Electronic Supplementary Material (ESI) for Biomaterials Science.

Electronic Supporting Information

Robust alginate/hyaluronic acid thiol-yne click-hydrogels with superior mechanical performance and stability as scaffolds for load-bearing soft tissue engineering

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1. EXPERIMENTAL SECTION

All chemicals were purchased from Sigma-Aldrich and cell culture reagents from ThermoFisher Scientific and were used without further purification unless stated otherwise.

1.1. Synthesis of HA-SH

HA-SH was prepared by modifying a previous published procedure.[1] Specifically, 1 g of HA sodium salt (Carbosynth, 40-50 kDa - FH01773) and 110 mg of 3,3'-dithiobis(propanoic dihydrazide) (DTPHY, Frontier Scientific) were dissolved in a 150 mM MES solution (100 mL) with occasional gentle swirling (final pH 4.1). Then, 180 mg of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC·HCl, Fluka) was added dropwise with stirring. The stirring was stopped, and the reaction was allowed to continue overnight. Then, 340 mg of TCEP-HCl (Fluorochem) were dissolved in 1 mL water and added; the reduction was left to proceed overnight. Finally, 2.4 g of NaCl were added to the solution, and the mixture was dialyzed against ultrapure water balanced to pH 4.5 with dilute HCl over 3 days, with 3-4 water changes every 24 h, to yield a solution of pure HA-SH sodium salt. After dialysis, the product was lyophilized to obtain a white solid (82% ± 8.7%). ^{1}H NMR (300 MHz; 298 K; D_2O): δ 2.80 (broad s, 2H, NHCOCH_2-), 2.62 (broad s, 2H, - CH_2SH), 1.93 (s, 3H, -NHCOCH_3).

1.2 Synthesis of 2-arm alkyne-functionalized PEG precursor ($\mathbf{2_{1A}}$)

All alkyne-functionalized PEG precursors were prepared by an already published procedure[2] that involves simple end group modifications highly efficient (Fischer esterification). To a suspension of 2-arm PEG_{1k}-OH (1.0 kg mol^{-1}, 10 g, 10 mmol) in benzene (75 mL) and toluene (75 mL), 2 drops of concentrated H_2SO_4 were added. The solution was heated to 80 °C with stirring to obtain a clear homogenous solution. To this solution, propiolic acid (2.8 g, 40 mmol) was added, and the solution was heated to reflux under Dean-Stark conditions. After no more water was collected in the condenser (*ca.* 20 h), the solution was allowed to cool down to room temperature, and the solvents were removed *in vacuo*. The resultant oil was dissolved in CH_2Cl_2 (100 mL) and washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL). The organic phase was dried ($MgSO_4$) and stirred with charcoal (*ca.* 0.1 g) for 30 minutes at 40 °C. The solvent was evaporated to collect the product ($\mathbf{2_{1A}}$) as a white sticky solid (70%). ^{1}H NMR (300 MHz; 298 K; $(CD_3)_2CO$): δ 4.33 (t, 2H, $^3J_{H-H}$ = 8 Hz, -CH_2OCO-), 3.87 (s, 1H, -CH≡CC(O)O-), 3.72-3.74 (m, 2H, -OCH_2CH_2O-), 3.58 (s, 2H, -OCH_2CH_2O-). ^{1}H NMR spectroscopy indicated ca. 91% conversion of the hydroxyl group to propiolate group. SEC (DMF): M_n = 2.2 kg mol^{-1} (ĐM = 1.13).

1.3 Synthesis of 3-arm alkyne-functionalized PEG precursor ($\mathbf{3_{1A}}$)

Similary, glycerol ethoxylate (1.0 kg mol^{-1}, 15 g, 15 mmol) was esterified with propiolic acid (6.3 g, 90 mmol) to collect the product (**$\mathbf{3_{1A}}$**) as a light yellow oil (Yield 75%). ^{1}H NMR (300 MHz; 298 K; $(CD_3)_2CO$): δ 4.32-4.35 (t, 2H, $^{3}J_{H\text{-}H}$ = 8 Hz, -C*H*$_2$OCO-), 3.88 (s, 1H, -C*H*≡CC(O)O-), 3.71-3.74 (m, 2H, -OCH$_2$C*H*$_2$O-), 3.58 (s, 2H, OC*H*$_2$C*H*$_2$O-). ^{1}H NMR spectroscopy indicated ca. 94% conversion of the hydroxyl group to propiolate group. SEC (DMF): M_n = 1.2 kg mol^{-1} ($Đ_M$ = 1.20).

1.4 Synthesis of 4-arm alkyne-functionalized PEG precursor ($\mathbf{4_{2A}}$)

4-arm PEG-tetrahydroxyl (2.0 kg mol^{-1}, 10 g) was purchased from JenKem Technology, USA, and it was esterified with propiolic acid (3.6 g) to collect the product (**$\mathbf{4_{2A}}$**) as a light yellow oil (Yield 64%). ^{1}H NMR (300 MHz; 298 K; $(CD_3)_2CO$): δ 4.32 (t, 2H, $^{3}J_{H\text{-}H}$ = 6 Hz, -C*H*$_2$OCO-), 3.89 (s, 1H, -C*H*≡CC(O)O-), 3.71-3.74 (m, 2H, -OCH$_2$C*H*$_2$O-), 3.58 (s, 2H, -OC*H*$_2$C*H*$_2$O-) 3.42 (s, 2H, -CC*H*$_2$O). ^{1}H NMR spectroscopy indicated ca. 98% conversion of the hydroxyl group to propiolate group. SEC (DMF): M_n = 4.2 kg mol^{-1} ($Đ_M$ = 1.08).

1.5 HA-SH:yne and ALG/HA-SH:yne hydrogel preparation

HA-SH:yne hydrogels (0.4 mL) were prepared in PBS at room temperature (*i.e.* 23 °C) considering four polymer content (*i.e.* 1.5, 2.5, 5, and 10% wt.%) and using 50:50 or 75:25 weight ratio of HA-SH to alkyne precursor. In a typical procedure, the corresponding amount of HA-SH was dissolved in 300 µL of PBS (adjustment of pH to 7.4 is required by adding 3-10 µL KOH 1M), while the alkyne-functionalized PEG precursor is dissolved in 100 µL PBS pH 7.4. Then, the alkyne solution is added to the thiol solution, and they are vigorously mixed by vortex for 5-10 seconds. Although the gelation times are fast, hydrogels were let to gel overnight.

ALG/HA-SH:yne hydrogels were prepared following a similar procedure. In this case, alginate (high viscosity alginate) was mixed with the thiol solution at different concentrations (10, 20, or 30 mg/mL) before adding the alkyne precursor. After gelation, the alginate network within the click-hydrogel was cross-linked by immersing the hydrogels in a $CaCl_2$ 150 mM solution for 10 minutes. The hydrogels were washed twice with water prior any further characterization. For swelling experiments, control alginate hydrogels were prepared as follows: alginate was dissolved in PBS (30 mg/mL), and the viscous solution was placed inside a dialysis membrane that was immersed in 150 mM $CaCl_2$ solution overnight (such amount of time ensured that all the alginate was cross-linked with no liquid core remaining). After that period, a solid alginate cylinder was obtained and cut into smaller pieces.

