Electronic Supplementary Material (ESI) for Materials Horizons.

Supporting Information

Freeform direct laser writing of versatile topological 3D scaffolds enabled by intrinsic support hydrogel

2PP structures for micro-Raman measurements

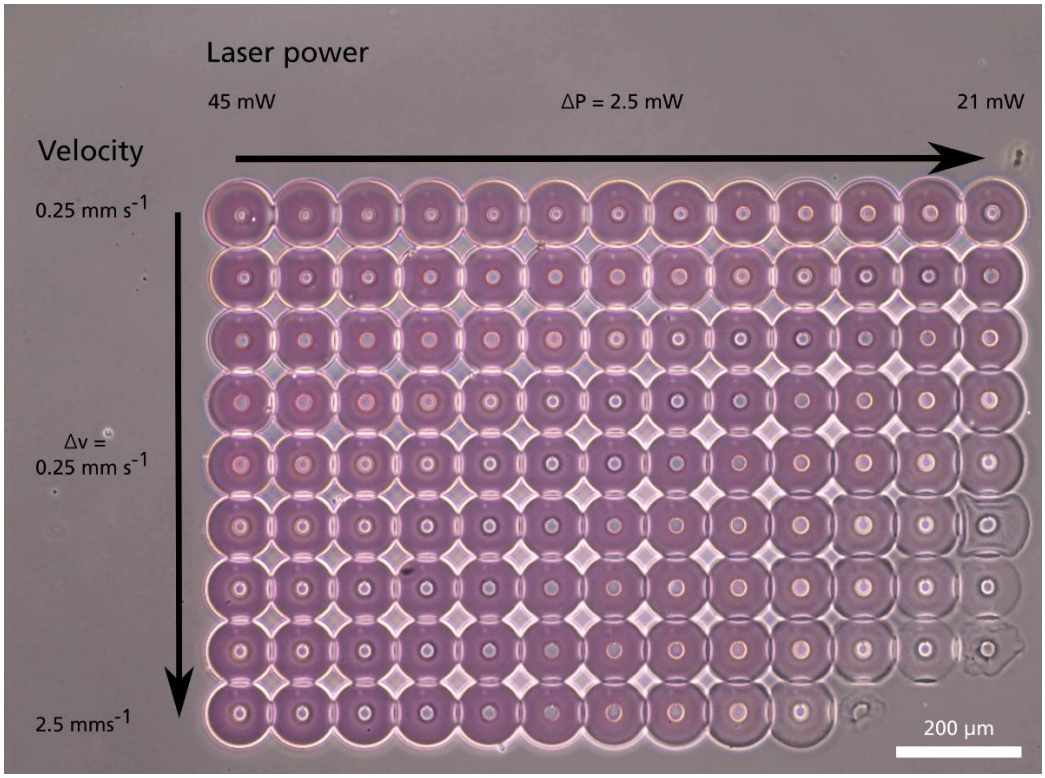

Figure S1: 13×9 array of cylinders structured via 2PP with different laser power and structuring velocity combinations in order to investigate the polymer properties with micro-Raman.

