Supporting Information

A single-component hydrogel bioink for bioprinting of bioengineered 3D constructs for dermal tissue engineering

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Experimental Section

Synthesis of methacrylate-functionalized pectin: Low methoxyl citrus pectin (Classic CU701) with galacturonic acid unit content of 86% and degree of methylation of 37%, was kindly provided by Herbstreith & Fox (Neuenbürg, Germany) and purified before chemical modification.[1] Pectin methacrylate (PECMA) was synthetized by reacting pectin with methacrylic anhydride (MA, Sigma-Aldrich). Pectin was dissolved in phosphate buffered saline (PBS, pH 7.4) at 1.25 wt% and stirred at room temperature (RT) until complete dissolution. To this solution, MA was added dropwise (0.5 mL/min) and left to react protected from light under vigorous stirring. The pH was periodically adjusted to 8.0 by the addition of 5 M NaOH. After 24 h, pectin was collected by twice precipitation with a ten-fold excess of cold acetone (4 °C), followed by drying at 40 °C in a vacuum oven. For further purification, the polymer was dissolved in ultrapure water (Millipore) and dialyzed (MWCO 3500, Spectra/Por®, SpectrumLabs) for 5 days. The final product was sterile filtered using 0.22 µm filter membranes (Steriflip® fillter unit, Millipore), frozen, lyophilized, and stored at -20 °C until further use. The extent of methacrylate substitution was determined by ^{1}H NMR recorded with a 400 MHz spectrometer AVANCE III (Bruker). Polymers were dissolved (8.5 mg/mL) in deuterated water (D_2O, Euriso-top) and transferred to NMR tubes containing a glass capillary loaded with 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP-d4, Euriso-top) as internal standard (δ = 0 ppm).

Conjugation of cell-adhesive peptide: The custom synthetized integrin-binding peptide CGGGGRGDSP (Genscript, cell-adhesive domain underlined) was conjugated to PECMA macromers through a base-catalysed thiol-Michael addition click reaction. For coupling the integrin-binding peptide, a solution of PECMA (1.0 wt%) was dissolved in triethanolamine (0.25 M, TEOA, Acros Organics) buffer at pH 8.25. After the addition of cell-adhesive peptide (64 mg/g polymer), the solution was protected from light and allowed to react under agitation and inert atmosphere. After 48 h, the solution was diluted with ultrapure water, purified by dialysis, and lyophilized. The coupling efficiency was quantified through the colorimetric DC Protein Assay (Bio-Rad) according to the manufacturer's instructions, with some modifications. Briefly, the macromer was dissolved in ultrapure water (1.0 wt%) and the solution (20 µL) transferred to a 96-well plate. To this solution, alkaline copper tartrate solution (25 µL) and dilute Folin reagent (200 µL) were then added, followed by mixing using a multi-channel micropipette. The solutions were allowed to react at RT for 30 min, and the protein content determined with a micro-plate reader (Synergy MX, BioTek) at 750 nm. Absorbance values were converted to peptide concentrations using a standard curve of free peptide in PECMA, and normalized against control polymer solutions that were subjected to the conjugation process but in the absence of peptide.

Preparation of pectin hydrogels: To produce photocrosslinked hydrogels, PECMA macromer was dissolved in 0.9 wt% NaCl containing 0.05 wt% of 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (I2959, Sigma) as radical photoinitiator. The solution was pipetted into a teflon plate with 750 µm spacers, covered with a SigmaCote treated glass slide, and exposed to UV light (7 mW/cm^2, 365 nm) using a BlueWave® 200 curing spot lamp (Dymax) for different durations (40–300 s). For the preparation of dual-crosslinked hydrogels two strategies were adopted: (1) photocrosslinked hydrogel samples were incubated in Dulbecco's minimum essential medium (DMEM, Gibco) with different concentrations of calcium chloride dihydrate (0–1.8 mM), at 37 °C in a humidified atmosphere of 5% CO_2; (2) PECMA macromer solutions (1.5 wt%) dissolved in 0.9 wt% NaCl containing I2959 were mixed with calcium chloride dihydrate at different concentrations (0–7 mM), yielding weakly crosslinked physical gels, followed by UV photopolymerization for different times (80–300 s).

Characterization of gel fraction, swelling ratio, microstructure and in vitro degradation: To determine the gel fraction, hydrogel samples (30 µL, n = 5) were lyophilized and weighted to determine the initial dry weight (W_{ID}). Dry samples were incubated in ultrapure water (1 mL) at 37 °C for 24 h and then were carefully removed from the solution, gently dabbed with a tissue paper to remove superficial water, and lyophilized to determine the final dry weight (W_{FD}). Gel fraction was determined based on the dry mass of hydrogels before (W_{ID}) and after (W_{FD}) incubation, according to Equation (1):

$$Gel\ \ fraction\ \ (\%) = \frac{W_{FD}}{W_{ID}} \times 100 \qquad (1)$$

The swelling ratio was determined by soaking freeze-dried hydrogels in ultrapure water (1 mL) at 37 °C for 24 h. Samples were collected, the hydrogel's superficial excess water was gently removed with a tissue paper, and the swollen weight (W_S) determined. Swelling ratio (*q*) was calculated by dividing the weight of hydrated hydrogel (W_S) by its initial dry weight (W_{ID}). The microstructure of hydrogels was observed by cryogenic scanning electron microscopy (cryoSEM). Immediately after preparation, hydrogel samples were frozen in slush nitrogen (N_2) and mechanically fractured to reveal the internal structure. After sublimation, samples were coated with gold/palladium and transferred to the cryoSEM microscope chamber (JEOL JSM 6301F/Oxford INCA Energy 350/Gatan Alto 2500). The degradation rate of hydrogels was enzymatically studied in vitro through the incubation of freeze-dried samples (n = 9) in ultrapure water (1 mL) supplemented with pectinase (0.05 mg/mL) from Aspergillus niger (Sigma-Aldrich) at 37 °C. At pre-determined time points, samples were gently washed with ultrapure water, freeze-dried and weighted (WD). The percentage mass loss of hydrogels was calculated using Equation (2):

$$Mass\ \ Loss\ \ (\%) = \frac{W_{ID} - W_D}{W_{ID}} \times 100 \qquad (2)$$

Characterization of hydrogel stiffness: The mechanical properties of hydrogels were analysed using a strain-controlled Kinexus Pro rheometer (Malvern). All tests were conducted with 4 mm diameter hydrogel samples (n = 5) in a humidified environment at physiological temperature (37 °C) to minimize hydrogel dehydration during analysis. To ensure an appropriate contact between the parallel plates, samples were

compressed at 10% of their initial height. Strain amplitude sweeps were conducted from 0.1 to 10% at 1 Hz, while frequency sweeps were carried out from 0.01 to 10 Hz at 0.5% strain, after determining the linear viscoelastic region.

