A Microstereolithography Resin Based on Thiol-Ene Chemistry: Towards Biodegradable Extracellular Constructs for Tissue Engineering

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SUPPORTING INFORMATION

CONTENTS

Experimental

Materials

5-Methyl-5-allyloxycarbonyl-1,3-dioxan-2-one (MAC) was synthesized as reported previously and recrystallized several times before use from toluene–diethyl ether (1 : 3 v/v).[1] All other solvents and chemicals were obtained from Sigma-Aldrich or Fisher Scientific and used as received.

General considerations

Polymerizations were performed under a nitrogen flushed atmosphere with pre-dried glassware using standard Schlenk line techniques. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-300 and DPX-400 spectrometer at 293 K. Chemical shifts are reported as δ in parts per million (ppm) and referenced to the residual solvent signal ($CDCl_3$: ^{1}H, δ = 7.26 ppm; ^{13}C, δ = 77.16 ppm). Gel-permeation chromatography (GPC) was used to determine the molecular weights and dispersities ($Đ_M$) of the synthesized polymers. GPC was conducted on a GPC50 supplied by Agilent and fitted with differential refractive index (DRI) detector. Samples were eluted through a column set consisting of a guard column (Varian Polymer Laboratories PLGel 5 μM, 50 × 7.5 mm) and two mixed D columns (Varian Polymer Laboratories PLGel 5 μM, 300 × 7.5 mm). The mobile phase was $CHCl_3$ at a flow rate of 1.0 mL min^{-1}, and samples were calibrated against Varian Polymer Laboratories Easi-Vial linear poly(styrene) standards (162–3.7 x 10^5 g mol^{-1}) using the software package Cirrus v3.3.

Organocatalytic ROP of 5-methyl-5-allyloxycarbonyl-1,3-dioxan-2-one (MAC)

In a typical experiment, MAC (95 g, 0.475 mol, 50 eq.), which had been dissolved in $CHCl_3$, passed over anhydrous $MgSO_4$ (1 g) and recovered *in vacuo* to remove excessive amounts of entrained water, was dissolved in $CHCl_3$ (950 mL) and introduced into a nitrogen flushed dry 2 L round bottomed flask equipped with magnetic stirrer bar. Separately, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (1.44 g, 9.46 x 10^{-3} mol, 1 eq.) and water (150 μL, 8.33 x 10^{-3} mol, 1.14 eq.) were combined and introduced into the MAC solution under nitrogen with stirring. The resulting solution was stirred at ambient temperature for 18 h before the DBU catalyst was quenched by addition of Amberlyst A15 H^+ acidic resin, filtered through a silica plug with ethyl acetate and precipitated twice from chloroform into ice cold hexanes. The resulting polymer was poured into a round bottomed flask, re-dissolved in $CHCl_3$ and the solvents removed in vacuo to yield a viscous, transparent and colourless liquid. Yield: 94.1 g (98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.90–5.82 (m, CH_{vinyl}), 5.32–5.21 (m, CH_2-$_{vinyl}$), 4.69–4.58 (m, OCH_2CHCH_2), 4.41–4.22 (m, OC(O)OCH_2), 3.78–3.70 (m, CH_2OH), 1.26 (s, CH_3), 1.22 (s, C(CH_3)CH_2OH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.9 (CC(O)O), 154.5 (OC(O)O), 131.8 (CH_{vinyl}), 118.6 ($CH_{2\text{-}vinyl}$), 68.7 ($OC(O)OCH_2$), 66.0 (OCH_2CHCH_2), 46.7 (CCH_3), 17.6 (CH_3). GPC ($CHCl_3$, RI): M_n ($Đ_M$) = 5 870 g mol^{-1} (1.39).

Stereolithography

The photolithographic resin was blended by the addition of 0.5 wt% Irgacure 784 photoinitiator and 0.25 wt% photoresist (Kalsec Durabrite® Oleoresin Paprika Extract NS) into a 1:1 molar mixture of poly(MAC) and pentaerythritol tetrakis(3-mercaptopropionate) (PTP) in the absence of ambient light. Microstereolithography (μSL) was carried out on a custom-built system described previously.[2] A blue LED light engine (Enfis Uno Air) with a peak output at 465 nm was used as the projector light source. The resin tray was a 5 mm glass plate coated with a 2 mm thick layer of silicone elastomer.

