

Synthetic 3D PEG-Anisogel Tailored with Fibronectin Fragments Induce Aligned Nerve Extension

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Supporting Information

Materials

The peptide sequences H-NQEQVSPLERCG-NH₂ (M_w = 1358.6 g/mole), H-NQEQVSPLRGDSPG-NH₂ (M_w = 1482.7 g/mole), and Ac-FKGGGPQGIWGQERCG-NH₂ (M_w = 1717.6 g/mole) are provided by Pepscan (Lelystad, NL). The eight arm PEG-vinylsulfone (PEG-VS, average M_w = 20,000 Da) by Jenkem technology USA Inc. Triethanolamine is supplied by Sigma Aldrich. For dialysis, Slide-A-Lyzer dialysis cassettes (MWCO = 3.5 kDa) are provided by ThermoFisher. Calcium chloride and Sodium chloride are purchased from Sigma Aldrich and Tris(hydroxymethyl) aminomethan provided by AppliChem. For protein production, BL21 Gold (DE3) E. coli are provided by Agilent Technologies. The GSTrap HP Column (5 mL) and PreScission protease are supplied by GE Healthcare Life Sciences. Yeast extract, Tryptone, and Imidazole are purchased from AppliChem. Isopropyl β D thiogalactopyranosid and phenyl methyl sulfonyl fluorid is supplied by Roth and lysozyme is provided by Sigma Aldrich. For cell experiments, L929 fibroblasts are obtained from DSMZ, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI) media, phosphate buffered saline (PBS) by Lonza, TrypLE™ Express Enzyme, and Antibiotic Antimycotic (100X) are supplied by gibco Thermo Fisher Scientific, fetal bovine serum by Biowest, thrombin from human plasma by Sigma Aldrich, human fibrinogen by Milan Analytica, Fibrogammin 1250 (FXIII) by CLS Behring, and recombinant brain derived neurotrophic Factor (BDNF) by PeproTech. For staining, Triton-X-100 is provided by Sigma Aldrich, 4', 6 diamidino 2 phenylindole (DAPI) and paraformaldehyde (PFA) by AppliChem, primary neurotubulin antibody TuJ1 by BioLegend, a secondary fluorescent goat anti mouse antibody by ThermoFisher, and CytoPainter Phalloidin-iFluor 488 Reagent for F actin filaments by Abcam. 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is provided by Promega. Bovine Serum Albumin (BSA) is supplied by Sigma Aldrich and a Fluo-4 Calcium Imaging Kit by ThermoFisher.

Methods

Analysis and characterization Instruments

A Bruker DPX 400 FT NMR spectrometer (400 MHz) at room temperature is used to obtain ^1H NMR spectra. For rheological characterization, a DHR 3 Rheometer with 20 mm conical geometry from TA Instruments is applied to determine the mechanical properties of the hydrogels. All measurements are performed at the truncation gap of 51 μm , a strain of 0.5 %, and a frequency of 0.5 Hz. To analyze the gelation time, measurements are performed for the duration of 30 minutes to ensure that the storage modulus of the sample reaches a plateau. Subsequently, a frequency sweep followed by an amplitude sweep is performed from 0.1 to 100 Hz and 0.1 to 1000 %, respectively, while keeping the amplitude or frequency constant at 0.5 % or 0.5 Hz. Dynamic light scattering (DLS) is used to determine the hydrodynamic radius of an sPEG-Q molecule. For cryo FE-SEM, swollen gels are frozen in liquid ethane for 1 min and transferred into liquid nitrogen. Hydrogel cross-sections are cut inside the FE-SEM pre-chamber and samples are visualized at 1 kV and 1 μA with FE SEM SU4800 (Hitachi Ltd. Corporation) after multiple steps of 5 min sublimation.

Cell experiments

Cell culture experiments are carried out with L929 mouse derived fibroblasts or isolated dorsal root ganglions (DRGs) from day 10 chicken embryos. DRGs are retrieved and used right away or dissociated into single neurons as previously described.¹ Fibroblasts (L929) are cultured in a basal medium consisting of RPMI 1640, supplemented with 10 % fetal bovine serum and 1 % amphotericin B at 37 °C, 5 % CO_2 , and 95 % humidity. Full DRGs or dissociated neurons are cultured in DMEM, containing 10% fetal bovine serum, 1 % Penicillin Streptomycin, and 10 or 15 ng/mL brain-derived neurotrophic factor (BDNF) for 2D or 3D experiments, respectively.