Assessment of hydrogel's biological behaviour: Human neonatal dermal fibroblasts (hNDFs) isolated from human neonatal foreskin samples (Coriell Institute for Medical Research, USA) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, Sigma) and amphotericin B (2.5 mg/L, Sigma). Cells were cultured in 5% CO_2 at 37 °C in tissue culture polystyrene flasks. To assess the behaviour of hNDFs within PECMA hydrogels, cells (passage 3 to 8) were trypsinized using a 0.05 wt% trypsin/ethylenediamine tetraacetic acid (EDTA) solution (Sigma) before reaching confluence (70–80%) and centrifuged at 1200 rpm for 5 min. The cell pellet was suspended and mixed within the PECMA solutions (1.5 wt%) containing 0.05 wt% of I2959 at a final density of 1×10^7 cells/mL. Cell-laden polymer solutions (20 µL) were pipetted onto a teflon plate with 500 µm spacers, covered with a SigmaCote treated glass slide and exposed to UV light (7 mW/cm^2). Cell-laden hydrogels were soaked in 0.9 wt% NaCl (0.5 mL) for 30 min, followed by the addition of culture media. At predetermined time periods, cellular hydrogels were collected and characterised for their metabolic activity, cell viability and cell morphology.

Preparation of bioink, assessment of rheological properties and bioprinting setup: PECMA low macromer biofunctionalized with the RGD-peptide was dissolved in 0.9 wt% NaCl containing I2959 (0.05 wt%) as radical initiator. After solubilization, $CaCl_2$ (5mM) was added dropwise under vigorous agitation as the ionic crosslinking agent, and the solution incubated at 25 °C and 1400 rpm (> 1 h) until G' stabilization. Then, hNDFs were detached from the tissue culture plate, centrifuged at 1200 rpm for 5 min, suspended in 0.9 wt% NaCl, and mixed within the bioink at a concentration of 1×10^7 cells/mL.

To determine the rheological properties, bioink formulations with $CaCl_2$ at 0, 1, 3, 5 or 7 mM were tested using a Kinexus Pro rheometer (Malvern). Viscosity tests were performed at 25 °C in a humidified environment to prevent evaporation, using a shear ramp test (1 to 1000 s^{-1} shear rate) over the course of 2 min. Two loading cycles with 1 min intervals were performed before the rheological data acquisition. To determine the

yield stress, bioinks were subjected to a shear stress ramp, ranging from 1 to 100 Pa (2 min) with plate–plate geometry (0.5 mm distance).

For bioprinting, the cell-laden bioink was loaded in a sterile syringe with a 23 G cylindrical nozzle (7018314, Nordson EFD) and printed using a commercial bioprinter Regemat 3D V1 (Regemat 3D, Spain). Proprietary software was used to automatically generate G-code to control the bioprinter to fabricate desired constructs. Immediately after bioprinting, constructs were exposed to UV light (7 mW/cm^2, 365 nm) using a BlueWave® 200 curing spot lamp (Dymax Corporation) for covalent crosslinking. Printed constructs were incubated in culture media and characterized for cell viability, metabolic activity, morphology and ECM deposition.

Determination of metabolic activity and cell viability: The metabolic activity of cells within PECMA hydrogels was assessed by the resazurin assay. Resazurin (resazurin sodium salt at 0.1 mg/mL, Sigma-Aldrich) was diluted (20 %v/v) in DMEM and incubated with the hydrogels for 2 h at 37 °C. Samples were measured using a microplate reader (Synergy MX; Biotek) at 530 nm (excitation) and 590 nm (emission). Cell viability was determined with the live/dead assay. CyTRAK OrangeTM (10 µM) and DRAQ7TM (3 µM) (Biostatus) were diluted in DMEM and used as dyes for live cell and dead cells, respectively. Both dyes were incubated for 10 min at RT before the visualization of hydrogels using a laser scanning microscope (CLSM, Leica TCS-SP5 AOBS, Leica Microsystems).

Morphology of bioprinted cells and ECM deposition: For observation of cell morphology/cytoskeletal structure and ECM deposition, immunofluorescence staining of F-actin, fibronectin, collagen type-I, laminin and nuclei was performed for the cell-laden hydrogels after predetermined time points of culture. Samples were fixed in 4 %v/v paraformaldehyde (PFA, Sigma) in PBS for 30 min, followed by extensive washing with PBS. Samples were permeabilized for 10 min with 0.2 %v/v Triton X-100 (Sigma) in PBS. Samples were incubated in blocking solution (1 w/v% bovine serum albumin in PBS) for 1 h at RT, and left overnight at 4 °C with appropriate primary antibodies. Then, samples were rinsed three times with PBS and incubated for 45 min with appropriate secondary antibodies and the conjugated probe phalloidin/Alexa Fluor® 488 (1:40, Molecular Probes-Invitrogen) for F-actin staining. For nuclei, hydrogel samples were counterstained with Vectashield with DAPI (Vector Labs) immediately before confocal

visualization (CLSM, Leica SP5AOBS, Leica Microsystems). For laminin staining, samples were incubated in blocking solution (1 w/v% bovine serum albumin and 4 %v/v FBS in PBS) for 1 h and incubated with the primary antibody according the steps described above. Primary and secondary antibodies are listed in the Table S1, ESI.

