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Near-Infrared-Light Regulated Angiogenesis in a 4D hydrogel

*Yijun Zheng**[a#], Zhijun Chen[b,c], Qiyang Jiang[a,d], Jun Feng[a,d], Si Wu[b,e], Aránzazu del Campo**[a,d]*

[a]INM – Leibniz Institute for New Materials, Campus D2 2, 66123 Saarbrücken, Germany [b]Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany. [c]Key Laboratory of Bio-based Material Science and Technology of Ministry of Education, Northeast Forestry University, Hexing Road 26, Harbin 150040, People's Republic of China. [d]Saarland University, Chemistry Department, 66123 Saarbrücken, Germany. [e]CAS Key Laboratory of Soft Matter Chemistry, Hefei National Laboratory for Physical Sciences at the Microscale, Key Laboratory of Optoelectronic Science and Technology, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei 230026, China.

[#]current address: School of Physical Science and Technology, ShanghaiTech University, Shanghai 201210, China.

Materials Synthesis and Charaterization: 4-Arm-PEG Maleimide 20K was purchased from Creative PEG work (North Carolina, USA). Cyclic RGDfC was purchased from Peptide International (Louisville, USA). The GCRDVPMSMRGGDRCG crosslinker was purchased from Proteogenix (Schitigheim, France). Poly(maleic anhydride alt-1-tetradecene) and 4,7,10-trioxa- 1,13- tridecanediamine were obtained from Sigma Aldrich, Germany. Solvents have p.a. purity and were used as purchased unless specified. HPLC analyses was performed with a HPLC JASCO 4000 (Japan) equipped with a diode array UV-Vis detector and fraction collector. UV/VIS spectra were recorded with a Varian Cary 4000 UV/VIS spectrometer (Varian Inc. Palo Alto, USA). TEM images were collected on a JEOL JEM1400 transmission electron microscope. A diode laser with a wavelength of 974 nm (type P976MF, PhotonTec Berlin GmbH) coupled with a 105-μm (core) fiber was used as the excitation light source. The output power of the diode laser was controlled by a laser driver (device type ds11-la12v08-pa08v16-t9519-254-282, OsTech GmbH i.G.). The output power density of the diode laser was measured using an optical power meter (model 407A, Spectra-Physics Corp.) and a NIR indicator (Newport, model F-IRC1). The fluorescence emission was measured with a fiber coupled spectrometer (Ocean Optics QEpro) in a home-made laser box due to safety issue. We used a blue-green filter (Schott BG38 glass filter) in order to block the much more intense excitation light at 974 nm.

Synthesis of cyclo[RGD(DMNPB)fC]: Cyclo[RGD(DMNPB)fC] peptide was synthesized according to a previous protocol.[1]

Synthesis of UCNP: The $NaYF_4$ core/shell upconverting nanoparticles (UCNPs) were synthesized according to previous procedure.[2-3] The details are provided below.

Synthesis of β-$NaYF_4$: 0.5 mol% Tm^{3+}, 30 mol% Yb^{3+} core nanoparticles. The $NaYF_4$:TmYb core nanoparticles were synthesized according to $Y(CH_3COO)_3 \bullet xH_2O$ (372 mg,1.4 mmol), $Yb(CH_3COO)_3 \bullet xH_2O$ (210 mg, 0.6 mmol) and $Tm(CH_3COO)_3 \bullet xH_2O$ (3.5 mg, 0.01 mmol) were

added to a 100 mL threeneck round-bottom flask containing octadecene (30 mL) and oleic acid (12 mL). The solution was stirred magnetically and heated to 120 °C under vacuum (heating rate: 3 °C/min) to form the lanthanide oleate complexes. The solution was degased at 120 °C for 15 min to remove residual water, acetic acid and oxygen. The temperature of the solution was then lowered to 50 °C and the reaction flask was placed under a gentle flow of Ar. During this time, a solution of ammonium fluoride (296 mg, 8.0 mmol) and sodium hydroxide (200 mg, 5.0 mmol) dissolved in methanol (20 mL) was prepared via sonication. Once the reaction mixture reached 50 °C, the methanol solution was added to the reaction flask and the resulting cloudy mixture was stirred for 30 min at 50 °C. The reaction temperature was then increased to ~70 °C and degased for 15 min to remove methanol in the reaction flask. Then, the reaction flask was placed under a gentle flow of Ar. Subsequently, the reaction temperature was increased to 300 °C (heating rate: 20 °C/min) under the Ar flow and kept at this temperature of 90 min. During this time the reaction mixture became progressively clearer until a completely clear, slightly yellowish solution was obtained. The mixture was allowed to cool to room temperature naturally. The nanoparticles were precipitated by the addition of ethanol (~80 mL) and isolated via centrifugation at 5000 rpm. The resulting pellet was dispersed in a minimal amount of hexane (5-10 mL) and precipitated with excess ethanol (~60 mL). The nanoparticles were isolated via centrifugation at 5000 rpm and then dispersed in hexane (10–15 mL) for the subsequent shell growth procedure.