1.6 Hydrogel characterization

Gel fraction (GF) and equilibrium water content (EWC). Hydrogels were fabricated as stated in the above procedure. To determine their gel fraction (GF), the hydrogels were lyophilized and their weights (W_g) recorded. The hydrogels were then allowed to swell in deionized water for 3 days, with frequent water changes to extract any unreacted alkyne precursor or HA-SH. After that period, the hydrogels were lyophilized, and their weights (W_r) were recorded again. All measurements were repeated in triplicate. GF is expressed as:

$$\text{Gel Fraction } (\%) = \frac{\mathrm{W_r}}{\mathrm{W_g}} \times 100\ \% \qquad (1)$$

To determine the equilibrium water content (EWC), hydrogels were allowed to swell in PBS (pH 7.4) for 24 hours, and their weight was recorded (W_s). Hydrogels were then lyophilized, and the weights recorded again (W_d). All measurements were repeated in triplicate. EWC is expressed as:

$$\text{EWC } (\%) = \frac{\mathrm{W_s} - \mathrm{W_d}}{\mathrm{W_s}} \times 100\% \qquad (2)$$

Swelling and degradation studies. Hydrogels were fabricated as stated in the above procedure and then placed in the corresponding solution (*i.e.* PBS, Ringer's solution or cell culture media) at 37 °C in an orbital shaker-incubator (Model ES-20, Grant Instruments (Cambridge) Ltd.) with a shaking speed of 80 rpm. The swelling solution was replaced regularly to remove unreacted alkyne PEG precursors and to prevent the build-up of solute concentration. At specific time points, the hydrogels were removed, gently blotted dry, and their weight was recorded. In case of degradation studies, hyaluronidase was added (*i.e.* 100 U mL^{-1}, 50 U mL^{-1}, or 10 U mL^{-1}) to a Ringer's solution prepared with a $Ca2^{+}$ concentration of 8 mM). Hence, the swelling and degradation was monitored by the percentage of weight of the hydrogel at each time point (W_t) compared to the weight before submersion (W_0), which is defined as:

$$\boldsymbol{Swelling\ Factor}\ (\%) = \frac{\boldsymbol{W_t}}{\boldsymbol{W_0}} \times \mathbf{100}\% \qquad (4)$$

Rheological characterization. All rheology was performed on an Anton Parr MCR 302 rheometer fitted with a parallel plate configuration (diameter of 50 mm or 8 mm) at 37 °C keeping the normal force constant at 0 N. During optimization, in a typical rheological test for gelation kinetics (*i.e.* evolution of storage moduli (G') and loss moduli (G") as a function of time; time

sweep), the HA-SH solution (with or without alginate) was spread over the lower plate at 37 °C. Then, the alkyne-functionalized PEG precursor was homogeneously distributed on top and carefully mixed with the thiol component. The upper plate was immediately lowered to a plate separation of 0.5 mm, and the measurement was started. A frequency of 5 Hz and a strain of 1% was applied to minimize interference with the gelation process and keep the measurement within the linear viscoelastic region. The normal force was also kept constant at 0 N. The gel point was determined by the cross-over between the G' and G". Similarly, to monitor the cross-linking process of the alginate with Ca^{2+} ions, 0.5 mL of $CaCl_2$ 150 mM were homogeneously distributed on top and carefully mixed with the ALG + HA-SH:yne system. Frequency sweeps were carried out on the gel formed from this experiment applying a constant strain of 0.5%, while the frequency was ramped logarithmically from 0.1 to 100 rad s^{-1}). Meanwhile, amplitude sweeps were conducted applying a constant frequency of 10 rad s^{-1}, while the strain was ramped logarithmically from 0.01% to 100%. All measurements were repeated in triplicate, and representative charts are shown.

The optimized ALG/HA-SH:yne hydrogels were characterized by frequency and amplitude sweeps as stated before, but using a parallel plate of 8 mm in diameter.

Uniaxial compressive tests. All uniaxial compressive testing was performed on a M100-1CT Testometric fitted with a load cell of 1 kN. Hydrogel samples, which were prepared as described above (n = 8-10), were tested either as prepared (*i.e.* after gelation overnight) or after being immersed in culture media for 1, 7, 14, and 21 days. A preload force of 0.1 N was set, and each test was carried out at a compression velocity of 5 mm min^{-1}. Each gel was subjected to 98% strain to determine the ultimate compressive stress and strain. Regarding cyclic compression, each gel was subjected to 30% strain at 5 mm min^{-1} for both the loading and unloading curves. Data was analyzed using Wintest analysis software.

Dynamic Mechanical Analysis (DMA). DMA (STARe System DMA 1, Mettler Toledo) was used to determine the mechanical stability of the ALG/HA-SH:2_{1A} click-hydrogels (*ca.* 10 × 6 mm, diameter × thickness) immersed in cell culture media for 18h at 37 °C. Uniaxial compression testing was performed at 1 Hz under 10% strain. After being equilibrated in cell culture media for four days at 37 °C, hydrogels were gently clamped and pressed together after mounting on the plates. All testing was conducted at 37 °C. Measurements were repeated on three samples.

Cryogenic Scanning Electron Microscopy. Cryogenic scanning electron microscopy (cryo-SEM) was performed on ZEISS SUPRA 55-VP equipped with cold stage and sample preparation chamber. HA-SH:2_{1A} and ALG/HA-SH:2_{1A} hydrogel samples were carefully placed on a stub and frozen in liquid nitrogen (-195 °C) under vacuum. The stub was then transferred to the cold stage (-125 °C) of the preparation chamber. There, the frozen sample was carefully surface fractured and sublimated at -95 °C for 15 min to reveal the cross-sectional surface. The temperature was then brought down to -125 °C, and the sample was sputter coated with platinum before being transferred under vacuum into the main SEM chamber (kept at -186 °C for imaging). The accelerating voltage was set at 2 kV to avoid burning the sample.

1.7 Biocompatibility studies and 3D cell encapsulation

Cytotoxicity of alkyne precursors and degradation products. To confirm that our click-hydrogels and their degradation products were non-cytotoxic, cell viability tests on MC3T3 (murine pre-osteoblasts) cells were undertaken. To that end, ALG/HA-SH:yne click-hydrogels were immersed in cell culture media at 37 °C for 35 days. Then, the cell media was recovered and centrifuged before being used for the cells study. Similarly, the three alkyne precursors were dissolved in cell media at 5 mg mL^{-1}. MC3T3 cells were obtained from Public Health England. Cells were cultured in 175 cm^2 tissue culture flasks using MEM alpha medium (Gibco), as advised by the supplier, with addition of 10% FBS and 1% pen/strep, at 37 °C, 5% CO_2. Cells were seeded on 12 well plates (2000 cells cm^{-2}) for viability assays. Cells were left to adhere and proliferate on the wells for 72 h, then incubated with the cell media containing the degradation products or the dissolved alkyne at different concentrations. Viability was measured after 24 h and/or 72 h using PrestoBlue metabolic assay (Invitrogen), and results are reported in comparison to controls (0 mL of alkyne/degradation products). Experiments were performed in triplicate.

3D encapsulation. The Y201 hTERT-immortalised human clonal mesenchymal stem cell (MSC) line (a kind gift from Prof Paul Genever, University of York) was cultured in MEM-α medium supplemented with 10% v/v FCS, 1% penicillin/streptomycin, 10 µM asc-2-phos, and 5 mL Glutamax. For 3D cell encapsulation, hydrogels (n=4) were prepared in PBS in 24-well cell culture inserts with a 0.4 µm pore PET membrane. Specifically, 400000 cells per gel (ALG/HA-SH:2_{1A}) were suspended with the alginate + HA-SH solution, followed by addition of the alkyne (2_{1A}) and mixing. Then, 100 µL of the solution was added into the inserts and let to gel. After 10 minutes, 100 µL of $CaCl_2$ was added on top of the gel, while 0.9 mL were added in the well. The alginate-based network was allowed to cross-link for 10 minutes. After that, fresh media was added on

top (100 µL) and around the hydrogel (900 µL), and cells were incubated up to 21 days. Cell viability was assessed using alamarBlue® viability assay at different time points (*i.e.* 24, 48, 72h, and 7 and 21 days) according to the manufacturer's recommendations. Fluorescence was measured using a BioTek FLx800 plate reader (λEx. = 540 nm, λEm. = 590 nm). Cell viability was also assessed on day 1 (24 hours), day 7 and day 21 using Live/Dead™ Viability/Cytotoxicity Kit (Invitrogen), including calcein AM for live cells (λEx. = 495, λEm. = 515) and ethidium homodimer for dead cells (λEx. = 528, λEm. = 617). The staining solution was prepared by dissolving calcein AM (0.5 µL mL^{-1}) and ethidium homodimer (2 µL mL^{-1}) in PBS. On the day of assay, hydrogels were washed with PBS, and the staining solution was added around (350 µL) and on top (150 µL) of the gels, which were then incubated for 30 minutes. Fluorescence imaging in three-dimensions was conducted with a Leica TCS SP5 AOBS Inverted confocal microscope. Z-stacks with an average thickness of 200 µm were collected from different zones of each sample, and the images were processed using ImageJ software (1.51u).