Additional Raman and indentation data

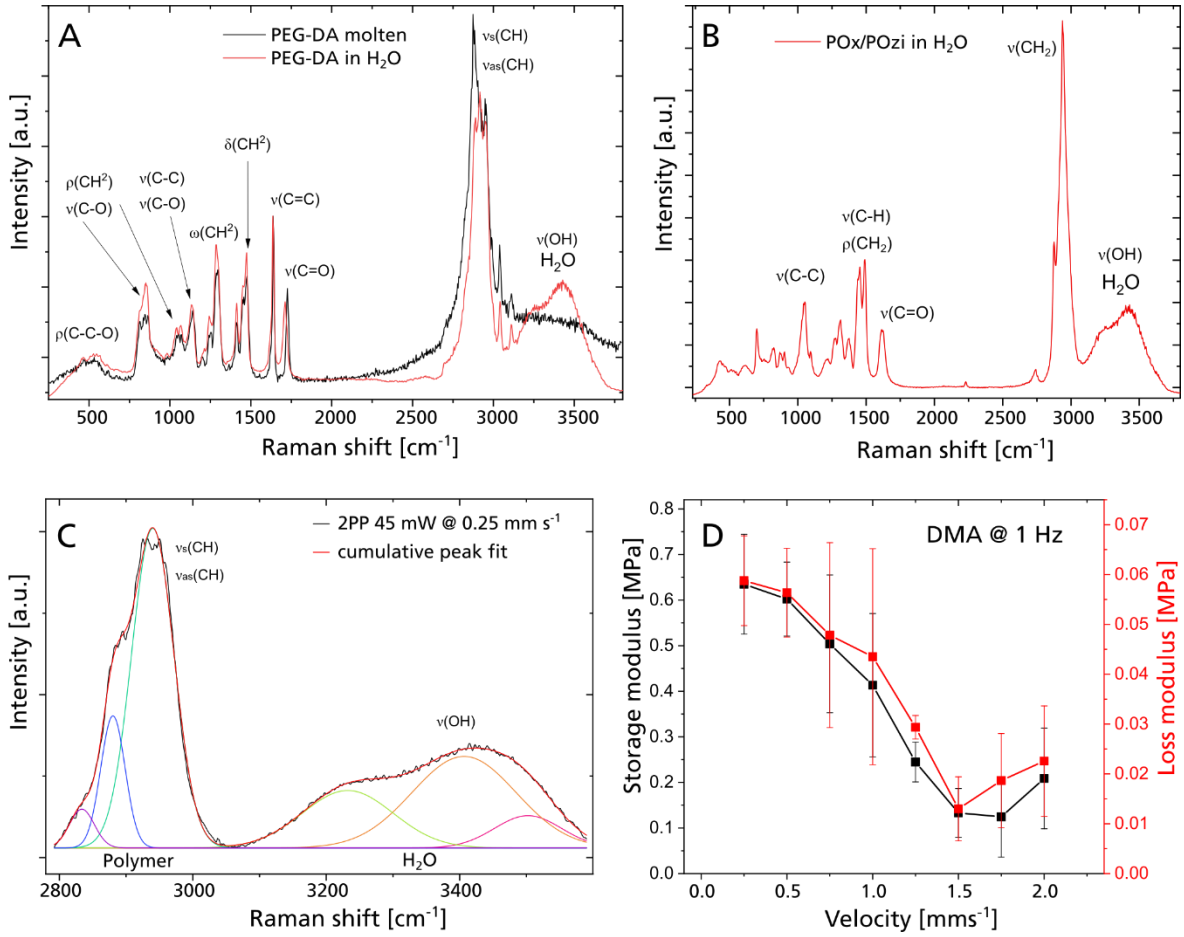

Figure S2: Micro-Raman spectra of PEG-DA (A), POx-b-POzi (B) and a 2PP structured cylinder with bands of polymer and H_2O fitted in order to determine their area ratios (C). D) Dynamic mechanical analysis of 2PP structured cylinders performed via nanoindentation.

Reference for Raman band assignment:

PEG-DA[1,2]

POx-b-POzi[3]

Water[4]

Dynamic mechanical analysis was performed 2 µm below the sample surface with a frequency of 1 Hz on identical 2PP structured cylinders used for Raman spectroscopy. The resulting dynamic modulus represents the viscoelastic properties of the material while the storage modulus E´ describes the elastic and the loss modulus E´´ the viscous fraction[5].