For the robotic spotter experiments, dissociated neurons are seeded onto the gels and cultured for 4 days in the presence of four different media compositions (10 ng/mL BDNF, 10 ng/mL BDNF plus anti-alpha 5

antibody, 10 ng/mL BDNF plus anti- α_v antibody, no BDNF). The anti- $\alpha_5\beta_1$ and anti- $\alpha_v\beta_3$ antibodies block the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, respectively, potentially inhibiting cell adhesion. After 4 days, extended neurites are fixed with 4% PFA for 15 min and stained with the primary neurotubulin antibody TUJ1 (1:250) for 4 h in PBS, containing 4% BSA. Sequentially, a secondary fluorescent goat anti-mouse antibody (1:100) is added for 1 h in PBS, including 3 washing steps for 10 minutes between each solution. Images are taken with fluorescent microscopy and the total length of neurite extension is quantified for random spots using the NeuronJ plugin of ImageJ. This experiment is repeated twice.

In the case of 3D PEG hydrogels, different cell-adhesive biomolecules (i.e. H-NQEQVSPL-RGD-SPG-NH₂, FNIII₉*-10/12-14, and full length fibronectin) are bound to the hydrogel. Both RGD and FNIII₉*-10/12-14 bear the FXIIIa recognition site NQEQVSPL to covalently bind to the hydrogel. Fibronectin is mixed within the hydrogel and retains via physical entanglement and potential interactions with the K and Q-peptides. L929 fibroblasts are mixed directly within the precursor solution (1,000 cells per 50 μ L) before gelation and cultured for 7 days. At day 7, an MTS assay is carried out for 1 h at 37 °C in order to study cell metabolism and proliferation. 100 μ L of the incubated MTS solution is analyzed in a Molecular Devices Spectramax M2 plate reader (BioTek) at a wavelength of 490 nm. For immunostaining, cells are fixed after 7 days in culture. Gels are washed twice in PBS for 20 min and fixed in 4 % PFA for 1 h. Cells are then washed 3 times for 20 min before they are permeabilized in 0.1 % triton-X-100 for another hour. After another washing circle, gels are blocked with 4 % BSA for 4 h and cells are stained using CytoPainter Phalloidin-iFluor 488 overnight. On the next day, samples are washed 4 times and the nucleus is stained with DAPI for 1 h. Finally, the samples are washed 4 times in PBS and stored in PBS until imaging with confocal microscopy.

DRGs are put on the bottom of an ibidi μ -slide 8 well plate and the precursor solution (20 μ L) is subsequently pipetted on top of the DRG. After 5 min of crosslinking at RT and another 20 min in the incubator at 37 °C, 300 μ L DMEM, supplemented with 15 ng/mL BDNF, is added to completely cover the

gel. To analyze neurite extension after 7 days, the neurons are fixed and stained with the primary neurotubulin antibody TUJ1 and a secondary fluorescent goat anti-mouse antibody. Therefore, after culture, the samples are washed 2 times for 30 minutes each, and subsequently fixed by means of 4 % PFA for 1 h. Afterwards, samples are washed 3 times, treated with 0.1 % triton-X-100 for 1 h, washed 3 times, blocked with 4 % BSA in PBS for 4 h, followed by the addition of the primary neurotubulin antibody TUJ1 (1:250) in 4 % BSA containing PBS overnight at room temperature. After washing 4 times with PBS for 30 minutes, the secondary antibody Alexa Fluor 633 anti-mouse (1:100) is added and incubated for 4 h at room temperature. Finally, samples are washed 4 times and stored in PBS until imaging with confocal microscopy.