2. Supplementary Data

2.1 Material synthesis

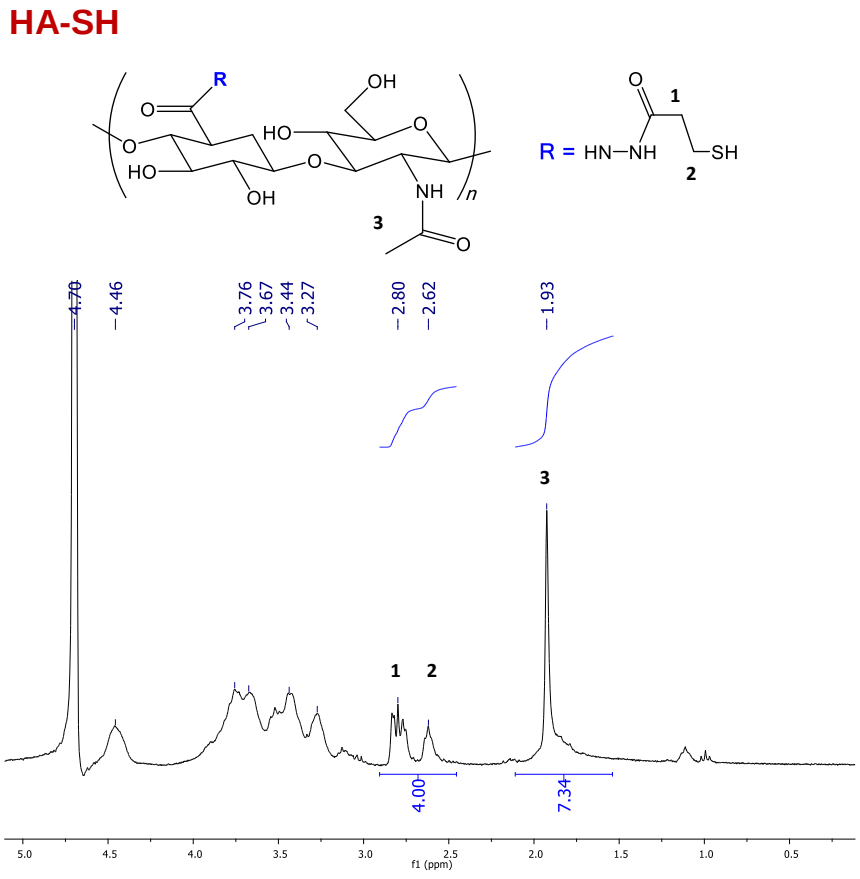


Figure S1. ^{1}H NMR spectrum of HA-SH in deuterated water (300 MHz, 298 K).

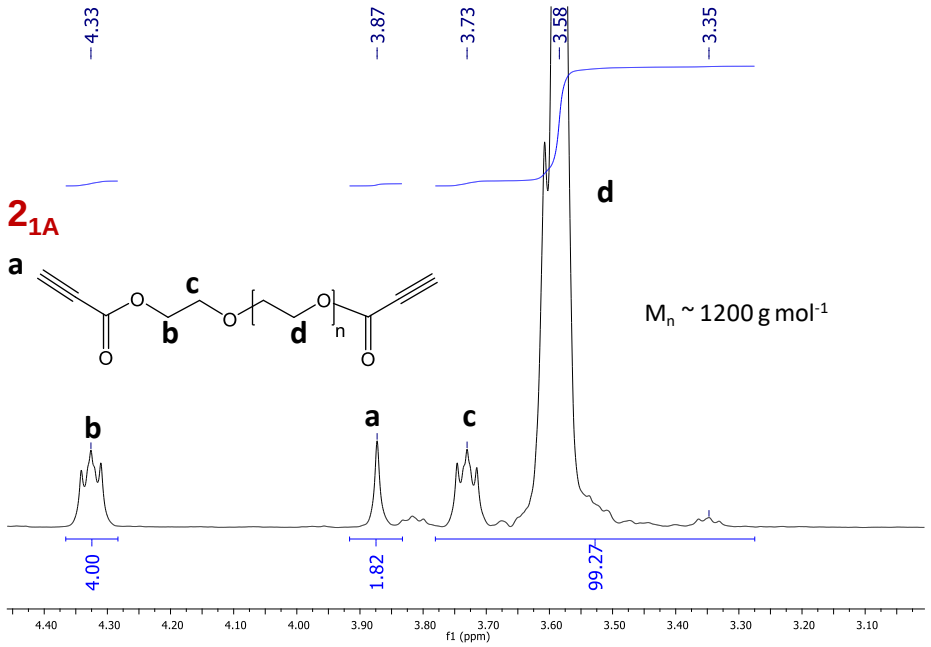


Figure S2. ^{1}H NMR spectrum of yne-terminated 2-arm PEG (2_{1A}, 1 kg mol^{-1}) in d_6-acetone (300 MHz, 298 K).

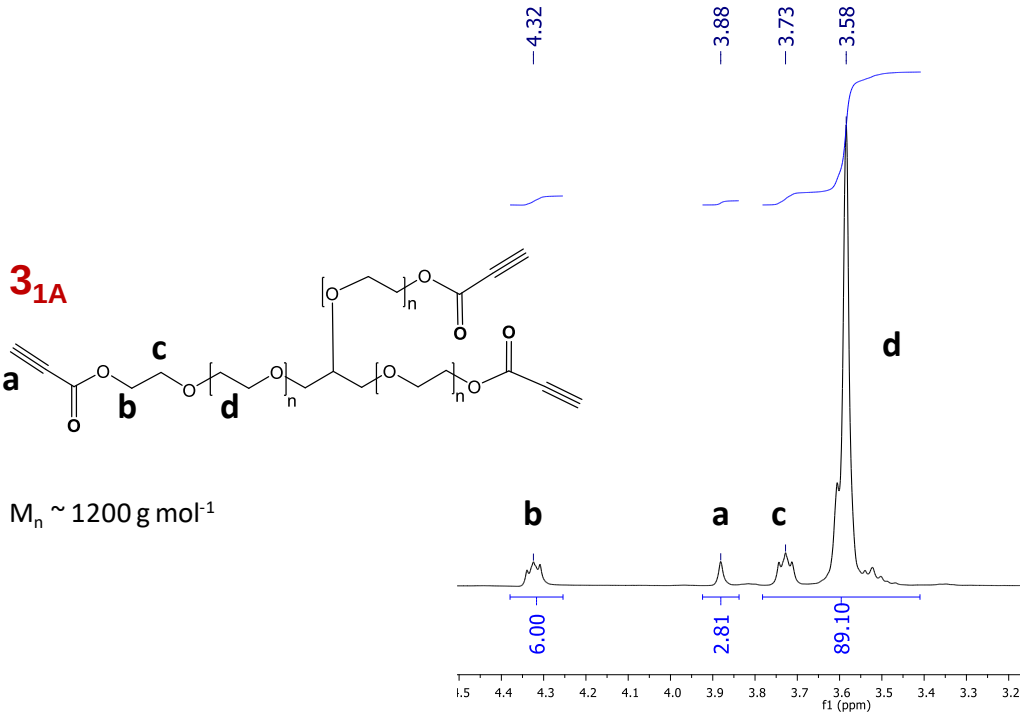


Figure S3. ^{1}H NMR spectrum of yne-terminated 3-arm PEG (3_{1A}, 1 kg mol^{-1}) in d_6-acetone (300 MHz, 298 K).