3D confocal image of 2PP structured hydrogel ring

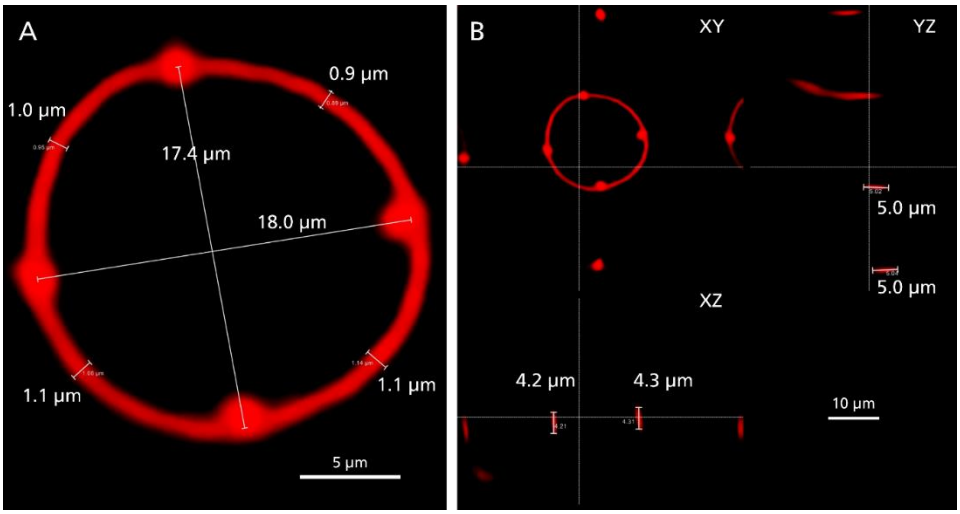

Figure S3: Confocal fluorescent image of a single hydrogel ring of Figure 3C. A) XY plane with ring diameter of (17.7 ± 0.3) µm and its XY line thickness of (1.1 ± 0.3) µm. B) All three planes with its Z line thickness of (5 ± 1) µm. Since the resolution of the microscopy image is limited by diffraction and the 3D image reconstruction depends on several experiment settings the numbers can only be regarded as assumption. Errors are based on theoretical resolution limit of the optical setup.

