Supporting Information

Enzymatically Crosslinked Poly(2-alkyl-2-oxazoline) Networks For 3D Cell Culture

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Experimental Section

Materials

Hydrazine monohydrate, 2-ethyl-2-oxazoline (EOXA), methyl para-toluenesulfonate (MeTs), 2-chloroethylammonium chloride, sodium sulfate anhydrous, sodium carbonate anhydrous, potassium hydroxide (KOH), 2,2'-(ethylenedioxy)diethanethiol (EDDT), trifluoroacetic acid (TFA), triethanolamine (TEOA) and barium oxide were purchased from Sigma Aldrich. Dimethyl 3,3'-dithiodipropionate was purchased from TCI chemicals. Methanol (MeOH), hydrochloric acid (HCl), glacial acetic acid, and triethylamine (TEA) were purchased from Merck Millipore. Dichloromethance (DCM, extra dry), acetonitrile (ACN, extra dry), methyl succinyl chloride and 2-methyl-2-oxazoline (MOXA) were purchased from Acros Organics. 2-Morpholinoethanesulfonic acid hydrate (MES hydrate), tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl), divinyl sulfone (DVS), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) and ethyl isonipecotate were purchased form Fluorochem. MOXA, EOXA and TEA were distilled over KOH prior to use and stored under argon. Methyl p-toluenesulfonate and ethyl isonipecotate were distilled under high vacuum over CaH_2 and stored under argon. All the other chemicals were used as received.

Synthesis of hydrogel precursors

3,3'-Dithiobis(propanoic dihydrazide) (DTPHY)

Dimethyl 3,3'-dithiodipropionate (25g, 104.9 mmol) was dissolved in 400 mL MeOH. Hydrazine monohydrate (30.5 mL, 629.4 mmol, 6 equiv) was added dropwise at 0° C within 1 hour and then stirred overnight under reflux. Then the reaction mixture was cooled down on ice and the precipitated product was vacuum filtered and washed with cold MeOH (20mL), cold water (20 mL) and cold MeOH (20 mL). The white precipitate was recrystallized once in MeOH and subsequently dried under high vacuum, yielding white crystals (21.25 g, 89.2 mmol, 85%).

^{1}H NMR (400 MHz, DMSO-d6) δ = 9.05 (s, 2H), 4.21 (s, 4H), 2.88 (t, 4H), 2.40 (t, 4H).

Methyl 3-(4,5-dihydrooxazol-2-yl)propanoate (MestOXA)

MestOXA was synthesised in accordance to the previously reported procedure (Figure S1a).[1]

Methyl-7-chloro-4-oxo-5-azaheptanoate

Methyl succinyl chloride (25.0 g, 166 mmol, 1 eq.) and 2-chloroethylammonium chloride (19.3 g, 166 mmol, 1 eq.) were suspended in dry DCM (200 mL). At 0 °C, TEA (53 mL, 380 mmol, 2.2 eq.) was added dropwise over a period of 1h. Then the reaction mixture was allowed to warm to ambient temperature and was stirred overnight before water (40 mL) was added. The organic phase was subsequently washed with 1 M HCl, water and brine and finally dried over anhydrous sodium sulfate. After filtration and solvent removal, the residue was purified on neutral alumina gel using DCM:MeOH (99:1) as eluent. The product appeared as a light yellow oil (26.48 g, 136.8 mmol, 83.3%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 6.33 (s, 1H), 3.65 (s, 3H), 3.61 – 3.49 (m, 4H), 2.64 (t, J = 6.8 Hz, 2H), 2.49 (t, J = 6.7 Hz, 2H). ^{13}C NMR (76 MHz, $CDCl_3$) δ = 173.45, 171.78, 51.94, 43.91, 41.36, 30.89, 29.30 ppm.

MestOXA

The synthesis of MestOXA was summarized in Figure S1a. Methyl 4-(2-chloroethyl)amino-4-oxobutanoate (26.48 g, 136.8 mmol, 1 eq.) and anhydrous sodium carbonate (11.6 g, 109.4 mmol, 0.8 eq.) were reacted (neat) in a 250 mL RB flask mounted on a rotary evaporator (40° C, 20 mbar) for 48 h until the absence of CO2 formation indicated full conversion. Subsequently dichloromethane was added, the reaction mixture filtered, and the solvent removed. The crude yellow oil was purified by distillation over barium oxide under reduced pressure, yielding a colorless oil (14.81 g, 94.4 mmol, 69%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.24 ppm (t, J = 9.73 Hz, 2 H), 3.82 (t, J = 9.16 Hz, 2 H), 3.70 (s, 3 H), 2.68 (t, J = 7.44 Hz, 2 H), 2.58 (t, J = 7.44 Hz, 2 H); ^{13}C NMR (76 MHz, $CDCl_3$): δ = 172.8, 167.0, 67.5, 54.4, 51.8, 30.1, 23.1 ppm.

PMOXA-$COOCH_3$

In an oven-dried two-neck RB flask was added 15 mL of dry acetonitrile, 2-Methyl-2-oxazoline (7.1 g, 83.4 mmol, 170 eq.) and MestOXA (2.31 g, 14.7 mmol, 30 eq.) under a N_2 atmosphere. After stirring the reaction mixture for 10 min at 0°C, Methyl p-toluenesulfonate (91 mg, 0.5 mmol, 1 eq.) was added under N_2 and stirred for another 10 min on ice. Then the mixture was heated to 70 °C and kept at this temperature for 48 h under stirring and within an Argon atmosphere. After this time, the polymerization was terminated by adding an excess of ethyl isonipecotate (800 uL,

5 mmol, 10 eq.) at room temperature, and left stirring for another 48 h under argon. The solvent was removed under reduced pressure, and the crude polymer dissolved in 100 mL of deionized water and purified by dialysis against ultrapure water using 1 kDa MWCO dialysis membranes for two days. The PMOXA-$COOCH_3$ (7.25 g, 77 % yield) was obtained after lyophilization as a white powder. The chemical structure and the purity of the synthesized polymer were determined by ^{1}H-NMR (400 MHz) (Figure S2) and the molecular weight measured by gel permeation chromatography (GPC) as reported in the Supporting Information (Table S1).