For 3D imaging, laser scanning confocal microscopy (SP8 Tandem Confocal, Leica Microsystems Inc.) is used. Therefore, a photodiode 405 (Dapi), an Argon laser adjusted to 488 nm (Alexa Fluor 488), a diode pumped solid state laser 561 (microgels/fibers), and/or a Helium-neon laser 633 nm (Alexa Fluor 633) is applied. The resulting emission is detected using a photomultiplier tube (microgels/fibers/actin) or a hybrid detector (nucleus/neurites) at a magnification of 10X performing a z stack combined with a sequential scan. Image processing is performed within the LasX software to stitch tile scans of one sample. Finally, a maximum projection image is obtained for further analysis.

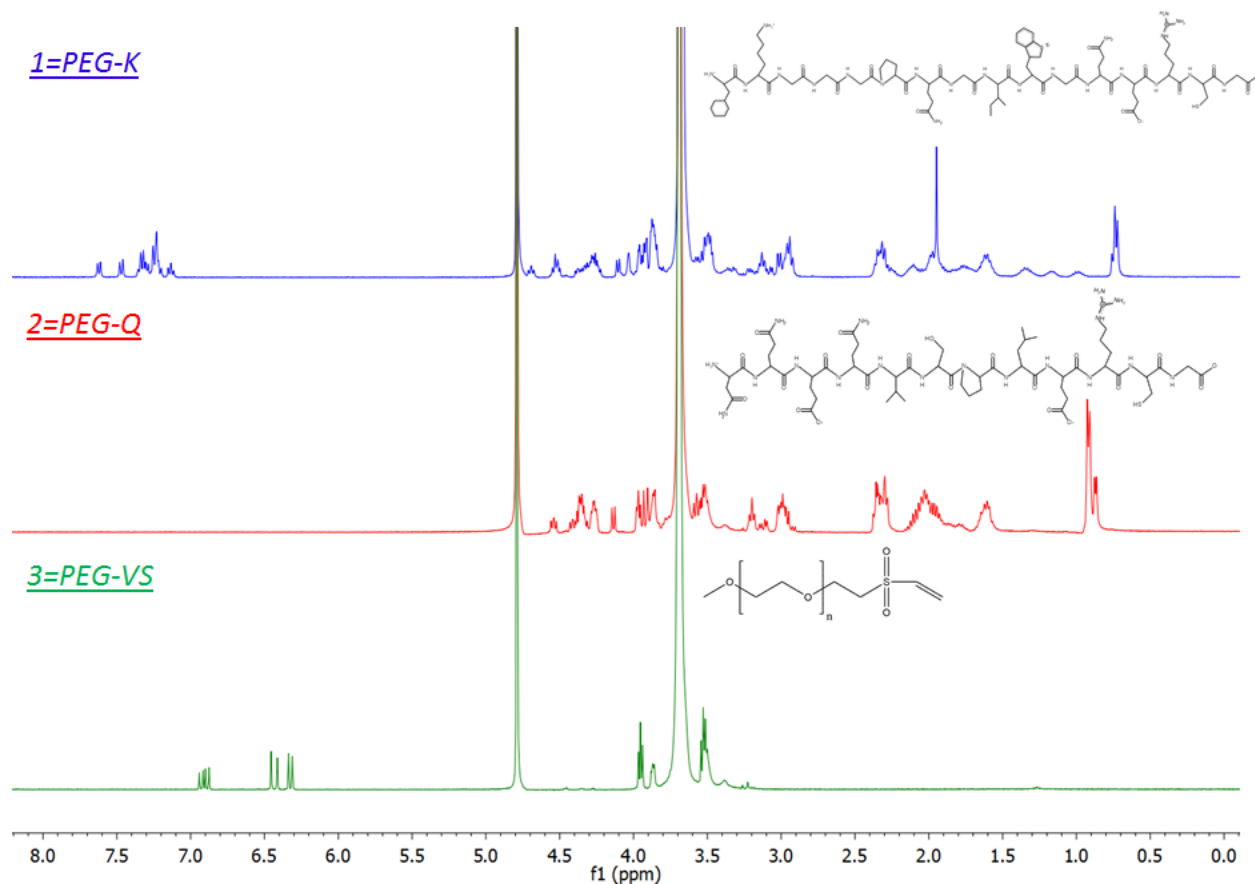


Figure S1. ^1H NMR of PEG-VS (bottom), PEG-Q (middle), and PEG-K (top) including its molecular structures. For PEG-K, aromatic peaks appear from 7.1 ppm to 7.7 ppm. The aromatic groups in the sPEG-K appear at 7.1 ppm to 7.7 ppm and are used to determine the functionalization. As there are no separable signals present in the PEG Q conjugate, the vanishing of the vinylsulfone signals (6.3 ppm ($=\text{CH}_2$), 6.4 ppm ($=\text{CH}_2$), and 6.9 ppm ($\text{SO}_2\text{CH}=\text{}$)) demonstrates successful functionalization.

^1H NMR (D_2O , 400MHz, δ); PEG VS: 6.91 (dd, 1H, Vinyl H), 6.43 (d, 1H, Vinyl H), 6.32 (d, 1H, Vinyl H), 4.79 (s, 1H, D_2O), 3.454 (m, 1H, PEG backbone); PEG Q: 4.79 (s, 1H, D_2O), 3.454 (m, 1H, PEG backbone). Yield: 91%; PEG K: 7.62 (s, 1H, Ar H), 7.47 (s, 1H, Ar H), 7.10 7.37 (m, 8H, Ar H), 4.79 (s, 1H, D_2O), 3.454 (m, 1H, PEG-backbone). Yield: 88.2%.