2PP processing window depending on RGD concentration

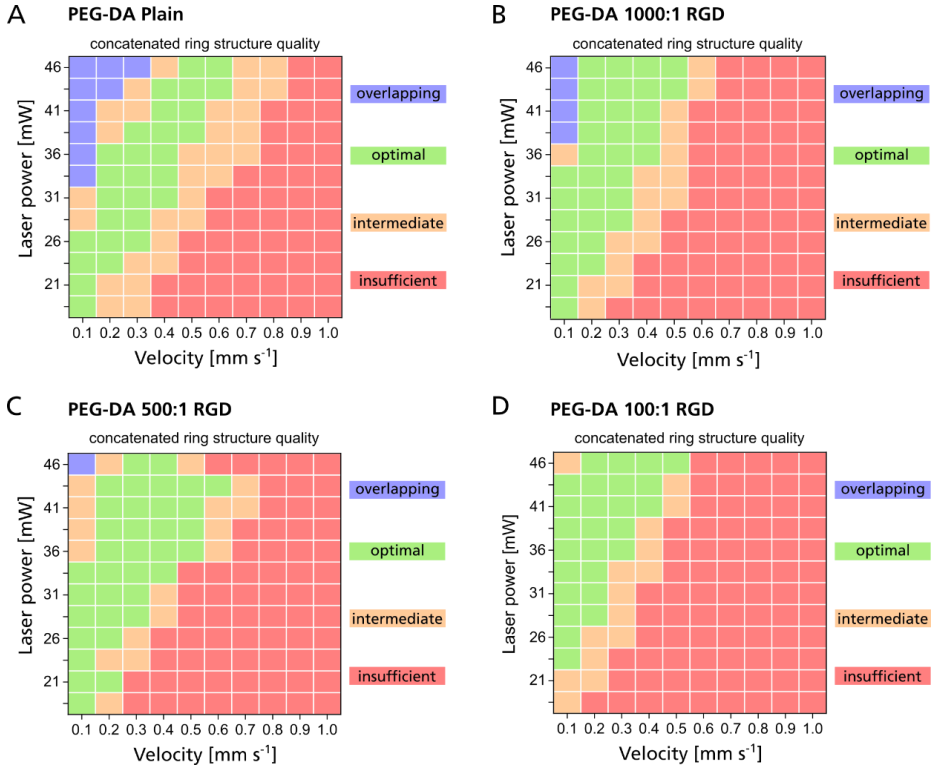

Figure S4: Determination of 2PP processing window in order to achieve 3D concatenated rings via a parameter search depending on the RGD concentration. A) 0 mol. % RGD, B) 0.1 mol. % RGD, C) 0.5 mol. % RGD and D) 1.0 mol. %. For optimal light dosage the appearance is as desired (green), while the rings are overlapping when it is too high (blue) and rings are not closed or distorted when it is too low (red). Intermediate structure qualities are classified in orange.

Proliferation of L929 Fibroblasts on 2PP structured discs

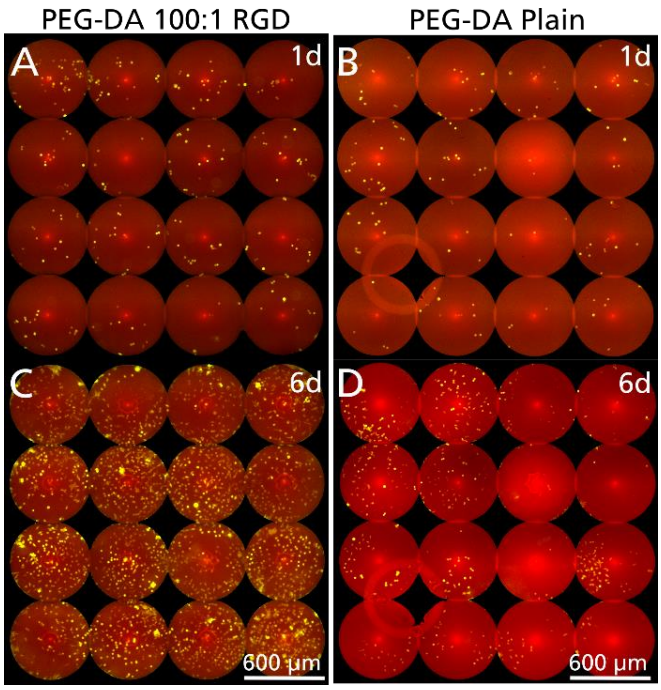

Figure S5: Dual fluorescent microscopy images of TurboGFP-labeled L929 Fibroblasts (yellow) cultured on 2PP structured 2D discs (red), made of RGD-modified PEG-DA (A,C) and without RGD modification (B,D). Images are aquired after 24 hours (A, B) and after 6 days (C, D) of cell seeding.

Table S1. Material composition of investigated PEG-DA hydrogels

	PEG-DA Plain	PEG-DA 1000:1 RGD	PEG-DA 500:1 RGD	PEG-DA 100:1 RGD
PEG-DA [mg]	499.6	499.6	499.6	499.6
POx-b-POzi [mg]	600.9	600.9	600.9	600.9
Bidist. H_2O [mg]	1093.5	1093.5	1093.5	1093.5
RGD [mg]	0	0.6	1.2	6
Rose Bengal [mg]	15	15	15	15

Positioning errors of linear XYZ stages

Table S2. Maximum position deviation of the axis system during an XZ circular motion. It decreases with increasing circle diameter and decreasing speed.

Velocity [mm s^{-1}]	max. Positioning error [µm]		
	XZ Ring diameter [µm]		
	5	10	25
0.05	0.05	0.05	0.01
0.10	0.10	0.10	0.06
0.20	0.13	0.16	0.13
0.40	0.45	0.26	0.26

Table S3. Maximum position deviation of the axis system during an YZ circular motion with a diameter of 20 µm. This increases with increasing velocity.

