Electronic Supplementary Information (ESI) for the manuscript

Redox-triggerable Firefly Luciferin-Bioinspired Hydrogels as Injectable and Cell-encapsulating Matrices

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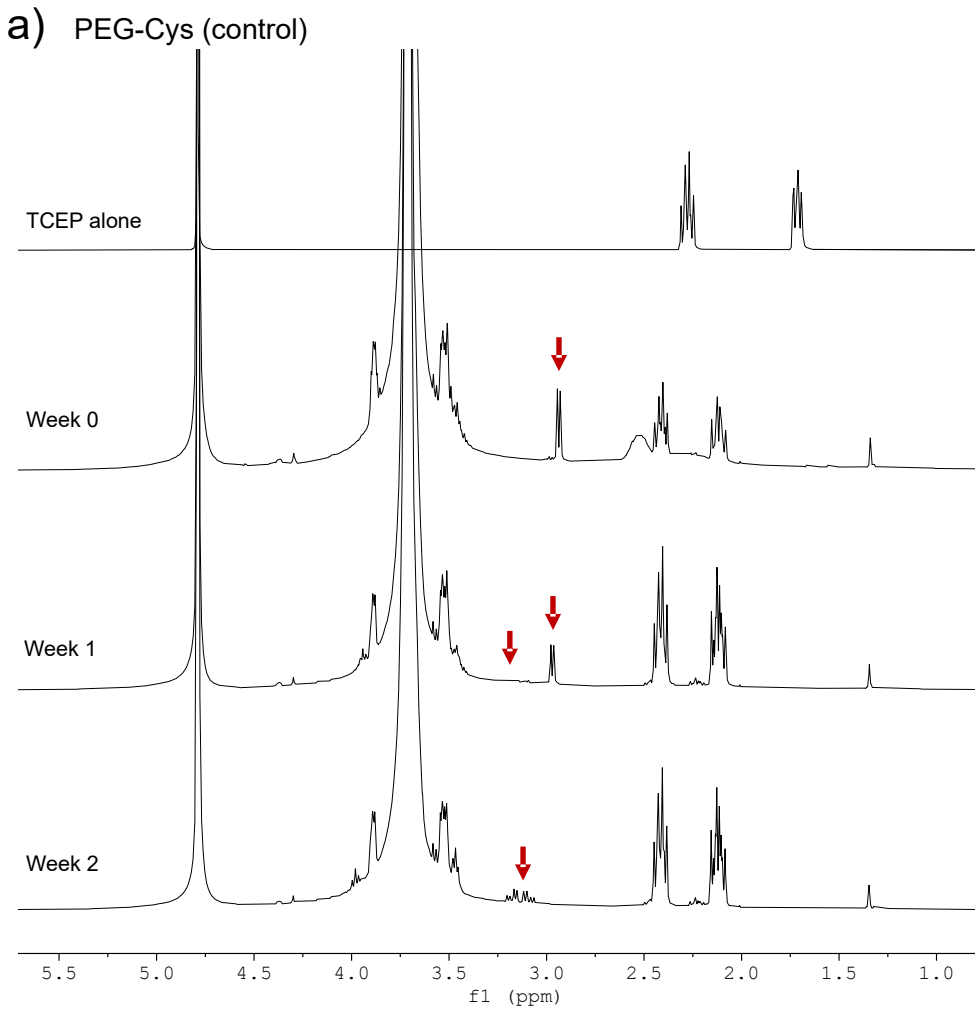
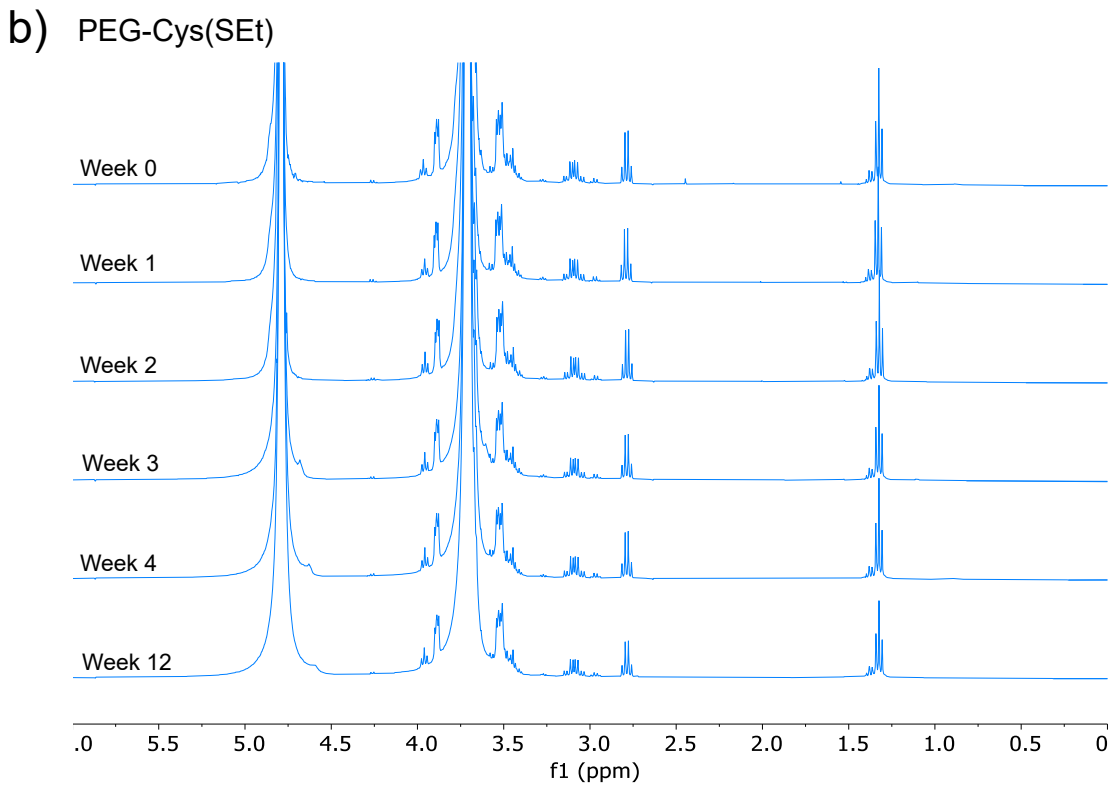

a) PEG-Cys (control)
TCEP alone
Week 0
Week 1
Week 2
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)


b) PEG-Cys(SEt)
Week 0
Week 1
Week 2
Week 3
Week 4
Week 12
.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
f1 (ppm)