Statistical analysis: Statistical analyses were performed in the GraphPad Prism 7.0 software. The results were reported as the mean ± standard deviation (SD). The non-parametric Mann–Whitney test was applied with 95% confidence interval and statistically significant differences marked with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

Supplementary Figures

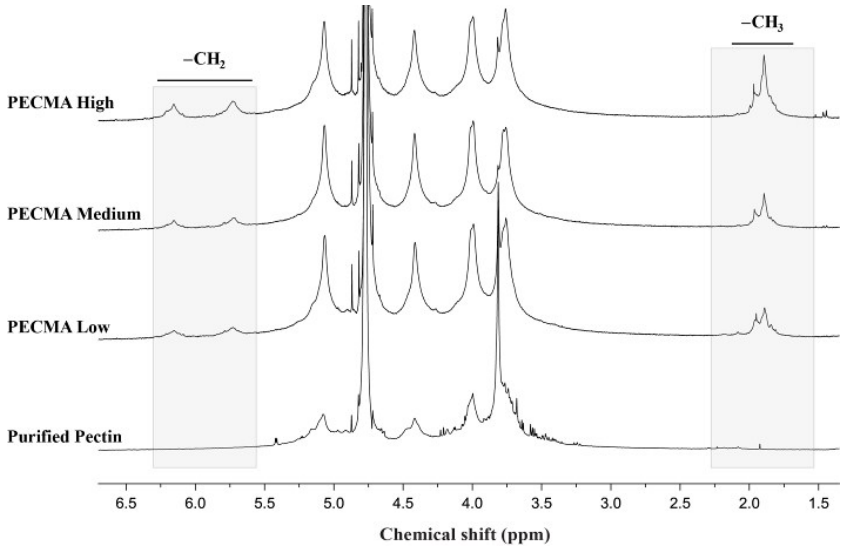

Figure S1. ^{1}H NMR spectra of purified pectin and pectin methacrylate (PECMA) with different degrees of methacrylation. New peaks corresponding to methyl ($–CH_3$) and methylene ($–CH_2$) groups are absent in the spectra of pure pectin, but present in the backbone of macromers modified with methacrylic anhydride.

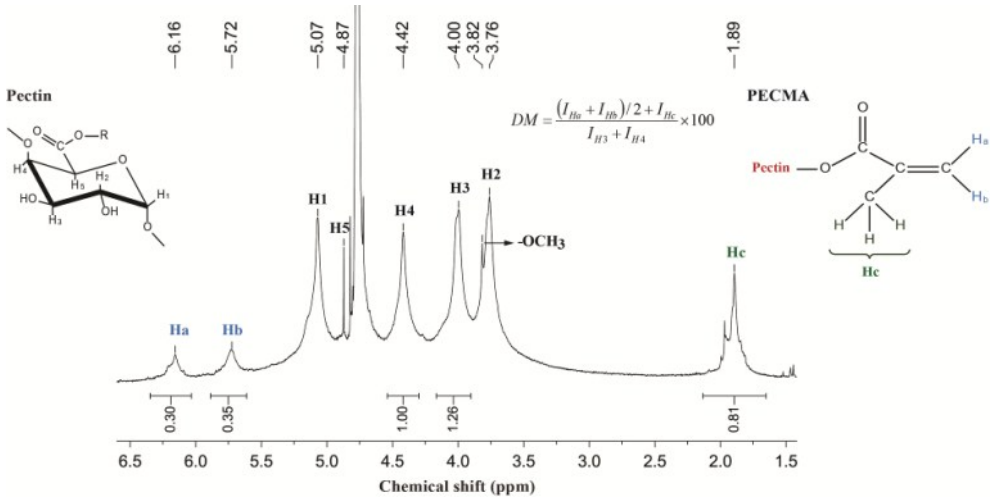

Figure S2. Representative ^{1}H NMR spectrum of PECMA detailing the peaks corresponding to native protons of pectin and new methacrylate function. The identification of native peaks in pectin structure was performed based on published data [2]. The degree of methacrylation (DM) was defined as the ratio of methyl ($–CH_3$) and methylene ($–CH_2$) groups of methacrylate function introduced in the polymer backbone to the pectin protons located in position 3 (H3) and 4 (H4). This value was calculated by the integration of peaks at 1.89 ppm (CH_3), 6.16 ppm (CH_2), 5.72 ppm (CH_2), 4.00 ppm (H3), and 4.42 ppm (H4).

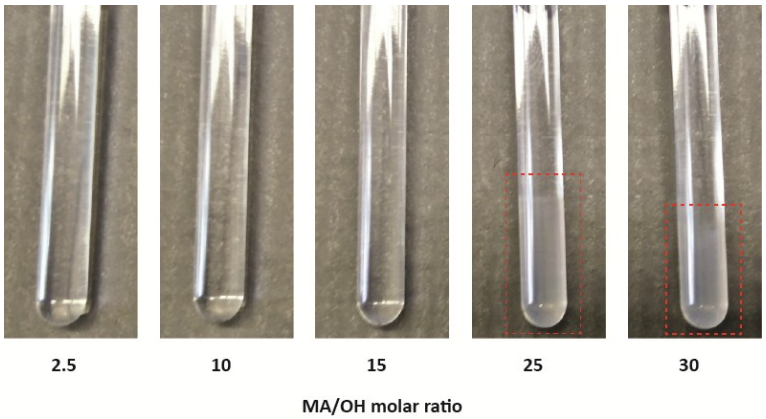

Figure S3. Macroscopic images of PECMA solutions in D_2O after chemical modification with different MA/OH molar ratios. For the ratios 2.5, 10 and 15, the solutions prepared showed complete dissolution, while using higher ratios it was possible to observe poor solubilization and polymer precipitation.

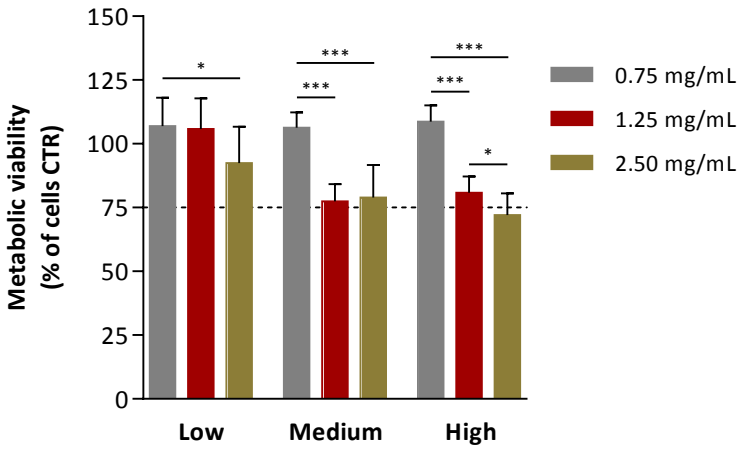

Figure S4. Effect of soluble PECMA macromer solutions prepared with varying degrees of methacrylation and polymer concentration on the metabolic activity of fibroblasts. Macromer solutions were dissolved in culture media at different concentrations and incubated with adherent fibroblasts cultured in monolayer for 24 h (*$P < 0.05$; ***$P < 0.001$).