3D (*10*, *3*)-*a* Network Production

The standard tessellation language (.stl) file of the structure produced by stereolithography was based on the (*10*, *3*)-*a* network described in previous literature.[3] The structure was produced on a 400 μm

base plate to ensure sufficient bonding to the glass build surface and using 100 μm layers and 10 s exposure per layer throughout.

Tensile Strength Measurements

Dog bone type samples measuring approximately 25.0 × 1.0 × 1.5 mm were produced *via* pouring resin into preformed stainless steel templates and subjected to photopolymerization in an EnvisionTEC Otoflash (1000 pulses) before being removed and subsequently postcured under UV irradiation for 1 h in a Metalight QX1 UV chamber. At ambient temperature and humidity, samples were axially loaded in a tensile testing machine (Deben MICROTEST) with a load cell capacity of 2 kN and crosshead speed of 0.1 mm min^{-1} with a premeasured grip-to-grip separation. Data were recorded from the instrument using Deben Microtest v5.5.14 and all values reported herein were obtained from an average of five repeat specimens.

Disc Production for Cell Seeding

Cell seeding discs were produced monolithically by varying the exposure time in a pool of the resin placed on the resin tray. The central, high optical transmission regions of the discs were exposed for 5 s, while the outer raised walls of the discs were exposed for 90 s. After production, excess free resin was removed by rinsing the constructs with isopropanol. The constructs were then subjected to post curing in a Metalight QX1 UV light box for 1 h before being washed with and stored in absolute ethanol.

Acquisition and Expansion of Bovine IVD cells

Bovine IVD tissue was obtained from bovine tails purchased from a local abattoir and cell isolated as previously described.[4] Briefly, discs were macroscopically dissected and cells extracted through digestion of the samples in 0.5% pronase (Merck Chemicals Ltd, UK) for 1 h followed by 0.5% collagenase type II (Invitrogen, UK) and 0.1% hyaluronidase (Sigma, UK) for 2-3 h at 37 °C. All digestion steps were performed in serum-free medium; DMEM (Sigma) supplemented with 10 μM ascorbic acid, 100 mM sodium pyruvate (Sigma) and 1 × Antibiotic Antimycotic solution (50 000 units penicillin, 50 mg streptomycin and 125 μg amphotericin, Sigma). The supernatant was passed through a 40 μm sieve to remove debris and IVD cells collected by centrifugation at 500 g for 5 min. Cells were cultured in complete medium (serum-free medium plus 10% foetal calf serum) at 37 °C, 5% humidified CO_2 and passaged when ~80 confluent.

Cell Seeding

Prior to cell seeding, the biomaterial constructs were washed in 70% ethanol (2 × 10 minutes), Dulbecco's phosphate buffered saline (PBS; Sigma, 2 × 30 minutes) and then overnight in complete medium. IVD cells were trypsinised (Sigma) and seeded onto constructs, placed in 24-well plates, at a density of 5000 cells cm^{-2} in 20 μL complete medium, ensuring all cells remained on surface of disc. Cells were incubated for 2 h at 37 °C, 5% CO_2, after which an additional 1 mL of culture medium was added to each well, taking care not to disturb adherent cells. Constructs were then cultured for up to 7 days at 37 °C, 5% CO_2, with media changes every 3-4 days.

Assessment of Cell Proliferation

Cell proliferation of IVD cells seeded onto the constructs versus standard monolayer conditions was investigated. This was assessed using a Cytotoxity Detection KitPLUS (LDH; Roche, UK) following manufacturer's recommended protocol for measuring cell proliferation. Fold change was calculated comparing number of viable cells after 5 days in culture versus the number of viable cells 24 h after plating (in triplicate).