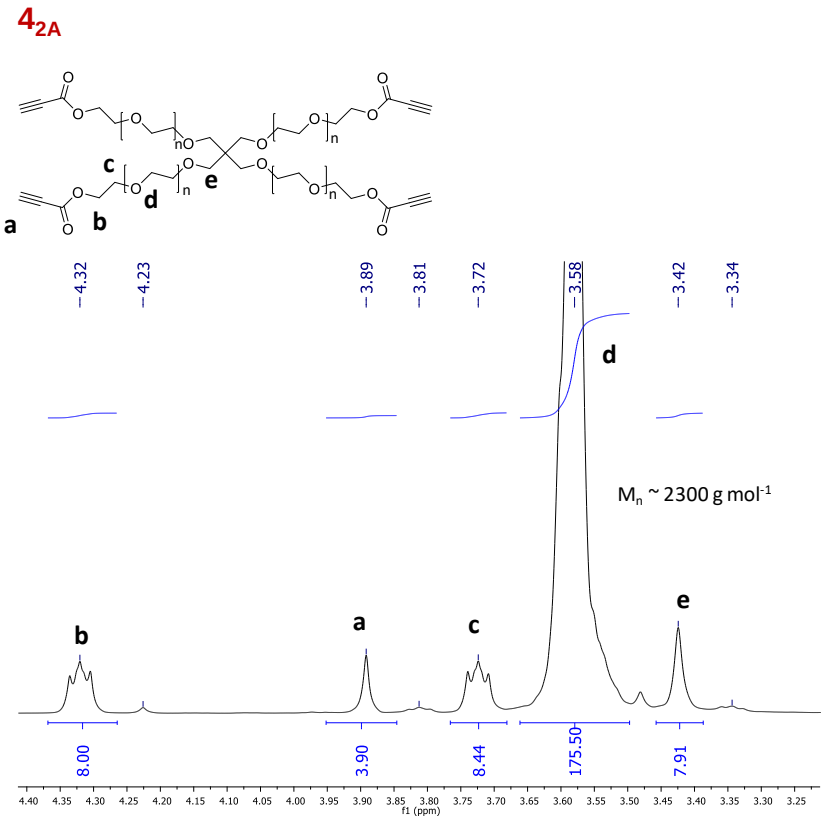


Figure S4. ^{1}H NMR spectrum of yne-terminated 4-arm PEG (4_{2A}, 2 kg mol^{-1}) in d_6-acetone (300 MHz, 298 K).

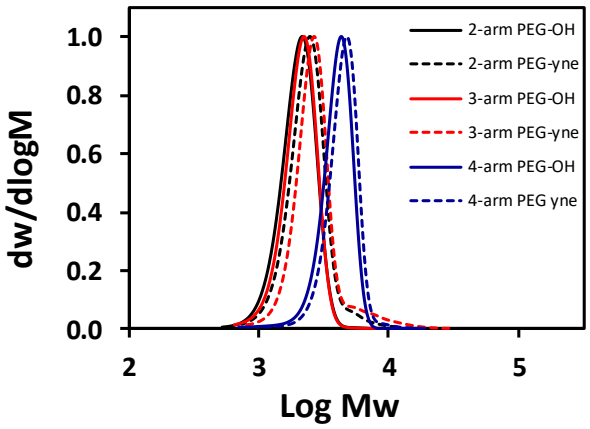


Figure S5. SEC chromatograms of alkyne-terminated PEG precursors (dashed lines) and starting PEG materials (solid lines). Molecular weight determined against poly(methyl methacrylate) using DMF (5 mM NH_4BF_4) as eluent.

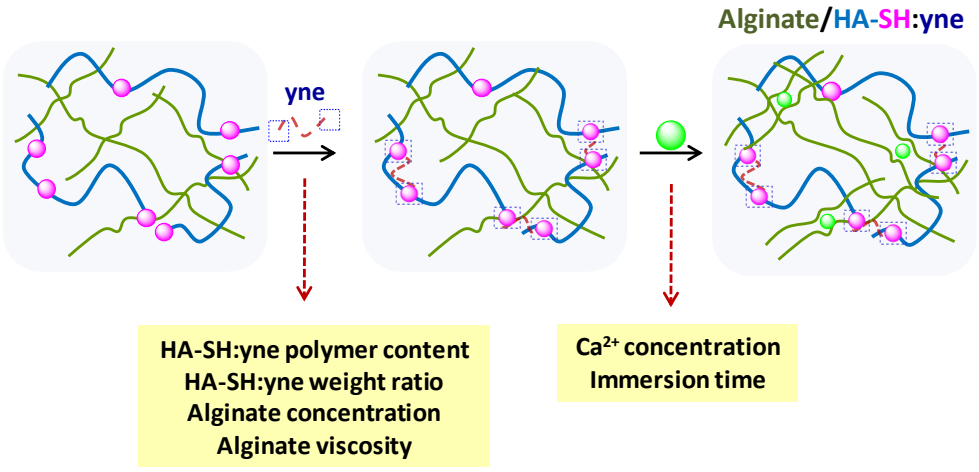
Table S1. Additional characterization data of alkyne-terminated PEG precursors.

Alkyne	End group conv.[a] (%)	M_n NMR[a] (kg mol^{-1})	M_n SEC[b] (kg mol^{-1})	$Đ_M$
2_{1A}	91	1.2	2.2	1.133
3_{1A}	94	1.2	2.5	1.196
4_{2A}	98	2.3	4.2	1.082

[a]Determined by ^{1}H NMR spectroscopy in deuterated acetone. [b]Determined by SEC analysis in DMF against poly(methyl methacrylate).

2.2 Optimization of HA-SH:yne and ALG/HA-SH:yne hydrogel preparation

HA-SH hydrogels were readily obtained in PBS pH 7.4 at room temperature (23 °C) by combining HA-SH with 2_{1A}, 3_{1A}, or 4_{2A}. Several parameters, such as the polymer content of the HA-SH:yne network or the weight ratio between HA-SH and the alkyne-terminated PEG were optimized to achieve the most adequate properties in terms of swelling response or viscosity, respectively, whereas the concentration of alginate as well as the crosslinking process were selected depending on the resulting mechanical improvement achieved.



Scheme S1. Schematics illustrating the variables optimized during the preparation of HA-SH:yne and ALG/HA-SH:yne click-hydrogels.

The table below (Table S2) summarizes the main outcome of the optimization process:

Table S2. Parameters considered during the optimization process of hydrogel preparation.

