmodified HA is characterized by comparing the methyl group belonging to the N-acetyl group (3 H per repeat unit, represented by a single peak at 2.1 ppm) to the hydroxyl (2 H per repeat unit, represented by a peak at 4.6 ppm). Specifically, the integrated areas under the peaks are compared to assess the correct proportion of methyl protons to the hydroxyl protons, establishing a baseline.

The degree of alkyne modification on the HA is characterized by comparing the methylene adjacent to the propargyl's amine to the methyl group. Specifically, the integrated signal of the methylene peak (2 H per propargyl, represented by a peak at 4.1 ppm) is compared to the methyl peak (3 H per repeat unit, represented by a single peak at 2.1 ppm). The degree of conjugation was determined by labeling the methyl peak (2.1 ppm) equal to 3.0 and integrating the methylene peak at 4.1 ppm. The value at this methylene peak was divided by 2 (theoretical maximum value) to determine the degree of alkyne modification.

The final degree of benzaldehyde modification on the HA is characterized by comparing the integrated spectra of the aldehyde proton (1 H per benzaldehyde, represented by a peak at 10.1 ppm) to the methyl group (3 H per repeat unit, represented by a single peak at 2.1 ppm). The degree of conjugation was determined by labeling the methyl peak (2.1 ppm) equal to 3.0 and integrating the aldehyde peak at 10.1 ppm. The value at this aldehyde peak represents the degree of modification with benzaldehyde.

BASIC PROTOCOL 5

3D CELL ENCAPSULATION IN HYALURONAN ELASTIN-LIKE PROTEIN GELS

Once both the ELP-HYD and HA-BZA materials have been prepared and their functionalization validated, they can be used to encapsulate cells in HELP gels. The procedure for encapsulation is detailed in this protocol and presented within Figure 2.

Materials

ELP-HYD (see Basic Protocol 1)
HA-BZA (see Basic Protocol 3)
10× isotonic PBS, sterile (see recipe)
Dulbecco's PBS (DPBS; e.g., Corning, cat. no. 21-031-CM)
Reagents necessary for cell culture dissociation (e.g., trypsin-EDTA)
Cell culture medium appropriate for cells of interest
Human mesenchymal stromal cells (hMSCs; e.g., Lonza, cat. no. PT-2501)

1.7-ml microcentrifuge tubes, sterile
Laboratory shaker
24-well plates (e.g., Fisher Scientific, cat. no. FB012929)