Synthesis of β-$NaYF_4$: 0.5 mol% Tm^{3+}, 30 mol% Yb^{3+} /β-$NaYF_4$ core/shell nanoparticles. $Y(CH_3COO)_3 \bullet xH_2O$ (479 mg, 1.8 mmol) was added to a 100 mL three neck round-bottom flask containing octadecene (30 mL) and oleic acid (12 mL). The solution was stirred magnetically and heated to 120 °C under vacuum (heating rate: 3 °C/min) and maintain at 120

°C for 15 min. The temperature of the reaction flask was lowered to 80 °C and the reaction flask was placed under a gentle flow of Ar. Then, the dispersion of NaYF4: 0.5 mol% Tm3+,

30 mol% Yb3+ core nanoparticles in hexane, which was synthesized by the procedure shown

above, was added to the flask. The resulting solution was heated to 110 °C (heating rate: 5°C/min) and degased for 15 min to remove hexane in the reaction flask. The reaction mixture was cooled to 50 °C and the flask was place under a gentle flow of Ar. Then, a solution of ammonium fluoride (259 mg, 7.0 mmol) and sodium hydroxide (175 mg, 4.4 mmol) in methanol (20 mL) was added. The resulting cloudy mixture was stirred at 50 °C for 30 min. The reaction temperature was then increased to ~70 °C and degased for 15 min to remove methanol in the reaction flask. Then, the reaction flask was placed under a gentle flow of Ar. Subsequently, the reaction temperature was increased to 300 °C (heating rate: 20 °C/min) and kept at this temperature for 90 min under the Ar flow. The mixture was allowed to cool to room temperature naturally. The nanoparticles were precipitated by the addition of ethanol (~80 mL) and isolated via centrifugation at 5000 rpm. The resulting pellet was dispersed in a minimal amount of hexane (5-10 mL) and precipitated with excess ethanol (~60 mL). The nanoparticles were isolated via centrifugation at 5000 rpm and then dried in the vacuum oven.

Preparation of aqueous solution of UCNPs: The aqueous solution of UCNPs was prepared according to a reference:[4] Poly(maleic anhydride alt-1-tetradecene) with number average molecular weight Mn = 7300 (corresponds to roughly 25 monomer units per polymer chain) and polydispersity 1.23 was purchased from Aldrich. This polymer becomes amphiphilic upon hydrolyzation of the anhydride functional groups. A 250 μL solution of poly(maleic anhydride alt-1-tetradecene) in chloroform (30 mg/mL) and a 100 μL dispersion of UCNPs in chloroform (10 mg/mL) were mixed and stirred for 2 h at room temperature. The UCNP dispersion was washed twice to remove the unreacted polymers with chloroform by two rounds of dilution and reconcentration through a centrifuge filter. After removal of chloroform, 4,7,10- trioxa- 1,13-tridecanediamine in chloroform (3 mg/mL) was then added to cross-link the polymer shell that had formed around each nanoparticle. The ratio of cross-linker molecules to polymer units was 1:10. The solution was sonicated for 20 min, the solvent was removed again by centrifuge, and the solid was dissolved in 200 μL TBE buffer (Tris-borate-EDTA) (pH 8-9) to get 5 mg/mL

UNCP-PMO solutions. After sonicating for 15 minutes, the nanocrystals dispersed completely and the solvent was removed by centrifuge and the nanoparticles were uptaken in 200 µL Hepes solutions. The nanocrystals solutions with a concentration of 5 mg/ mL were optically clear. The particles were characterized by transmission electron microscopy (TEM). The solutions were stable for at least 1 week (i.e., no precipitation occurred).