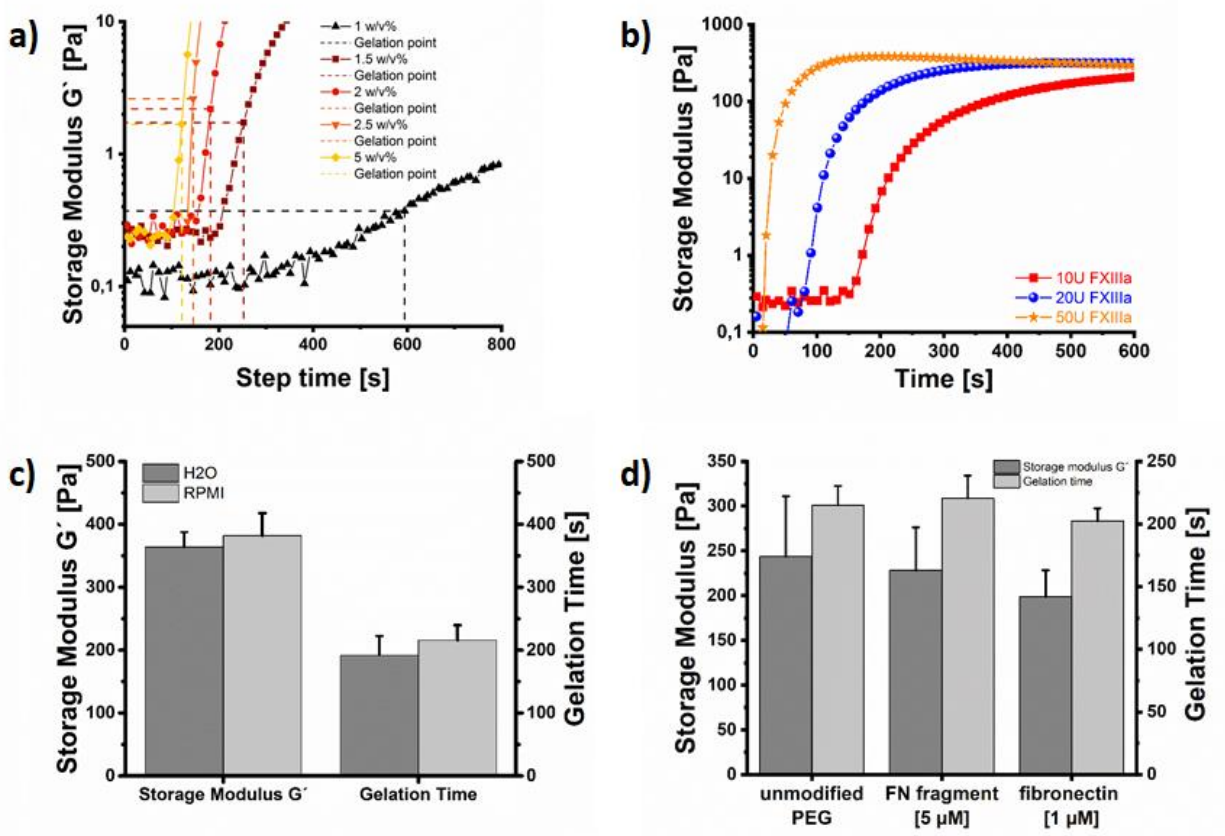


Figure S2. (a) Different gelation points for samples with different sPEG content, ranging from 1 to 5 w/v%. (b) Storage modulus for samples consisting of 2 w/v% sPEG using different enzyme concentrations, ranging from 10 to 50 U/mL. (c) 2 w/v% sPEG hydrogels prepared in water or media, showing no significant differences in both stiffness and gelation time. (d) Influence