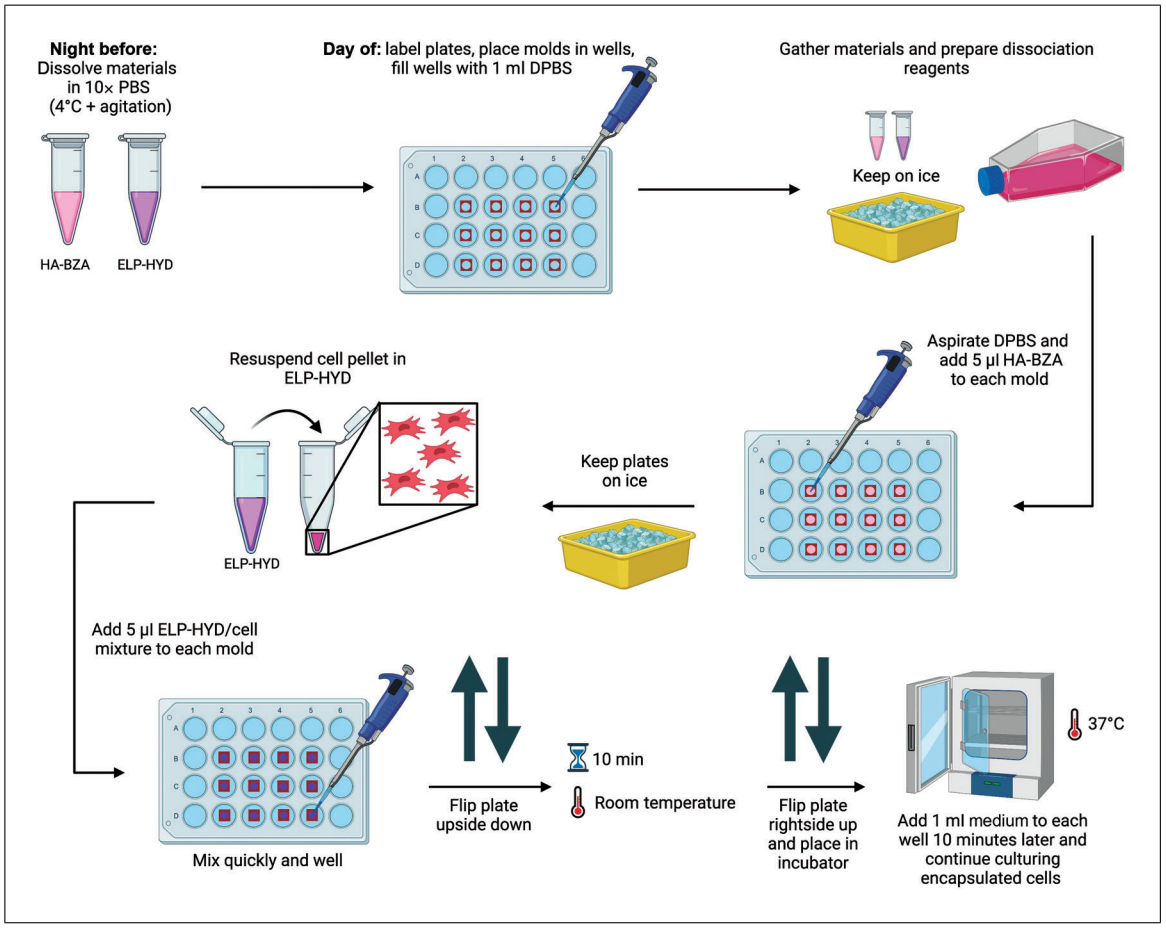

Figure 2 Overview schematic of cell encapsulation in hyaluronan elastin-like protein (HELP) gels. Prepare hyaluronic acid–benzaldehyde (HA-BZA) and ELP-hydrazine (ELP-HYD) in advance and keep on ice. Once HA-BZA has been dispensed into the molds, make sure that the plates are kept on ice. Resuspend the cells in ELP-HYD before quickly adding the ELP-HYD/cell mixture to the molds with HA-BZA. Mix each mold quickly but carefully, changing pipette tips for each mold. Immediately flip the plate upside down and incubate at room temperature for 10 min. Flip the plate over again before allowing it to incubate at 37°C for 10 min. After this 10-min incubation period, add medium to the wells, and proceed with normal culturing practices. DPBS, Dulbecco's phosphate-buffered saline.

0.8-mm-thick silicone sheet for molds (e.g., Electron Microscopy Science, cat. no. 70338-10)
No. 2 12-mm glass coverslips (e.g., Fisher Scientific, cat. no. 22-293-232P)
Microcentrifuge
37°C, humidified, 5% CO_2 incubator

Preparation of materials and reagents

1. The night before encapsulation, dissolve ELP-HYD and HA-BZA in sterile 10× PBS in microcentrifuge tubes at 4°C with agitation.

   Materials can be dissolved the morning of the planned encapsulation, but allocate at least 3 to 4 hr for them to dissolve. If the desired final concentration for ELP-HYD or HA-BZA is 1% (w/v), then the stocks should be prepared at 2% (w/v). ELP-HYD stock solutions for the ∼3000 Pa gel (ELP-RGD and ELP-RDG) should each be prepared at 4% (w/v). Typically, ∼30% excess material should be prepared to account for pipetting loss. To maintain sterility while measuring materials, ELP-HYD and HA-BZA should be transferred into sterile, tared microcentrifuge tubes using sterile tweezers in a tissue culture hood.

2. Place necessary number of 24-well plates in the tissue culture hood. Label with the appropriate information, and place 4-mm-diameter, autoclaved, sterile silicone molds, mounted on No. 2 12-mm glass coverslips, in each well. Add 1 ml sterile DPBS to each well, and leave in the hood until it is time to add HA-BZA in a later step.

3. Place full ice bucket into the tissue culture hood.

   Make sure to spray the full ice bucket thoroughly with 70% ethanol. Keep ELP-HYD and HA-BZA refrigerated at 4°C or on ice until needed.

4. Prepare necessary cell culture dissociation reagents and medium.

   The dissociation reagents and medium used will depend on the cell type. For the sample data shown in this protocol, hMSCs were dissociated using trypsin-EDTA. Alternatively, other commercially available cell dissociation solutions can be used. To prepare the hMSC cell culture medium, a combination of fetal bovine serum, penicillin-streptomycin, and Dulbecco's modified Eagle medium with high glucose was used (Madl et al., 2016). The hMSCs were passaged at ∼70% to 80% confluency, used between passages three and six, and seeded at a cell density of ∼3000 cells/cm^2. It is recommended that required volumes of the cell culture reagents are aliquoted and either brought to room temperature in the tissue culture hood or placed in a warm water bath for at most 30 min before use.

5. Gather gel reagents (ELP-HYD and HA-BZA). Make sure to keep them on ice.

Cell dissociation and encapsulation

6. To begin casting gels, aspirate DPBS from each well. Load each mold with 5 µl of the appropriate HA-BZA solution. From this point on, keep 24-well plates on ice.

   Use the pipette tip to ensure that the HA-BZA has been dispersed evenly across the mold. To limit condensation within the mold, wait until the HA-BZA has been added before placing the plate on ice. However, it is important to keep the plate on ice after this point since small volumes of water in the HA-BZA solution can rapidly evaporate at room temperature. The total gel volume for the 4-mm-diameter molds is 10 µl.

7. Dissociate cells following the supplier's protocol, and determine cell count. Keep cells on ice during subsequent steps.

8. Prepare cells for encapsulation by adding the correct volume of cell solution to a microcentrifuge tube. Centrifuge 5 min at 200 × *g*, room temperature, to pellet the cells.