Parameter	Options	Criteria	Final value	Figure
HA-SH:yne polymer content (%wt.)	1.5% 2.5% 5% 10%	Viscosity of the solution: impact on cell distribution	2.5%	Fig S6
HA-SH:yne weight ratio	50:50 75:25	Swelling response	75:25	Table S3 Fig S7
Alginate concentration	5 mg mL^{-1} 10 mg mL^{-1} 20 mg mL^{-1} 30 mg mL^{-1} 40 mg mL^{-1}	Improvement on the mechanical performance	3 mg mL^{-1}	Fig S8/S9
Alginate *(viscosity)*	Low High	Improvement on the mechanical performance	High	Fig S8/S9
Ca^{2+} concentration	100 mM 150 mM	Improvement on the mechanical performance	150 mM	Fig S8/S9
Immersion time	10 min 1 h 6 h	Improvement on the mechanical performance	10 min	Fig S8/S9

In short, the polymer content of the click-hydrogels was firstly determined according to the viscosity of the resulting scaffold. Hence, bearing their final application in mind, stem cells were encapsulated within HA-SH:4_{2A} hydrogels displaying different polymer content and incubated for 24 hours. After that period, viable cells were stained with Live/Dead™ Viability/Cytotoxicity Kit (Invitrogen) and observed under a confocal microscope with the only purpose to determine if they were homogeneously distributed within the 3D matrix. As it was expected, the higher viscosity of the HA-SH solution at 5% and 10% wt.% prevented cells to be uniformly dispersed before gelation, thus forming clusters within the hydrogel (Figure S6). In contrast, a much more homogeneous cell distribution was achieved for HA-SH:4_{2A} hydrogels at 1.5 and 2.5 wt.% polymer content. In addition to that, handling more concentrated precursor solutions is difficult, and we also anticipated that the diffusion of oxygen and nutrients through such compact and cross-linked networks would be in detriment to cell growth over time. Although at this stage we did not evaluate the relation between polymer concentration and cell growth, Chung *et al.*

reported that articular chondrocytes photoencapsulated in methacrylated HA-based hydrogels exhibited enhanced tissue growth when prepared with lower macromonomer concentration.[3]

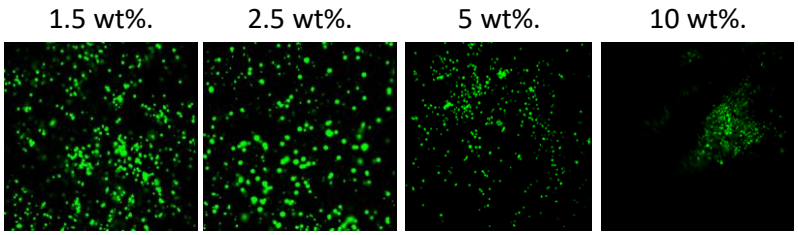


Figure S6. Images from 3D encapsulated cells after 24h of incubation for HA-SH:4_{2A} at different polymer content.

Regarding the HA-SH:yne weight ratio, 75:25 click-hydrogels exhibited a less pronounced swelling when immersed in PBS at 37 °C in comparison to 50:50 (Table S3) because of the less content of hydrophilic PEG within the hydrogel matrix, albeit all the compositions tested at this stage of the optimization were very stable, their mechanical integrity lasting for more than 21 days. However, although some expand-fit response is desirable for the hydrogel to fill perfectly the void it is injected into, high swelling factor (SF) values can induce some undesirable effects when the systems are used *in vivo*. Therefore, we targeted a SF between 110-130% for a better performance of the hydrogel.

Table S3. Swelling factor (SF, %) values determined during the optimization stage for click-hydrogels after being immersed 72 hours in PBS at 37 °C. (* Value after 48 hour of immersion).

Polymer content (wt%.)	Alkyne	HA-SH:yne (weight ratio)	SF (%) at 72 h
2.5	2_{1A}	50:50	195 ± 45
2.5		75:25	103 ± 0.7
2.5	3_{1A}	50:50	156 ± 0.7
2.5		75:25	118 ± 6
5		50:50	269 ± 38
7.5		50:50	228 ± 33
10		50:50	234 ± 6
10		75:25	151 ± 5
10	4_{2A}	50:50	163 ± 12
10		75:25	102 ± 2
5*		75:25	99 ± 8
2.5*		75:25	87 ± 1
2.5		50:50	97 ± 12
1.5*		75:25	75 ± 12

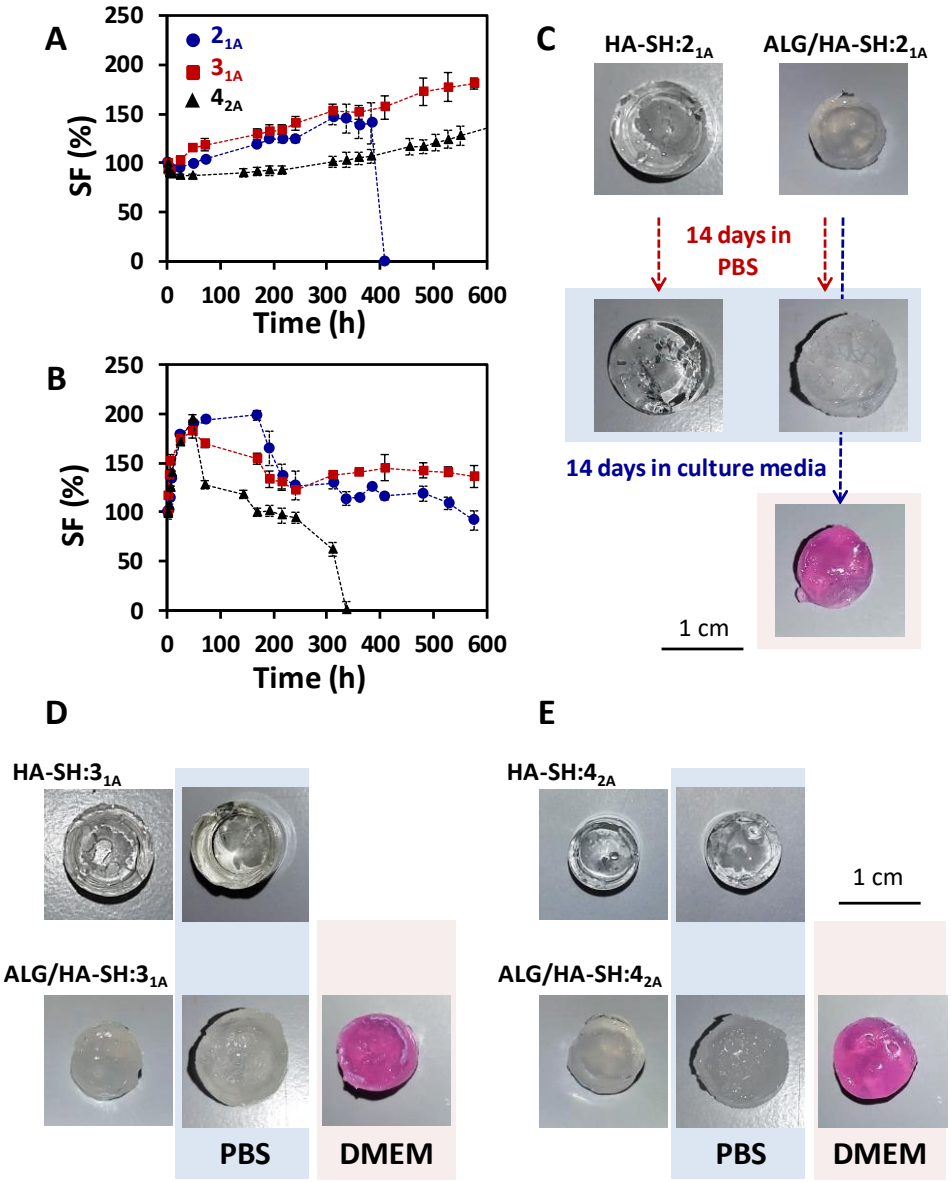


Figure S7. Swelling profiles for click-hydrogels immersed in PBS at 37 °C for A) HA-SH:yne (2.5 wt%. 75:25 SH:yne) and B) ALG/HA-SH:yne (2.5 wt%. 75:25 SH:yne). Images takes to show the volume change induced by swelling (14 days of immersion in PBS or cell culture media) for C) HA-SH:2_{1A}, D) HA-SH:3_{1A} and E) HA-SH:4_{2A} with and without alginate.