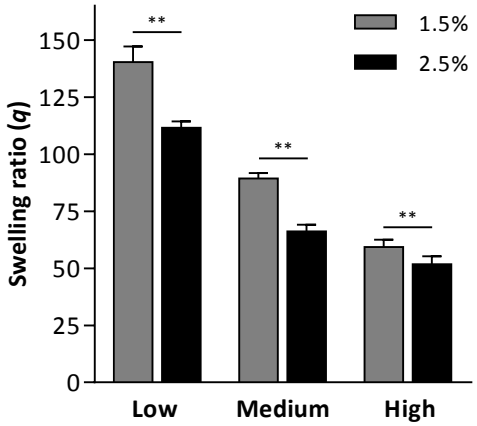

Figure S5. Swelling ratio of PECMA hydrogels with varying degrees of methacrylation tested at 1.5 and 2.5 wt% concentrations. Hydrogels were photocrosslinked by exposure to UV light for 160 s and then incubated in ultrapure water at 37 °C for 24 h (**P < 0.01).

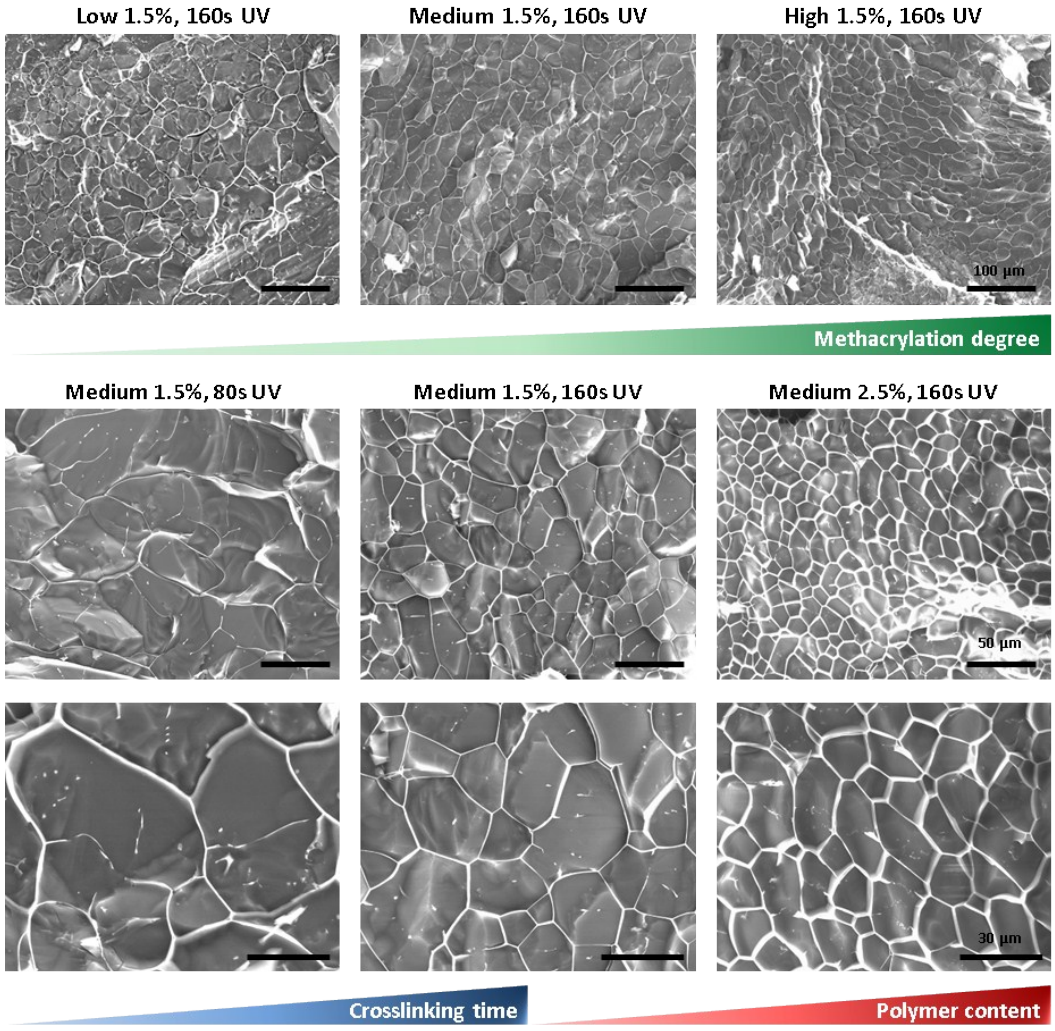

Figure S6. Influence of methacrylation degree, crosslinking time and polymer concentration on the microstructure of PECMA hydrogels visualized by CryoSEM analysis.