   The optimal encapsulation cell density will vary based on the cell type. For hMSCs, we often use a final encapsulation density of 1000 cells/µl.

9. Resuspend cell pellet in an appropriate volume of ELP-HYD at twice the desired final cell density. Use a 200-µl pipette, and mix 20 to 25 times to ensure the cells are homogeneously distributed in the ELP-HYD. Keep tube on ice during the mixing process.

10. To encapsulate the cells, add 5 µl cell/ELP-HYD mixture to each mold, keeping the plate on ice. Cast each gel one at a time with a fresh pipette tip. Use the following procedure to thoroughly mix the ELP-HYD/HA-BZA/cell solution within the mold: swirl the pipette tip 10 times clockwise, 10 times counterclockwise, 10 times clockwise, and 10 times counterclockwise (40 total swirls).

11. Once the final mold has been filled, flip plate so that the gels are suspended upside down. Note the time. Incubate gels for 10 min at room temperature. Flip plate back over, and incubate for 10 min at 37°C.

    The purpose of flipping the plate is to prevent potential cell settling within the hydrogel before gelation. Due to the quick gelation time and small volume of the gels, surface tension is sufficient to maintain the gel solution within the mold as the plate is flipped. The surface tension from the PBS wash in step 2 keeps the coverslip adhered to the well.

12. Add 1 ml cell culture medium to each well. Pipette carefully along the side of the well to avoid disrupting the gel.

13. Continue culturing encapsulated cells within the hydrogels in a 37°C, humidified, 5% CO_2 incubator. Replace cell culture medium as needed.

    Cells can be characterized at different time points as desired; for example, the 3D culture is compatible with common assays including characterization of cell viability (live/dead staining), DNA quantification, metabolism, gene/protein expression, and morphology (Hunt et al., 2021; LeSavage et al., 2022; Navarro et al., 2022; Shayan et al., 2023; Wang et al., 2017).

REAGENTS AND SOLUTIONS

MES buffer

50 ml ultrapure water
1.95 g MES hydrate (0.2 M final; e.g., Sigma-Aldrich, cat. no. M2933)
438 mg NaCl (0.15 M; e.g., Fisher Scientific, cat. no. BP358-212)
Adjust pH to 4.5
Store long-term at room temperature for up to 6 months

Isotonic PBS, 10×

1 L ultrapure water
11.5 g sodium phosphate dibasic (81 mM final; e.g., Fisher Chemical, cat. no. S374-500)
2.28 g sodium phosphate monobasic (19 mM final; e.g., Fisher Bioreagents, cat. no. BP329-1)
3.51 g NaCl (60 mM final; e.g., Fisher, cat. no. BP358-212)
Adjust pH to 7.4
Run through a 0.22-µm filter
Store long-term at room temperature for up to 12 months

COMMENTARY

Background Information

Here, we elaborate further on the chemistry of the synthesis reactions and applications of this method. In addition, we present anticipated challenges and potential solutions to help the reader troubleshoot the synthesis and encapsulation protocols.

The modification of ELP with hydrazine is accomplished via HATU chemistry. Through a HATU-mediated amidation reaction, a

Table 2 Troubleshooting Guide for Synthesis and Encapsulation Protocols

Problem	Possible cause	Solution
Difficulty weighing and transferring materials (ELP-HYD, HA-BZA) into reaction vessels	Buildup of static electricity[a]	Use an anti-static gun to neutralize any static when weighing out ELP and HA
Unable to withdraw DMF and DMSO from respective containers	Failure to use nitrogen-flushed syringes to withdraw DMF and DMSO	To prevent a vacuum from forming, inject a volume of nitrogen gas into the container equivalent to the volume of DMF or DMSO being removed
Difficulty filtering ELP-HYD and HA-BZA solutions	Clogged filter	Dilute solutions with cold ultrapure water before filtering and lyophilization[b]
Under-modification occurring at the point of HA-alkyne modification	EDC may be hydrolyzed	Use fresh EDC; keep EDC stored in desiccant and minimize exposure to air
Molds not adhering to glass coverslips	Residual material or debris on the mold	Consider plasma cleaning molds and coverslips with a plasma asher for 30 s before quickly placing molds into coverslips[c]
Low cell viability after encapsulation	Some cells may be more sensitive than others to being left on ice and in suspension for prolonged periods of time	Try limiting amount of time cells spend in suspension on ice postdissociation

BZA, benzaldehyde; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ELP, elastin-like protein; HA, hyaluronic acid; HYD, hydrazine.

[a]In lyophilized states, ELP and HA derivatives are prone to interference from buildup of static electricity.

[b]It is not unusual to require >1 filter as they can quickly become clogged.

[c]We use a Pico (low-pressure plasma system) plasma surface treatment machine (version A) by Diener electronic.