Preparation of UCNPs embedded PEG hydrogel: The PEG thiol/maleimide system was selected to form the UCNP embedded hydrogel.[5] The enzymatic degradable peptide sequence GCRDVPMSMRGGDRCG (VPM) was introduced for selective cleaving by matrix metalloproteinases (MMPs). PEG hydrogels functionalized with adhesive ligands were prepared in a one-pot reaction by mixing 4-arm PEG maleimide with RGD peptide and the degradable dithiol peptide as cross-linker at pH 8. A polymer content of 4 wt% PEG maleimide, 5 mg/mL UCNP-PMO, 1 mM of RGD ligand and 2.8 mM of crosslinking peptide were selected from previous reports.

Characterization of Young's Modulus of the hydrogels: The 4-arm PEG Maleimide 20K (10 wt%, 10 µL) was mixed with RGD (5 mM, 5 µL) and kept at 37°C for 30 min. Hepes solution with or without UCNP (5 µL, pH 8) was added to this mixture and the drop of mixture (8 µL) was placed on lower plate of a Rheometer (TA, DISCOVERY) (8 mm) in parallel plate geometry. The VPM (5 mM, 2 µL) was added to the above mixture and mixed with pipette tip. The head plate was adjusted above the sample and the area surrounding the drop was sealed with silicone oil to avoid evaporation. The stiffness was measured by time sweep at 37°C, with 0.5 N applied strain. The elastic modulus was calculated from the obtained storage modulus (G´) by using following expression. $E = 2Gx(1+v)$, where the poisson number was taken as $v = 0.5$.

Swelling ratio measurement: The swelling ratio was determined by weighting the swollen hydrogel (W_s) and the hydrogel after drying at 40°C for 24h (W_d). The swollen ratio (SR) was calculated after three replicates by $SR=(W_s-W_d)/W_d$.

NIR irradiation of PEG hydrogel modified with caged Fluorescein: The 4-Arm-PEG Maleimide (10 wt%, 10 μL) was mixed with NH_2-PEG-SH (3.5 K, 2 mM in HEPES, 4 μL) and incubated for 15 min at 37°C. The CMNB-caged Fluorescein (10 mg/mL in DMSO, 1 μL) was added to above solution and incubated further for 15 min at 37°C. Hepes solution with or without UCNP (25 mg/mL) was added (5 μL). The solution was placed in Ibidi® 15 well angiogenesis slide and mixed with VPM (5 mM, 2 μL) and allow to gelate at 37°C for 30 min. The sample was placed under the 974 nm illumination (10 W/cm^2) for different time intervals (0-10 min). Fluorescence images were taken by Zeiss Axio Observer epi-fluorescence microscope.

Cell culture: Fibroblast L929 cell line were cultivated at 37° C in 5% CO_2 in RPMI medium (Gibco) supplemented with 10 % fetal bovine serum (Invitrogen) and 1 % P/S (Invitrogen).

For cell spreading experiment, L929 1 × 10^5 mL^{-1} cells were directly suspended with 4-PEG-maleimide solution during polymerization.

HUVECs (Promocell) were cultured in M-199 basal medium (Gibco, 11043-023) and supplemented with penicillin/streptomycin (Gibco, 15140-122), ECGS supplement (Promocell, C-30160), sodium heparin (Sigma, H3149) and 20 % fetal bovine serum (FBS, Gibco, 10270) as previously described.[6] HUVEC were used between passages 2–6.

For Angiogenesis experiment, HUVECs (3 × 10^7 mL^{-1} cells) were directly suspended in 4-PEG-maleimide solution during polymerization.

NIR activated spreading of fibroblasts: 4-Arm-PEG Maleimide (10 μL 10 wt%) was mixed with cyclo[RGDfC] (5 μL 5 mM) and incubated for 30 min at 37 °C. HEPES buffer (5 μL) with or without UCNP-PMOs (25 mg/mL) was added. The cells suspended in the medium were added to the solution in 1:3 volume ratio, and 8 μL drops were placed in Ibidi® 15 well angiogenesis slide. 2 μL 5 mM VPM peptide was added, quickly mixed by pipetting, and the mixture was allowed to gelate for 15 min at 37 °C and 5% CO_2. Medium was added to each well, and the gels were incubated for 30 min at 37 °C and 5% CO_2. The samples were exposed

to NIR laser (974 nm, 10 W/cm^2) for 12 mins (12 cycles of 1 minute exposure followed by 1 min delay time). The cultures were cooled with a home-made peltier cooling unit placed below the culture plate. The temperature was maintained between 20-37^{o}C during irradiation. The cells were imaged after every 12-24 h and kept in culture for 1-2 days. The cell culture medium was exchanged by fresh medium right after irradiation and daily.

