

Supplementary Information

Functionalised type-I collagen as hydrogel building blocks for bio-orthogonal tissue engineering applications.

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	matrix	7
3. Figures		9
4. References		10

Experimental section

1. Materials

Freeze dried porcine collagen was purchased from NIPPON meat packers, Japan, sodium hydroxide, triethanolamine, methacrylic anhydride, *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich, St.Louis, USA. Acrylic anhydride was purchased from abcr GmbH Germany, 8 arm thiol [Poly ethylene glycol (tri-pentaerythritol)] and 4 arm thiol [Poly Ethylene Glycol (pentaerythritol)] molecular weight 10,000 Da were purchased from JenKem Technology, USA. Rat-tail collagen type-I was purchased from BD biosciences, UK.

2. Methods

2.1 Synthesis of methacrylated collagen

Freeze dried collagen was dissolved in Milli Q water and gently stirred. The pH of collagen solution was increased to pH 10 using 2N NaOH and methacrylic anhydride at a molar ratio of 5:1 (with respect to number of lysine amine groups in collagen) was added subsequently drop-wise at room temperature to modify the pristine collagen with reactive functional groups (Scheme 1). The reaction mixture was dialyzed against distilled water (pH 10) using 12-14kDa cutoff dialysis tubing (Spectrum Laboratories,

Inc., CA, US) for 2-3 days to remove reaction by-products and lyophilized for 3-4 days to and stored at 4°C until further use.

2.2 Tri-nitro benzene sulfonic acid (TNBS) assay

The extent of modification of MAC and AC was quantified using TNBS (2,4,6-trinitrobenzenesulfonic acid) calorimetric assay, by previously established protocols.^{1,2} Briefly, 2mg of dry sample was mixed with 1ml of 4wt% NaHCO₃ (pH 8.5) and 1 ml of 0.5wt% TNBS solution at 40°C under mild shaking. After 4 hours of reaction, 3 mL of 6M HCl solution was added and the mixture was heated to 90°C to dissolve any sample residuals. Then the solutions were cooled and extracted three times with anhydrous diethyl ether to remove the unreacted TNBS species. UV absorbance of samples was recorded using Shimadzu UV-Vis spectrophotometer (UV-2450) against a blank, prepared by the above procedure, except that the HCl solution was added before the addition of TNBS. The content of free amino acid groups and degree of functionalization (F) were calculated as follows:

$$\frac{\text{moles (Lys)}}{\text{g(collagen)}} = \frac{2 \cdot \text{Abs}_{346} \cdot 0.02}{1.46 \cdot 10^4 \cdot b \cdot x} \quad (1)$$

$$F = 1 - \frac{\text{moles (Lys) modified collagen}}{\text{moles (Lys) pristine collagen}} \quad (2)$$

where Abs₍₃₄₆₎ is the absorbance value at 346nm, 1.4×10⁴ is the molar absorption coefficient for 2, 4, 6-trinitrophenyl lysine (l. mol⁻¹ . cm⁻¹), *b* is the cell path length (1cm), *x* is the sample weight and *moles (Lys) modified collagen* and *moles (Lys) collagen* represent the lysine molar content in functionalized and pristine collagen, respectively.

2.3 Nuclear Magnetic Resonance

Structural properties and the degree of methacrylation of collagen lysine amines of collagen were also analyzed by ¹H NMR spectroscopy, using a 500 MHz Varian Inova NMR spectrometer equipped with a cryoprobe. Briefly, 3 mg MAC and pristine collagen was dissolved in 3 ml of deuterium oxide. Sodium 3-(trimethylsilyl)-2,2',3,3'-tetradeuteriopropionic acid was added to reference chemical shifts. In order to remove air bubbles, the dissolved samples were centrifuged at 1700 rpm, 20°C for 10 min. The degree of modification of collagen lysine amines was quantified from a protocol defined by earlier methods³. The ¹H NMR spectra were normalized to signals of phenylalanine sidechains (6.9-7.5 ppm) to obtain to

collagen concentrations. Subsequently, the lysine methylene signals (2.8-2.95 ppm) of pristine collagen and MAC were integrated to determine the degree of functionalization using,

$$F = \left(1 - \frac{A(\text{Lysine methylene of MAC})}{A(\text{Lysine methylene of pristine collagen})}\right) \times 100\% \quad (3)$$

where $A(\text{lysine methylene of MAC})$ and $A(\text{lysine methylene of pristine collagen})$ are the integrated intensities corresponding to functionalized and pristine collagen, respectively.