PEOXA-$COOCH_3$

In an oven-dried two-neck RB flask was added 15 mL of dry Acetonitrile, 2-ethyl-2-oxazoline (9.23 g, 93.1 mmol, 170 eq.) and MestOXA (2.58 g, 16.4 mmol, 30 eq.) under a N_2 atmosphere. After stirring the reaction mixture for 10 min at 0°C, Methyl p-toluenesulfonate (102 mg, 0.55 mmol, 1 eq.) was added under N_2 and stirred for another 10 min on ice. Then the mixture was heated to 80 °C and kept at this temperature for 48 h under stirring and within an Argon atmosphere. After this time, the polymerization was terminated by adding an excess of ethyl isonipecotate (800 uL, 5 mmol, 10 eq.) at room temperature, and left stirring for another 48 h under argon. The solvent was removed under reduced pressure, and the crude polymer dissolved in 100 mL of deionized water and purified by dialysis against ultrapure water using 1 kDa MWCO dialysis membranes for two days. The PEOXA-$COOCH_3$ (9.45 g, 80 % yield) was obtained after lyophilization as a white powder. The chemical structure and the purity of the synthesized polymer were determined by ^{1}H-NMR (400 MHz) (Figure S3) and the molecular weight measured by gel permeation chromatography (GPC) as reported in the Supporting Information (Table S1).

PMOXA- and PEOXA-COOH

The methyl and ethyl ester of the side chains and the end groups of the synthesized copolymers were hydrolyzed with 1 M NaOH for 24 h at room temperature. Glacial acetic acid was added until pH 6 was reached before the polymer was purified by dialysis in the same way as before and finally lyophilized to yield a white powder.

PMOXA- and PEOXA-VS

The synthesis of PMOXA- and PEOXA-VS was summarized in Figure S1b. In a typical reaction, PMOXA-COOH (2g, n(-COOH) = 2.8 mmol, 1 eq., 12% substitution degree of –COOH) was dissolved in 20 mL MES hydrate buffer (150 mM) and the pH was found to be 4.5. Subsequently, 3,3'-Dithiobis(propanoic dihydrazide) (1.33 g, 5.6 mmol, 2 eq.) and EDC (1.6 g, 8.4 mmol, 3 eq., predissolved in 5 mL of H_2O) was added one after the other under stirring and the reaction was left for 4 h. TCEP-HCl (2.4 g, 8.4 mmol, 3 eq.) was then added to react overnight. Then the polymer was purified by dialysis against acidified ultrapure H_20 (pH 3) for two days, yielding the thiolated PMOXA-SH. The purified PMOXA-SH inside the dialysis membrane was directly transferred to a dropping funnel and then added dropwise within 1 h to a DVS (16.5 g, 140 mmol, 50 eq.) in 100 mL TEOA (300mM, pH 8.0) buffer solution, which was previously bubbled with N_2 for 10 min, under high stirring and under N_2 atmosphere. After the addition was completed, the reaction mixture was left for another 1 h under stirring and then dialyzed against ultrapure H_20 for 2 days. The PMOXA-VS (2.35 g, 75 % yield) was obtained after lyophilization as a white powder. The chemical structure and the purity of the synthesized polymer were determined by ^{1}H-NMR (400 MHz) (Figure S4).

PMOXA- and PEOXA-peptide conjugates

PMOXA-LPETG

PMOXA-VS (330 mg, n(-VS) = 0.33 mmol, 1 eq.) was dissolved in 10 mL TEOA buffer (300 mM, pH 8) and the solution bubbled with N2 for 10 min. Then a 10 mL solution of GCRELPETGG (Mw 1017 g/mol, 500 mg, 0.5 mmol, 1.5 eq.) in H_2O was added dropwise within 30 min. under stirring and left reacting overnight at room temperature under an N2 atmosphere. Subsequently, the resultant solution was purified by dialysis against ultrapure water using 3.5 kDa MWCO dialysis membranes for 24 h.

PMOXA-GGGG

PMOXA-VS (374 mg, n(-VS) = 0.4 mmol, 1 eq.) was dissolved in 10 mL TEOA buffer (300 mM, pH 8) and the solution bubbled with N_2 for 10 min. Then a 10 mL solution of GGGGLERCL (Mw 860 g/mol, 520 mg, 0.6 mmol, 1.5 eq.) in H_2O was added dropwise within 30 min. under stirring and left reacting overnight at room temperature under an N2 atmosphere.
Subsequently, the resultant solution was purified by dialysis against ultrapure water using 3.5 kDa MWCO dialysis membranes for 24 h.

PEOXA-LPETG

PEOXA-VS (380 mg, n(-VS) = 0.38 mmol, 1 eq.) was dissolved in 10 mL TEOA buffer (300 mM, pH 8) and the solution bubbled with N_2 for 10 min. Then a 10 mL solution of GCRELPETGG (Mw 1017 g/mol, 580 mg, 0.57 mmol, 1.5 eq.) in H_2O was added dropwise within 30 min. under stirring and left reacting overnight at room temperature under an N_2 atmosphere. Subsequently,

the resultant solution was purified by dialysis against ultrapure water using 3.5 kDa MWCO dialysis membranes for 24 h.

PEOXA-GGGG

PEOXA-VS (400 mg, n(-VS) = 0.4 mmol, 1 eq.) was dissolved in 10 mL TEOA buffer (300 mM, pH 8) and the solution bubbled with N2 for 10 min. Then a 10 mL solution of GGGGLERCL (Mw 860 g/mol, 520 mg, 0.6 mmol, 1.5 eq.) in H_2O was added dropwise within 30 min. under stirring and left reacting overnight at room temperature under an N_2 atmosphere. Subsequently, the resultant solution was purified by dialysis against ultrapure water using 3.5 kDa MWCO dialysis membranes for 24 h.

Peptide synthesis

Peptides were synthesized by standard Fmoc solid phase supported peptide synthesis (SPPS) on an automated peptide synthesizer (Prelude, Protein Gyros Technologies) using Rink amide resin. Peptide cleavage from the resin and full removal of the protecting groups was performed manually using 2.5 % EDDT, 2.5% TIPS, 2.5% H_20, 92.5% TFA cleavage solution. The cleavage was performed for 2h at room temperature. Then the peptide was precipitated in cold diethyl ether twice before it was purified by preparative RP-HPLC using C18 column with 50-90% ACN/H_2O linear gradient over 20 min. The purity of the peptides was confirmed by analytical RP-HPLC on an Agilent 1260 infinity instrument, using a Poroshell EC-C18, 2.7 um bead size, 4.6 x 100 mm column with 10-90% ACN/H_2O linear gradient, with monitoring of the absorbance at 214 nm. The identity of the peptide was confirmed by electrospray ionization mass spectroscopy (ESI-MS) on a Water instrument equipped with a single quadrupole detector SQ 2.

