Thiol-ene alginate hydrogels as versatile bioinks for bioprinting

Huey Wen Ooi,[1] *Carlos. Mota,*[1] *A. Tessa ten Cate,*[2,3] *Andrea Calore,*[1,4] *Lorenzo Moroni,*[*1] *Matthew B. Baker*[*1]

[1]Department of Complex Tissue Regeneration, MERLN Institute for Technology-Inspired Regenerative Medicine, Maastricht University, 6211 LK Maastricht, the Netherlands.

[2]TNO, P.O. Box 6235, 5600 HE Eindhoven, the Netherlands

[3]Brightlands Materials Center, P.O. Box 18, 6160 MD Geleen, the Netherlands

[4] Department of Biobased Materials, Faculty of Engineering and Sciences, Maastricht University, Brightlands Chemelot Campus, Urmonderbaan 22, 6167 RD Geleen, the Netherlands

GPC Analysis of functionalized alginate

Aqueous GPC measurements were made on a Shimadzu Prominence LC equipped with RI and PDA detector. Measurements were performed using water (100 mM Sodium Nitrate) as the mobile phase on a Tosoh. G4000PWXL column (7.8 x 300 mm) and a flowrate of 0.4 mL/min. utilizing poly(ethylene glycol) as molecular weight standards.

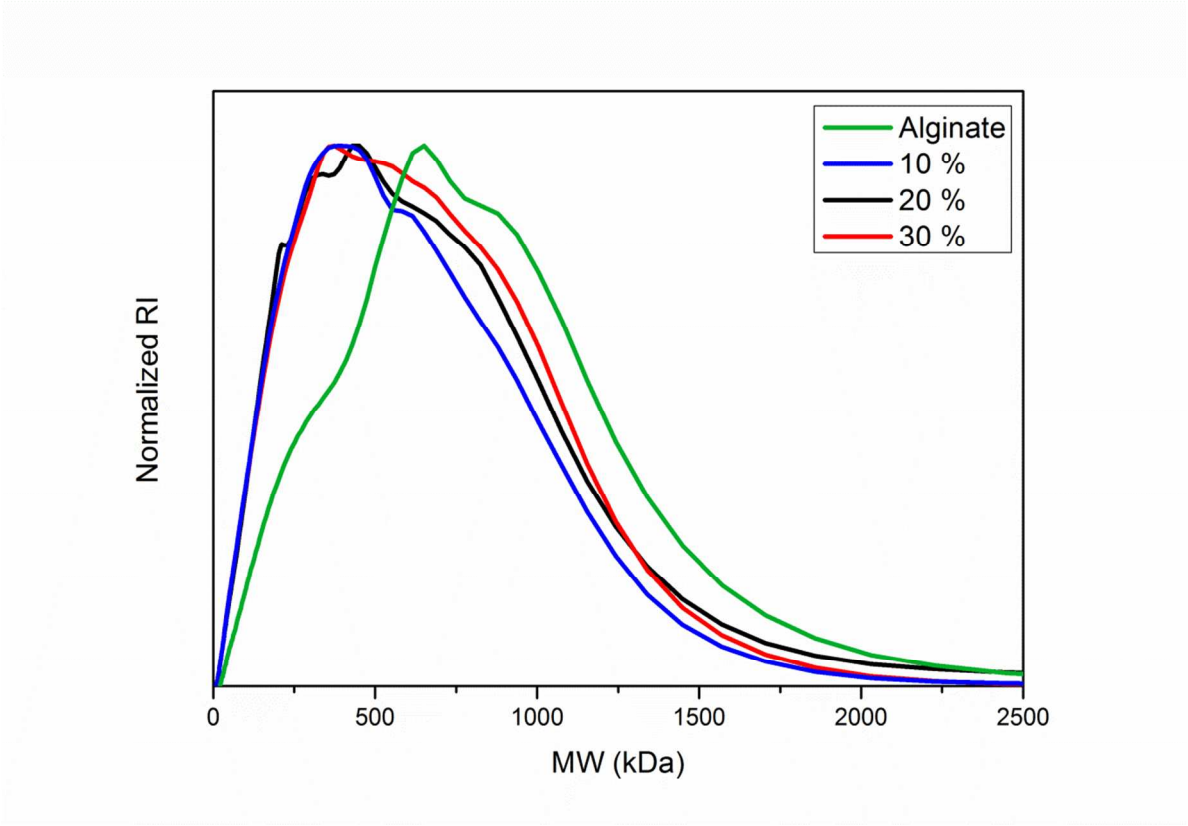


Figure S1. GPC traces in H_2O of alginate samples before and after reaction with different equivalents of norbornene methylamine (10, 20, and 30 mol %).