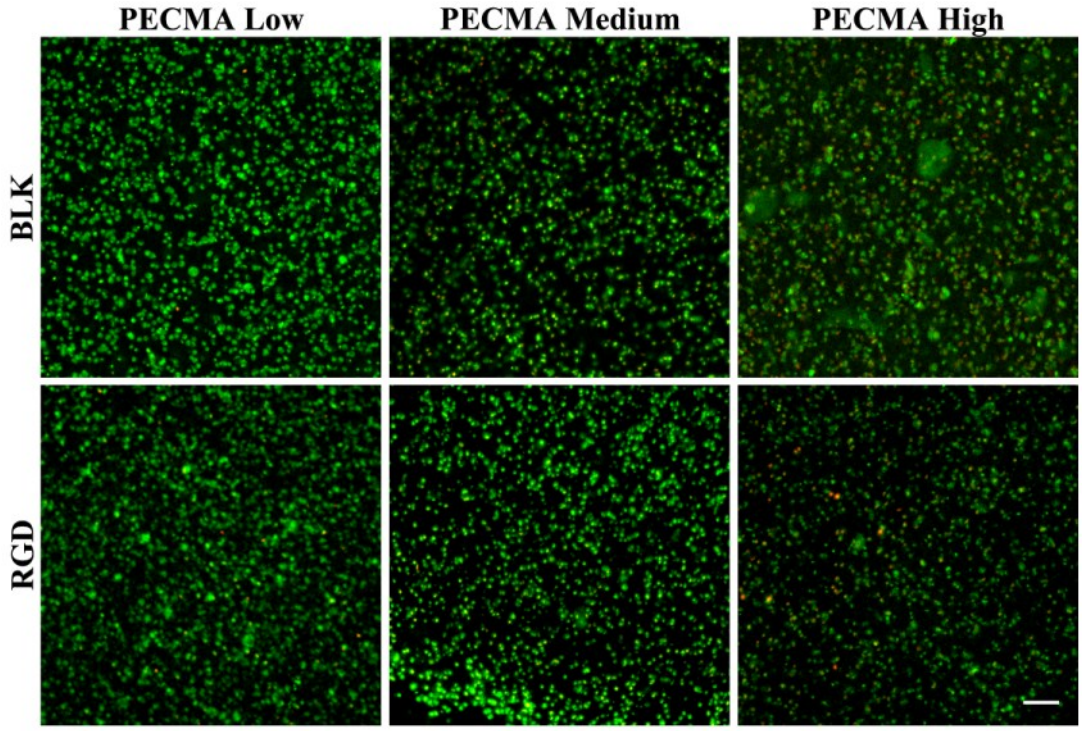

Figure S7. Effect of tethered RGD-peptide on the viability of fibroblasts entrapped within dual-crosslinked PECMA hydrogels (1.5 wt%, 160 s UV) after 24 h. CyTRAK OrangeTM is coloured in green (live cells) and DRAQ7TM is coloured in red (dead cells). Scale bar is 100 µm.

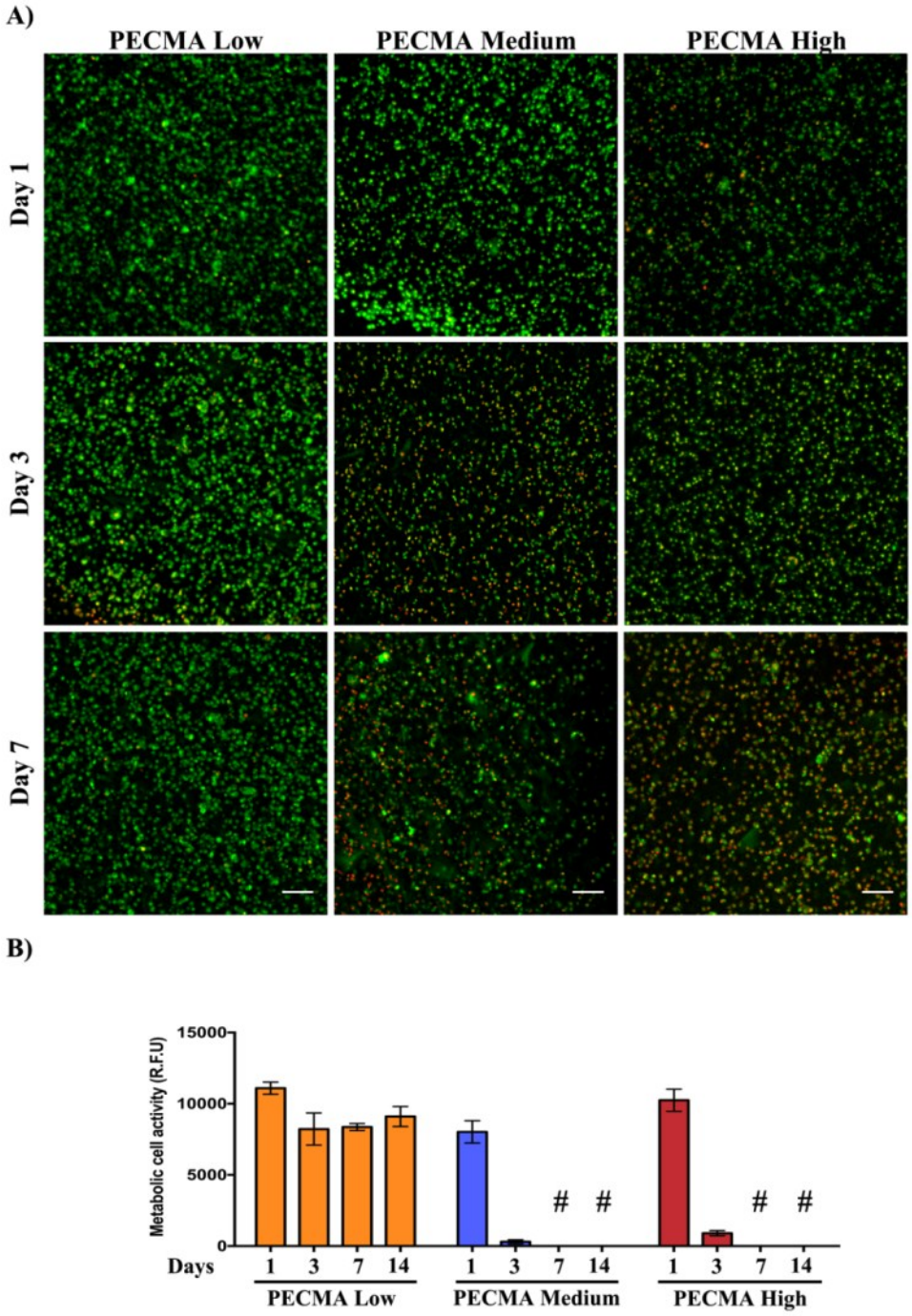

Figure S8. A) Live/Dead staining confocal images of fibroblasts entrapped within RGD-functionalized, dual-crosslinked PECMA hydrogels (1.5 wt%, 160 s UV) with varying degrees of methacrylation throughout 7 days of culture. CyTRAK OrangeTM is coloured in green (live cells) and DRAQ7TM is coloured in red (dead cells). Scale bars are 100 µm. (B) Metabolic activity of fibroblasts within the dual-crosslinked hydrogels (# represents residual levels of metabolic activity; RFU indicates Random Fluorescence Units). Fibroblasts entrapped within PECMA low hydrogels remained metabolically active throughout 14 days of culture, while cells cultured within stiffer PECMA medium and PECMA high hydrogels displayed a significant reduction on the metabolic activity at day 3.