LC-MS (ESI-TOF): for GCRELPETGG [M + H]+ calculated: 1017.47, found: 1017.8; for GGGGLERCL calculated: 860.43, found: 860.7.

NMR Spectroscopy

NMR spectra were recorded on a *Bruker* Avance *DRX-400* at room temperature, using DMSO-d6 as solvent for DTPHY, $CDCl_3$ for Methyl-7-chloro-4-oxo-5-azaheptanoate, MestOXA and PAOXA-$COOCH_3$ and D_2O for random-PAOXA-VS and PAOXA-peptide conjugates.

Gel Permeation Chromatography (GPC)

An Agilent 1100 GPC/SEC unit was used equipped with two PFG linear M columns (PSS) connected in series with an Agilent 1100 VWD/UV detector operated at 230 nm, as well as a DAWN HELEOS 8 multi-angle laser light scattering (MALS) detector followed by an Optilab T-rEX RI detector, both from Wyatt. Samples were eluted in hexafluoroisopropanol (HFIP) with 0.02 M K-TFAc at 1 mL/min at room temperature. Absolute molecular weights were evaluated with Wyatt ASTRA software and dn/dc values based on our analytical setup (dn/dc (PMOXA) = 0.2498 mL/g, dn/dc (PEOXA) = 0.2283 mL/g).

Production of Sortase

Pentamutant Sortase A was produced as previously described.[51] Briefly, the plasmid including SA pentamutant $(eSA)^2$ was used in the expression vector pET29 kindly shared by Prof. David Liu through Addgene (#75144). Electrocompetent E. coli BL21(DE3) were transformed with the plasmid and grown on an LB-agar selection plate containing kanamycin (50 ug/mL). An individual colony with good protein expression was selected and inoculated (180 rpm at 37 °C) overnight in

12 mL LB media with kanamycin (50 ug/mL). The culture was diluted to 1 L of LB media containing kanamycin and incubated at 37°C, 180 rpm until OD600=0.8, which was typically reached after 3.5 hours. Following the induction of pentamutant SA expression by IPTG (0.2mM) for 22 hours at 16 °C, cells were pelleted by centrifugation (8000 rpm, 15 minutes). Cells were lysed by resuspension in BugBuster master mix (BB, Merck 71456) and left with gentle shaking for 30 min at ambient temperature. Finally, lysates were cleared from the cell debris by centrifugation (20000 g for 30 min at 4°C) and the supernatant was collected and filtered at 0.45 um. SA was then isolated using a His-Trap HP affinity column (GE Healthcare) mounted on a preparative HPLC (Agilent 1260 infinity) using TBS + 1 mM bM with 10 mM imidazole as an eluent (10 min isocratic, then gradient from 10 to 250 mM imidazole over 30 min). The protein was re-concentrated at 4500 rcf and 4°C with vivaspin centrifugal filters with 10 kDa molecular weight cut-off (MWCO). Finally, the protein was dialyzed against TBS + 1 mM bM at 4°C with a 1 kDa MWCO membrane, with 4 buffer changes over 6 hours each, filtered at 0.2 mm, aliquoted and stored at - 80°C.

PAOXA hydrogel formation by SA-mediated crosslinking

The gelation kinetics for PAOXA gels crosslinked via SA, denoted PAOXA-SA, was characterized by rheometry. For clarification, the notation PMOXA/PEOXA-SA includes the two components PMOXA/PEOXA-GGGG and PMOXA/PEOXA-LPETG in equal amounts. Aliquots of PMOXA/PEOXA-GGGG and PMOXA/PEOXA-LPETG were solubilized at 10% (w/v) in sterile filtered TRIS buffered saline (TBS, 50 mM TRIS, 150 mM NaCl, pH 7.5). The two solutions were then combined in equal volume to form 10% (w/v) PMOXA/PEOXA-SA stock solutions. Hydrogel formation was performed at 2.5% and 5% (w/v) PMOXA/PEOXA-SA with additional

10 mM $CaCl_2$. To trigger gelation, SA in varying concentration of 1, 5, 10, or 15 µM was added to the PMOXA/PEOXA-SA polymer-peptide solution and then thoroughly mixed.

Cell culture

Human chondrocytes were isolated from non-arthritic articular cartilage obtained from surgical knee operations (patient consent and ethical approval were obtained with Swiss ethics approval number: ID PB_2017-00510) and kept in phosphate-buffered saline (PBS) supplemented with 10 µg mL^{-1} gentamycin (Gibco). The cartilage specimens were minced into 1-3 mm^3 pieces with a sterile blade and washed with Dulbecco's modified eagle medium (DMEM) (Glutamax, high glucose) (Invitrogen) with 10 µg/mL gentamycin. Minced cartilage tissue was digested with 0.1 % collagenase (Sigma) in DMEM supplemented with 10 % foetal bovine serum (FBS) (Invitrogen) overnight at 30 °C with gentle shaking. Digested tissue was filtered through a 100 µm and then a 40 µm cell strainer. The filtered solution of chondrocytes was centrifuged at 500 ×g for 10 min and washed twice with growth medium (DMEM containing 10 % FBS, 50 µg/ mL l-ascorbic acid -2-phosphate (Sigma) and 10 µg/mL gentamycin). The chondrocytes were seeded at a density of 3000 cells/cm2 and expanded in growth medium to passage 2 before encapsulation in the hydrogels.

Shear Moduli Measurements and Gelation Kinetics

After the addition of the SA to the polymer-peptide solution, the gel precursor was quickly loaded on an Anton Paar MCR 301 rheometer equipped with a 20 mm plate-plate geometry probe and metal floor, pre-warmed to 37 °C and with humidified chamber. Then the probe was quickly lowered to measuring position of 200 µm. The gelling was monitored at 1 Hz with 4% strain.

hAC encapsulation in hydrogels

Primary human articular chondrocytes were trypsinized and resuspended at 15 x 106 cells/mL in the 5% (w/v) PMOXA/PEOXA-SA polymer solution. SA was then added and the suspension quickly mixed to trigger the gelation. Subsequently they were cast in 4 mm diameter (50 uL volume) UV-sterilized PDMS cylindrical molds adhered to 24 well plate. The hydrogels were crosslinked for 15 min at 37° C before adding expansion medium, supplemented with gentamicin (10 ug/mL), ascorbic acid (50ug/mL), and 10% FBS. The gels were then incubated for 3 weeks in a controlled humidified chamber (37° C, 5% (v/v) CO2), and the culture medium was replaced two times per week.