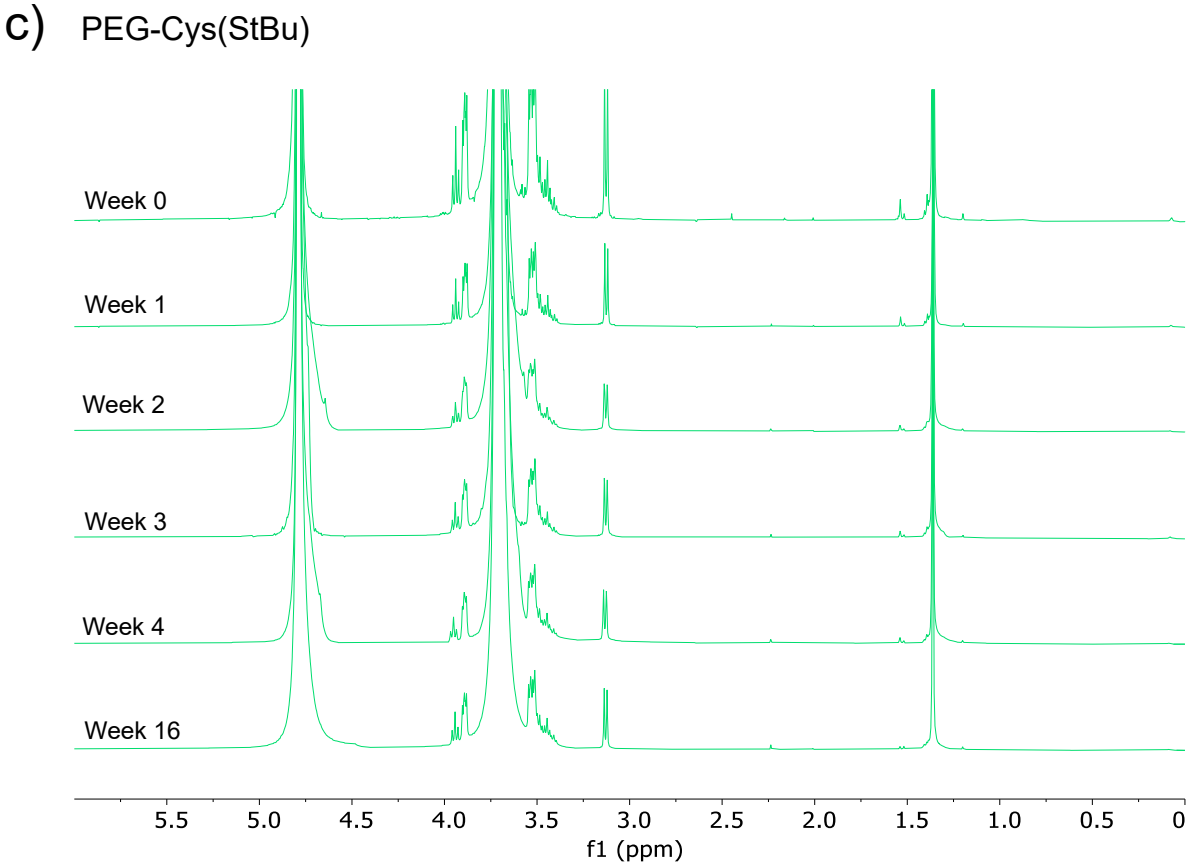


Figure S1. Stability study of aqueous solutions of PEG-Cys and the different PEG-Cys(SR) macromers upon storage at room temperature, as measured by ^{1}H NMR spectroscopy. The macromer solutions were prepared at 1 mM (20 mg/mL) concentration in d-PBS at 25°C and ^{1}H spectra were recorded at increasing aging times up to 16 weeks. During the aging period, samples were stored in a closed NMR tube, at room temperature and exposed to normal light conditions in the laboratory. **a)** PEG-Cys macromer showed spectral changes in the aliphatic region that are consistent with the formation of disulfide bonds. After 1 week of ageing, the doublet at 2.9 ppm corresponding to the methylene next to the free thiol group decreased in intensity and a new multiplet appeared at 3.2-3.0 ppm (relevant signals indicated with arrows). The formed multiplet was attributed to the newly formed methylene next to the derived disulfide. Complete conversion from thiol to disulfide was evident after 2 weeks ageing. Note that this sample contained 1 eq. of TCEP, needed to dissolve PEG-Cys in the aqueous medium and to ensure that all free thiols are present in the beginning of the experiment. In contrast, both **b)** PEG-Cys(SEt) and **c)** PEG-Cys(StBu) macromers evidenced no changes in the spectra over ageing time, indicating high stability of the precursor solutions.

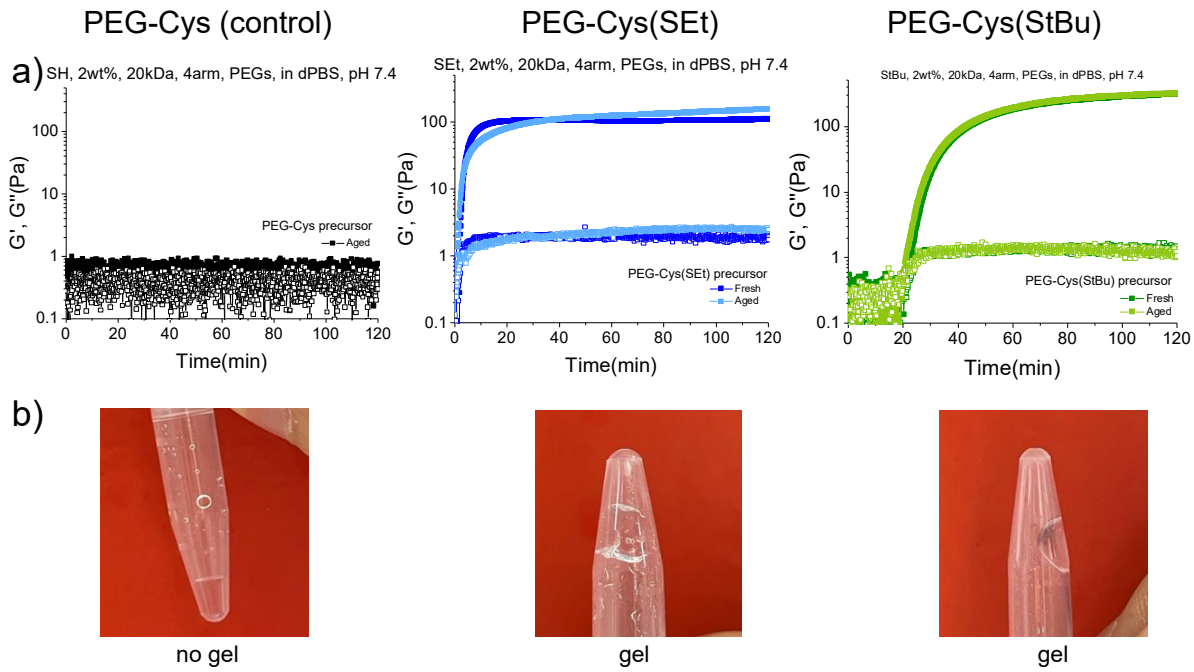


Figure S2. Reactivity study of 4-weeks aged aqueous solutions of the PEG macromers. After the NMR study, the aged PEG precursor (at 2 wt% concentration in d-PBS, pH 7.4, at 25°C) was mixed with PEG-CBT precursor in presence of 1 eq. TCEP, and the formation of a hydrogel was followed by **a)** time sweep experiment via oscillatory rheometry and **b)** a macroscopic gelation test. Aged PEG-Cys solution (left panel) did not form a gel, demonstrating that aged PEG-Cys precursor turned unreactive due to oxidation. In comparison, both PEG-Cys(SEt) (central panel) and PEG-Cys(StBu) (right panel) aged solutions formed a gel which gelation kinetics and final G' is very similar to those hydrogels prepared from fresh solutions of precursor. This demonstrates that PEG-Cys(SR) chemical reactivity is not affected by ageing. Time sweep experiments and macroscopic gelation tests were performed according to the protocols presented in the experimental section.

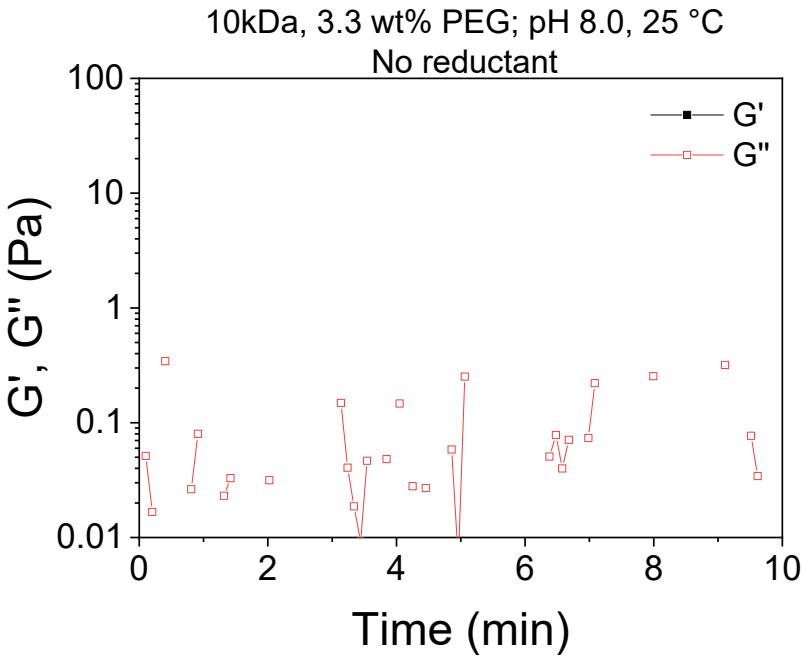


Figure S3. Time sweep experiment demonstrating the absence of redox-triggered gelation when PEG-Cys(StBu) is mixed with PEG-CBT in the absence of a reductant. Rheological curves showing the shear storage (G′) and loss (G″) moduli as a function of time. 10 kDa PEG, 3.3 w% polymer content, in HEPES pH 8.0, at CBT:Cys(StBu):TCEP (1:1:0) molar ratio, T= 25°C.