Boc-protected hydrazinoacetic acid is conjugated onto the ELP to achieve Boc-protected ELP (Fig. 1A). The amidation reaction proceeds through activation of the carboxylic acid on tri-Boc by the coupling agent HATU. The HATU molecule generates an active ester that can react with the amine on the lysine residue to form an amide bond between the ELP and tri-Boc (Vrettos et al., 2017). In a subsequent deprotection step, ELP is dissolved in a mixture of TFA and DCM (50:50 ratio) to remove the Boc protective groups, revealing the ELP-HYD product (Fig. 1A). TFA allows the ELP to solubilize while tri-isopropylsilane aids in the deprotection.

In the HA modification with alkyne, we use EDC to activate the carboxylic acid of HA with NHS (Fischer, 2010). The activated ester is easily consumed by propargylamine through a nucleophilic attack of the carboxyl group, resulting in HA-alkyne (Fig. 1B). In the second step, HA-alkyne is further modified using copper click chemistry to append benzaldehyde moieties to the alkyne site, resulting in HA-BZA (Fig. 1B; Moses & Moorhouse, 2007). The EDTA chelates excess copper ions, sequestering them to quench the reaction.

Along with the culture of various individual cell types, the HELP system also works for the culture of human organoids (Hunt et al., 2021). Other published variations of the HELP system involve changing the concentration (Zhu et al., 2017) or molecular weight of HA, bioconjugating with aldehyde, or using oxidation reactions to form aldehyde (Suhar et al., 2022). In addition, the lower critical solution temperature can be modulated by adding additional hydrophilic or hydrophobic moieties (Navarro et al., 2022). All of these variations in formulation are expected to impact the final hydrogel mechanical properties and cellular interactions.

Critical Parameters

HA is hygroscopic, and therefore it is important that it be left in its closed container and brought to room temperature before opening. This will help prevent condensation and water absorption. Make sure to do this at all steps of the HA modification (e.g., also bring

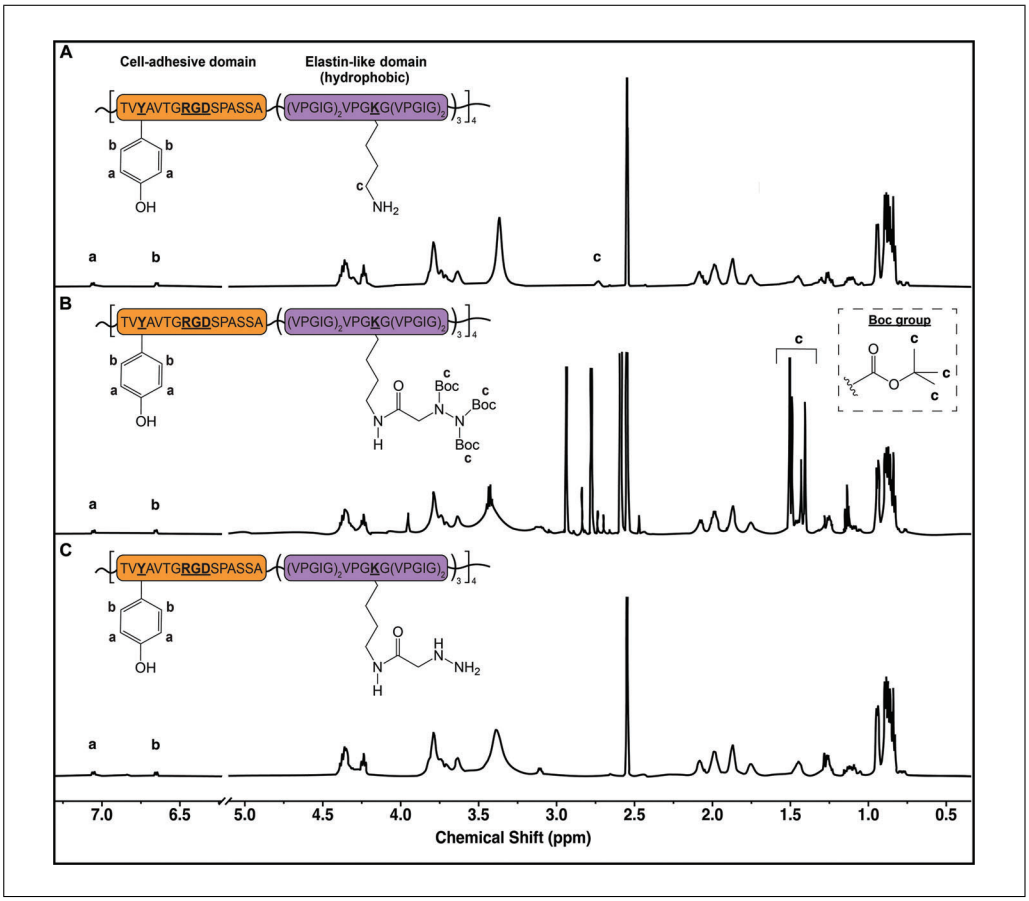

Figure 3 Representative nuclear magnetic resonance (NMR) spectra for the different stages of the elastin-like protein (ELP) modification resulting in ELP-hydrazine (ELP-HYD). (**A**) NMR spectrum for unmodified ELP, depicting tyrosine peaks at 6.6 ppm (2 H per tyrosine) and 7.1 ppm (2 H per tyrosine) and the lysine peak at 2.8 ppm (2 H per lysine). (**B**) NMR spectrum for Boc-protected ELP after hexafluorophosphate azabenzotriazole tetramethyl uronium–mediated amidation of the lysine. The Boc peak is depicted at 1.5 ppm (27 H per hydrazinoacetic acid). (**C**) NMR spectrum for ELP-HYD demonstrating the deprotection of the three Boc groups on the hydrazinoacetic acid through the disappearance of the Boc peak at 1.5 ppm.