NIR-activated angiogenesis in 4D: 4-Arm-PEG Maleimide (10 µL 10 wt%) was mixed with cyclo[RGDfC] (4.2 µL 5 mM), VEGF (Promokine, C-64424, 1 µL, 500 ng mL^{-1}) and incubated for 30 min at 37 °C. HEPES buffer (5 µL) with or without UCNP-PMOs (25 mg/mL) was added. The cells suspended in the medium were added to the solution in 1:3 volume ratio, and 8 µL drops were placed in Ibidi® 15 well angiogenesis slide. 2 µL 5 mM VPM peptide was added, quickly mixed by pipetting, and the mixture was allowed to gelate for 15 min at 37 °C and 5% CO_2. Medium was added to each well, and the gels were incubated for 30 min at 37 °C and 5% CO_2. The samples were exposed to NIR laser (974 nm, 10 W/cm^2) for 12 mins (12 cycles of 1-minute exposure followed by 1 min delay time). The cultures were cooled with a home-made peltier cooling unit placed below the culture plate. The temperature was maintained between 20-37^{o}C during irradiation. The cells were imaged after every 12-24 h and kept in culture for 1-2 days. The cell culture medium was exchanged by fresh medium right after irradiation and daily.

For the patterned activation, a photoresist mask was fixed below the cell culture plate. NIR laser was then shined from the bottom of the plate, through the mask, for 12 min (12 cycles of 1 min exposure followed by 1 min delay time). For deep tissue irradiation, pork skin tissue with 2.5 mm thickness was placed above the cell culture plate at a distance of 1 mm. The cell culture was irradiated from the top, through the tissue, for 40 min (40 cycles of 1 min exposure followed by 1 min delay time). The tissue was taken out for ice cooling after every 1 min of exposure to prevent over-heating. The cells were imaged after 24 h.

For imaging, samples were fixed with 4% PFA solution for 2 h, washed with PBS and blocked with 1% BSA solution for 1h. The cells were permeabilized with 0.5% Triton for 1 h, actin fibers were stained with TRITC-phalloidin (1:200 in water) and nucleus was stained by Hoechst 33342, Trihydrochloride, Trihydrate (1:500 in water, life technology). The samples were washed with PBS and imaged by Zeiss LSM 800 confocal microscope.

Cellular aspect ratio measurements: For cellular aspect ratio measurements, three random light microscopy images at 20x magnification were taken from each gel using an inverted microscope. To quantify cellular spreading, the maximum orthogonal length and width of each cell was measured using NIH ImageJ, and the aspect ratio calculated as the longer length divided by the shorter length. Each image produced ≥ 3 measurements from randomly selected cells, or n≥45 for each sample. The measured aspect ratios were then sorted into bins to form histograms of spreading for each formulation.

Live/dead Staining: Encapsulated cells were visualized for viability using a fluorescent live/dead staining kit (FDA/PI) and imaged with Zeiss LSM 880 microscope. For assessment of viability, three random images of each gel at 20x magnification were taken through both the live (FITC) and the dead (TRITC) filters. Cell viability was then assessed by counting the total number of live and dead cells in each image and calculating the ratio of live cells to total cells. For all cellular images, each construct was first viewed from the top to the bottom surface to ensure uniform cellular morphology throughout the construct volume and that images were obtained from the interior of the gel.

Statistics: Statistical differences were determined by Student's t-test where appropriate, with significance indicated by $p<0.05$. All data is presented as a mean ± standard deviation.