2.4 Circular Dichroism

All spectra were performed on a Chirascan™ CD Spectrometer, Applied Photophysics Ltd., (Surrey, UK). Briefly, a quartz cell of 0.1 cm path length was used to record the CD spectra's of collagen and modified collagen samples between 180-260 nm at a scan rate of 1nm/s. A spectrum of double distilled water was subtracted from collagen and modified collagen spectra. Rpn (Ratio of positive to negative band) was calculated from the resulting spectra for collagen before and after modification.

2.5 Fabrication of hydrogels and sol-gel characterization

A T-piece syringe mixing system established in our lab was used to fabricate the hydrogels (16). Briefly, 500mg (pH 6.7-7) of methacrylated collagen (MAC) 10% (w/w) was taken in glass syringe and mixed with multi-arm PEG thiols (4arm and 8arm) at different functional ratios to fabricate hydrogels. Multi-arm PEG thiols were dissolved in deoxygenated water before its use. Three different formulations of MAC and 4 arm PEG thiols (4A) at functional ratios 1:0.5(1); 1:1(2) and 1:2(3) with the increasing functional equivalents of thiols to MAC, denoted as M4A1, M4A2 and M4A3 respectively to form discrete gels. Similarly four different formulations of MAC and 8 arm PEG thiols (8A) at functional ratios 1:0.5(1); 1:1(2); 1:2(3) and 1:4(4) with the increasing functional equivalents of thiols to MAC, denoted as M8A1, M8A2, M8A3 and M8A4 respectively to form discrete gels. In order to improve the gelation time of hydrogels, 0.05 M TEOA was added before addition of thiols in the syringe system to increase the pH to 8-8.2 of the final formulation. All the reaction mixture in the syringe was mixed between 25-30 cycles in order to fabricate homogenous hydrogels.

2.6. Determination of sol-gel fraction

To characterize the sol-gel fraction, all the fabricated hydrogels were cut into pieces (n=3) and dried. The dried weights (W_0) were obtained gravimetrically and incubated in ddH₂O on an orbital shaker for 24hr to remove the sol fraction. Further the gels were dried again under vacuum to obtain constant weight (W_t). The gel fraction was calculated using the following equation 4 and 5.^{4,5}

$$\text{Sol fraction (\%)} = \left[\frac{W_o - W_t}{W_o} \right] \times 100 \quad (4)$$

$$\text{Gel fraction (\%)} = 100 - \text{sol fraction} \quad (5)$$

2.7 Rheology

Mechanical properties of hydrogels were assessed using parallel plate rheometry (AR 2000 rheometer, TA instruments, Inc., UK). Fabricated hydrogels were punched in cylindrical shape (1mm thick, 10mm diameter) and bulk modulus (G') and viscous modulus (G'') measurements were recorded at a frequency range of 1-10Hz at 25°C using 8mm aluminum plate geometry. The gap was adjusted starting from the original sample height and compressing the sample to reach the sample reach a normal force of 0.3N. Rheological measurements were made on hydrogels after 24h post gelation. The storage modulus (G') values from the frequency dependent measurement were used to determine the ξ (Mesh size) and M_c (average molecular weight between crosslinks) using equation 6 and 7 respectively.⁶

$$\xi = \left(\frac{G' NA}{RT} \right)^{-1/3} \quad (6)$$

$$M_c = \frac{C \rho RT}{G' p} \quad (7)$$

where C is the final polymer concentration (5% w/v), ρ is the density of water at 298 K (997 kg m⁻³), R is the molar gas constant, $G' p$ is the peak value of G' , NA is the Avogadro constant and T is temperature (298 K).

In situ gel kinetics was also measured using AR2000 Advanced Rheometer (TA Instruments) with a custom made parallel plate titanium geometry of 19mm diameter was used for the rheological characterization of the hydrogels as described earlier.⁷ Gel components, methacrylated collagen and PEG thiols (M4A2 and M8A3) were pre-mixed as described in earlier section. The formulations without any TEOA catalyst will be denoted as M4A2.1 and M8A3.1 respectively. A total volume of 1 mL of the resulting material was injected into a custom-made cylindrical aluminum plate as described by authors. Acrylated collagen (AC) was synthesized using the same method like MAC synthesis using acrylic anhydride as a reactant. It was mixed with multi-arm PEG thiols 4A and 8A to form A4A2 and A8A3 gels. Similarly, the formulations without any TEOA catalyst will be denoted as A4A2.1 and A8A3.1 respectively. Oscillatory stress sweeps were performed on hydrogels shortly after mixing and thereafter

at different time intervals over 10 h to monitor the curing process. The first measurement was recorded 15 min after mixing and the normal force (0.3N), temperature (25°C), and frequency (0.1 Hz) was kept constant. The samples were covered with Parafilm M and kept in moist condition between measurements. The rates of gelation at different time intervals were compared via the normalized elastic modulus, $G'_r = [G'(t) - G'_0] / [G'_\infty - G'_0]$, where G'_0 is the elastic modulus at the starting point and G'_∞ is the equilibrium elastic modulus after complete gelation. G'_∞ is the average of the last 10 G' points obtained from frequency sweep measurements.⁸