The low mechanical strength of the HA-SH:yne hydrogels (Fig. S8 and S9) was overcome by adding into the system an interpenetrated non-covalently cross-linked network based on alginate. Hence, in order to achieve the best possible improvement regarding the mechanical response of the gels under compression, we optimized the alginate used (*i.e.* low viscosity or high viscosity), the alginate concentration in the hydrogel, the Ca^{2+} concentration in the cross-linking solution, and finally the immersion time for cross-linking. Again, Table S2 summarizes this optimization process, while Figure S8 and S9 display the preliminary mechanical results derived from it.

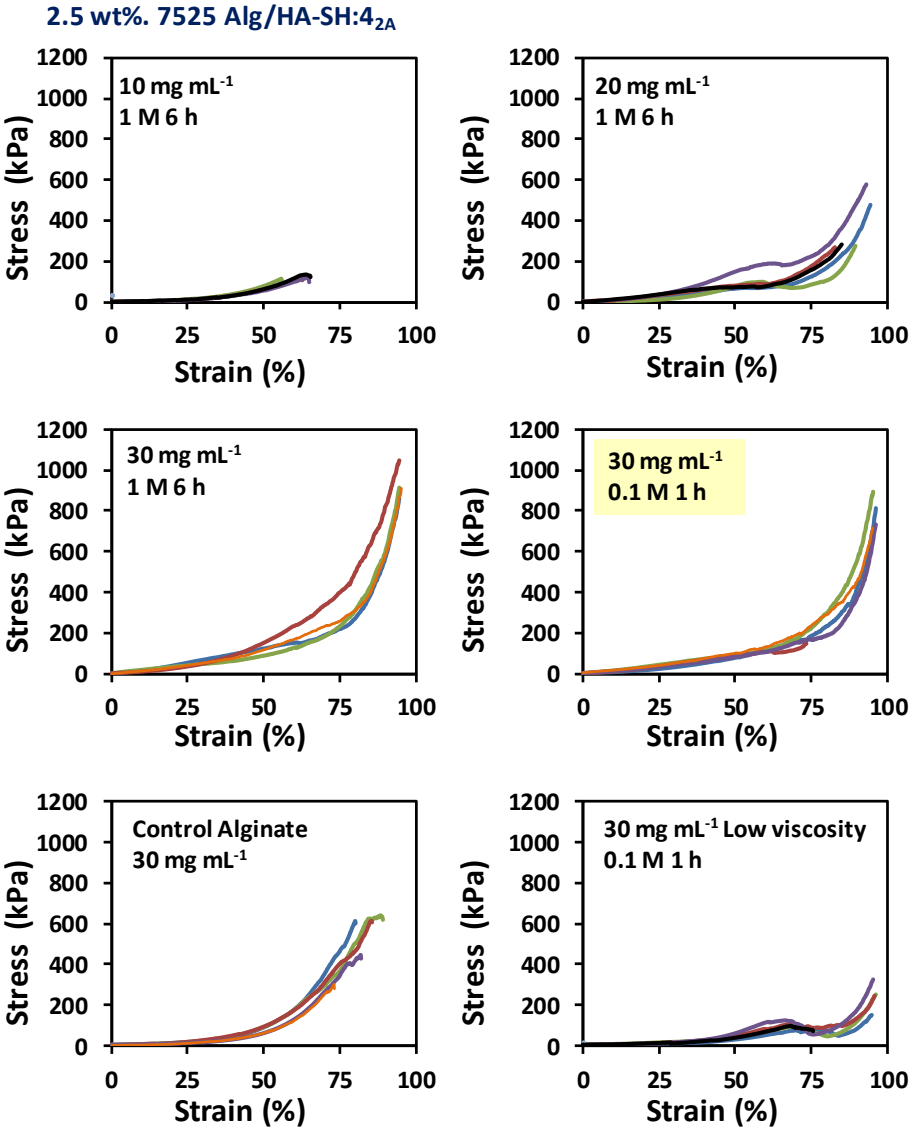


Figure S8. Representative compressive stress-strain curves recorded for ALG/HA-SH:4$_{2A}$ click-hydrogels prepared with varying amounts of alginate and crosslinking parameters.

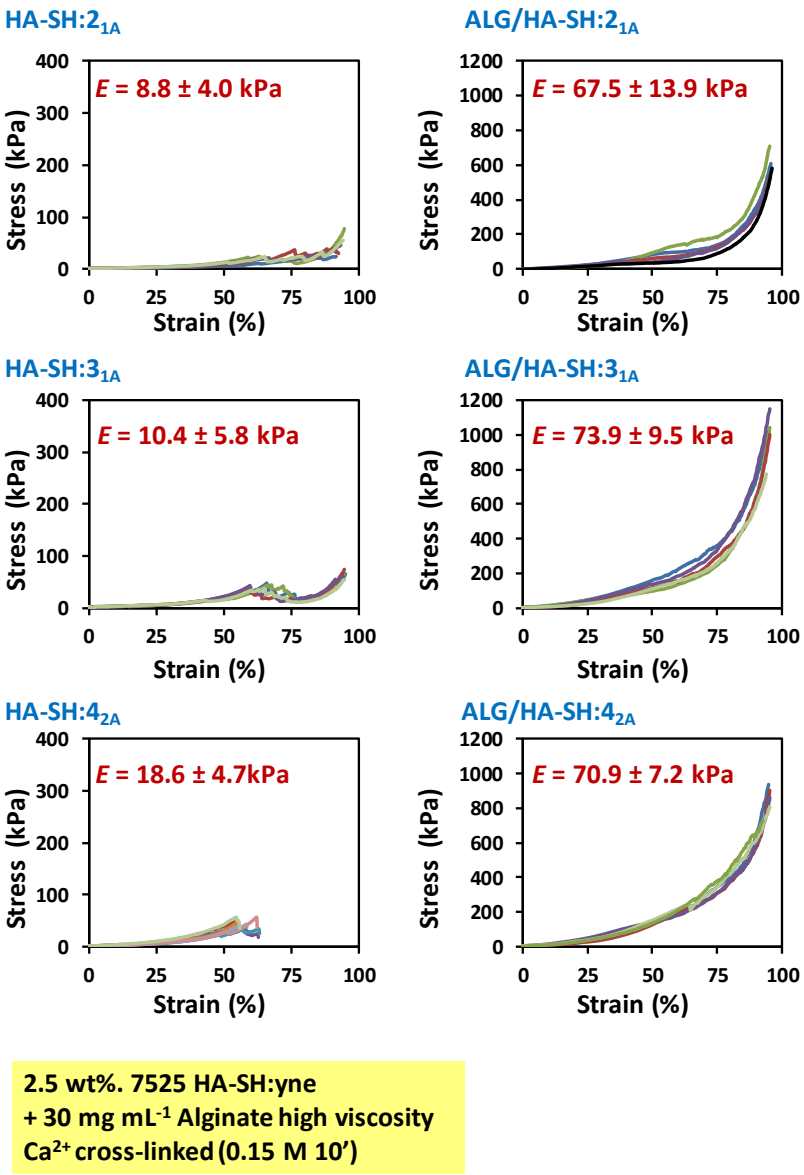


Figure S9. Representative compressive stress-strain curves recorded for HA-SH:yne (left column) and ALG/HA-SH:yne (right column) click-hydrogels prepared with the optimized alginate concentration and cross-linking parameters. Each graph displays the mean Young's moduli determined from the linear range of the curve (below 10% strain).