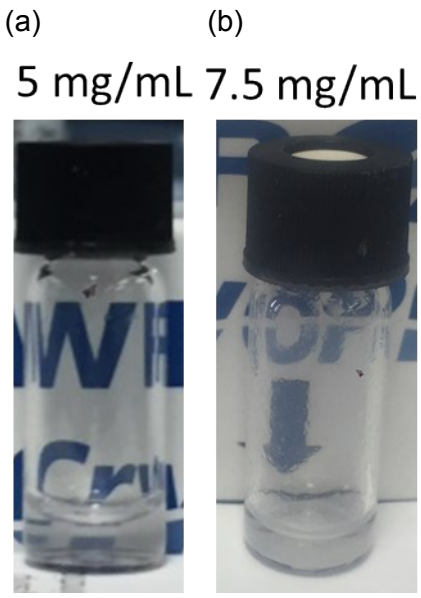


Figure S1. Photograph of the UCNP-PMO water solution at two different concentrations: (a) 5 mg/mL and (b) 7.5 mg/mL after ligand exchange.

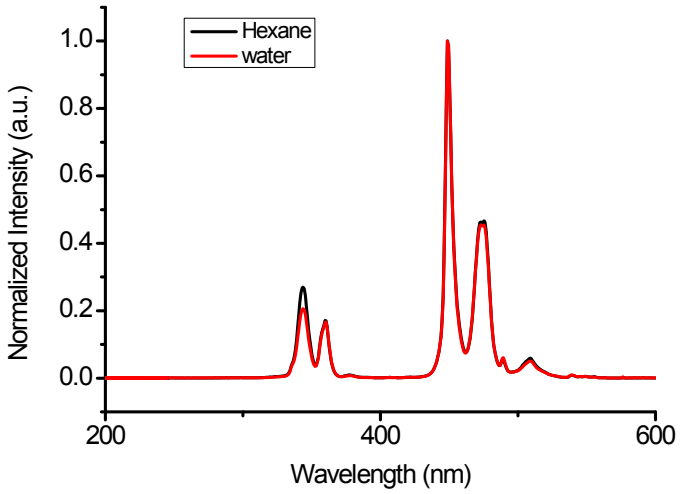


Figure S2. Normalized Emission spectrum of UCNPs in hexane (black) before ligand exchange, and in water (red) after ligand exchange to obtain UCNP-PMO. Solution concentration was 5 mg/mL and excitation occurred with LED laser at 974 nm (10 W/cm^2).

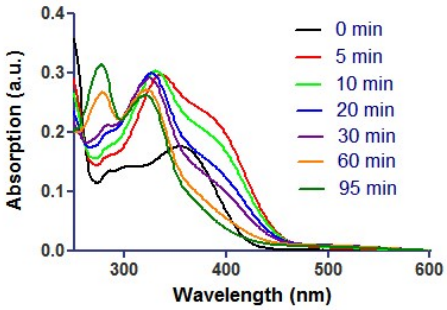


Figure S3. (a) UV-Vis spectra of 1 mM cyclo[RGD(DMNPB)fC] solution in water after irradiation at increasing exposure times at 360 nm (2.7 mW/cm^2) using a LUMOS 43 LED (Atlas Photonics Inc). For irradiation, the quartz cuvette Quartz High Precision Cell (Art. No. 115-10-40, 200 µl) was used.

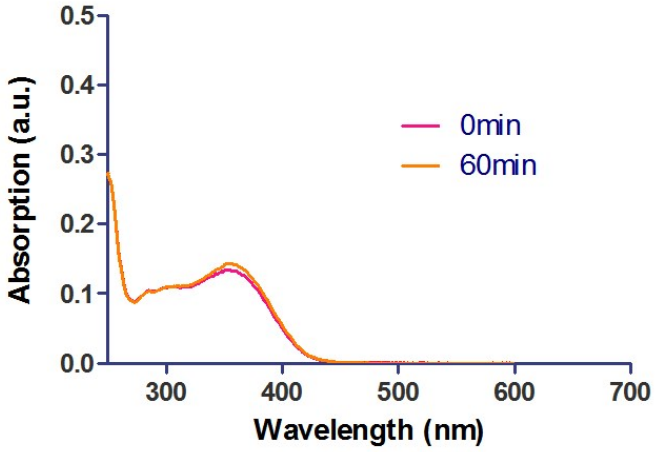


Figure S4. UV-Vis spectra of 1 mM cyclo[RGD(DMNPB)fC] solution in water with increasing exposure times after irradiation at 974 nm (10 W/cm^2). For irradiation, the quartz cuvette Quartz High Precision Cell (Art. No. 115-10-40, 400 µl) was used.