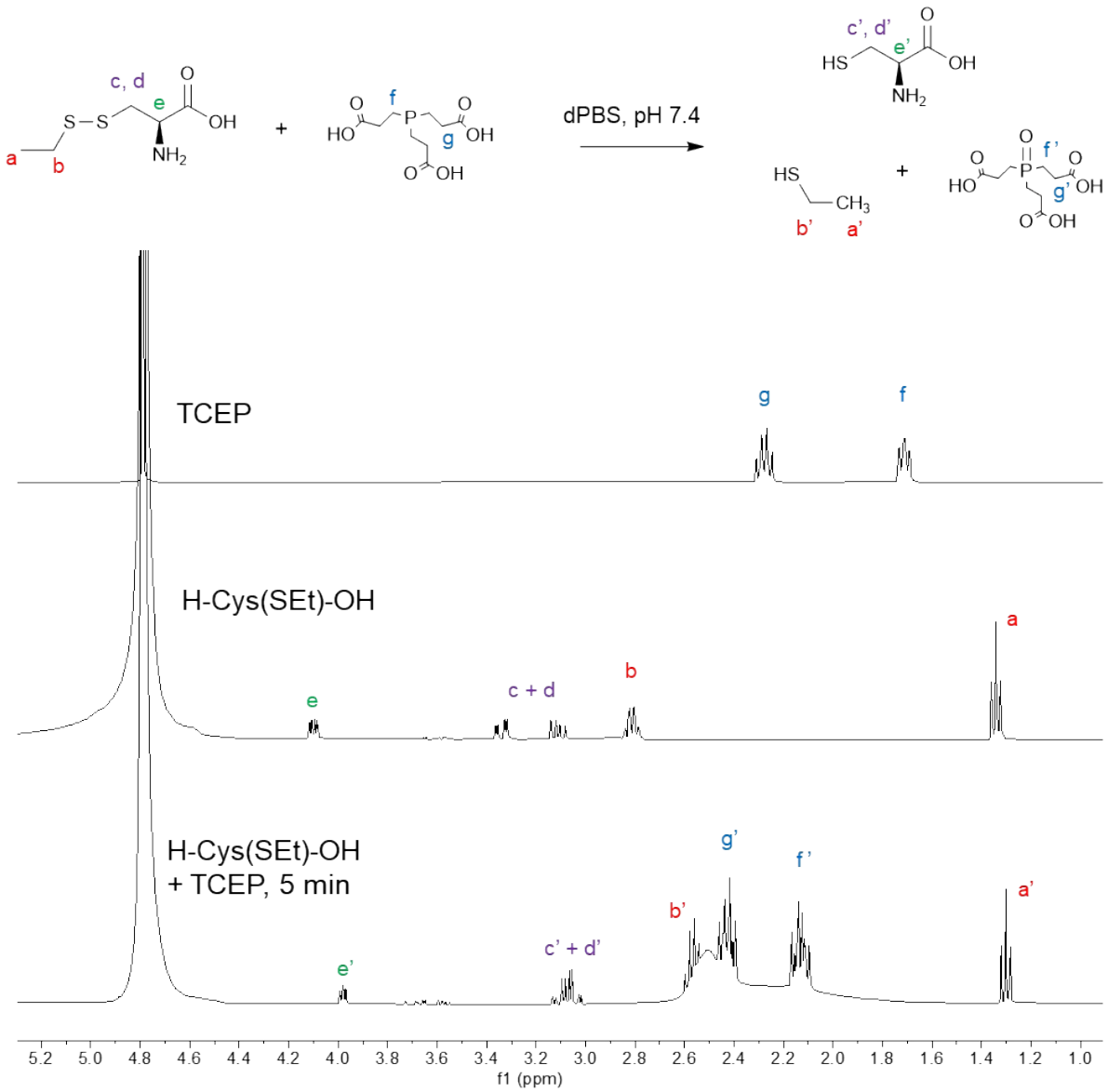


Figure S4. Deprotection reaction of H-Cys(SEt)-OH amino acid with 1 eq. TCEP, monitored by ^{1}H NMR. Conditions: d-PBS pH 7.4, T= 25°C, (1:1) molar ratio. 1 eq. of H-Cys(SEt)-OH was mixed with 1 eq. TCEP in an NMR tube, the sample was immediately loaded into the NMR spectrometer, and a spectrum was recorded 5 min after mixing. At this reaction time, the spectrum evidenced the complete cleavage of the disulfide group.

In the following, chemical shifts before and after TCEP addition are informed, ^{1}H-NMR (400 MHz, dPBS), δ [ppm]:

Before addition of TCEP

H-Cys(SEt)-OH, δ = 4.10 (m, 1H, chiral -CH, *e*); 3.36-3.32 (m, 1H, -CH) and 3.14-3.08 (m, 1H, -CH) (*c*+*d*); 2.84-2.78 (q, 2H, -*CH*$_2$CH$_3$, *b*); 1.36-1.32 (t, 3H, -CH$_2$*CH*$_3$, *a*).

Triphenylphosphine, TCEP, δ = 2.31-2.25 (m, 2H, -CH$_2$, *g*) and 1.74-1.69 (m, 2H, -CH$_2$, *f*).

After TCEP addition, 5 min of reaction.

H-Cys-OH formed, δ = 3.98 (m, 1H, chiral -CH, *e'*); 3.10-3.03 (m, 2H, -CH$_2$, *c'*+*d'*).

HSEt formed, δ = 2.60-2.54 (q, 2H, -*CH*$_2$CH$_3$, *b'*); 1.32-1.28 (t, 3H, -CH$_2$*CH*$_3$, *a'*).

Triphenylphosphine oxide, δ = 2.46-2.40 (m, 2H, -CH$_2$, *g'*) and 2.17-2.10 (m, 2H, -CH$_2$, *f'*).

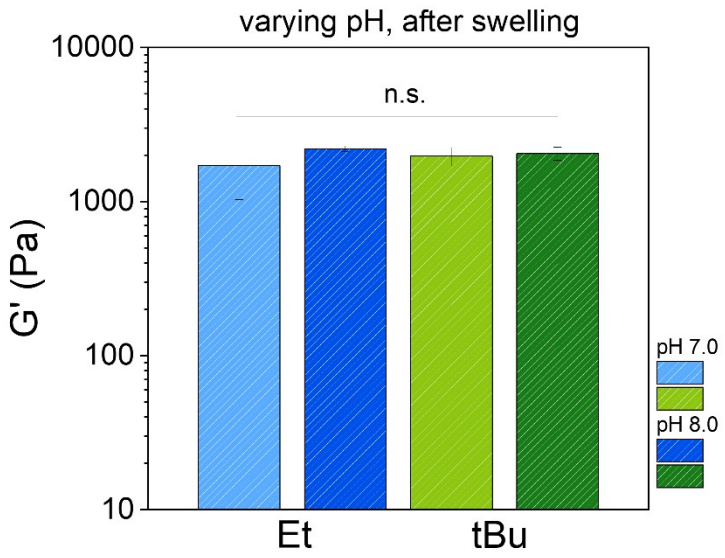


Figure S5. pH regulation does not affect final mechanical strength of CBT-Cys(SR) gels, after swelling. Gel composition: 20 kDa PEGs, 5 wt% polymer content, in HEPES, at (1:1) TCEP:Cys(SR) molar ratio, at T= 25 °C. Specific pH values are indicated in each case. In all cases, data are plotted as mean ± SD, n=4. Statistical significance analysis was performed by ANOVA followed by the post-hoc Tukey test (*$p < 0.05$ used for statistical significance; n.s. = not significant).

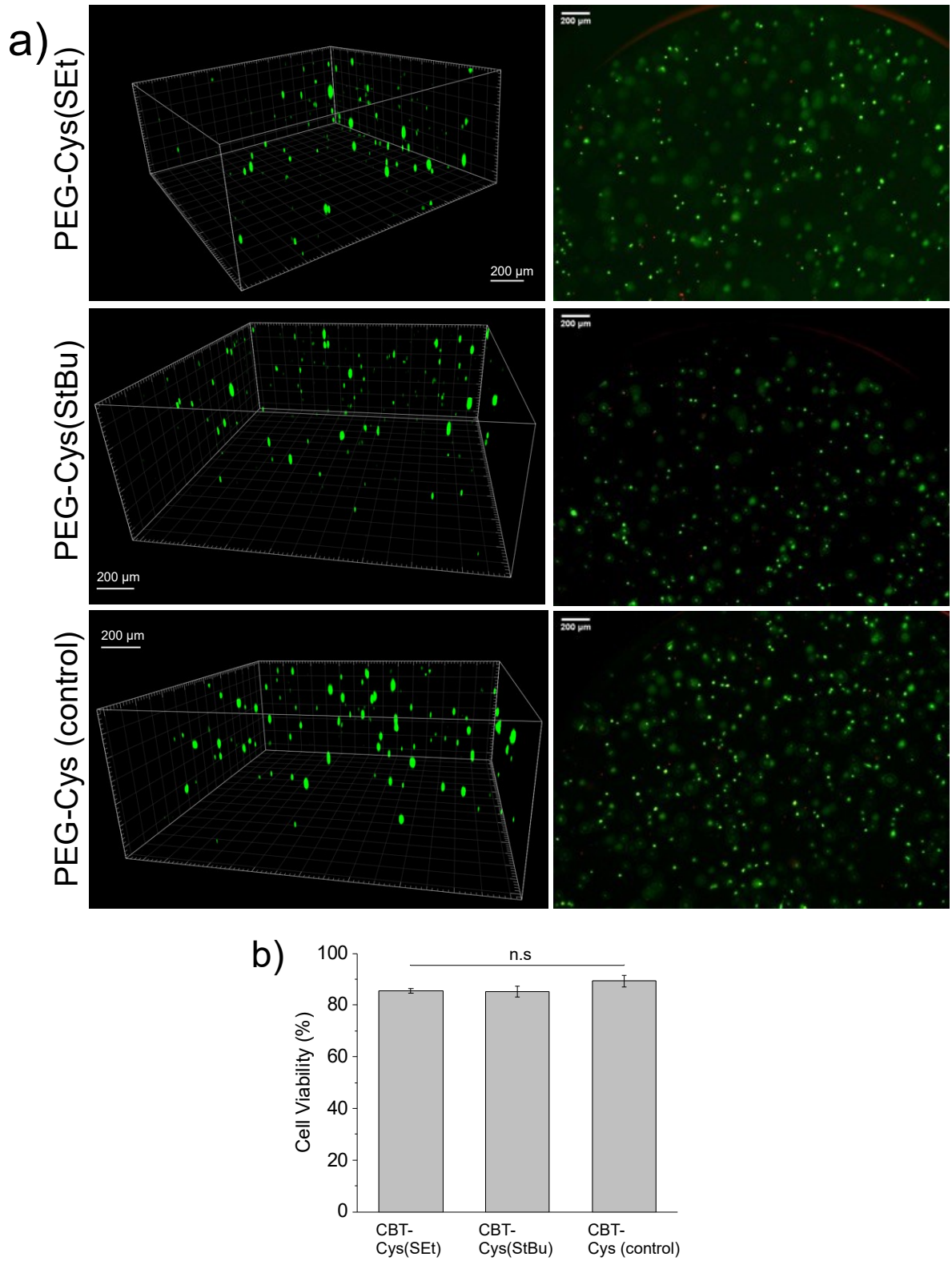


Figure S6. Study of cell viability of encapsulated hMSCs in CBT-Cys(SR) hydrogels biofunctionalized with cell-adhesive cyclo(RGDfK(C)) peptide, at 3 days post-encapsulation. **a)** Z-stack fluorescence images showing homogeneous distribution of embedded cells (left panel) and representative images showing post-encapsulation hMSCs survival after live (green, calcein AM)/ dead (red, ethidium homodimer-1) staining (right panel). **b)** Corresponding quantification of cell viability, at varying protecting groups. The diverse CBT-Cys(SR) gels are

compared to a CBT-Cys control gel. Scale bars: 200 μm. Statistical analysis was performed by ANOVA followed by post-hoc Tukey test (*$p < 0.05$ used for statistical significance; n.s. = not significant). Gel composition: 20 kDa, 3 wt% PEGs, 0.03 wt% (0.5 mM) cyclo(RGDfK(C)), starting cell density per gel was 5000 cells.