Fluorescence microscopy and cytocompatibility

Hydrogels were washed once with warm PBS and then incubated for 1 h at 37° C in medium containing 4 μM calcein AM and 20 μM propidium iodide (PI). Samples were then washed once with PBS, covered with fresh medium and imaged on a confocal microscope (Carl Zeiss, AG/LSM 510) with 494 nm (green, Calcein) and 528 nm (red, PI) excitation filters.

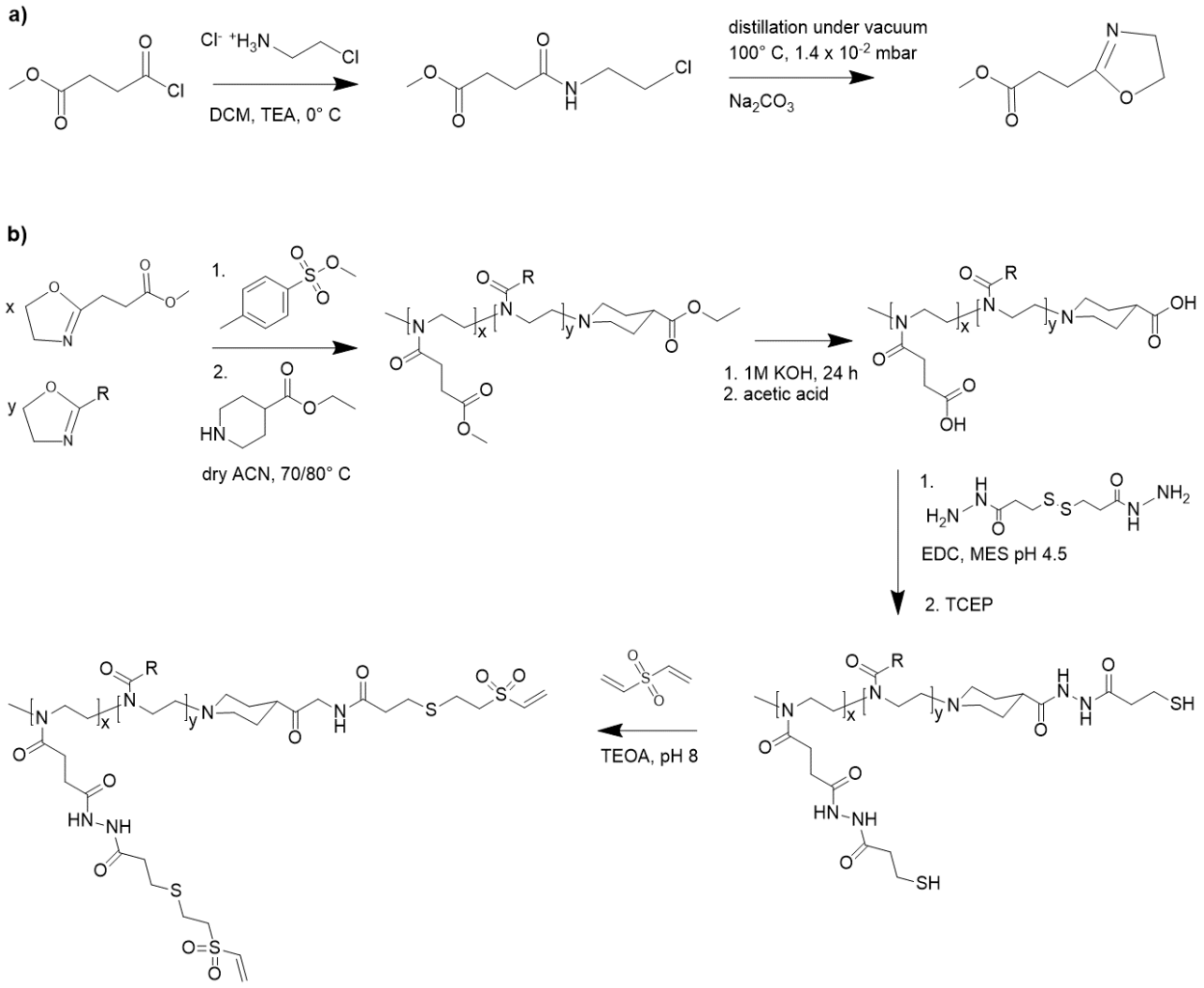

Figure S1. (a) Synthesis of methyl 3-(4,5-dihydrooxazol-2-yl)propanoate (MestOXA). (b) Synthesis of the vinylsulfonated random copolymer (PAOXA-VS).