2.3 General characterization of HA-SH:yne hydrogels

Table S4. Gelation time (GT, seconds; determined by vial tilt method), gelation fraction (GF, %), and equilibrium water content (EWC, %) for click-hydrogels at 2.5 wt%. with a 75:25 SH:yne ratio. ALG/HA-SH:yne were prepared incorporating alginate at 30 mg mL^{-1}.

System	GT (seconds)	EWC (%)	GF (%)
HA-SH:2_{1A}	118 ± 5	96.9 ± 0.10	66.7 ± 4.2
Alg/HA-SH:2_{1A}	N/A	96.3 ± 0.40	78.0 ± 0.9
HA-SH:3_{1A}	58 ± 7	96.4 ± 0.20	62.6 ± 3.2
Alg/HA-SH:3_{1A}	N/A	95.3 ± 0.10	76.8 ± 1.5
HA-SH:4_{2A}	23 ± 8	96.2 ± 0.30	67.5 ± 2.9
Alg/HA-SH:4_{2A}	N/A	95.9 ± 0.20	77.0 ± 4.3

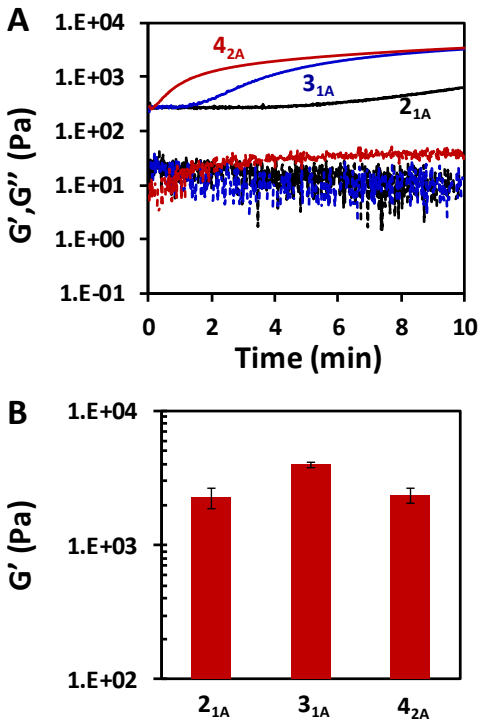


Figure S10. A) Representative gelling curves (time sweeps) as monitored by rheometry for HA-SH:yne hydrogels (2.5 wt%. 75:25 SH:yne). Gelation takes place very fast, being G' > G'' since the beginning. B) Mean values of the final storage moduli (G') after 30 minutes.

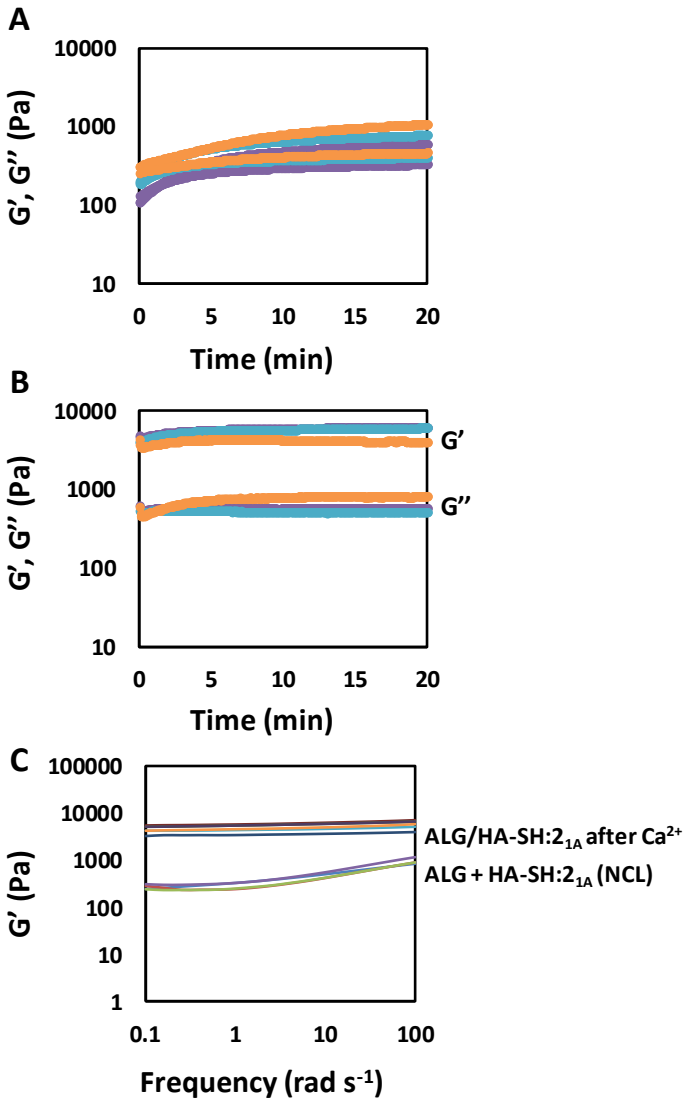


Figure S11. A) Thiol-yne network formation assessed by time sweep (5 Hz and 1% strain) for ALG+HA-SH mixture after adding alkyne precursor 2_{1A}. B) Non-covalently cross-linking of the alginate-based network assessed by time sweep (5 Hz and 1% strain) for ALG+HA-SH:2_{1A} after adding $CaCl_2$ cross-linking solution (0.15 M for 2 minutes before lowering the top plate). C) Frequency sweeps curves for ALG+HA-SH:2_{1A} and ALG/HA-SH:2_{1A} click-hydrogels.

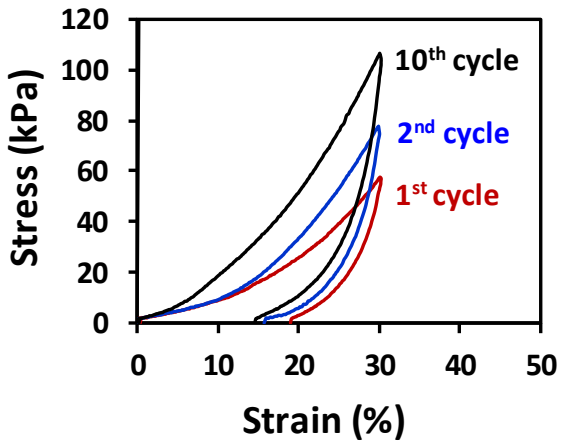


Figure S12. Representative compressive cyclic stress-strain curves of ALG/HA-SH:2_{1A} click-hydrogels after being immersed in cell culture media for 4 days.