XZ Ring diameter: 20 µm	
Velocity [mm s^{-1}]	max. Positioning error [µm]
0.2	0.17
0.4	0.34
0.6	0.42
0.8	0.54
1.0	0.56
1.2	0.61
1.4	0.66
1.6	0.72
1.8	0.74
2.0	0.79

Experimental Section

Hydrogels PEG-DA and POx-bPOzi: Poly(ethylene glycol) diacrylat (PEG-DA) with a molecular weight of 700 g mol^{-1} was used and purified by Sephadex G-25 column chromatography and stored under the exclusion of light. The polymers forming the thermogelling sacrificial print support was reported previously and characterized in detail.[6,7]

Synthesis of RGD: The RGD-peptide was synthesized by solid-phase fmoc peptide synthesis using an automated microwave peptide synthesizer (Liberty BlueTM, CEM Corporation, USA). Shortly, Fmoc-L-Lysine-Wang resin was loaded into a 30 ml perfluoralkoxy reaction vessel with a glass frit in the bottom (CEM Corporation, USA). Deprotection was done using 10 % piperidine in 0.1 M oxyma (v/v) at 75 °C for 3 min. Subsequently, a 5 M excess of the amino acid compared to the functional group was dissolved in DMF and mixed with 0.1 M oxyma (v/v). Initial coupling was performed for 25 min at 25 °C followed by incubation at 57 °C for 2 min. In a second coupling step the mixture was incubated at 75 °C for 5 min. After cleavage from the resin the peptide was purified by reversed phase chromatography using an FPLC system (GE Healthcare Äkta Explorer, Life sciences, Freiburg, Germany) with a Luna C18 100A column (21.2 mm × 250 mm, Phenomenex Inc., Torrance, CA). Successful peptide synthesis was analyzed using ESI-MS.

Rheology measurement: The rheological evaluation was carried out using a mixture of PEG-DA and PMeOx-b-PnPrOzi (DP: 200) hydrogel. For complete dissolution and homogenizing the solution was shaken at 4 °C overnight to yield a transparent solution at 4 °C and a physical hydrogel at room temperature. Rheological properties were investigated using the rheometer Physica MCR-301 (Anton Paar, Austria) using a plate-plate geometry (25 mm diameter) equipped with a solvent trap and Peltier element for temperature control. To investigate the viscoelastic properties amplitude sweeps from 0.01 % to 500 % strain deformation and a fixed angular frequency of 10 rad s^{-1} at 23 °C were performed.

Composition of hydrogel samples: The photoinitiator Rose Bengal (4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetraiodofluorescein disodium salt, Sigma-Aldrich Chemie GmbH, Germany), and the freeze dried maleimidohexanoic acid modified RGD-motif (6-Maleimidohexanoic acid – serine (S) – tyrosine (Y) - arginine (R) – glycine (G) – aspartic acid (D) – glycine (G)) were first dissolved in bidistilled water separately and then added to the poly(ethylene glycol) diacrylate (PEG-DA, 700 g mol^{-1}, Sigma-Aldrich Chemie GmbH, Germany) – diblock copolymer poly(2-methyl-2-oxazoline)-b-poly(2-n-propyl-2-oxazine) (POx-b-POzi) – water formulation and stirred at 4 °C for 2 days. Four formulations with different RGD-motif concentrations were prepared (0 mol. %, 0.1 mol. %, 0.5 mol. %, 1.0 mol. %, relating to PEG-DA), see Table S1.