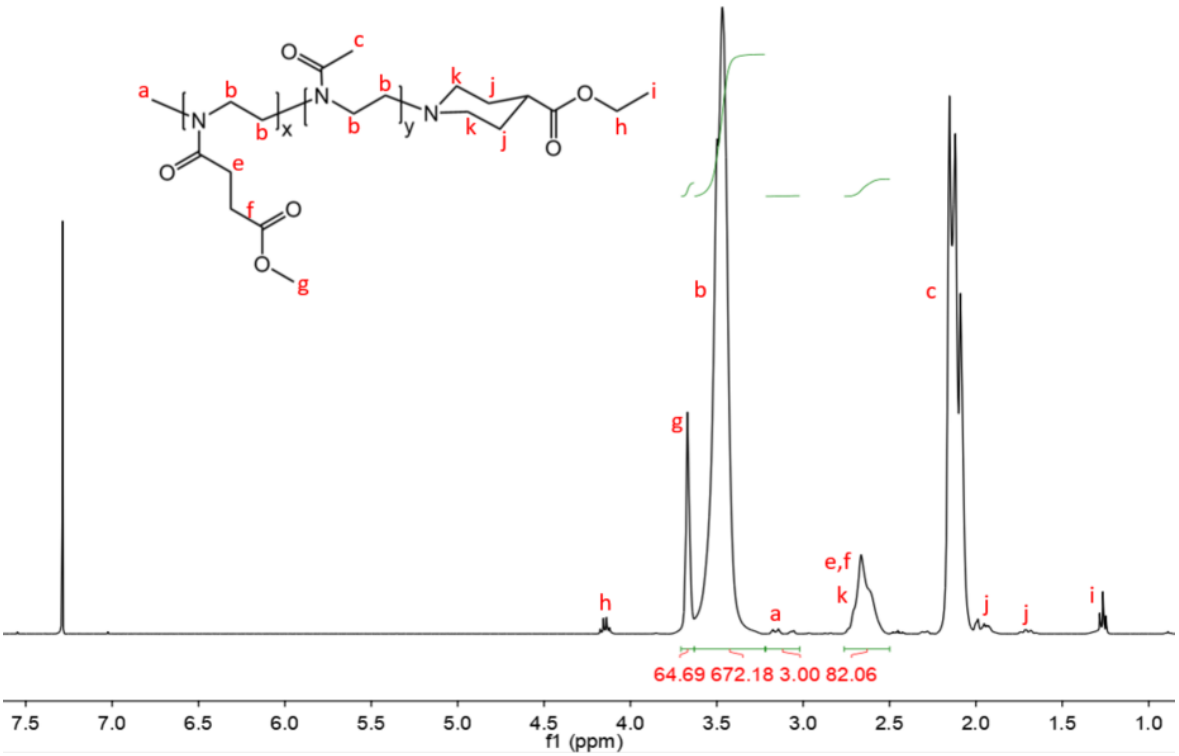

Figure S2. ^{1}H-NMR (400 MHz) spectrum of PMOXA-COOCH$_3$ in CDCl$_3$.

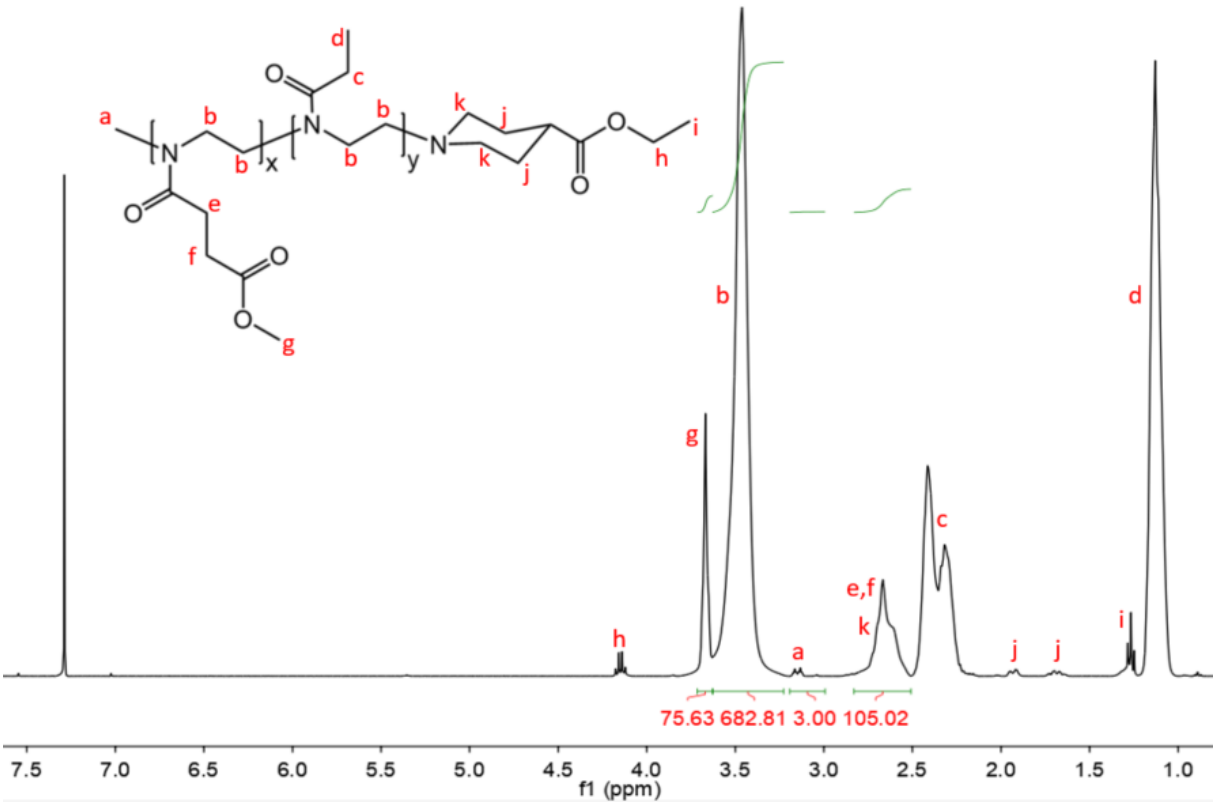

Figure S3. ^{1}H-NMR (400 MHz) spectrum of PEOXA-COOCH$_3$ in CDCl$_3$.