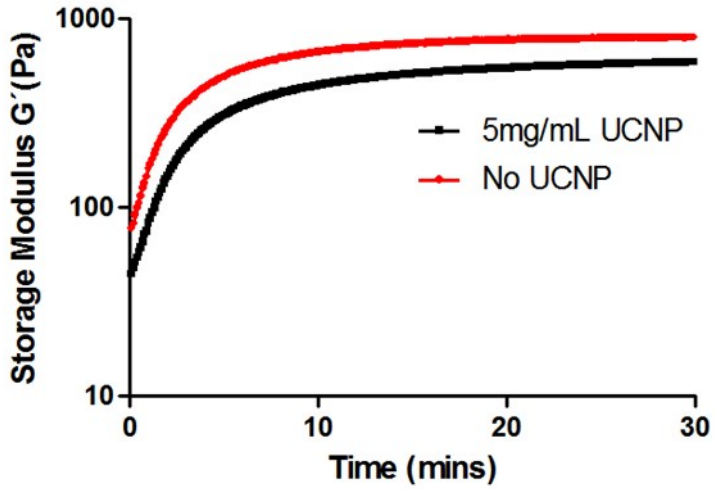


Figure S5. Polymerization kinetics of PEG hydrogel followed by dynamic mechanical analysis at 37^{o}C. The graph shows the increase in the shear modulus (G') over time.

Table S1. Physicochemical properties of 4 wt% PEG hydrogel with and without UCNPs.

Properties	Without UCNP	with 5 mg/mL UCNP
Elastic Modulus (G´) at 37 °C (Pa)	2080 ± 314	1860± 105
Swelling ratio (mg water/mg gel)	130±17	145±23

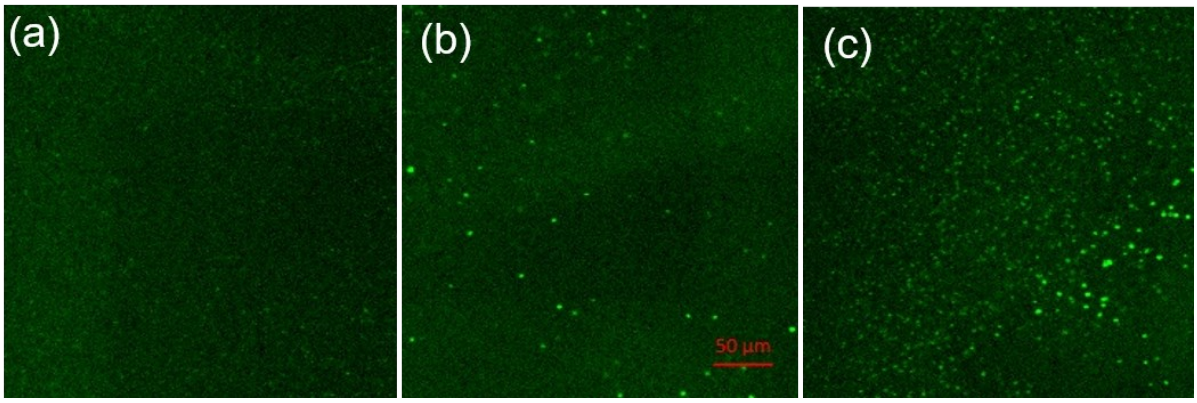


Figure S6. Fluorescence images of PEG hydrogel embedded with different concentration of UCNP. (a) 5 mg/mL; (b) 7.5 mg/mL and (c) 10 mg/mL.

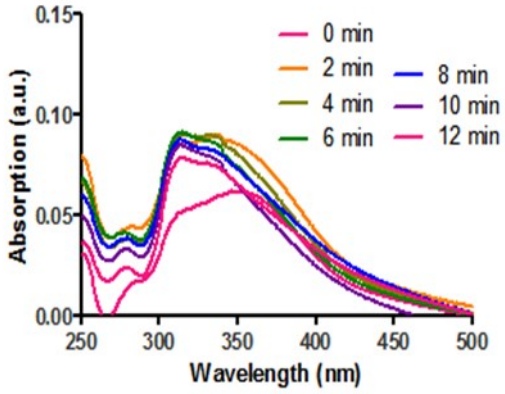


Figure S7. UV−vis absorption spectra of a solution of 4 wt% PEG-MAL functionalized with 1 mM cyclo[RGD(DMNPB)fC] and 5 mg/mL UCNPs after exposure at 974 nm at increasing illumination times.