Fabrication of 2PP samples: High precision microscope cover glasses (borosilicate glass, (170 ± 5) µm thickness, No. 1.5H, A. Hartenstein, Germany) served as substrates for samples intended for 2PP structure characterization. They had been silanized with 3-(trimethoxysilyl)propyl methacrylate (ABCR GmbH, Germany) beforehand in order to improve the adhesion between substrate and hydrogel structure. A drop of hydrogel formulation was placed between the cover slip and a microscope slide with a 100 µm PET foil serving as spacer. 3D scaffolds intended for live cell measurements were structured directly onto the #1.5H glass bottom of a 35 mm ibidi® µ-Dish (ibidi GmbH, Germany), which was also silanized with 3-(trimethoxysilyl)propyl methacrylate. All samples were fabricated by two-photon polymerization (2PP) initiated by a pulsed femtosecond Laser (Chameleon-Ultra II, Coherent Inc., USA) at a wavelength of 705 nm, focused with a 60x water objective (CFI Plan Apo VC 60x C WI 1.2, Nikon Instruments Inc., Japan). The 3D movement of the objective focus inside the resin was performed via air-bearing, direct drive linear stages with high resolution (± 0.2 µm axial / ± 0.5 µm lateral) (X: ABL80030, Y: ABL15030, Z: ABL10100, Aerotech Inc., USA). The motion control of the linear stages is based on G-Code, which in turn was generated via a self-developed python software code allowing adjusting the overall structure size, the ring diameter, the ring periodicity and the number of layers in z-direction. In order to ensure a constant Z-position relative to the substrate a self-implemented autofocus routine was performed several times for large samples. To prevent temperature related laser power shifts an own developed self-adjusting power regulation was performed after each layer. After 2PP structuring the ibidi chamber was placed upside down in a large bidistilled water bath for 15 – 30 min. During this development step the non-illuminated part of the resist is dissolved in excess water and the hydrogel structure swells due to water uptake, with uncrosslinked PEG-DA (sol fraction) also extracted at this point. The chamber is turned again and transferred into fresh bidistilled water for an additional wash and to replace the water inside the chamber. Subsequently, the chamber could be sealed for storage or fluorescent microscopy imaging and was only opened again for cell seeding.

Fluorescent confocal 3D measurement: 3D confocal images were captured using a Leica TCS SP8X (Leica Microsystems GmbH, Germany) equipped with a white light laser SuperK Extreme EXW (NKT Photonics GmbH, Germany) and a 20x multi immersion and 63x water immersion objectives (HC PL APO CS2 20x/0.75 IMM, HC PL APO CS2 63x/1.2 water, Leica Microsystems GmbH, Germany). The Rose Bengal dye was excited at 570 nm with a fluorescent detection range from 575–795 nm, while the GFP fluorophore was excited at 488 nm and detected from 493–540 nm. For time-lapse measurements, every 10 min an image was acquired. The 3D images were post processed using a three dimensional median filter with a radius of three pixels.

Raman measurement and determination of Hydrogel/H_20 Raman band ratio: For Raman measurements an array of cylinders (diameter: 100 µm, height: 15 µm, XY-step width: 0.5 µm, Z-step width: 1.0 µm) were structured while varying the laser power (45 – 21 mW, ΔP: 2 mW) and the process speed (0.25 – 2.5 mm s^{-1}, Δv: 0.25 mm s^{-1}) for each cylinder (Figure S1). Micro Raman spectroscopy was conducted with the Alpha 300A (WITec Wissenschaftliche Instrumente und Technologie GmbH, Germany) and a 100x Oil objective (CFI Achro 100x/1.25 Oil, Nikon, Japan). The excitation laser has a wavelength of 532 nm and spectral gratings with 1800 lines mm^{-1} and 600 lines mm^{-1} respectively, were used during detection. At each investigated sample position, 10 measurements were accumulated with an integration time of 5 s each. For baseline correction and peak deconvolution the software OriginPro (Version 2019, OriginLab Corporation, USA) was used (Figure S2C). The peak integrals for all water related and PEG-DA related bands, were summed up separately and the area ratio of both components were calculated. For direct comparison, the spectra were normalized to the maximum value of the water related Raman band.