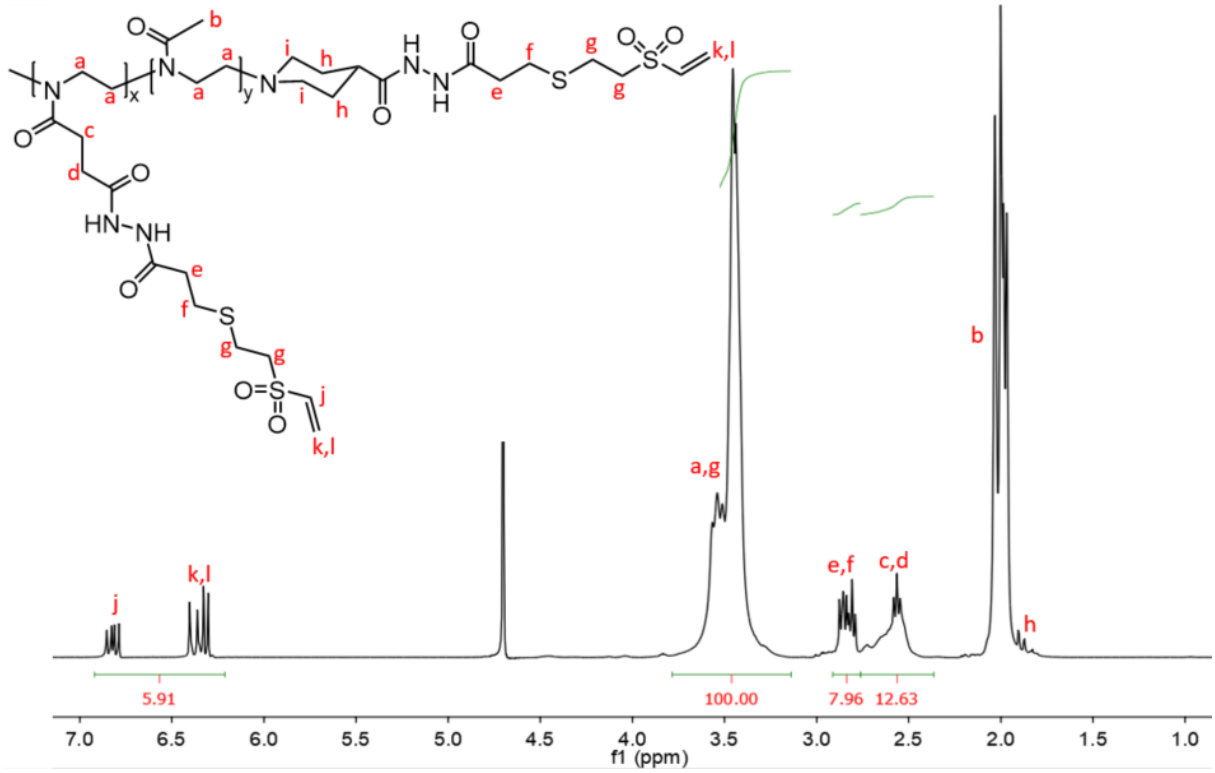

Figure S4. ^{1}H-NMR (400 MHz) spectrum of PMOXA-VS in D_2O.

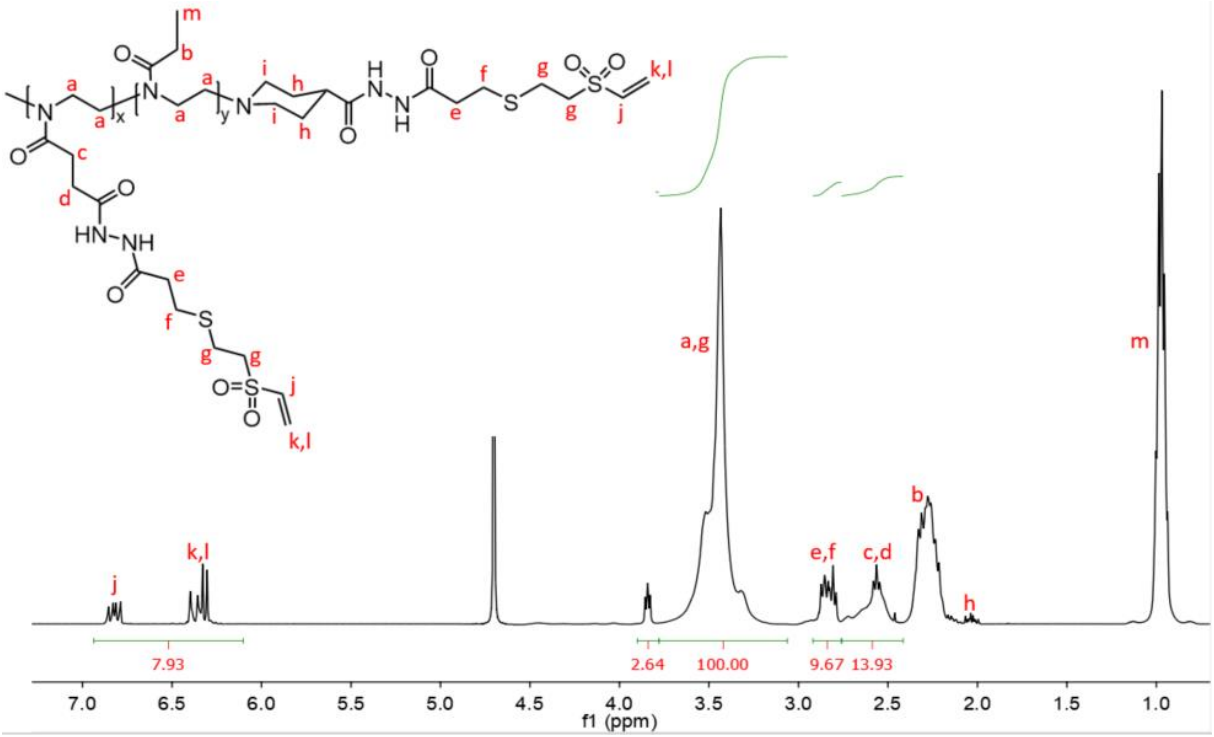

Figure S5. ^{1}H-NMR (400 MHz) spectrum of PEOXA-VS in D_2O.