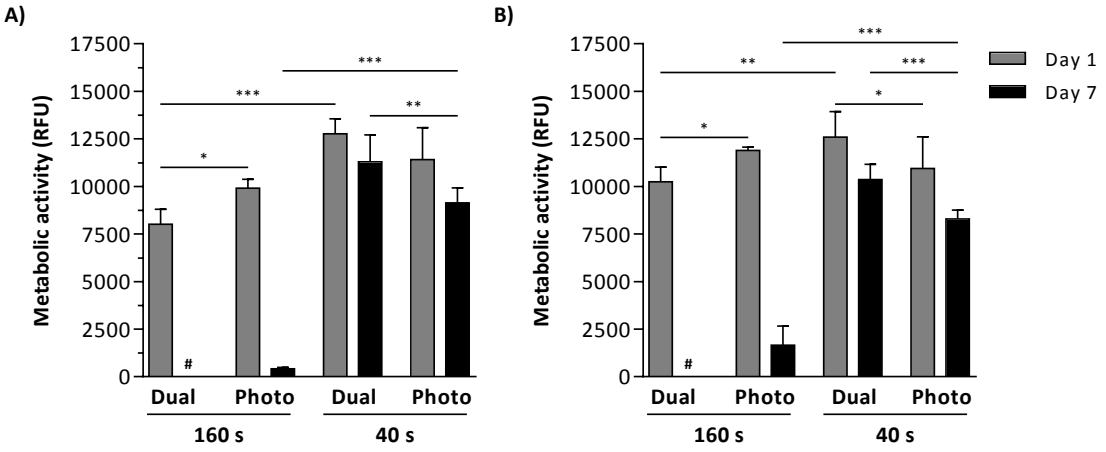

Figure S9. Metabolic activity of fibroblasts embedded within RGD-functionalized PECMA medium (A) and PECMA high (B) hydrogels (1.5 wt%) photocrosslinked for 80 s and 40 s, respectively. Both formulations were also photocrosslinked for 160 s for comparison. Chemical hydrogels were incubated in culture media containing calcium chloride (1.8 mM), yielding a dual crosslinked hydrogel (designated as "Dual"). Cell-laden hydrogels were also incubated in culture media without calcium chloride (designated as "Photo"). # indicates residual levels of metabolic activity; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

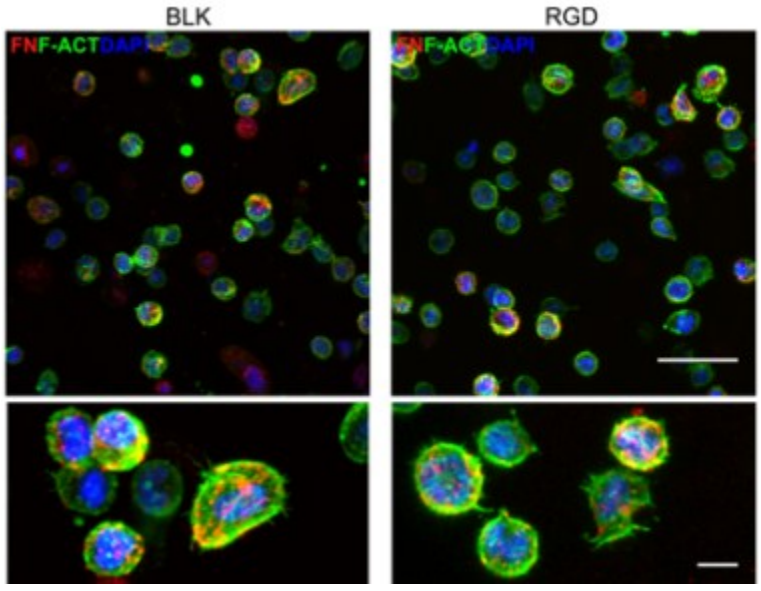

Figure S10. Morphology and spatial distribution of fibroblasts embedded within dual-crosslinked PECMA low hydrogels, with or without (BLK) tethered RGD-peptide, after 14 days of culture showing DNA (blue), F-actin (green) and fibronectin (red). Scale bars represent 50 µm on the top images and 10 µm on the bottom images with higher magnification.

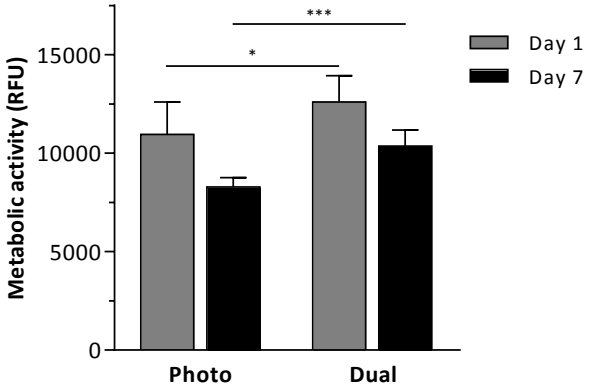

Figure S11. Metabolic activity of hNDFs embedded within photo- or dual-crosslinked RGD-PECMA high hydrogels (1.5 wt%, 40 s UV) for 7 days. *$P < 0.05$; ***$P < 0.001$.

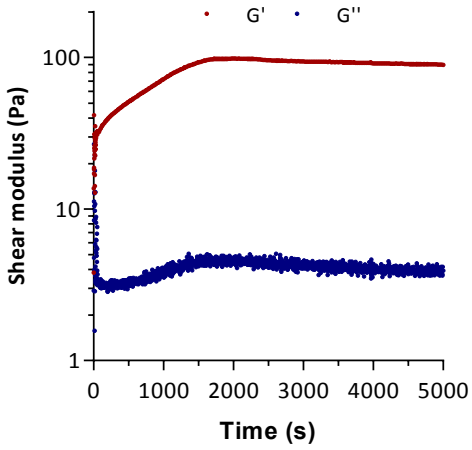

Figure S12. Gelation profile of PECMA low bioink at 1.5 wt% with 5 mM $CaCl_2$. Gelation occurred within 1 h upon the addition of $CaCl_2$. The elastic (G') and viscous (G'') modulus increased after the addition of $CaCl_2$ and remained stable approximately after 1 h of reaction.