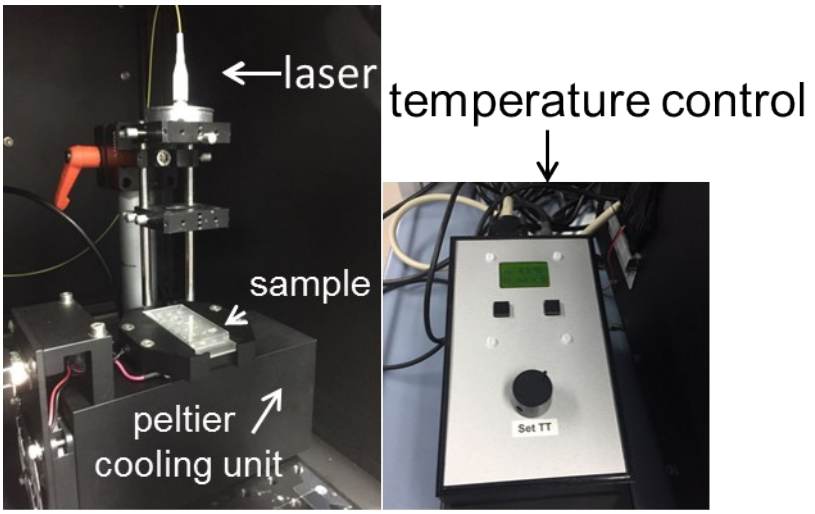
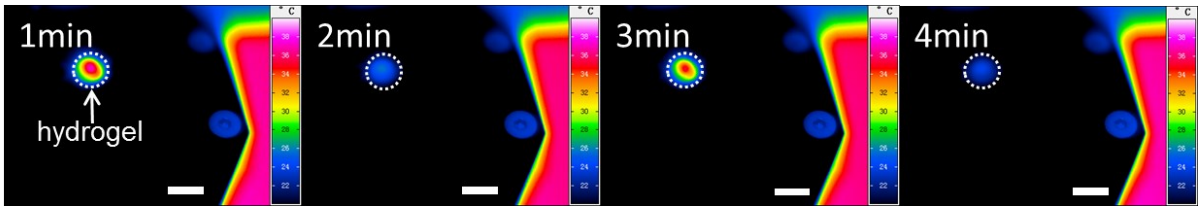


Figure S8. Photograph of the home-made peltier cooling unit used during exposure equipped with a temperature control system for cooling.

(a)



(b)

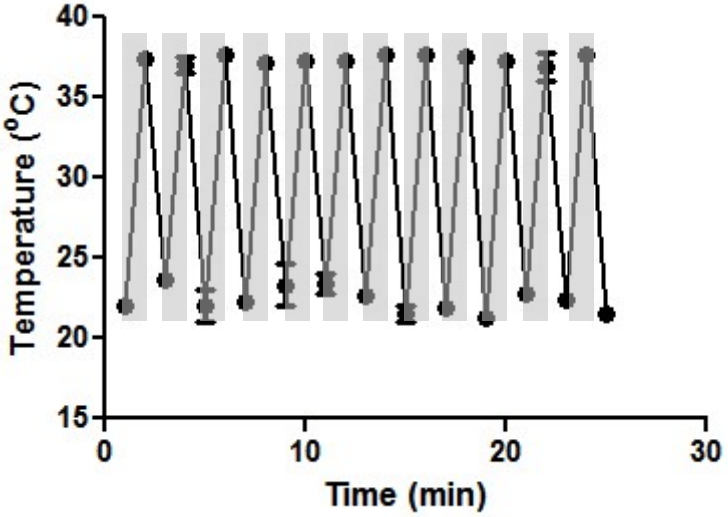


Figure S9. (c) IR images depicting the temperature variation in the hydrogel with 5 mg/mL UCNP-PMOs during two 1 minute exposure/1 minute delay cycles at 974 nm (10 W/cm^2). Scar bar: 5 mm (c) Temperature profile of hydrogel with 5 mg/mL UCNPs as a function of laser irradiation time.