HA-alkyne and HA-BZA to room temperature before use) to prevent inaccuracies in mass.

At the beginning of ELP-HYD modification, it is key that the ELP is completely dissolved in DMSO before proceeding because this protein is not soluble in DMF, which is the optimal solvent for the HATU reagent. Likewise, it is important that tri-Boc is completely dissolved in the DMF so that its carboxylic acid group can be activated with HATU.

Another potential pitfall can occur during the ether precipitation step of the ELP-HYD modification. It is extremely important that ether-grade conical tubes that can withstand the 18,000 × *g* centrifugation speed used. It is also recommended that the tubes are only filled to about four-fifths of their maximum volume to prevent leakage of ether during centrifugation. For this protocol, that would be ∼35 ml. To efficiently ensure that the tubes are balanced before centrifugation, it is beneficial to add a glass pipette volume full of ELP solution dropwise (allowing for a greater surface area–to-ether ratio) to each tube of ether, switching back and forth until all ELP has been added. When decanting supernatant after centrifugation, caution should be taken as the pellet may be loose. While pouring supernatant into a waste container, the cap of the tube can be used to cover the opening, allowing just a small stream of liquid to flow out. At the same time, the tube can be slowly turned by hand.

An essential characteristic of ELP to factor in throughout this entire process is its lower critical solution temperature. Due to this property, maintaining ELP at cold temperatures around 4°C enhances its solubility. This is relevant during dialysis, where it is important that the water is prechilled to 4°C before the dialysis bag is placed into the bucket. If the water

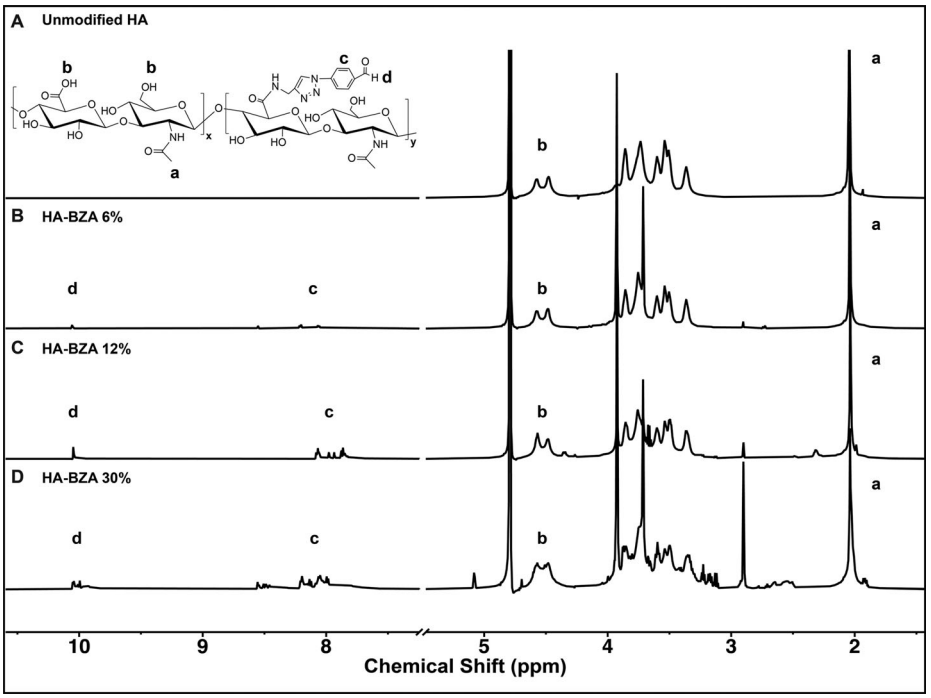

Figure 4 Representative nuclear magnetic resonance (NMR) spectra for (**A**) unmodified hyaluronic acid (HA) and (**B–D**) modified HA, after click chemistry addition of the benzaldehyde moiety, resulting in (B) 6% HA-benzaldehyde (HA-BZA), (C) 12% HA-BZA, and (D) 30% HA-BZA. The degree of benzaldehyde modification on the HA is characterized by comparing the methyl group at 2.1 ppm (3 H per repeating unit) to the aldehyde group at 10.1 ppm (1 H per benzaldehyde).

is at room temperature, there is a risk that the ELP will precipitate out of solution. This also applies to cell encapsulation where it can be detrimental to the experiment if the ELP-HYD precipitates out of solution before the crosslinking reaction occurring, hence the emphasis on keeping solutions and plates on ice.