Experimental Section

Materials and Methods

Chemicals and solvents had p.a. purity and were used as received, unless otherwise stated. 4-arm (molar masses of 10 and 20 kDa) star-polyethylene glycol (PEG) polymers end-functionalized with amine groups (PEG-NH_2) were purchased from Jenkem (USA), Boc-Cys(SEt)-OH · DCHA was purchased from Bachem AG (CH), all other chemicals were purchased from Merck (Darmstadt, DE), ABCR (Karlsruhe, DE), Sigma Aldrich (DE), AcrosOrganics (BE) and Thermo Fischer Scientific (DE). PEG-CBT and PEG-Cys were synthesized according to our reported protocol.[1] PEG-Cys(SEt) and PEG-Cys(StBu) macromers were synthesized following the protocols described below.

20 mM HEPES buffer solutions were freshly prepared at pH 8.0 and 7.0, with and without the following reductants: dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP) or glutathione (GSH). Deuterated PBS (d-PBS) was prepared by dissolving NaCl, KCl, Na_2HPO_4 and KH_2PO_4 in deuterium oxide, followed by pH adjustment to 7.4 with NaOD and DCl.

Thin layer chromatography (TLC) plates (ALUGRAM® SIL G/UV254) were obtained from Macherey-Nagel (Düren, DE). TLC plates were observed under 254 or 365 nm light. Purification of modified polymers was typically performed by dialysis against acetone and water. Spectra/Por 3 dialysis tubing (molecular weight cut-off MWCO= 3.5 kDa) from Spectrum Chemical (USA) was used.

Solution ^{1}H-NMR and ^{13}C-NMR spectroscopy was performed on a Bruker Avance 300 MHz. Unless otherwise stated, all measurements were taken at 298 K and the solvent residual peak (5.32 ppm for CD_2Cl_2, 2.05 ppm for acetone-d_6 or 4.79 ppm for D_2O ppm) was employed as internal reference. Deuterated solvents were obtained from Deutero GmbH (Kastellaun, DE). Chemical shifts (δ) are given in parts per million. The following abbreviations are used: s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, dd-double doublet. The degree of

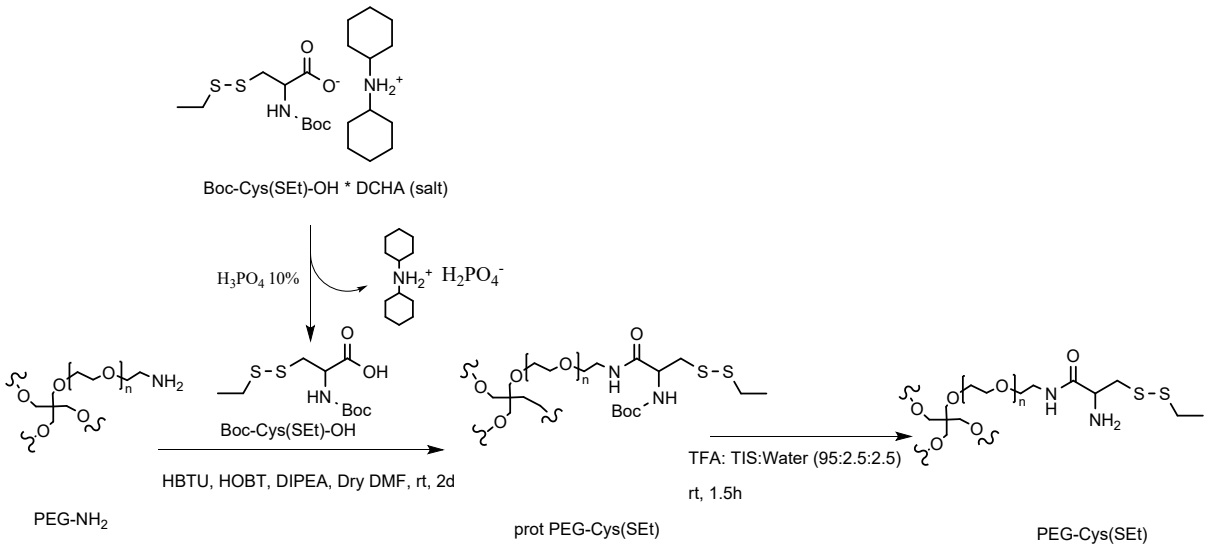
substitution of PEG polymer was calculated by end-group determination. The integral of the signal corresponding to the PEG backbone (3.70-3.40 ppm) was set to 220 H or 440 H (for a 10 or 20 kDa macromer, respectively) and compared to the integral of the protons corresponding to the incorporated molecule. Functionalization degrees of >80% and yields of >85% were obtained in all cases. Data was analyzed and plotted with MestReNova.

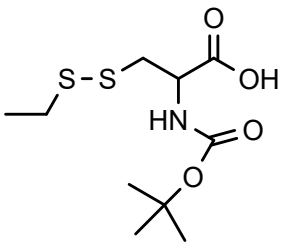
HPLC purification of the compound was performed with a Agilent Infinity II HPLC equipped with a diode array, UV/Vis detector and fraction collector. Polaris C18 columns were used for semi-preparative (250 × 21.2 mm) runs. Solvent gradients using a combination of the following eluents were used: solvent A (MilliQ water + 0.1% formic acid) and solvent B (100% ACN + 0.1% formic acid), typically over 30 min duration.

Electrospray ionization mass spectrometry (ESI-MS) was recorded with a 1260 Infinity Liquid Chromatography/Mass Selective Detector (LC/MSD) (Agilent Technologies, DE) and Quadrupole Time-of-Flight (Q-TOF) with a 6545 Accurate-Mass Quadrupole Time-of-Flight (LC/Q-TOF-MS) (Agilent Technologies, DE) using electrospray ionization.

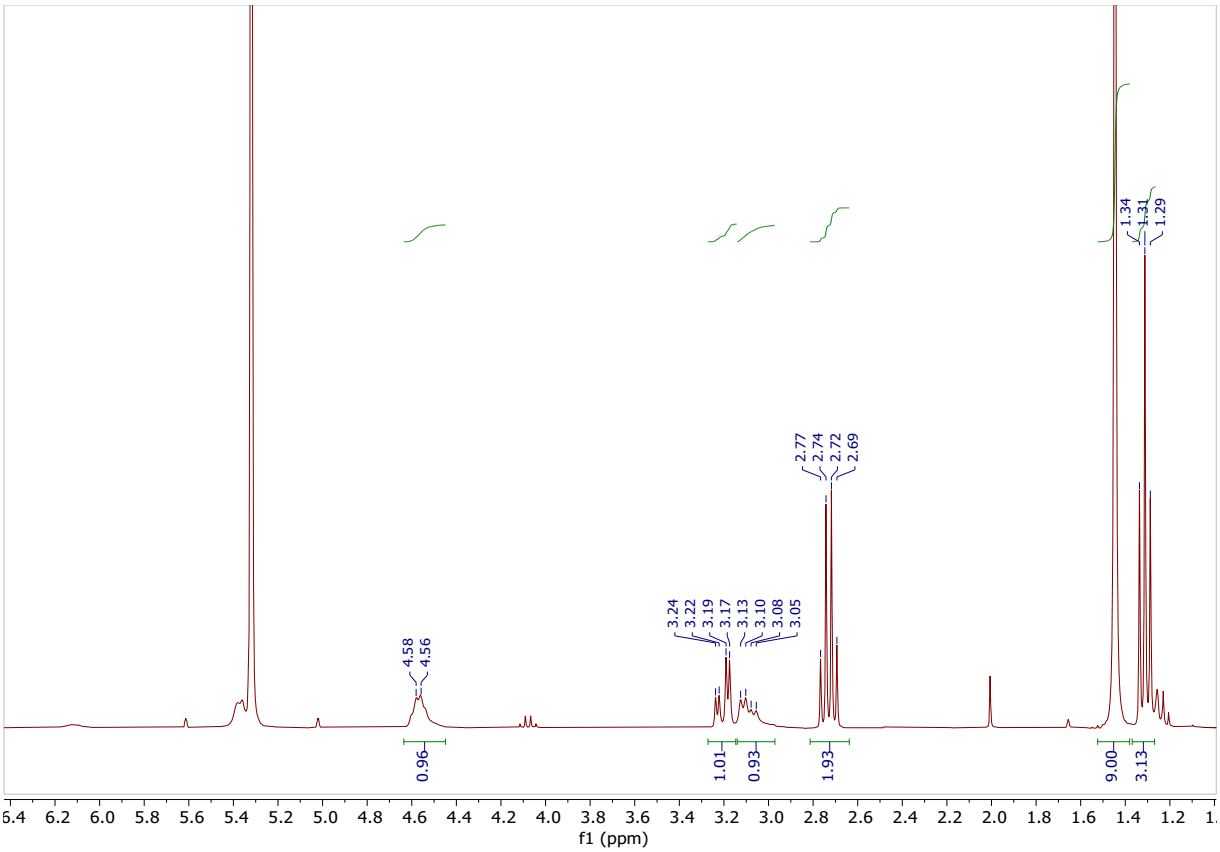
Chemical synthesis of reactive precursors