2.8 Collagenase assay

Enzymatic degradation of hydrogels was done using Type-I-Collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St.Louis, USA). Hydrogels resulted from formulations M8T3 and M8T4 were used in this study along with native collagen (5%) cross-linked with EDC-NHS serving as control. The control samples were fabricated according to the protocol mentioned earlier.⁹ Hydrogels of 1mm thickness were cut into small pieces of 6mm diameter were placed in a vial containing 5U/mL collagenase solution in 0.1M tris-HCL (pH 7.4) and 5mM CaCl₂. Further the samples were incubated at 37°C and the collagenase solution was changed at every 8 hours and the sample weights were measured at different time points. Enzymatic degradation of samples relative to their original weight were measured as function of time using the following equation where W_0 is the original or initial weight of sample and W_t is the weight of degraded sample at certain intervals.⁹

$$\text{Residual mass} = \frac{W_t}{W_0} \times 100\% \quad (8)$$

2.9 Cryo-Scanning electron microscopy (SEM)

Low temperature scanning electron microscopy (Cryo-SEM) was carried out in a Tescan (Vega II - XMU) with cold stage sample holder at -50°C using a backscattered electron detector (BSE) and secondary electron detector (SED). A 6 mm circular piece of the M8T4 hydrogel formulation was blotted and sectioned prior imaging. The images shown correspond to representative cross sections of the material. In all cases fast speed scanning was used and no sample burning was observed.

2.10 *In vitro* biocompatibility of human corneal epithelial cells (HCEC)

Green fluorescence protein (GFP) transfected HCECs were seeded on pre-polymerized top of the hydrogel surface to evaluate the biocompatibility of the material. A stable GFP-HCEC cell line was established by the method earlier.¹⁰ The fabricated M8T4 hydrogels were cut into 6mm hydrogel discs and fitted into 96 well plates followed by sterilization with 3X antibiotic solution consisting of 300 U/ml

penicillin and 300 µg/mL streptomycin. Proliferation of HECs on hydrogel surface was evaluated by seeding five thousand cells on top of the hydrogel. Cells were also seeded on tissue culture plate and pristine collagen cross-linked with EDC-NHS⁹ were serving as positive control along with an hydrogel made from 8 arm PEG thiol cross-linked with 8 arm PEG maleimide (Mn= 41600, Creative PEGWorks, NC, US) serving as negative control. The seeded cells were maintained in Keratinocyte-serum free media (KSFM; Life Technologies, Invitrogen, Paisley, UK containing 50µg/mL bovine pituitary extract and 5 ng/ml epidermal growth factor) within a humidified 37°C incubator with 5% CO₂. Photomicrographs of the cells were taken at Day 1, 3 and 5 using a fluorescence microscope (AxioVert A1, Carl Zeiss, Göttingen, Germany). Three different areas of 1290×965 µm² each were sampled for cell counts.

2.11 3-D *in vitro* cardiac progenitor cell encapsulation in hydrogel matrix

Murine cardiac progenitor cells (CPCs) were isolated from 3-week-old C57BL/6 mouse hearts using a Millipore Cardiac Stem Cell Isolation kit (Millipore, Darmstadt, Germany), following the manufacturer's protocol and with prior ethical approval from the Djurförsöksetiska Nämnden Linköping (Animal Ethical Committee, Linköping). They were cultured and maintained in DMEM/ F12 + GlutaMAX™-I (1X) media containing 10% FCS, 1% PEST, 1X ITS (Insulin, Transferrin, Selenium), 0.5% DMSO and EGF (20ng/mL). For encapsulation, 1×10⁶ cells were mixed inside 1 ml of M8T4 hydrogel matrix (n=3 samples). Unmodified type I rat tail collagen that had been neutralized with HEPES buffer and 1N NaOH to pH 7.0 and into which CPCs were mixed and then thermogelled at 37°C served as controls (n=3).^{11, 12} Both sets of encapsulated cells were maintained in a humidified, 37°C incubator with 5% CO₂ and supplemented with culture media. On day 3 of culture, the composite cell-hydrogel matrices were harvested, washed with 1X sterile PBS and incubated at 37°C with calcein AM/ethidium homodimer (4/2 µM, Live Dead, Invitrogen, Grand Island NY) for 20-30 min to evaluate cell viability. The stained constructs were imaged using confocal microscope (LSM700, Carl Zeiss, Germany). 3-D stacking and Z-sectioning was also performed using LSM700 software to assess the infiltration of cells on the hydrogel matrix.