Young's modulus measurements: For indentation measurements cylinders with a diameter of 150 µm and a height of 40 µm were structured via 2PP. Mechanical analysis was performed using a Pavone nanoindenter (Optics11 Life, Amsterdam, The Netherlands) at three spots each. The spherical probe has a diameter of 40 µm and was indented two µm (< 10 % of the sample thickness) into the flat sample surface within 4 s. This loading curve was used to determine the Young´s modulus using the Hertz contact model with the Optics11 DataViewer V2.0.27 assuming a Poisson ratio of 0.5 due to the high water content of the hydrogel. The indentation was followed by a 10 s relaxation phase and 5 periods of a 1 Hz sinusoidal oscillation for calculation of the storage and loss modulus. For indenter measurements on concatenated ring structures, fields of 500 µm × 500 µm were fabricated via 2PP. These were constructed from rings with a diameter of 20 µm and a total of four layers. Loading curve with an indentation depth of 18 µm was obtain in 6.5 s.

Cell Culture: The L929 cell line, stable transfected with TurboGFP (L cell, L-929, derivative of Strain L), was obtained from the chair of Tissue Engineering and Regenerative Medicine, Würzburg, Germany. The growth medium used was HyCloneTM (Fisher Scientific GmbH, Germany) supplemented with 10 % FBS (FBS Gold, Bio&SELL GmbH, Germany) and 1 % penicillin-streptomycin (Sigma-Aldrich Chemie GmbH, Germany). The cells were incubated in tissue culture flasks (T75, Greiner Bio-One GmbH, Germany) at 37 °C in humidified air containing 5% CO_2.

Flip Over Assay: The flip over assay samples consists of a 4 × 4 array of discs with a diameter of 600 µm each and a thickness of 10 µm. They were structured in a continuous helical movement with a XY-step width of 0.5 µm and a Z-step width of 1.0 µm at a velocity of 1.5 mm s^{-1}. Before cell seeding of TurboGFP-labeled L929 with a concentration of 5000 cells cm^{-2}, the samples were sterilized inside a 35 mm ibidi µ-Dish with 70% ethanol solution for 15 min, washed with sterile bidistilled water for 15 min and incubated at 37°C in medium for at least 1 h. 24h after cell seeding, the samples were flipped inside the µ-Dish and the 2PP samples with attached cells were hanging overhead, while the glass substrate rests on top of the rim inside the dish. Microscopy images were performed on an inverted microscope (Eclipse Ti, Nikon Instruments Europe BV, Japan) after 24h (before and after flipping of the sample), 48 h, 4 days and 6 days after seeding. The mechanical impact of the flipping procedure after day 1, did not affect the cell number significantly for both sample variations, which indicates strong cell adherence to the polymer surface. The excitation light was supplied by a mercury lamp (Intensilight CHGFI, Nikon Instruments Inc., Japan) combined with a F36-504 optical filter set (AHF Analysentechnik AG, Germany) for the GFP channel and F36-504 (AHF Analysentechnik AG, Germany) for the Texas Red channel. Brightfield images were acquired using a halogen lamp (12 V 100 W, HLX6462, Nikon Instruments Inc., Japan). A 10x air objective (Plan Fluor 10x Ph1 DL) was used in combination with a 1000 × 1000 pixel EM-CCD camera (C9100, Hamamatsu Photonics K.K., Japan). For cell counting, all three channels were processed with a 2 px median filter. A 50 px Gaussian convoluted background subtraction was performed on the GFP channel using the Fiji Plugin BioVoxxel. The Texas Red channel (Fluorescent image of the 2PP 2D discs) was then used as an image mask in order to separate parts of the image exhibiting the glass substrate and consequently only count cells adherent on the hydrogel discs. The GFP channel was then binarized and adjacent cells were separated via a watershed algorithm before counting with Fijis Particle Analyser. These measurements were conducted three times each, for the PEG-DA 100:1 RGD and the PEG-DA Plain formulation as reference. Results were subjected to a statistical analysis (unpaired two samples T-test) resulting in a significant difference in cell number for day six ($p = 0.00839$).