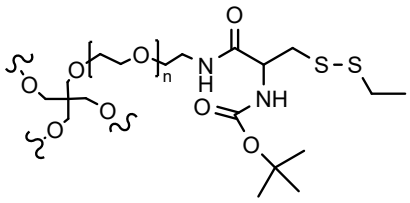
Synthesis of PEG-Cys(SEt) macromers



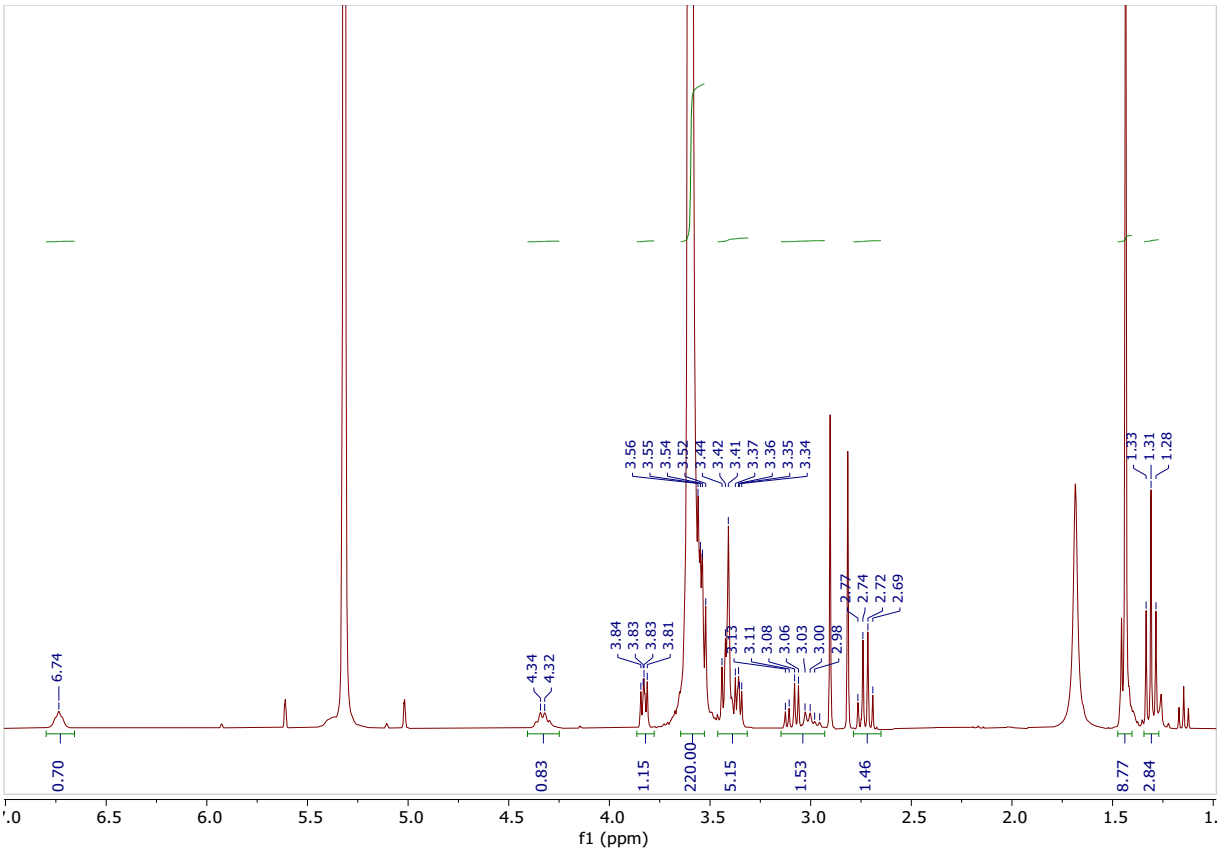


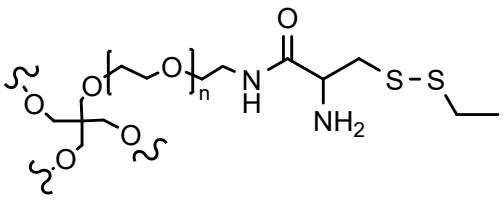
Synthesis of Boc-Cys(SEt)-OH free amino acid: a protocol indicated by the manufacturer was followed.[2] Ethyl acetate (2.3 mL) was cooled down in an ice-salt bath to 0 °C and Boc-Cys(SEt)-OH*DCHA (432 μmol, 200 mg) was added as solid (it partially dissolved). To this mixture, phosphoric acid (10 % v/v, 2.5 mL) was added until a clear solution was formed. The reaction mixture was kept at 0 °C for 5 min in falcon tube without stirring, at this point the pH of the aqueous phase was around 3 and phase separation was observed. After separation of the two liquid layers, the organic layer was separated and extracted with phosphoric acid (10 % v/v, 1 mL) and water (1 mL, three times). Completion of reaction was checked by TLC in 60:40 hexane/ethyl acetate. The organic layer was dried over sodium sulfate, filtrated, and evaporated in vacuo. A yellow oil-like compound was obtained and checked by ^{1}H-NMR (DCM-d_2) to prove disappearance of the DCHA signals at (3.29-3.27 ppm, 2.03-1.99 ppm, 1.82-1.79 ppm, 1.65 ppm, and 1.24-1.21 ppm). ^{1}H-NMR (300 MHz, DCM-d_2, δ [ppm]) =5.38-5.36(m, 1H, -NH); 4.57(m, 1H, chiral -CH); 3.24-3.17 (m, 1H, -CH); 3.13-3.05 (m,1H, -CH); 2.67-2.77 (q, 2H, -CH_2CH$_3$); 1.42 (s, 9H, Boc); 1.34-1.29 (t, 3H, -CH$_2$$CH_3$).



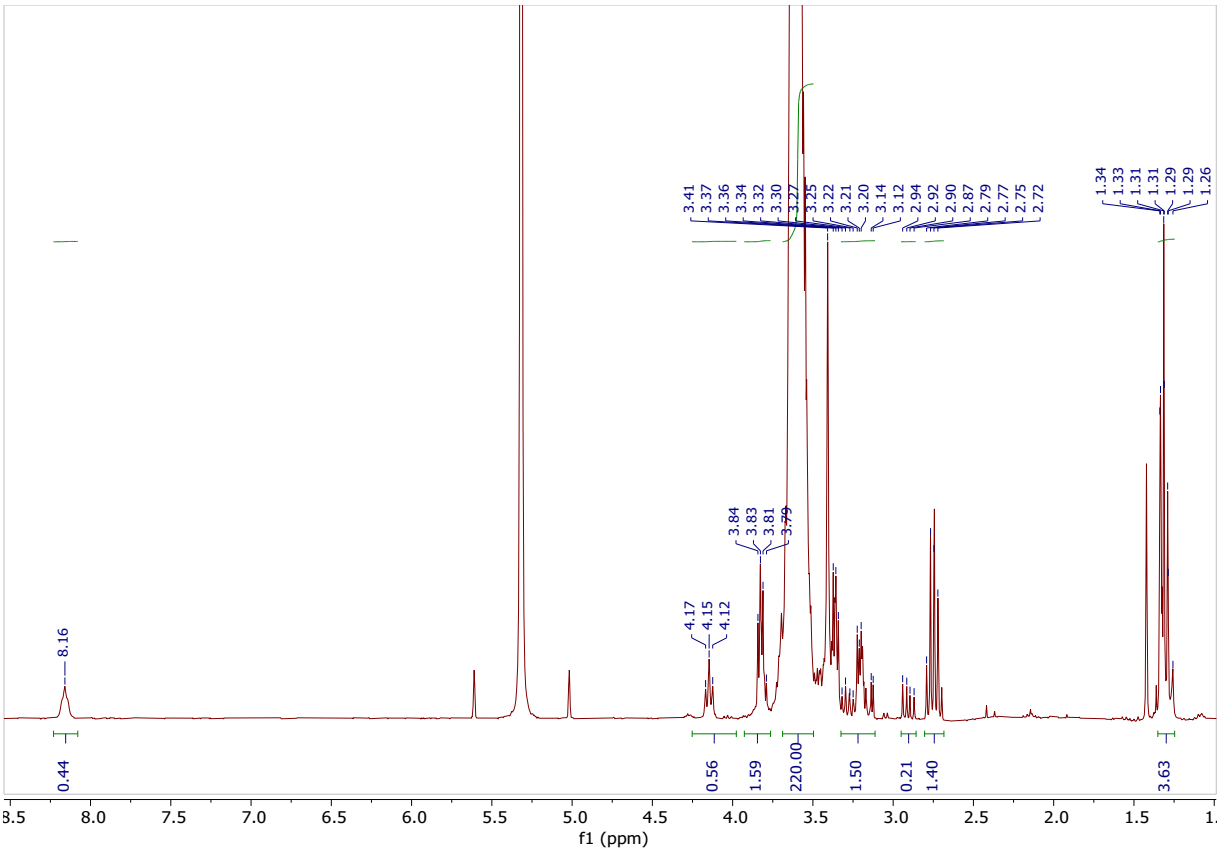


Synthesis of PEG-Cys(SEt)-Boc: HBTU (149 µmol, 56.4 mg, 8 eq.), HOBT (149 µmol, 20.1 mg, 8 eq.), Boc-Cys(SEt)-OH (149 µmol, 42 mg, 8 eq.) and DIPEA (470.7 µmol, 82 µL, 25.3 eq.) were dissolved in DMF (4 mL) and added to a solution of the PEG-amine (18.6 µmol, 186 mg, 1 eq.) in DMF (2 mL). The mixture was stirred at room temperature for 2 days. The polymer was precipitated in cold diethyl ether and the white precipitation was recovered by centrifugation (1 °C, 4000 rpm, 30 min). The solid was re-dissolved in dichlomethane and precipitated in cold diethyl ether again. The product was checked by ^{1}H-NMR and the substitution degree was calculated to be 85%. 150 mg of product was obtained (yield> 99 %). ^{1}H-NMR (300 MHz, DCM-d_2, δ [ppm]) = 7.95 (s, -NH); 6.73 (s, -NH); 4.32 (m, chiral -CH); 3.84-3.34 (m, PEG chain); 3.13-3.06 (m, -CH); 3.03-2.96 (m, -CH); 2.76-2.69 (q, -CH_2CH_3), 1.42 (s, Boc); 1.31 (t, -CH_2CH_3).