of protein addition to a 2 w/v% PEG hydrogel. No significant differences can be identified.

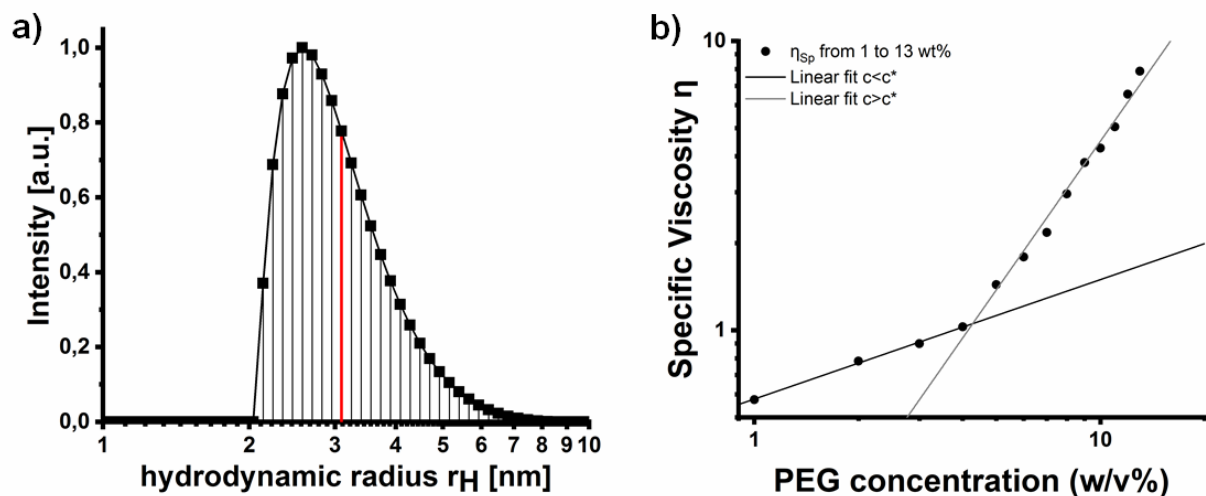


Figure S3. (a) DLS measurement of sPEG-Q. The red line indicates the averaged value for the hydrodynamic radius. (b) Specific viscosity plotted against the polymer concentration. The intersection of both linear fits ($c < c^*$, $c > c^*$) indicates the critical concentration c^* .