Assessment of Cell Viability and Morphological Analysis

To assess cell viability, media was removed from the IVD cell-seeded constructs following 5 days culture, the constructs washed with PBS and stained using Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) following the manufacturer's recommended protocol. For morphological analysis, constructs were removed from media after 7 days in culture, washed in PBS and fixed in 4% paraformaldehyde for 15 minutes at 4 °C. Constructs were treated with 0.25% Triton-X100 in PBS (5 minutes) then blocked in 1% bovine serum albumin (BSA) for 1 h at room temperature. Constructs were incubated with 1:100 (in 1% BSA) mouse monoclonal Vincullin primary antibody (Abcam, UK) for 1 h at 4 ˚C followed by 1:300 goat-anti mouse secondary antibody conjugated to Alexa Fluor® 488 (Invitrogen) for 1 h, room temperature. One unit of Phalloidin per sample (Alexa Fluor 594, Invitrogen) was then added for 20 minutes at room temperature. Constructs were incubated with a 5 μM solution of DRAQ5™ (Biostatus, UK) at 37 °C for 15 minutes to visualise the nuclei, after which the constructs were washed in PBS and resuspended in PBS for imaging. Images were collected on a Leica TCS SP5 AOBS upright confocal microscope using ×20 and ×63 dipping objectives. ImageJ software was used to create maximum intensity projections of each optical stack gathered using the confocal software.

Figures, Schemes and Tables:

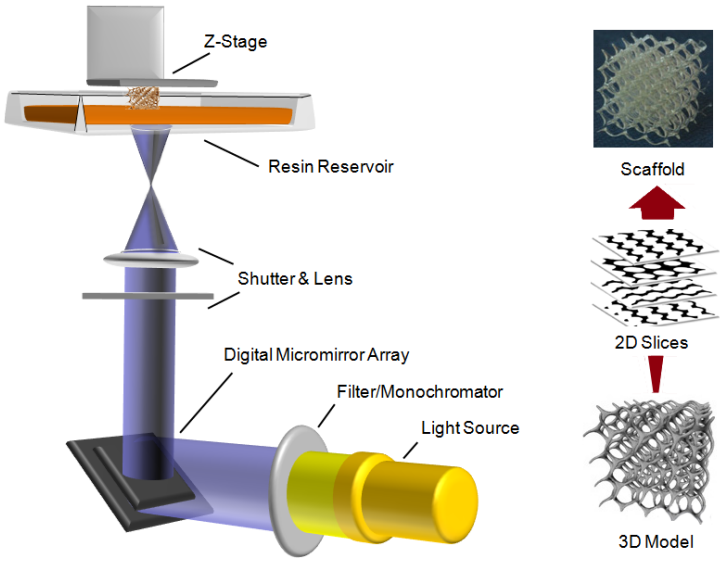


Fig. S1. Representative schematic of 3D microstereolithography process.

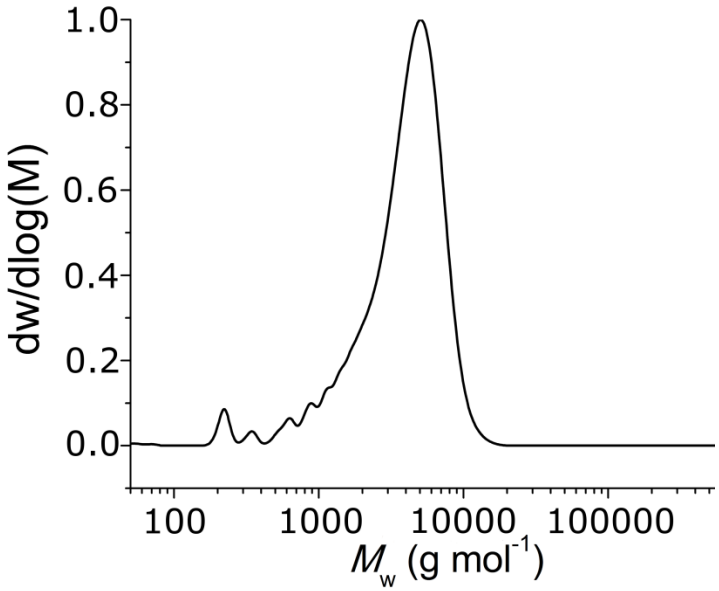


Fig. S2. GPC chromatogram of PMAC.