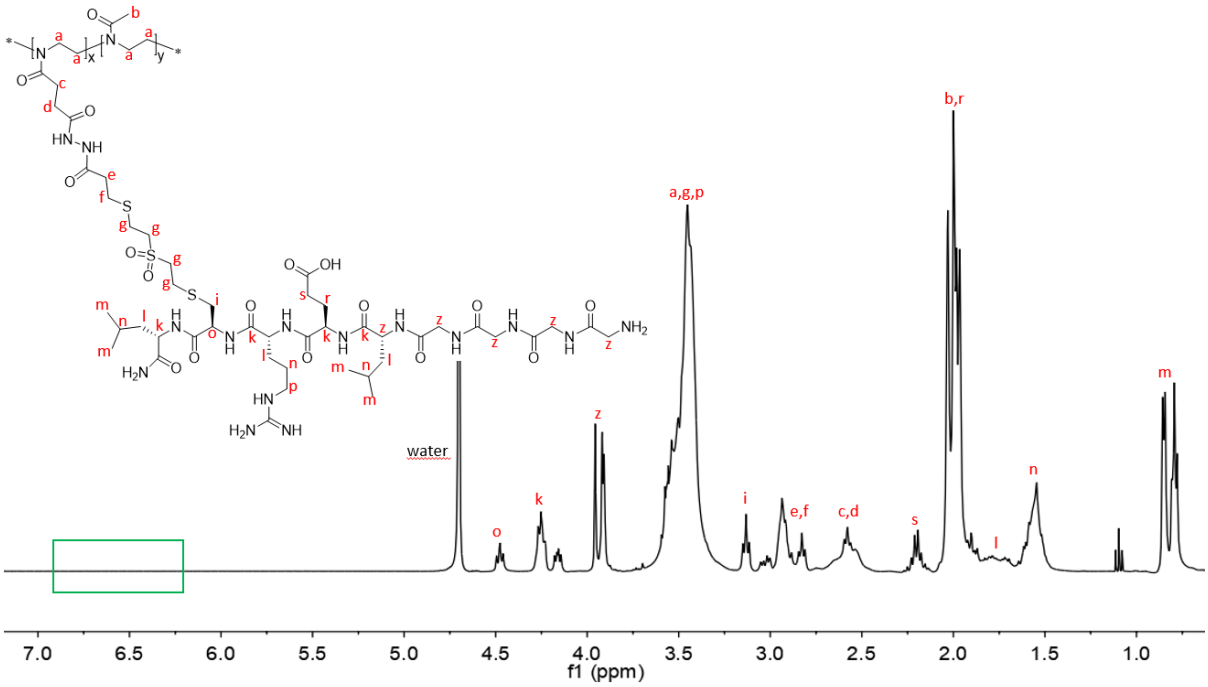

Figure S6. ^{1}H-NMR (400 MHz) spectrum of PMOXA-GGGG in D_2O. Highlighted in the green box is the full disappearance of the peaks corresponding to the protons of the VS group.

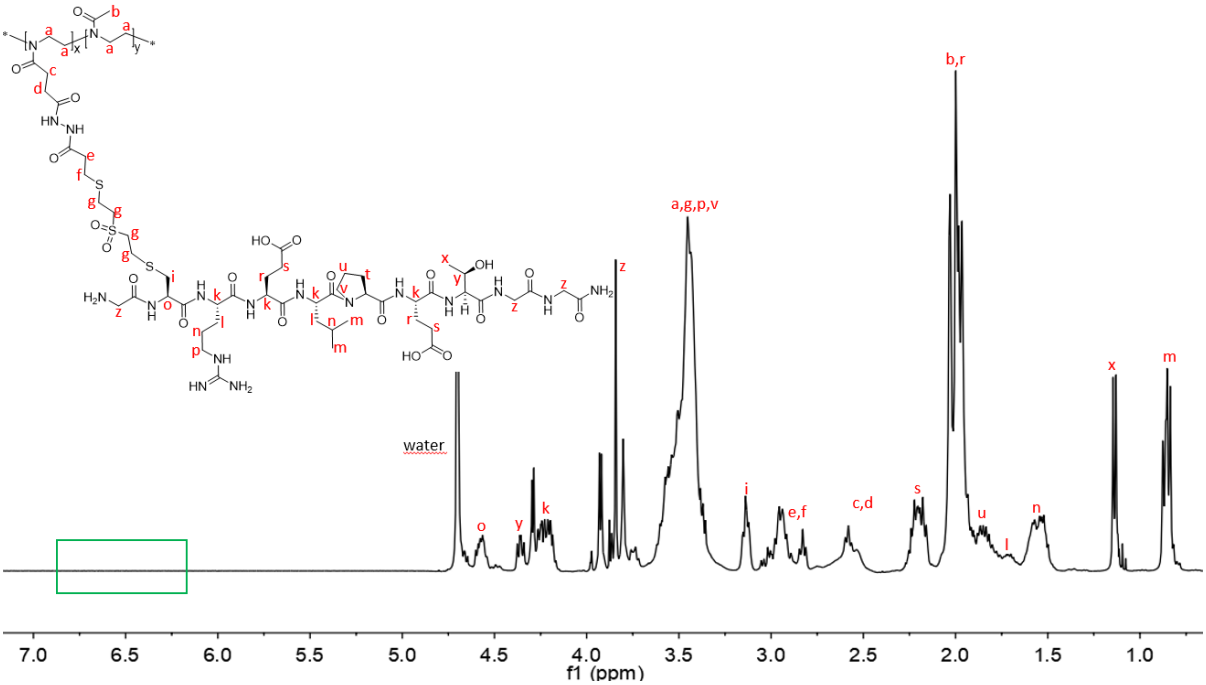

Figure S7. ^{1}H-NMR (400 MHz) spectrum of PMOXA-LPETG in D_2O.