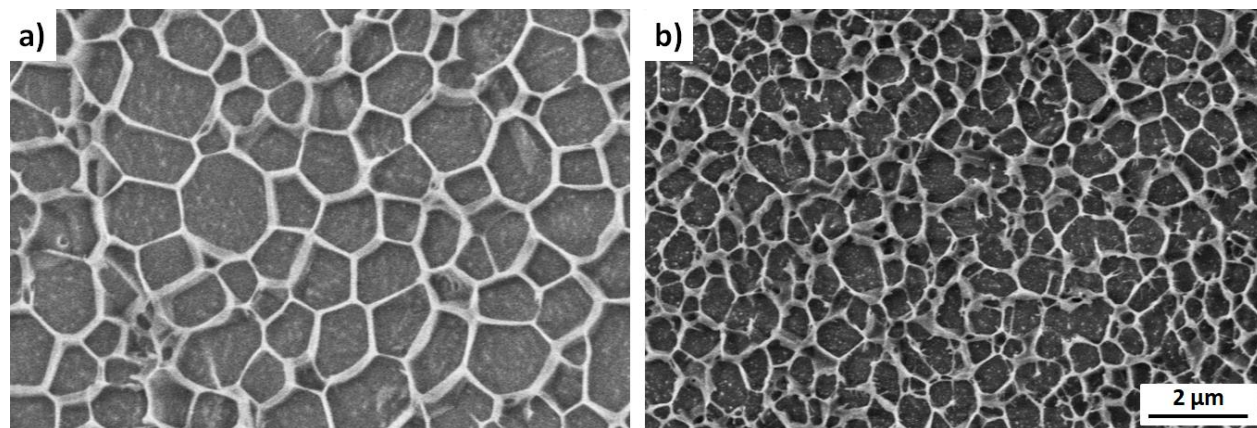


Figure S4. Morphology of a 1 w/v% sPEG hydrogel after different stages of sublimation. (a) Superficial structure similar to a honeycomb after 3 x 5 min sublimation. (b) Intense sublimation revealing the 3D structure inside of the hydrogel, indicating a homogeneous morphology throughout the hydrogel.

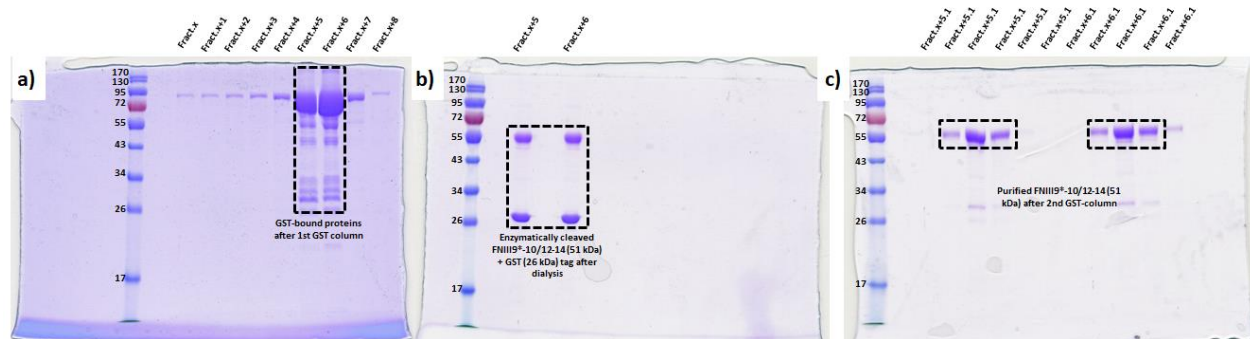


Figure S5. (a-c) SDS-PAGE gels after first purification using a GST affinity chromatography (a), enzymatic cleavage (b), and final purification (c).