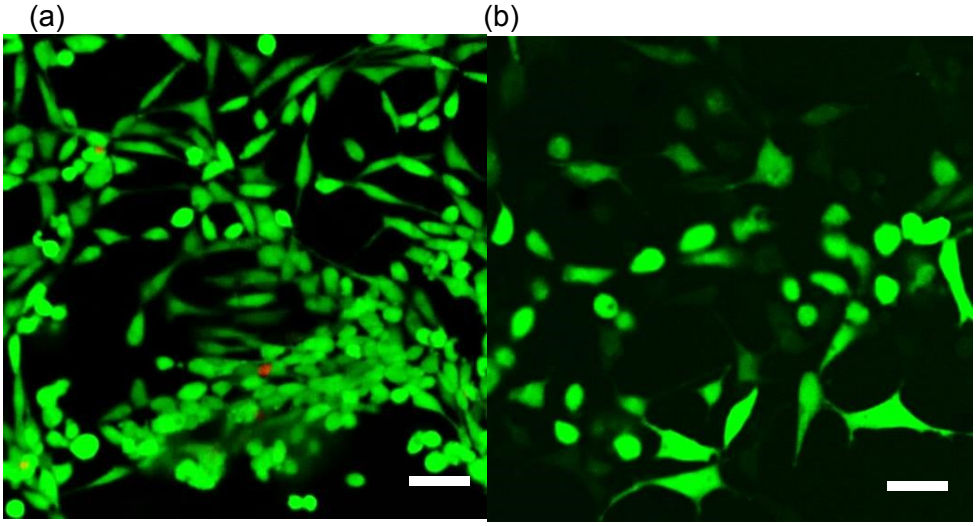


Figure S10. Fluorescence images of live-dead staining of L929 fibroblasts encapsulated in PEG hydrogels modified with cyclo(RGDfC) modified with (a) and without (b) UCNP-PMAOs (5 mg/mL). Cells were labelled 24h after culture.

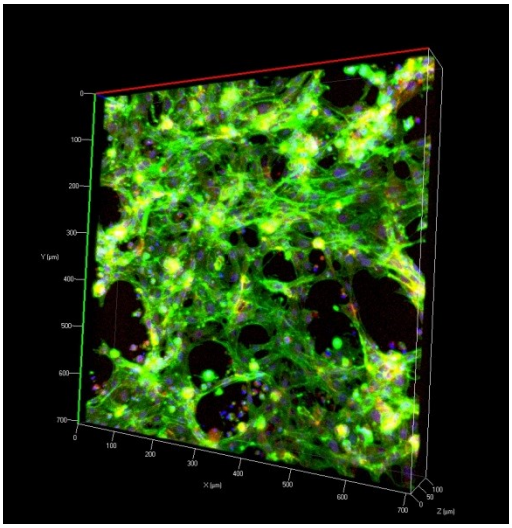


Figure S11. Z-stack fluorescence image of HUVEC vascular network formed within a cyclo(RGDfC) functionalized PEG maleimide hydrogel and VEGF after 24 h. Nucleus was stained by DAPI (blue), actin fibers with Phalloidin (green), and cell body with PECAM-1 (red).

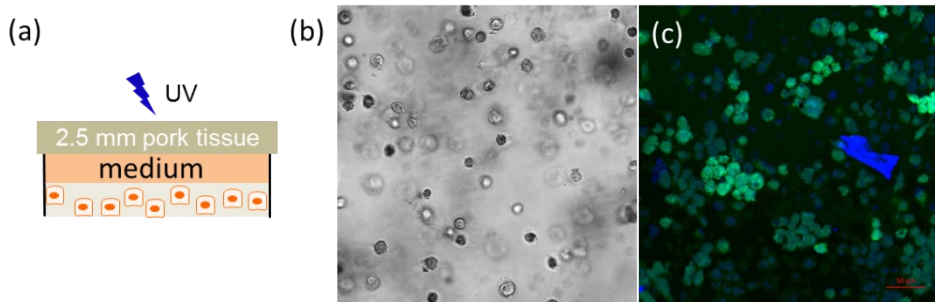


Figure S12. a) Illustration of in-deep tissue UV irradiation by covering the substrate with 2.5 mm pork tissue (b) L929 cell image after irradiation with 360 nm light for 40 mins (5 mW/cm^2).(c) HUVEC after irradiation with 360 nm light for 40 mins (5 mW/cm^2)

Reference:

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