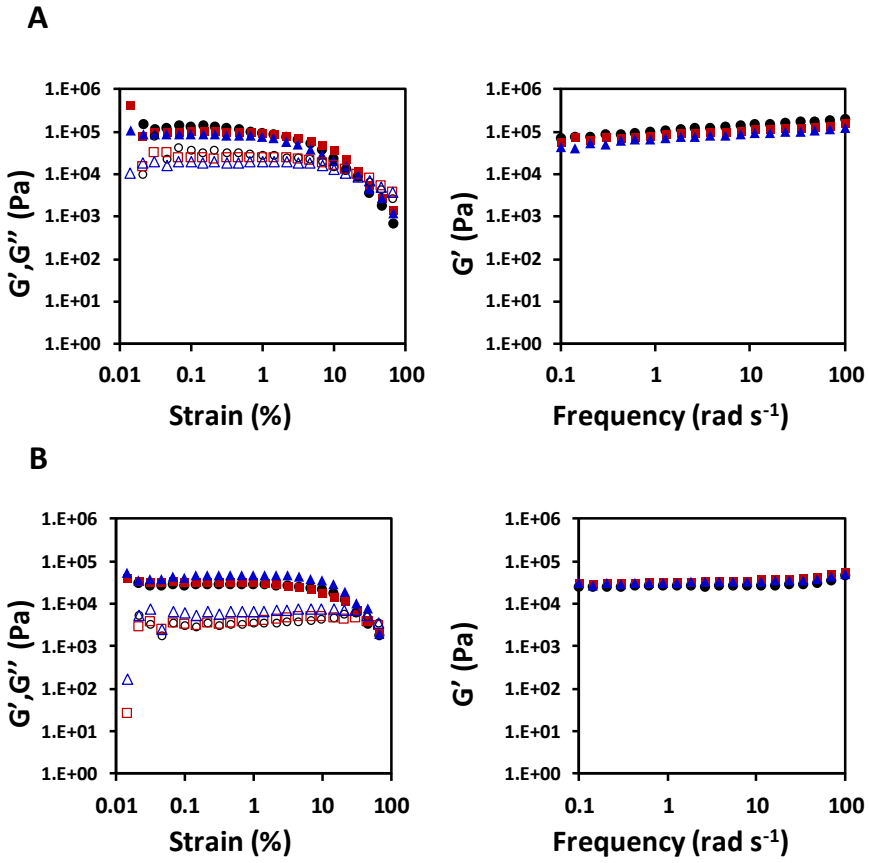


Figure S13. Amplitude (left column) and frequency sweeps (right column) recorded for bulk ALG/HA-SH:2_{1A} click-hydrogels (2.5 wt%. 75:25 thiol:yne) prepared with the optimized alginate concentration and cross-linking parameters: A) as prepared and B) after being immersed in cell culture media at 37 °C for four days (n = 3 samples for each system).

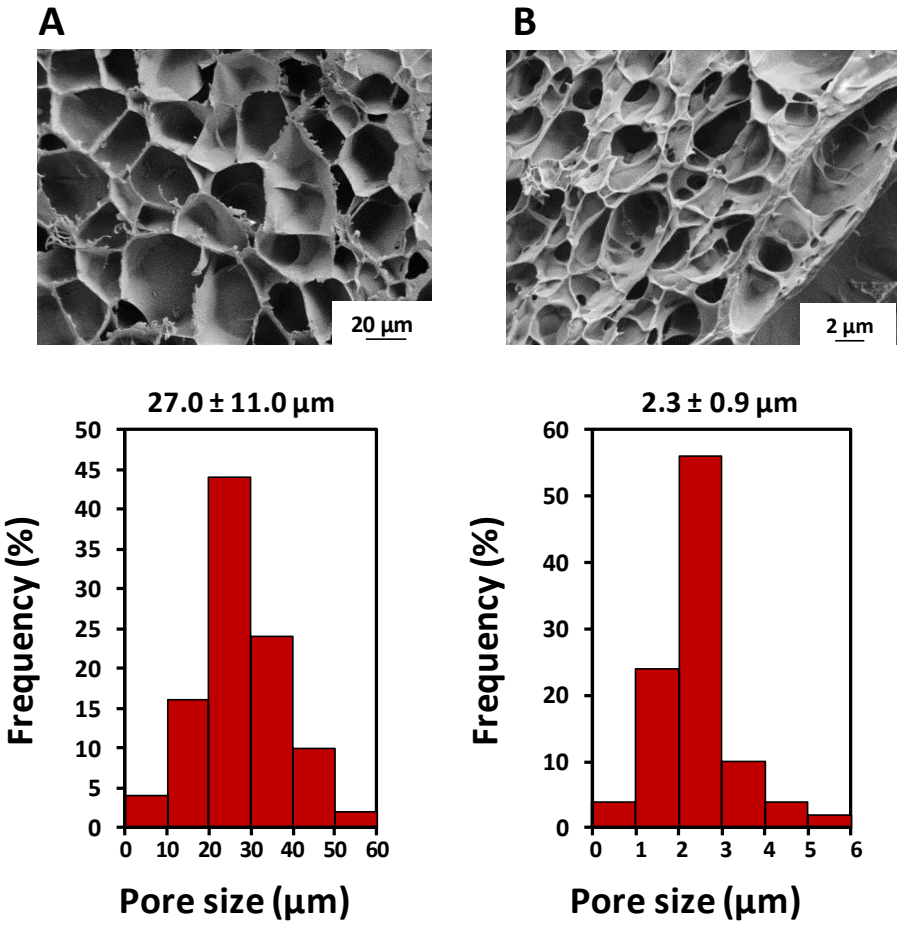


Figure S14. Cryo-SEM images taken for A) HA-SH:yne and B) ALG/HA-SH:yne click-hydrogels and pore size distribution determined from the images.

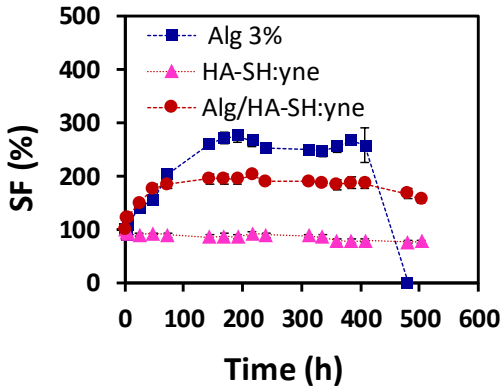


Figure S15. Swelling profiles monitored during the optimization stage for click-hydrogels immersed in PBS at 37 °C: HA-SH:2_{1A} hydrogels (2.5 wt%.) immersed in PBS + 0.1 M Ca^{2+} to check the role played by the cross-linking cation.

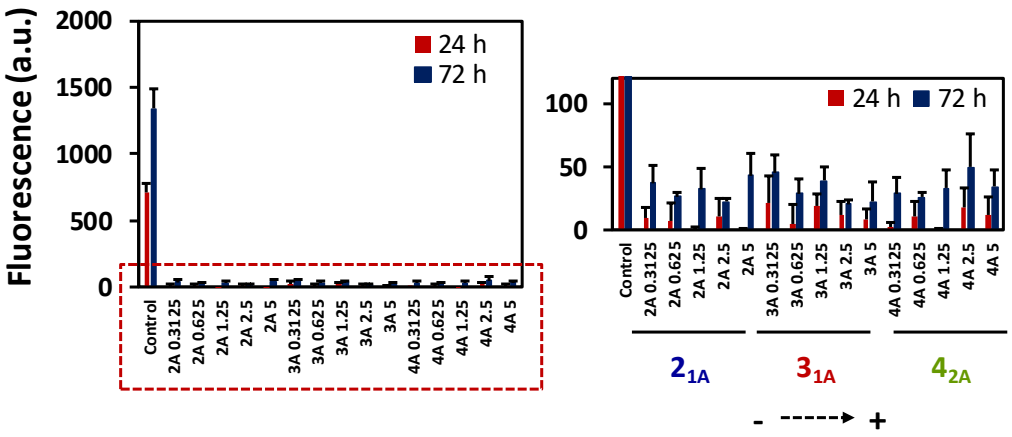


Figure S16. Cytotoxicity of the alkyne PEG precursors dissolved in cell culture media at different concentrations (5, 2.5, 1.25, 0.63, and 0.31 mg mL^{-1}). In comparison to the control (0 mg mL^{-1}), the relative viability of cells in contact with the alkyne precursors is less than 1.2%.

3. References

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2. L. J. Macdougall, M. M. Pérez-Madrigal, M. C. Arno and A. P. Dove, *Biomacromolecules*, 2018, **19**, 1378-1388.
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