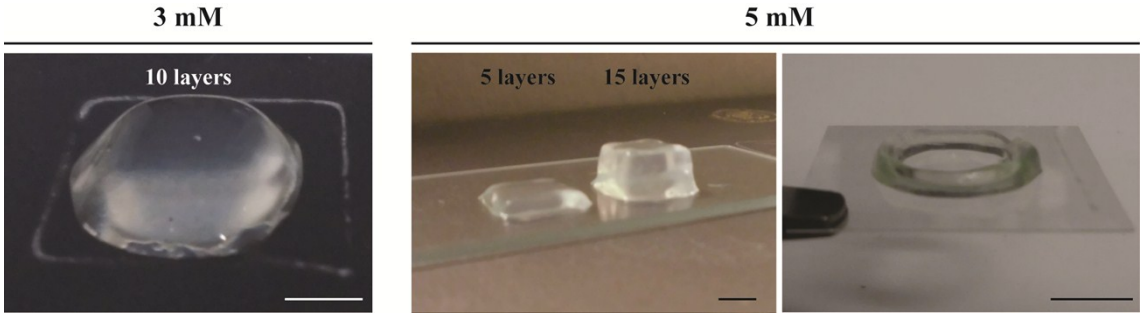

Figure S13. The effect of calcium chloride concentration on the shape fidelity printed 3D constructs using PECMA low macromer solutions (1.5 wt%). Images of constructs (8 (L) × 8 (W) mm^2) printed with bioink solutions containing either 3 or 5 mM $CaCl_2$ (scale bar: 4 mm). Hollow tube construct (10 mm Ø, 10 layers) printed with the bioink solution containing 5 mM $CaCl_2$ (scale bar: 5 mm).

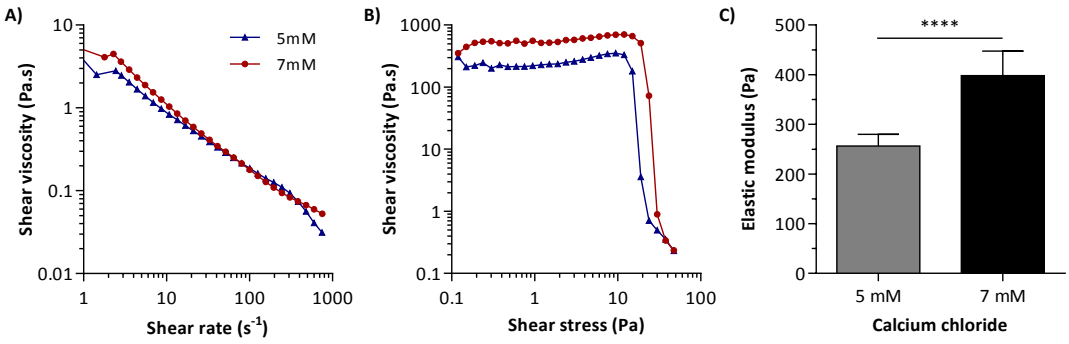

Figure S14. Effect of $CaCl_2$ concentration (5 mM and 7 mM) on the rheological and mechanical properties of printable bioinks prepared at 1.5 wt%: A) viscosity, B) yield stress curves, and C) elastic modulus of 3D hydrogel constructs (1.5 wt%, 160 s UV) determined upon 24h of incubation in culture medium. Yield stress (5 mM: 9.16 Pa; 7 mM: 10.92 Pa) and elastic modulus (5 mM: 256.2 ± 23.8 Pa; 7 mM: 398 ± 49.6 Pa) increase as a function of $CaCl_2$ concentration. ****$P < 0.0001$.

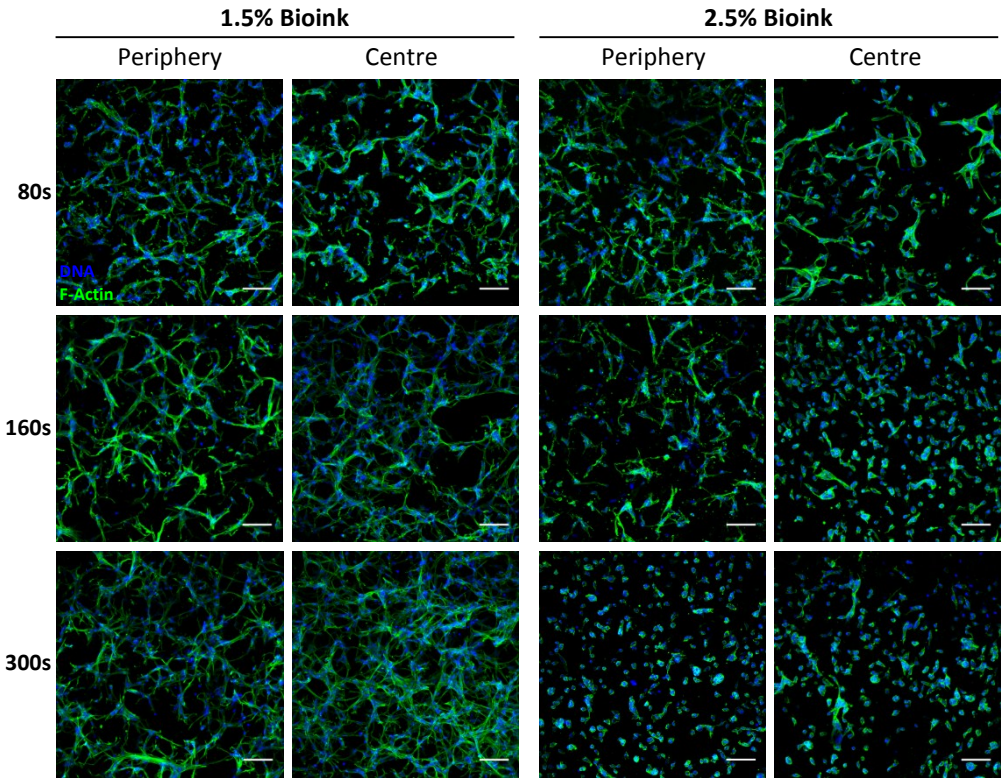

Figure S15. Effect of bioink concentration and UV photocrosslinking time on the morphology of dermal fibroblasts within bioprinted 3D constructs at day 7 of culture (scale bar: 100 µm).