To ensure sterility of ELP-HYD and HA-BZA solutions, it is important to prevent cracking of the tubes during the freezing steps. We have found that the tubes that come with the Steriflip filter unit do not withstand the −80°C freezing step well and often crack. Therefore, it is recommended to filter the solution into a regular, sterile 50-ml conical tube, freeze at −80°C, and then quickly replace the lid with a Steriflip filter top (e.g., Millipore Sigma, cat. no. SCGP00525) immediately before lyophilization. To avoid thawing, consider transferring the frozen solution to the tissue culture hood in a box of dry ice. It is also recommended that the conical tube only be filled to about 50% to 60% of its total capacity to prevent cracking. If cracks do occur in the tube during freezing, thaw the sample at 4°C in a beaker, collect the solution, and repeat the filtration process.

During HA-alkyne synthesis, adjusting the pH after adding propargylamine is a key step to ensure that the desired degree of modification is achieved. This is especially important for the reactions that require lower molar equivalents. A pH of 6 is the optimal pH for the reaction to occur. As pH increases beyond this point, NHS linkages become more prone to hydrolysis. Additionally, it is very important to make sure that NHS is added before EDC. In the absence of NHS, EDC hydrolyzes and degrades quickly. Having the NHS protecting group present will provide a stabilizing effect. Given the susceptibility of EDC to hydrolyzation, it should be stored in desiccant, and effort should be taken to minimize the time it is exposed to air. Otherwise, EDC can take on water over time and form clumps.

During HA modification with azido-BZA, supplementation of PBS with beta-cyclodextrin is necessary as it functions as a phase-transfer agent for the azido-BZA small molecule that will be added in a subsequent step. Beta-cyclodextrin has a pocket into which the hydrophobic benzene group of the small molecule can fit, allowing the azido tail to protrude. This facilitates the reaction

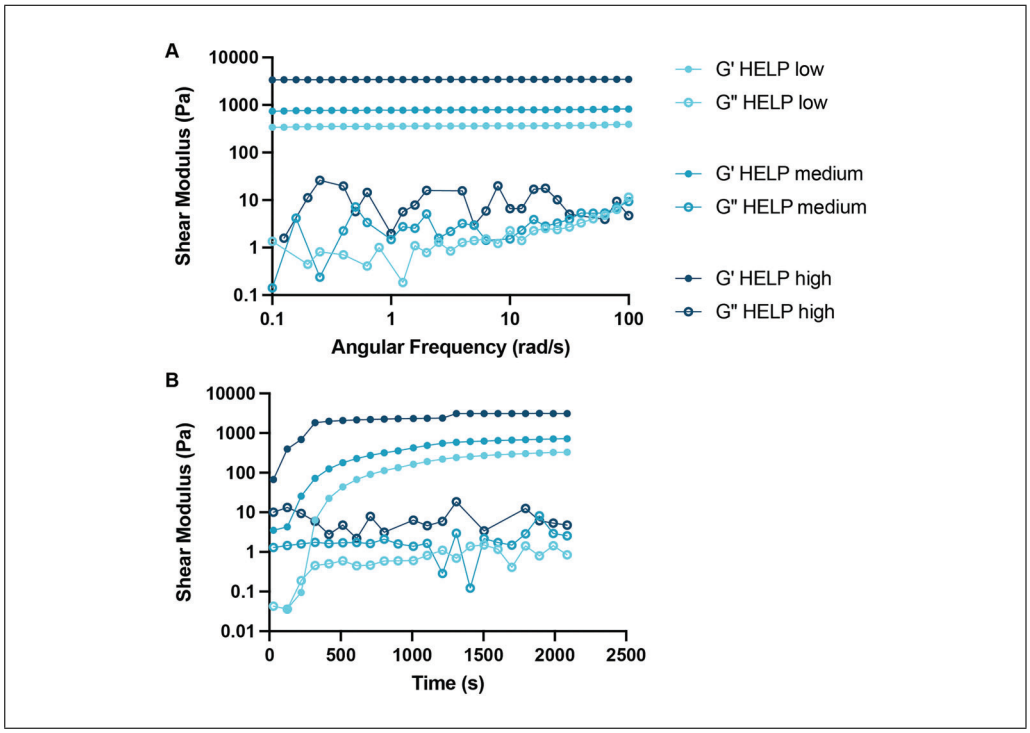

Figure 5 Rheological characterization of hyaluronan elastin-like protein (HELP) gels of varying stiffness. Representative frequency (**A**) and time sweeps (**B**) of HELP low (G' ~300 Pa), medium (G' ~800 Pa), and high (G' ~3000 Pa) stiffness formulations. By altering the degree of modification of the hyaluronic acid (HA)-alkyne and HA-benzaldehyde and the final ELP-hydrazine concentration, a range of stiffnesses can be achieved. Samples were evaluated on a stress-controlled TA Instruments ARG2 Rheometer with a 20-mm cone-on-plate geometry. Frequency sweeps were conducted over an angular frequency range of 0.1 to 100 rad/s and a strain of 1%. Time sweeps were performed at an angular frequency of 1 rad/s and a strain of 1%.

and increases its efficiency by solubilizing the small molecule in the aqueous phase. It is important that isotonic 10× PBS is used because these reactions are pH sensitive, and the acidic HA can interfere with the overall pH. Copper sulfate acts as a catalyst for the click reaction. Sodium ascorbate functions as a protecting agent to prevent copper from being oxidized so that the reaction can continue to occur. It is important to add sodium ascorbate to the reaction vial first to prevent the copper sulfate solution from degrading the HA.