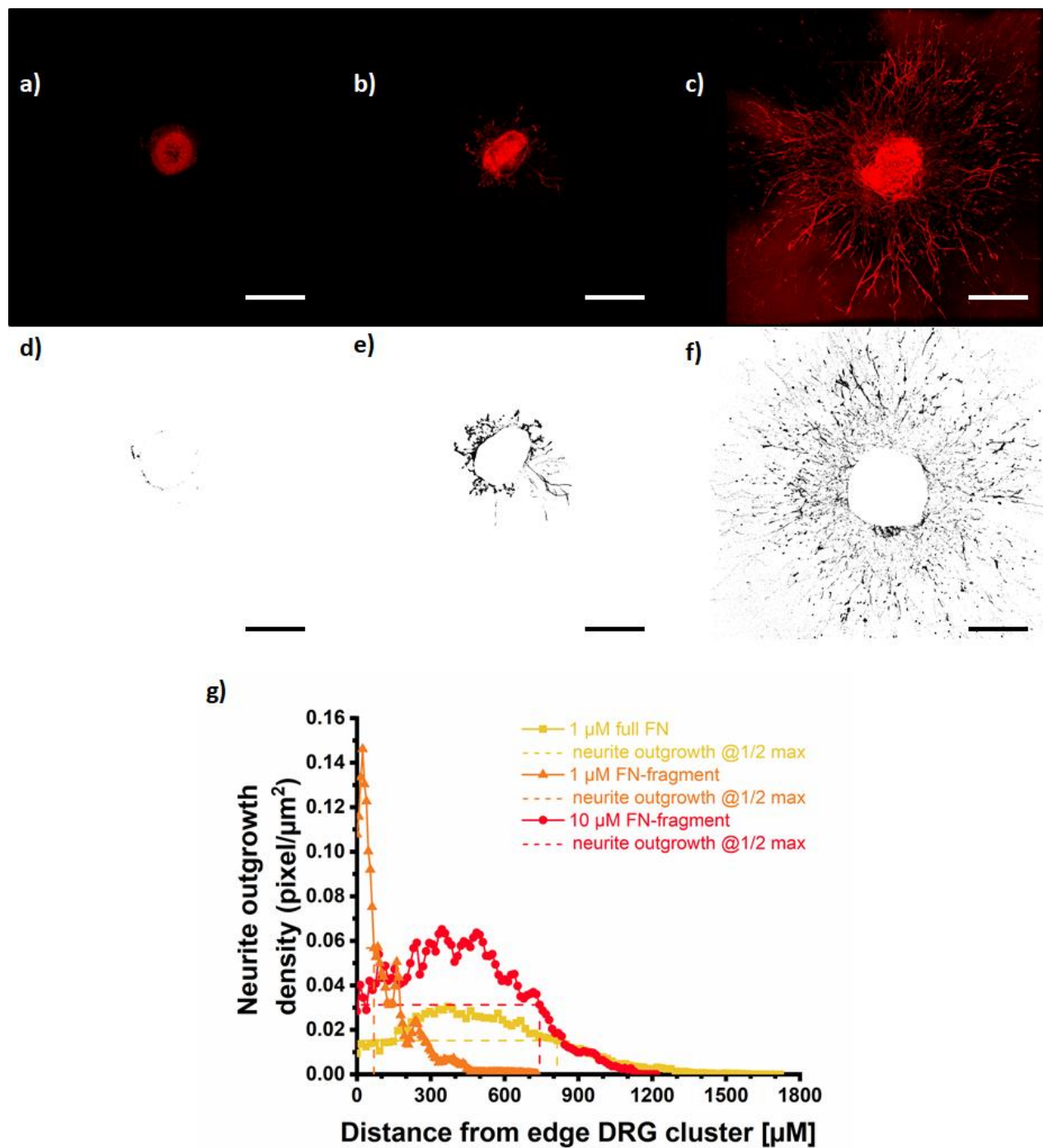


Figure S6 (a-c) Neurite outgrowth inside different biofunctionalized 1 w/v% PEG hydrogels, showing unmodified sPEG (a), 1 μM FNIII9*-10/12-14 (b), and 10 μM FNIII9*-10/12-14 (c). (d-f) Exemplary binary images after thresholding using Otsu's method for unmodified sPEG hydrogels (d), 1 μM FNIII9*-10/12-14 (e), and 10 μM FNIII9*-10/12-14 (f). (g) Neurite outgrowth determination as distance at which the pixel density drops to 1/2 of the maximum pixel density: 1 μM fibronectin (yellow square),

1 μM FNIII9* 10/12 14 (orange triangle), and 10 μM FNIII9* 10/12 14 (red circle). Scale bar is 500 μm . Images represent a series of three replica each.