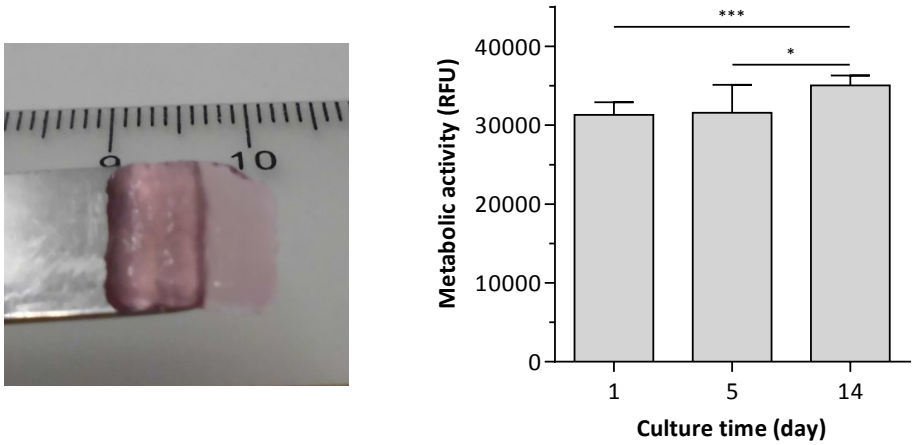

Figure S16. Photograph of printed the 3D construct (8 (L) × 8 (W) × 0.9 (H) mm^3) at day 14 of culture and metabolic activity of dermal fibroblasts embedded within construct throughout the culture period (n = 1, two independent experiments).

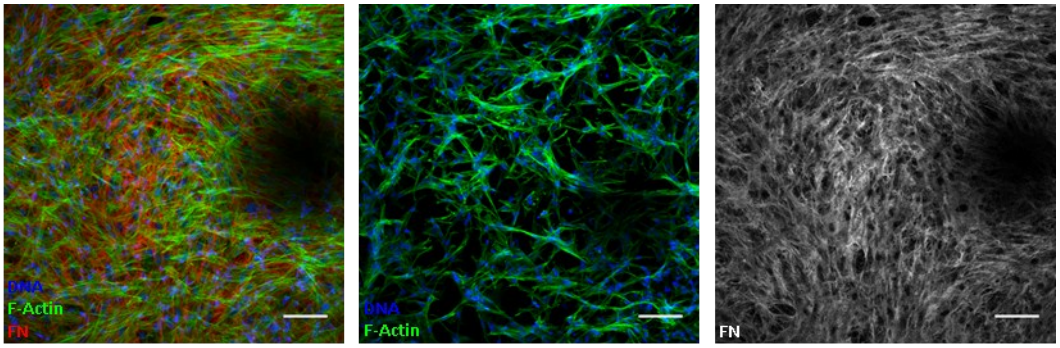

Figure S17. Morphology of embedded fibroblasts and fibronectin network within manually deposited hydrogels, stained for nuclei (blue), F-actin (green) and fibronectin (FN). Scale bars represent 50 µm.

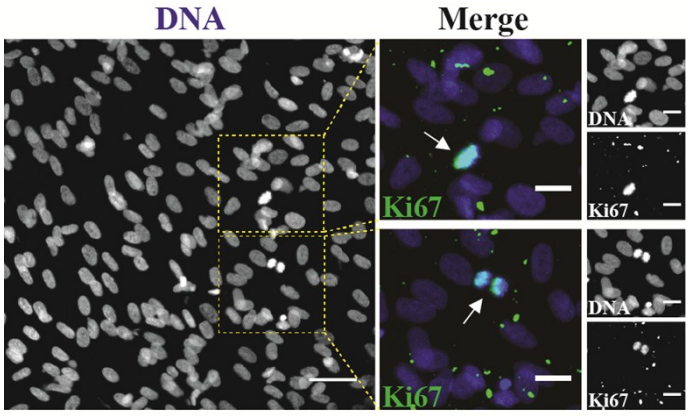

Figure S18. Confocal microscopy images showing the presence of proliferative fibroblasts (Ki67 positive, green) within the printed 3D constructs. The boxes mark the magnified areas in the panels on the right stained for nuclei (blue) and Ki67 (green). Scale bars represent 50 µm for the left image and 10 µm for magnified images.

Movie S1. 3D reconstruction of confocal microscopy images of manually deposited dual-crosslinked cell-laden PECMA low hydrogel after 14 days of culture, showing cell spreading throughout the construct. Cells are stained for nuclei (blue) and F-actin (green).

Movie S2. 3D reconstruction of confocal microscopy images of bioprinted dual-crosslinked cell-laden PECMA low 3D hydrogel constructs after 14 days of culture, showing spread cells surrounded by a fibronectin-rich mesh network (nuclei (blue), F-actin (green), fibronectin (red)).

Table S1. Primary and secondary antibodies used for detection of cell morphology, proliferation and ECM protein deposition.

Target	Primary antibody	Dilution	Supplier and reference number	Secondary antibody	Dilution	Supplier and reference number
Fibronectin	Rabbit anti-fibronectin	1:400	Sigma-Aldrich f3648	Alexa Fluor 594 goat anti-rabbit	1:1000	Invitrogen A11072
Collagen-I	Rabbit anti-collagen type-I	1:200	Rockland 600-401-103-0.1	Alexa Fluor 594 goat anti-rabbit	1:500	Invitrogen A11072
Laminin	Rabbit anti-laminin	1:100	Sigma-Aldrich L9393	Alexa Fluor 594 goat anti-rabbit	1:500	Invitrogen A11072
Proliferation	Rabbit anti-Ki67	1:200	Abcam ab15580	Alexa Fluor 647 donkey anti-rabbit IgG	1:500	Invitrogen A31573
Keratinocytes	Rabbit anti-cytokeratin	1:100	Dako Z0622	Alexa Fluor 488 donkey anti-rabbit	1:1000	Invitrogen A21206
Fibroblasts	Mouse anti-vimentin	1:100	Santa-Cruz Biotechnology sc-6260	Alexa Fluor 488 donkey anti-mouse	1:1000	Invitrogen A21202

References

1. R. F. Pereira, C. C. Barrias, P. J. Bartolo and P. L. Granja, *Acta Biomater*, 2018, **66**, 282-293.
2. H. Winning, N. Viereck, L. Nørgaard, J. Larsen and S. B. Engelsen, *Food Hydrocolloids*, 2007, **21**, 256-266.