During the cell encapsulation process, it is important to consider when the plate should and should not be on ice. To limit condensation within the molds, which may affect the final gel concentration, wait until HA-BZA has been added before placing the plate on ice. However, it is important to keep the plate on ice after this point since small volumes of water in the HA-BZA solution can rapidly evaporate at room temperature. Recall that the behavior of ELP-HYD has a strong temperature dependence and should also be kept on ice as much as possible.

Lastly, it is imperative to pipette and mix carefully to avoid air bubble formation. Air bubbles can interfere with gelation and cause cell membrane damage. At the same time, it is important to move quickly and begin mixing immediately, as gelation occurs rapidly, especially with the high-stiffness gels.

Troubleshooting

Potential problems that could arise throughout the protocol, their possible causes, and how to address them are discussed in Table 2.

Understanding Results

NMR demonstrates successful modification of ELP and HA

H^1 NMR was run on samples of ELP at various stages of the ELP-HYD modification. Before modification, a peak representing the protons of the methylene group adjacent to the amine on the lysine residue is evident on the NMR spectrum at 2.8 ppm (Fig. 3A). At the next stage, the NMR spectrum of the ELP with Boc-protected hydrazine contains a distinct peak at around 1.5 ppm corresponding

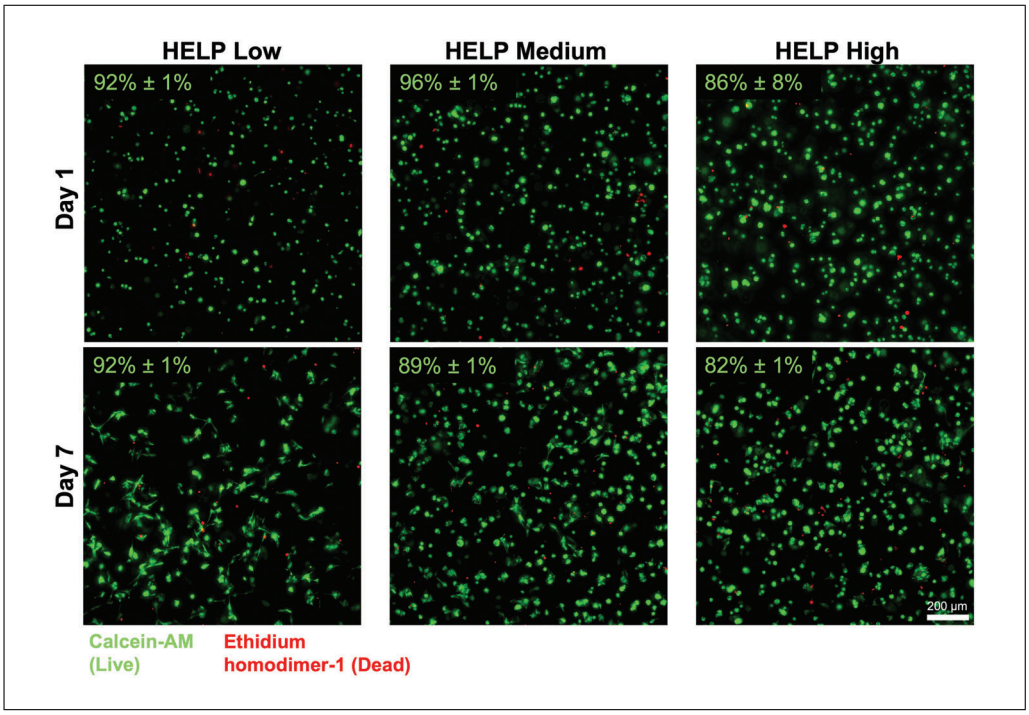

Figure 6 Live/dead staining of human mesenchymal stromal cells encapsulated in hyaluronan elastin-like protein (HELP) gels of low (G' ~300 Pa), medium (G' ~800 Pa), and high (G' ~3000 Pa) stiffness formulations after 1 and 7 days. Cells in HELP gels exhibited high viability under a range of hydrogel stiffnesses.