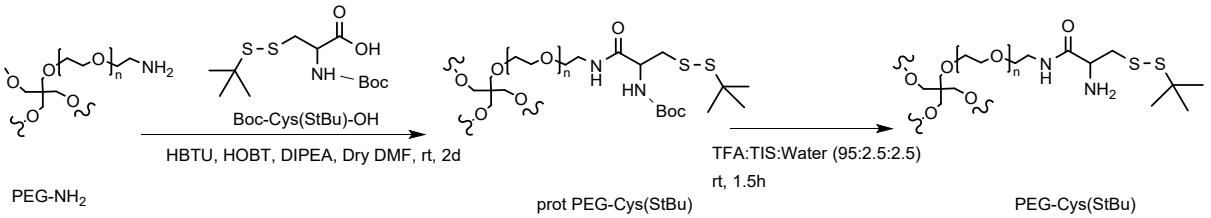


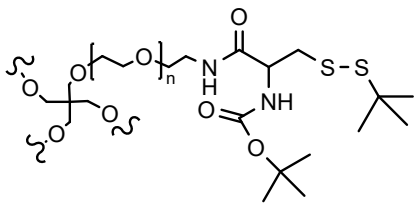


Synthesis of PEG-Cys(SEt): The protected macromer PEG-Cys(SEt)-Boc (180 mg) was dissolved in TFA: TIS: water (95: 2.5: 2.5) (4 mL) and reacted for 1.5 h at room temperature. Under these conditions that include TIS as scavenger, the Boc group was selectively cleaved while the disulfide bond that protects the thiol rest remained stable.[3, 4] TIS can be easily removed due to its volatility. The crude was evaporated under nitrogen stream to half of the initial volume, followed by addition of water (4 mL), dialysis (acetone ×2, acetone/water ×1, water ×2) and lyophilization. A yellow-white solid polymer was obtained (167 mg, yield=90 %), which was checked by ^{1}H-NMR in DCM-d_2, observing complete removal of the Boc protecting group. ^{1}H-NMR (300 MHz, DCM-d_2, δ [ppm]) = 8.27 (s, -NH); 4.17-4.12 (m, chiral -CH); 3.84-3.34 (m, PEG chain); 3.32-2.87 (m, -CH_2); 2.79-2.72 (m, -CH_2CH$_3$); 1.34-1.29 (m, -CH$_2$CH_3).



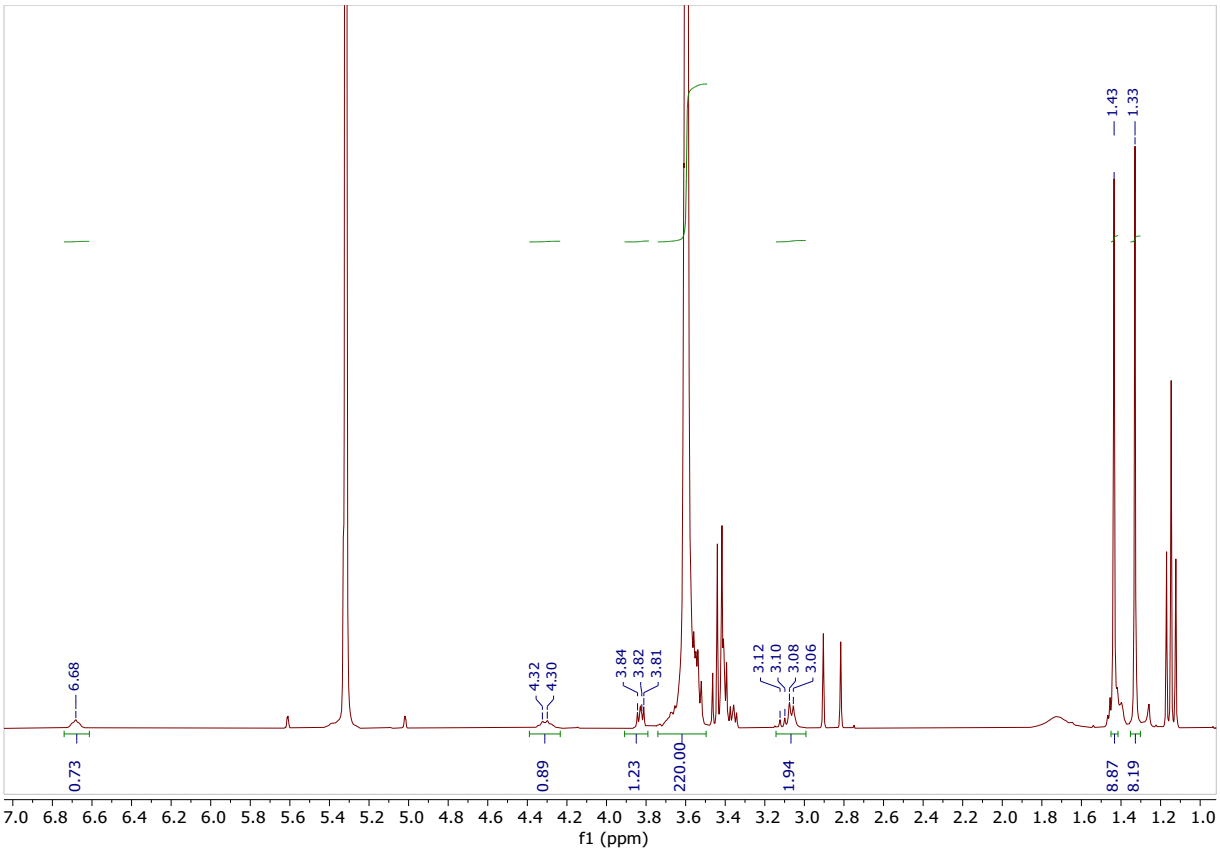
Synthesis of PEG-Cys(StBu) macromers

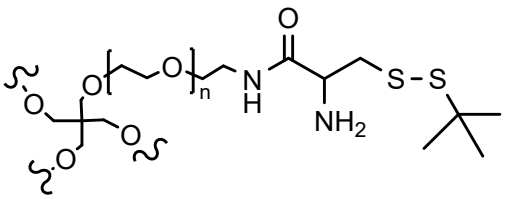




Synthesis of PEG-Cys(StBu)-Boc: HBTU (223 µmol, 84.5 mg, 12 eq.), HOBT (223 µmol, 30.1 mg, 12 eq.), Boc-Cys(StBu)-OH (1.223 mmol, 69.2 mg,12 eq.) and DIPEA (705 µmol, 123 µL, 38.4 eq.) were dissolved in DMF (3 mL) and added to a solution of PEG-NH_2 (18.6 µmol, 186 mg, 1 eq.) in DMF (2 mL). The mixture was stirred at room temperature for 2 days. The polymer was precipitated in cold diethyl ether and recovered by centrifugation