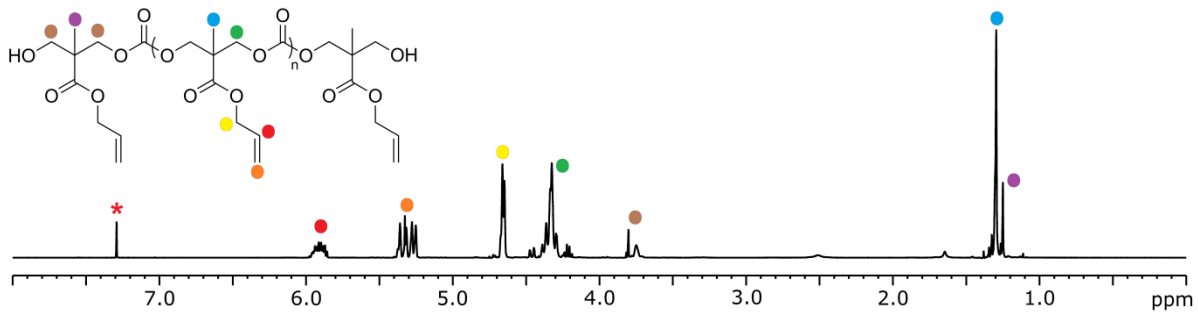


Fig. S3. ^{1}H NMR spectrum ($CDCl_3$, 298K; * = residual $CHCl_3$ peak at δ = 7.26 ppm) of PMAC.

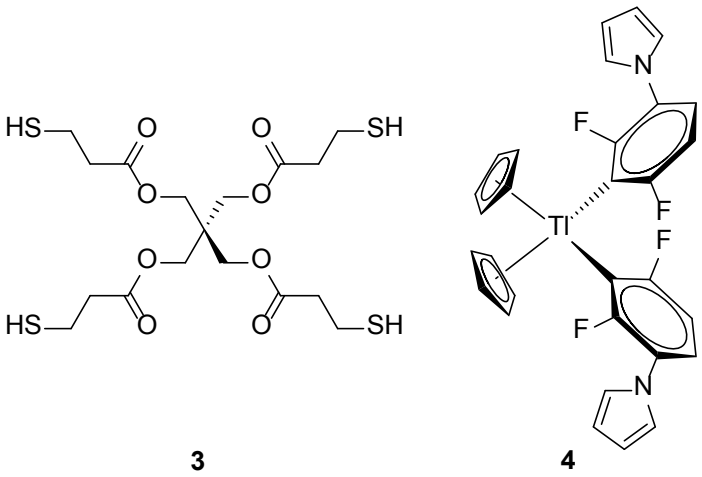


Fig. S4. Chemical representation of the multi-arm thiol cross-linker pentaerythritol tetrakis(3-mercaptopropionate) (**3**) and photoradical initiator Irgacure 784 (**4**) used in the production of the μSL resin.

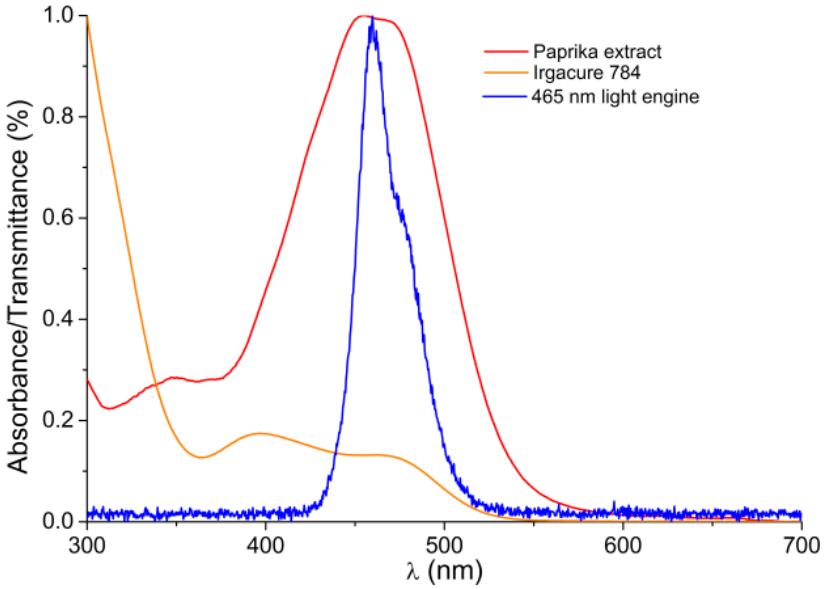


Fig. S5. UV/Visible absorbance spectrum of Kalsec Durabrite® Oleoresin Paprika Extract NS (red), Irgacure 784 (orange) and the transmission spectrum of the 465 nm light engine (blue) employed in this study.