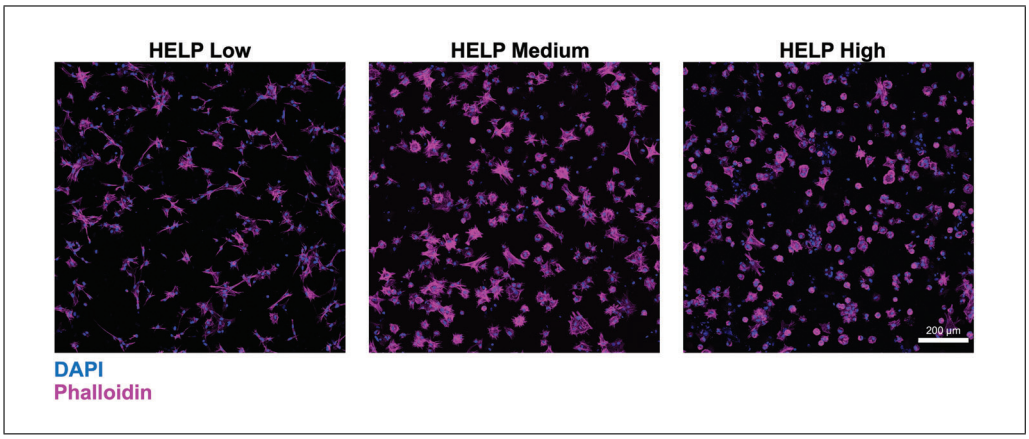

Figure 7 Visualization of altered cell morphology of human mesenchymal stromal cells encapsulated in hyaluronan elastin-like protein (HELP) gels of low (G' ~300 Pa), medium (G' ~800 Pa), and high (G' ~3000 Pa) stiffness formulations after 1 week of culture. Cells appear more extended in the HELP low condition. The level of extension decreases as gel stiffness increases. Cell nuclei are stained with DAPI (blue). F-actin filaments are stained with phalloidin (magenta).

to the Boc groups (Fig. 3B). Lastly, to confirm successful deprotection of the hydrazine group, and thus ELP-HYD modification, the disappearance of this Boc peak is observed in the spectrum of the final ELP-HYD product (Fig. 3C).

To characterize the final degree of benzaldehyde modification of HA, H^1 NMR was again used. As expected, the baseline spectrum for unmodified HA does not include an aldehyde peak at 10.1 ppm (Fig. 4A). By measuring the protons on the aldehyde group at 10.1 ppm relative to the protons of the methyl group at 2.1 ppm, it was determined that HA-BZA with modifications of 6% (Fig. 4B), 12% (Fig. 4C), and 30% (Fig. 4D) were successfully synthesized.

HELP gels can be tuned to a wide range of stiffnesses

To demonstrate that the HELP system can be synthesized to create gels with a broad

range of stiffnesses, oscillatory shear rheology was conducted on the three different HELP formulations (HELP low, medium, and high) that were introduced in Basic Protocol 3. HELP low, medium, and high have storage moduli (G′) of ∼300, 800, and 3000 Pa, respectively (Fig. 5A). Gelation occurred within minutes for HELP low and within seconds for HELP medium and high gels, with a stable gel formed after 20 min (Fig. 5B). The gels were crosslinked *in situ* on the rheometer stage, and measurements of storage (G′) and loss (G″) moduli were taken throughout the entire gelation process. The plateau storage modulus was reached 30 min after mixing the two hydrogel components together. Previous work in the laboratory has demonstrated that diffusivity is not significantly altered in HELP gels that fall within this range of stiffness (LeSavage et al., 2022).

HELP gels support the viability of encapsulated cells

Live/dead staining of encapsulated hMSCs shows that all three HELP formulations support high cell viability at both 1 and 7 days postencapsulation (Fig. 6).

HELP gel stiffness affects cell morphology

hMSCs encapsulated in HELP low, medium, and high gels were stained for DAPI (nuclei) and phalloidin (actin filaments) to assess cell morphology in gels of different stiffnesses (Fig. 7). Cells appear more extended in HELP low, and this extension decreases as the gels get stiffer in HELP medium and high.

Time Considerations

Starting with unmodified ELP, the modification of ELP with hydrazine takes on average 10 days. The HA-alkyne synthesis takes ∼1 week. It takes an additional week to complete the HA modification with azido-BZA. These time ranges all include the required dialysis, freezing, and lyophilization steps. If sufficient space and resources are available, ELP-HYD and HA-BZA can be synthesized concurrently. The NMR procedures require less than a day to perform, and once the materials are dissolved, the encapsulation can be completed in a few hours. Make sure to take into consideration the time required to grow the relevant cells or organoids to confluency.

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Author Contributions

Meghan E. Hefferon: Data curation; formal analysis; investigation; methodology; project administration; visualization; writing—original draft; writing—review and editing. **Michelle S. Huang:** Conceptualization; formal analysis; investigation; resources; writing—review and editing. **Yueming Liu:** Conceptualization; formal analysis; investigation; resources. **Renato S. Navarro:** Formal analysis; investigation; resources; visualization; writing—original draft. **Narelli de Paiva Narciso:** Formal analysis; investigation; resources; writing—review and editing. **Daiyao Zhang:** Formal analysis; investigation. **Giselle Aviles-Rodriguez:** Resources. **Sarah C. Heilshorn:** Conceptualization; funding acquisition; supervision; writing—review and editing.

Conflict of Interest

S.C.H, R.A.S, and D.R.H are inventors on a patent application (no. PCT/US2021/057925) submitted by the Board of Trustees of Stanford University.

Data Availability Statement

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

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