3D Cell Migration: For all 3D cell migration samples, a ring diameter of 20 µm was used to generate two different sample variations: Assembling the 3D ring scaffold with a periodicity of 40 µm, all rings are physically connected (welded configuration). Using a ring periodicity of 27 µm however, the polymerization volume of each ring do not meet and the rings are loosely interconnected (concatenated configuration). Four layers of XYZ-rings were structured, with the first layer buried half inside the substrate to ensure a high contact area and a strong adhesion. Every sample consists of four squares made of connected rings parallel to four squares made of concatenated rings. The overall size of one square is 750×750 µm. Before cell seeding of TurboGFP-labeled L929 with a concentration of 5000 cells cm^{-2}, the samples were sterilized inside a 35 mm ibidi µ-Dish with 70% ethanol solution for 15 min, washed with bidistilled sterile water for 15 min and incubated at 37°C in medium for at least 1 h.

Cardiomyocytes:

Sample preparation: Both variations of ring scaffolds, were placed in a well of a 6 well plate (657160_100, Greiner, Kremsmünster, Austria) and carefully flowed with 5 ml of 70 % EtOH (1.00974.2511, Merck, Darmstadt, Germany) for 15 min. After two washing steps with 5 ml of DMEM-F12 (21331020, Gibco, Waltham, USA), the scaffolds in the wells were coated with matrigel (356231, Corning, Corning, USA) in a concentration of 0.0083 mg cm^{-2} at 37 °C for at least one hour. Directly before addition of the cells, matrigel solution was removed and replaced with 5 ml TS medium per well (see medium composition in[8])

hiPSC Differentiation: The hiPSC line IBMT1 was reprogrammed at Fraunhofer IBMT (BioSample ID: SAMN05335455) and cultured on growth factor-reduced matrigel (356231, Corning, Corning, USA) in 60 mm culture dishes (Nunc, Waltham, USA). Cells were dissociated with TrypLE (#12563011, Gibco, Waltham, USA) at a confluency of 70 – 80 % and resuspended in mTeSR1 medium (#8580, StemCell, Vancouver, Canada) supplemented with 10µM Rock Inhibitor (#ab120129, Abcam, Cambridge, UK). Cell count was determined with NucleoCounter® NC-200TM (Chemometec, Allerod, Denmark). The single cell suspension was cultured over three days at 37 °C and 5 % CO_2 at a start concentration of 7.5 x 105 IBMT1 cells ml^{-1} in mTeSR1 medium in a CEROTube (#2800005, OLS OMNI Life Science, Bremen, Germany) of the suspension bioreactor CERO (OLS OMNI Life Science, Bremen, Germany) with daily medium change over visual-based sedimentation. After three days, the formed aggregates were differentiated for ten days as described by Fischer et al.[8] to hiPSC derived cardiomyocytes. On day ten, the aggregates were transferred onto one side to the scaffold, made of welded rings, and onto the other side of the scaffold with concatenated rings.

Cardiomyocyte dissociation: hiPSC derived cardiomyocytes were washed twice with DPBS (-/-) (#14190250, Gibco, Waltham, USA) and dissociated with TrypLE at 37 °C until a homogeneous single cell suspension was reached. The reaction was stopped with TS medium (see medium composition in Fischer et al.). After 2 min centrifugation at 300 g (Multifuge X1R, Heraeus, Waltham, USA), the supernatant was removed and the pellet resuspended in TS medium again.

Cardiomyocyte cultivation: Dissociated cardiomyocyte cell suspension was carefully dropped on the welded rings scaffold and the cardiomyocyte aggregates were dispensed directly over the scaffold with the concatenated rings using wide bore tips (613-0999, VWR, Radnor, USA). Samples were cultivated at least 15 days at 37 °C and 5 % CO2 (Heracell 240, Heraeus, Waltham, USA) with microscopic observation after one, four, seven and eleven days. Medium change was also performed every two to three days.

References

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