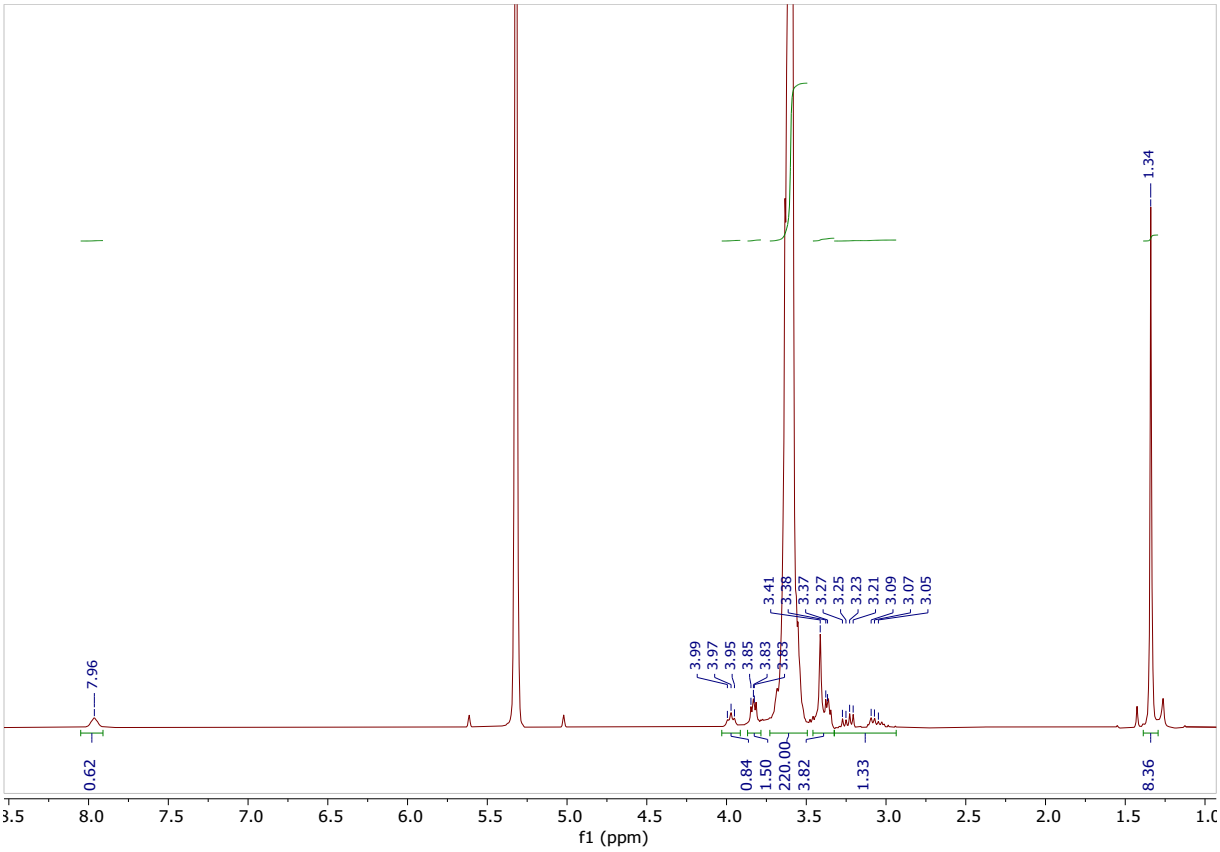
(1 °C, 4000 rpm, 30 min). The solid was re-dissolved in dichlomethane and precipitated in cold diethyl ether again. The crude was checked by ^{1}H-NMR and the substitution degree was calculated to be 95 %. 190 mg of product was obtained (yield= 90 %). ^{1}H-NMR (300 MHz, DCM-d_2, δ [ppm]) = 6.76 (s,1H, -NH); 4.31 (m, chiral -CH); 3.84-3.34 (m, PEG chain); 3.10-3.06 (m, -CH_2); 1.42 (s, Boc); 1.33 (s, -StBu).

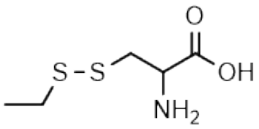




Synthesis of PEG-Cys(StBu): PEG-Cys(StBu)-Boc (186 mg) was dissolved in TFA: TIS: water (95: 2.5: 2.5) (4 mL) and reacted for 2 h at room temperature. The solvent was evaporated under nitrogen stream to half of the initial volume, followed by addition of water (5 mL), dialysis (acetone ×2, acetone/water ×1, water ×2) and lyophilization. A pale yellow solid polymer was obtained (180 mg, yield= 95 %) and checked by ^{1}H-NMR in DCM-d_2 to prove the complete removal of Boc protecting group. ^{1}H-NMR (300 MHz, DCM-

d_2, δ [ppm]) = 7.96 (s, -NH); 3.99-3.95 (m, chiral -CH); 3.83-3.41 (m, PEG chain); 3.27-3.23 (m, -CH_2); 3.27-3.21 (m, -CH_2); 1.34 (s, -StBu).





Synthesis of H-Cys(SEt)-OH amino acid: Boc-Cys(SEt)-OH (40 mg) was synthesized following the above mentioned protocol, then dissolved in TFA: TIS: water (95: 2.5: 2.5) (1 mL) and reacted for 1 h at room temperature. The crude was dropped into cold diethylether, followed by centrifugation (3000 rpm, 30 min). An off-white precipitate was obtained and further purified by preparative HPLC (method: 0B-50B, 270 nm, ret.time = 16.7 min) and evaporated to afford 5 mg of H-Cys(Et)-OH as a white solid compound (yield = 19%). ^{1}H-NMR (400 MHz, d-PBS) δ [ppm] = 8.47 (m, 2H, -NH2); 4.10 (m, 1H, chiral -CH); 3.36-3.32 (m, 1H, -CH); 3.14-3.08 (m,1H, -CH); 2.84-2.78 (q, 2H, -*CH_2*CH_3); 1.36-1.32 (t, 3H, -CH_2*CH_3*).

Preparation of PEG precursor solutions

Precursor solutions were freshly prepared. PEG-CBT precursors and PEG-Cys(SR) (R= Et, tBu) precursors were dissolved in 20 mM HEPES. PEG-Cys precursors was dissolved in 20 mM HEPES containing 1 equiv. of TCEP per Cys equiv., unless otherwise stated. A TCEP:Cys molar ratio of (1:1) was kept to prevent disulfide formation between free Cys groups. After dissolving the polymers in the corresponding buffer, the solution was vortex-mixed, ultrasonicated (ca 5 s) and centrifuged (13000 rpm, 30 s) to eliminate bubbles. The final pH of the precursor solutions was verified with an Elite pH Spear Pocket Testers (Thermo Scientific, NL).

Study of stability and reactivity of PEG-Cys(SR) precursors in aqueous solution

20 kDa PEG-Cys(SR) macromers (R=H, Et, tBu) were dissolved in deuterated PBS buffer (d-PBS) to reach 24 mg mL^{-1} concentration and measured by ^{1}H NMR. ^{1}H NMR spectra were recorded at selected aging times t=0, 1, 2, 3 weeks, up to 16 weeks. During the aging period, samples were stored in a closed NMR tube, at room temperature and exposed to normal light conditions in the laboratory. The evolution of the proton signals corresponding to disulfide side chains was monitored. After NMR recording was complete, aged PEG-Cys(SR) samples were tested for their ability to form hydrogel. To this end, the aged precursor solution (1 mM) was mixed at (1:1) volume with freshly prepared 1 mM PEG-CBT solution (in 1x PBS buffer containing 4 mM TCEP, pH 7.4) to reach 2 wt% polymer content. The formation of a hydrogel was checked visually (vial inversion test) after 1 h and 1 day of mixing.

After completion of NMR experiments, the PEG-Cys(SR) aged precursor solution was mixed with PEG-CBT solution directly on the rheometer plate and TCEP solution was added. A final polymer content of 2 wt% was reached, and molar ratio Cys(SR):CBT:TCEP of 1:1:1 was kept.

The gelation kinetics and final mechanical strength of a gel was followed by time sweep studies by oscillatory rheometry (see further details below).

Study of disulfide cleavage of H-Cys(SEt) amino acid with TCEP by ^{1}H NMR

H-Cys(SEt)-OH was dissolved in d-PBS, pH 7.4, at 25 °C to reach 10 mg/mL (~50 mM) concentration. Separately, TCEP solution in the same solvent and at the same concentration was prepared. ^{1}H NMR was recorded on both separate samples. Then, equal volumes of H-Cys(SEt)-OH and TCEP solutions were mixed in the same NMR tube at (1:1) Cys: TCEP molar ratio). The sample was mixed gently and immediately loaded into the NMR spectrometer, and a spectrum was recorded 5 min after mixing.

Hydrogel preparation and characterization

Estimation of gelation time of CBT-Cys(SR) hydrogels by the "pipetting" macroscopic test

A macroscopic test previously reported by our group[5] was followed to estimate the gelation time of hydrogels. PEG-precursors solutions at a given concentration and pH were prepared as detailed above.

CBT-Cys(SR) gels (R= Et, tBu)

15 µL of 6.6 wt% PEG-CBT solution and 15 µL of 6.6 wt% PEG-Cys(SR) (R= Et, tBu) solution were placed in a plastic Eppendorf vial at (1:1) CBT-Cys(SR) molar ratio, followed by addition of 10 µL of reductant solution (1 equiv. of reductant per Cys(SR) group) and a stopwatch was started. The curing solution was continuously mixed with pipette (size of pipette tip= 2-200 µL, 53 mm; from Eppendorf epT.I.P.S.®, Germany) until the gelling solution stopped flowing. Time was measured using a Rotilabo-Signal-Timer TR 118 (Roth, Germany) stopwatch. Gelation time was estimated in this test as the time elapsed between the mixing of

the two components (PEGs and reductant) and the moment when pipetting of the mixture was no longer possible. The final polymer concentration was 5 wt%.

CBT-Cys gels

Gels were prepared as above, except that 20 µL of 5 wt% PEG-CBT was mixed with 20 µL of 5 wt% PEG-Cys precursor and no extra reductant solution was added. The final polymer concentration was 5wt%.

Rheology of hydrogels during in situ crosslinking (before swelling)

The rheological properties of hydrogels were measured on a Discovery HR-3 rheometer or on a Discovery HR20 rheometer (both from TA Instruments, USA) using 12 mm parallel plates, a Peltier stage temperature control system. Precursor solutions were prepared as above.