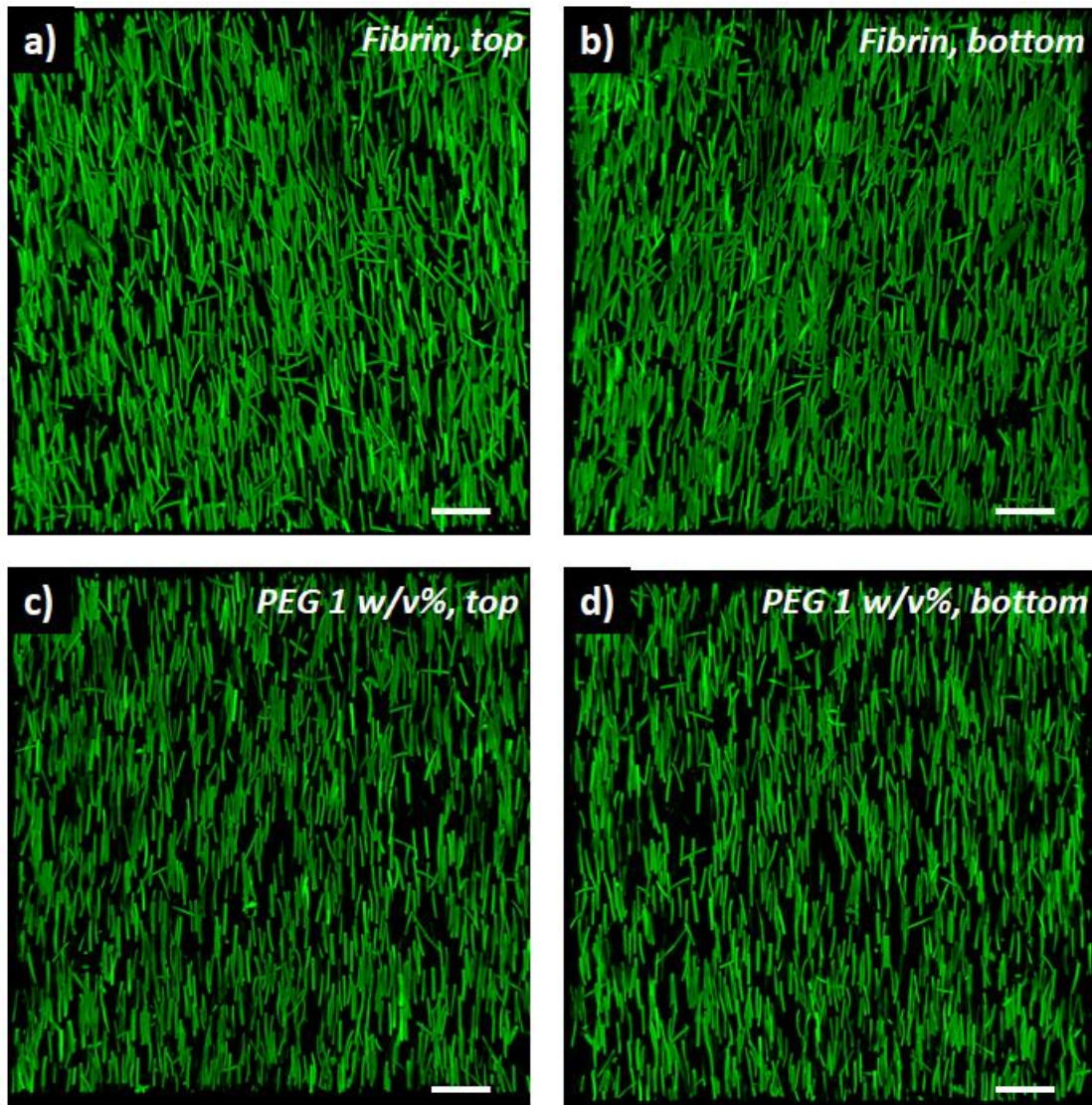


Figure S7. (a-b) 1 v/v% μ -gels in 4 mg/mL fibrin gels with images taken from the top (a) and bottom (b) of the gels. (c-d) 1 v/v% μ -gels in 4 mg/mL 1 w/v% PEG hydrogels with images taken from the top (c) and bottom (d) of the gels. Microgels are highly aligned and do not sink to the bottom. Scale bar is 100 μm . Green color: fluorescein.

1. Rose, J. C.; Camara-Torres, M.; Rahimi, K.; Kohler, J.; Moller, M.; De Laporte, L., Nerve Cells Decide to Orient inside an Injectable Hydrogel with Minimal Structural Guidance. *Nano Lett* **2017**, 17, (6), 3782-3791.