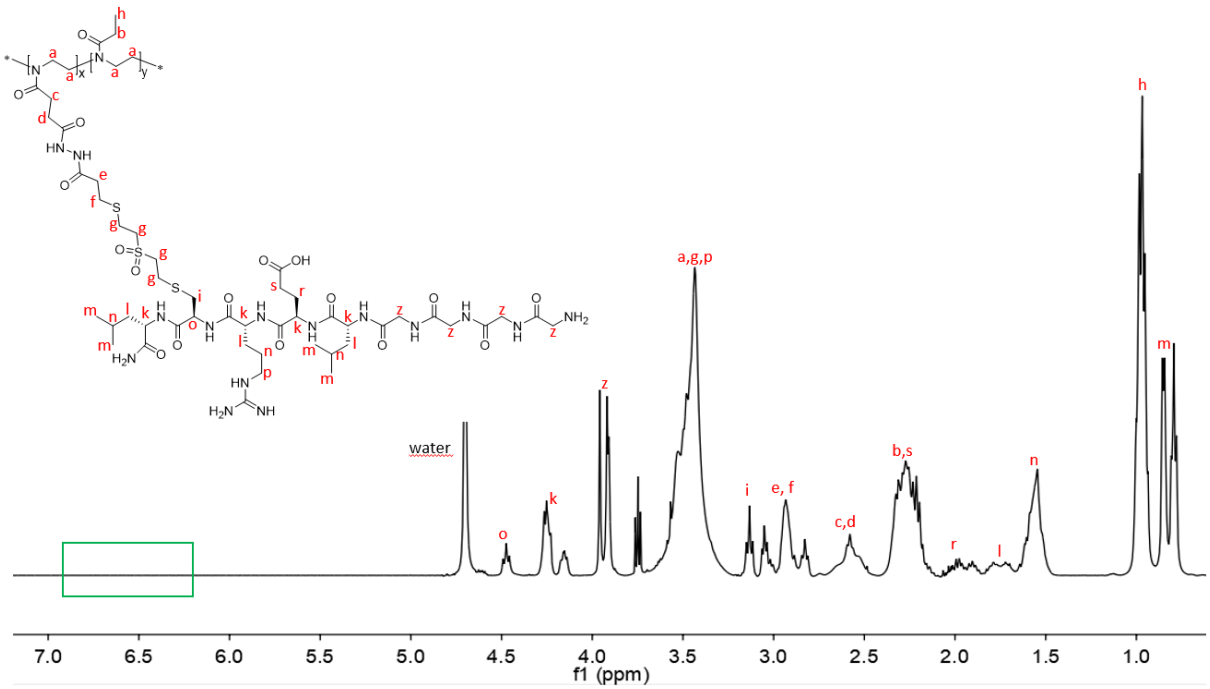

Figure S8. ^{1}H-NMR (400 MHz) spectrum of PEOXA-GGGG in D_2O.

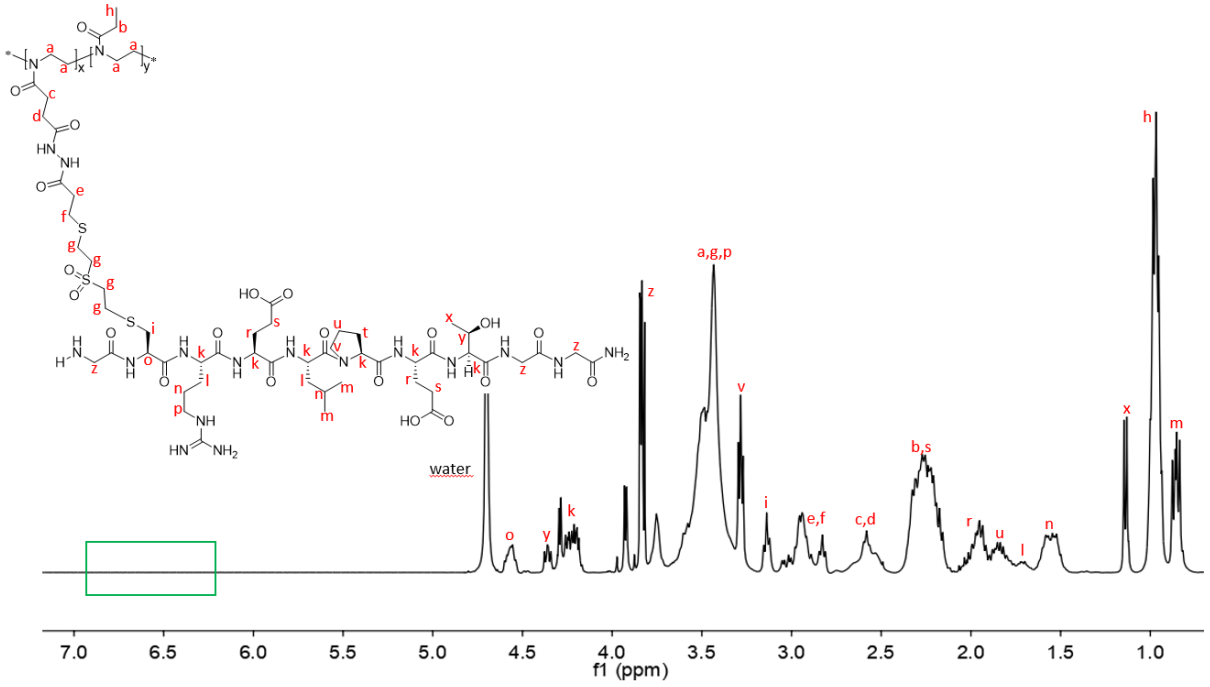

Figure S9. ^{1}H-NMR (400 MHz) spectrum of PEOXA-LPETG in D_2O.

Table S1. Molecular weights and polydispersity index (PDI) of the PMOXA-$COOCH_3$ and PEOXA-$COOCH_3$ measured by HFIP GPC[a] and determined by ^{1}H-NMR spectroscopy[b].

	M_n (g/mol)[a]	M_w (g/mol)[a]	M_n (g/mol)[b]	PDI[a]
PMOXA- $COOCH_3$	11800	13000	15800	1.10
PEOXA-$COOCH_3$	16900	19300	18400	1.14

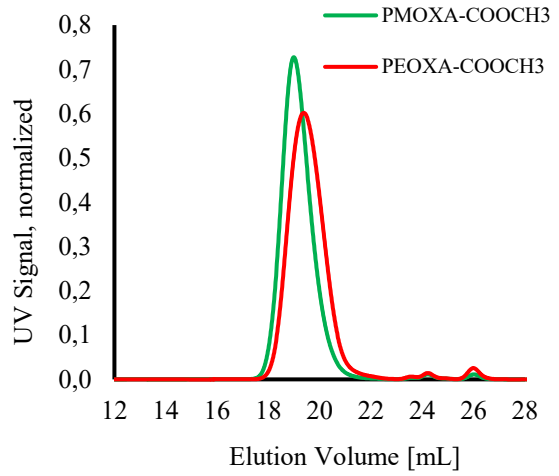

Figure S10. GPC elugrams of PMOXA-$COOCH_3$ (green trace) and PEOXA-$COOCH_3$ (red trace).

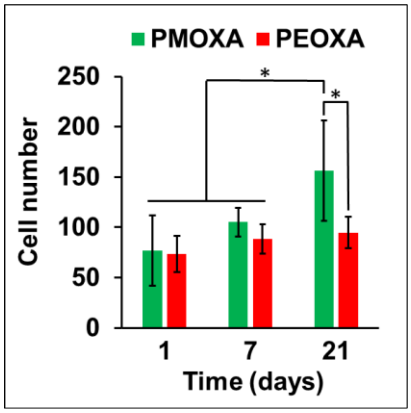

Figure S11. Cell proliferation of hACs encapsulated in PMOXA or PEOXA hydrogels after 1, 7, and 21 days of culture. Cell number as counted per image. Asterisk (*) represents significant difference ($p < 0.05$). SD n=6

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(2) Chen, I.; Dorr, B. M.; Liu, D. R. A General Strategy for the Evolution of Bond-Forming Enzymes Using Yeast Display. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (28), 11399–11404.