3. Figures

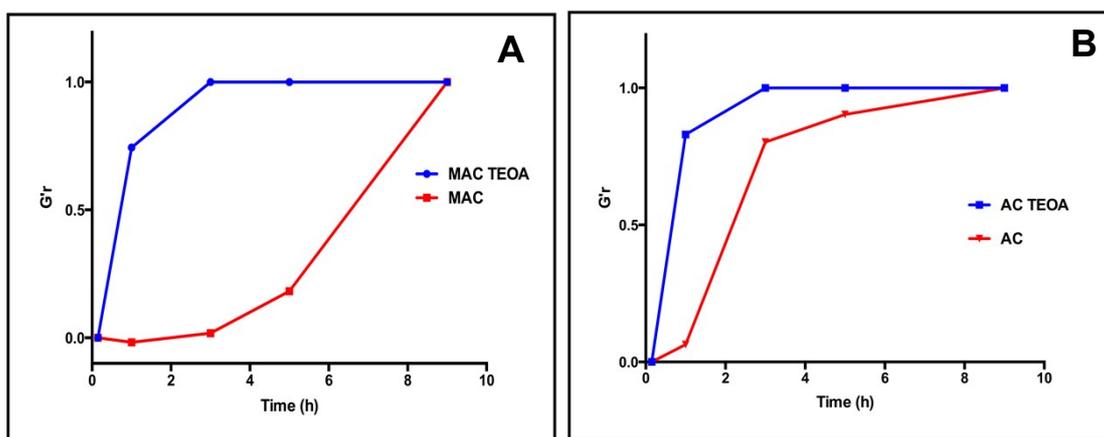


Figure S1. *In situ* gelation kinetic graphs of 4 arm PEG thiol cross-linked with (A) MAC with and without TEOA (M4A2 and M4A2.1 formulations) and (B) AC with and without TEOA (A4A2 and A4A2.1) formulations.

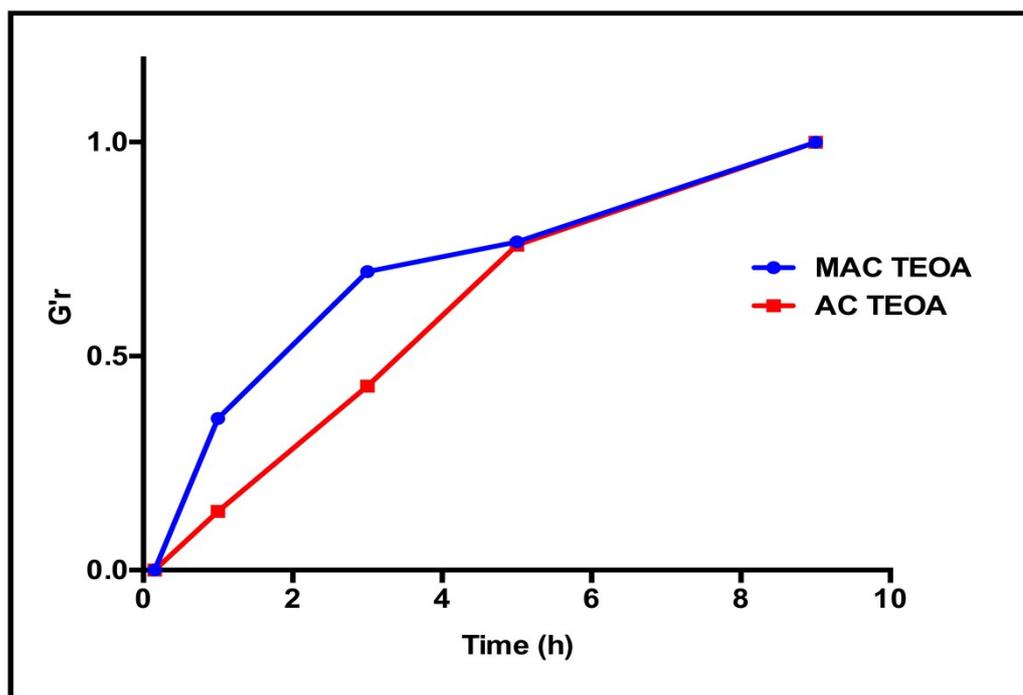


Figure S2: *In situ* gelation kinetic graph of 8 arm PEG thiol cross-linked with MAC and AC with TEOA (M8A3 and A8A3 formulations).

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