CBT-Cys(SR) hydrogels (R=Et, tBu)

15 µL of 6.6 wt% PEG-CBT solution and 15 µL of 6.6 wt% PEG-Cys(SR) solution, and the resulting solution was loaded on the lower plate, followed by addition of 10 µL of reductant solution, and mixed with pipette tip directly on the plate. The upper plate was approached to reach a gap size of 300 µm, and the sample was sealed with silicon oil to avoid evaporation during measurement, unless otherwise stated. The total time required for sample loading and start of measurement was approximately 1 min. Strain sweeps (0.1 to 1000% strain at frequency = 1 Hz) and frequency sweeps (0.01 to 100 Hz at strain = 1%) were performed to determine the linear viscoelastic regime. Time sweep measurements were carried out within such regime using the following parameters: starting gap of 300 µm, controlled axial force (0.0±0.1 N), frequency 1 Hz, strain 1%, temperature = 25°C, unless otherwise stated. In order to capture the first moments of the gelation processes of fast curing gels, additional time sweep measurements were performed without the use of an oil trap; this enabled to decrease the time required to set the experiment down to 30 s. Such time sweep measurements were conducted for only 10 min

to avoid drying effects. Gelation time was estimated from the time sweep curves as the time at which G'= G''. The final polymer concentration was 5 wt%

CBT-Cys gels

Gels were prepared similarly, except that 20 µL of 5wt% PEG-CBT was mixed with 20 µL of 5wt% PEG-Cys precursor and no extra reductant solution was added. The final polymer concentration was 5 wt%.

Rheology characterization of hydrogels after swelling

CBT-Cys(SR) hydrogels (R=Et, tBu)

20 µL of 6.25 wt% PEG-CBT solution and 20 µL of 6.25 wt% PEG-Cys(SR) solution were placed in a PDMS cylindrical mold (8 mm diameter), quickly mixed with 10 µL of reductant solution to reach a 5 wt% hydrogel, and allowed to crosslink in a humid chamber at room temperature for 2 h. The obtained gels were carefully demolded and swollen for 24 h in 20 mM HEPES at the corresponding pH value. Swollen hydrogels (ca 9-10 mm diameter) were loaded to the rheometer and measured using upper plate geometry of 8 mm diameter with rough surface to ensure a good contact with the swollen gel. Time sweep measurements were carried out for 3 min to avoid sample´s evaporation, using the following parameters: starting controlled axial force 0.05 N, variable starting gap (depending on sample´s thickness, typically 700-1000 µm), frequency 1 Hz, strain 1%, temperature = 25°C.

CBT-Cys gels

Gels were prepared similarly, except that 25 µL of 5 wt% PEG-CBT solution was mixed with 25 µL of 5 wt% PEG-Cys solution, no extra reductant solution was added. The final polymer concentration was 5 wt%.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). For each condition, typically 3 independent experiments were performed. A one-way analysis of variance (ANOVA) with a Tukey test of the variance was used to determine the statistical significance between groups. The statistical analysis was performed to compare different groups and significant difference was set to $*p < 0.05$.

Cell studies

Cell culture

Bone marrow derived human mesenchymal stem cells (hMSCs, Poietics, Lonza) were cultured in basic medium (αMEM (Gibco) containing 10% fetal bovine serum (FBS) (PAN-Biotech), 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Gibco)) and incubated at 37°C and 5% CO_2 in a humidified environment. For experiments of 1 day culture, cells at passage 3 and 4 were used and single cell dissociation before encapsulation was performed using accutase (StemPro™Accutase™, Gibco).

For experiments of 3 days culture, a different batch of stem cells was used, and small modifications were introduced. Bone marrow derived hMSCs were isolated from bone marrow as described previously[6] and cultured in basic medium (αMEM (Gibco) containing 10% fetal bovine serum (FBS) (PAN-Biotech), 2 mM L-glutamine, 0.2 mM ascorbic acid, 1 ng/mL basic fibroblast growth factor, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Gibco)) and incubated at 37°C and 5% CO_2 in a humidified environment. For these experiments, cells at passage 5 were used and single cell dissociation before encapsulation was performed using trypsin-EDTA(Gibco).

Hydrogel preparation for 3D hMSC culture

CBT-Cys and CBT-Cys(SR) hydrogels with bioactivity (i.e., cell-adhesive) were prepared by adapting previously reported protocols.[1]

CBT-Cys(SR) hydrogels (R=Et, tBu)

Precursor solutions of 4-arm, 20 kDa PEG-CBT (80 mg mL^{-1}, 8 wt%), PEG-Cys(SR) (70 mg mL^{-1}, 7 wt%) and solution of cyclo(RGDfK(C)) (3.23 mg mL^{-1}, 4.6 mM) were prepared by dissolving the lyophilized polymer in sterile 20 mM HEPES buffer pH 8.0 inside a sterile laminar flow and used directly without further filtration. PEG-CBT stock solution (2 µL, 8 wt%) was mixed with cyclo(RGDfK(C)) (1 µL, 4.6 mM) and incubated for 30 min at 37 °C. The hMSC cell suspension ($5x10^5$ cells mL^{-1}, cell density leading to a value within the typical range of $3x10^5$ - $3x10^7$ cells mL^{-1}) in serum free DMEM (1 µL) was added to the above solution, followed by the addition of 2 µL of PEG-Cys(SR) solution. Total 6 µL of resulting mixture were placed in an Ibidi 15-µwell angiogenesis slide. Immediately, the reductant solution (4 µL, 7 mM) was added to the µ-well, carefully mixed with pipette tip and allowed to crosslink for 15 min at 37 °C and 5% CO_2. After gelation, basic medium for hMSCs (45 µL) was added to rinse the gel (2X, 10 min each), and medium was replenished again. Final gel composition was 1.6 wt% PEG-CBT, 0.03 wt% (0.46 mM) cyclo(RGDfK(C)), 1.4 wt% PEG-Cys(SR), and cell density was 5000 cells per gel (for 1 day culture) or 2000 cells per gel (for 3 days culture).

CBT-Cys hydrogels

Precursor solution of 4-arm, 20 kDa PEG-CBT (53.3 mg mL^{-1}, 5.3 wt%) and solution of cyclo(RGDfK(C)) (3.23 mg mL^{-1}, 4.6 mM) were prepared by dissolving the lyophilized polymer in sterile 20 mM HEPES buffer pH 8.0 inside a sterile laminar flow and used directly without further filtration. Precursor solution of 4-arm, 20 kDa PEG-Cys (28 mg mL^{-1}, 2.8 wt%) prepared in sterile HEPES buffer pH 8.0 containing 1 equiv. of TCEP per Cys equiv. PEG-CBT stock (3 µL, 5.3 wt%) was mixed with cyclo(RGDfK(C)) (1 µL, 4.6 mM) and incubated for 30

min at 37 °C. The hMSC cell suspension ($5x10^5$ cells mL^{-1}, cell density leading to a value within the typical range of $3x10^5$ - $3x10^7$ cells mL^{-1}) in serum free DMEM (1 µL) was added to the above solution and 5 µL of resulting mixture were placed in an Ibidi 15-µwell angiogenesis slide. Immediately, the solution of PEG-Cys (5 µL, 2.8 wt%) was added to the µ-well and mixed carefully with pipette tip. The curing and washing steps followed the same procedure as mentioned above. Final gel composition was 1.6 wt% PEG-CBT, 0.03 wt% (0.46 mM) cyclo(RGDfK(C)), 1.4 wt% PEG-Cys(SH), and cell density was 5000 cells per gel (for 1 day culture) or 2000 cells per gel (for 3 days culture).

Live/dead assays

For experiments at 1 day culture, hMSCs were cultured in CBT-Cys and CBT-Cys(SR) hydrogels after 1 day and cell culture medium was removed. Samples were incubated for 5 min with fluorescein diacetate (40 µg mL^{-1}) and propidium iodide (30 µg mL^{-1}) in PBS, washed 2X with PBS (10 min each) and imaged with Zeiss Axio Observer microscope with corresponding filter settings and using a 10X air objective. For experiments at 3 days culture, samples were incubated for 30 min at 37 °C with calcein AM (4 mM) and ethidium homodimer-1 (6 mM) in αMEM medium, washed once with PBS and imaged with a digital fluorescence microscope (EVOS FL Imaging System, ThermoFisher) with corresponding filter settings and using 4X and 10X air objectives. Cells were kept in PBS and imaging was done at normal cell culture conditions (at 37 °C and 5% CO_2 in a humidified environment) in a climate chamber connected to the microscope within 1 h of staining. Excitation parameters were adjusted to use minimum light intensity in order to maintain cytocompatibility. For each sample, imaging was performed across different z-stacks in three different wells per condition, and at least 500-600 individual cells were counted manually using Image J (NIH) to calculate the percentage viability of each sample.

Statistical analysis. All experiments were performed in quadruplicates. Cell viability data were expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) with a Tukey test of the variance was used to determine the statistical significance between groups. The statistical analysis was performed to compare different groups and significant difference was set to $*p < 0.05$.

Study of cell density distribution across the gel at 3 days culture

Experiments were performed in triplicates. To study the homogeneity of cell encapsulation in CBT-Cys and CBT-Cys(SR) hydrogels, hMSCss were encapsulated following the same protocol as described above. Cells were cultured (at 2000 cells per gel, to have a lower cell density for visualization) for 3 days. Samples were incubated at 37 °C for 30 min with calcein AM (4 mM) and washed once with PBS. Samples were kept in culture medium and imaged with Zeiss LSM 880 confocal microscope with a 10X air objective, using Z-stack and tile scan mode with 5 µm per stack. The cell distribution across the gel was qualitatively analyzed by constructing 3D representation of z-stack images using Imaris image analysis software (Oxford Instruments, UK).

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