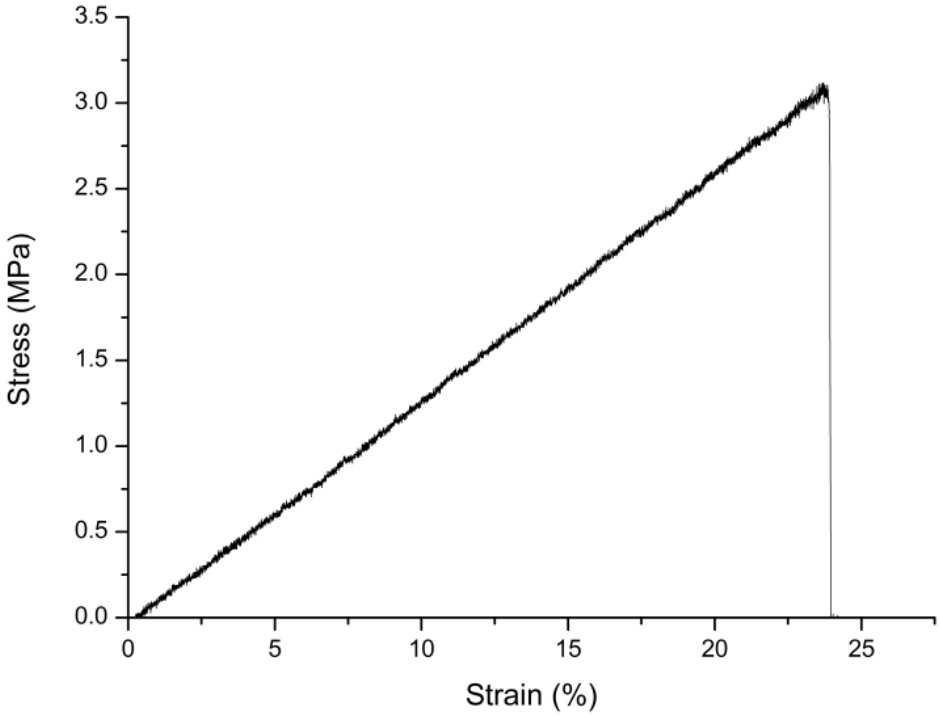


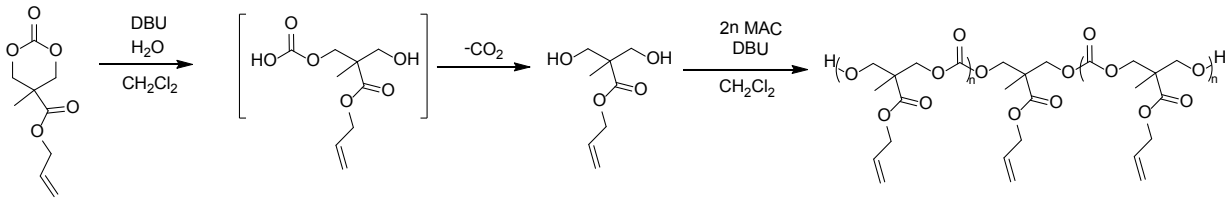
Fig. S6. Typical stress *vs.* strain curve of a dog bone sample of cured resin. Typical dimensions for specimens subjected to tensile testing were 25.0 × 1.0 × 1.5 mm.

Fig. S7. Typical cell seeding plate produced to assess the viability of IVD cells.

Table S1. Cure thicknesses as a function of photoresist (paprika extract) through a drop of resin on a glass slide after 10 seconds exposure to the blue light engine source used in this study. Irgacure 784 and viscosity modifier (propylene carbonate) were held at 0.5 wt% and 10 wt% respectively across the sample range.

Paprika extract (wt%)	Cure depth (μm)
0.00	– [a]
0.10	254
0.20	150
0.25	124
0.30	99
0.40	82
0.50	– [b]

[a] The entire layer of resin was cured in this time (> 500 µm) and as such was not measured. [b] The film formed was too weak to be measured by the profileometer due to insufficient crosslinking.



Scheme S1. Synthesis of PMAC by ring-opening polymerisation using water as the initiator.

References.

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2. S. J. Leigh, C. P. Purssell, J. Bowen, D. A. Hutchins, J. A. Covington and D. R. Billson, *Sens. Actuators, A*, 2011, **168**, 66-71.
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