Estimation of G and M blocks

Based on high temperature NMR (370 K), estimation for G- and M-blocks are 74 % and 26 %, respectively. Calculations carried out on estimations of block compositions were based on report by Grasdalen[1] and Penman et al.[2]

Estimation of G and M blocks was derived as followed:

For the determination of the alginate G and M block compositions

The mol faction of G and the doublet frequency F_{GG}

$$F_G = \frac{I_A}{I_B + I_C}$$

$$F_{GG} = \frac{I_C}{I_B + I_C}$$

The mol fraction of M is then derived from

$$F_G + F_M = 1$$

And the relationship between the doublets is

$F_{GG} + F_{GM} = F_G$ and $F_{MM} + F_{MG} = F_M$

Another way to look at this "blockiness" is $\eta = \frac{F_{MG}}{F_M \times F_G}$ where 0 – 1 indicate blockiness; 1 indicates perfect randomness, and > 1 indicates alternative blocks

Integration (*I*)

I_A	1
I_B	0.6205
I_C	0.7262
F_G	0.7426
F_{GG}	0.5392
$F_G + F_M$	1.0000
F_M	0.2574
F_{GM}/F_{MG}	0.2033
F_{MM}	0.0541
η	1.0635

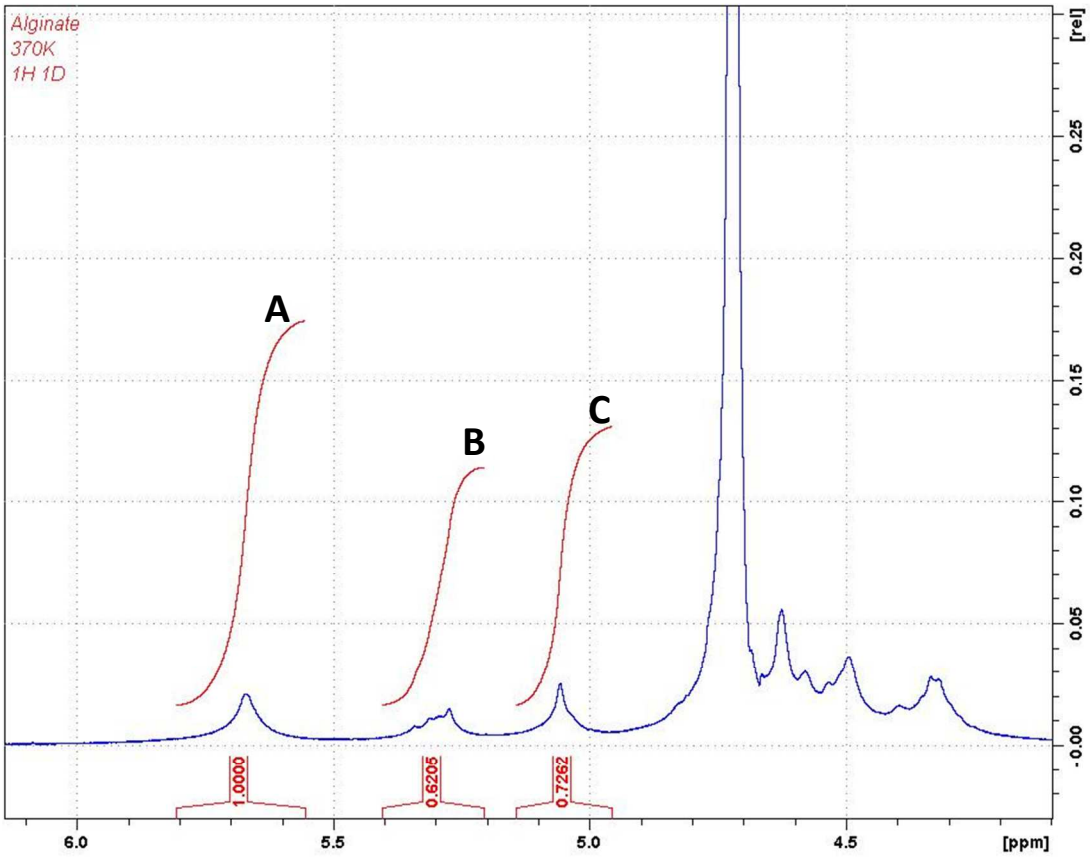


Figure S2. Example of a purified alginate spectrum used for the estimation of M/G block ratio.

Synthesis of Alg-norb and thiol-ene reactions

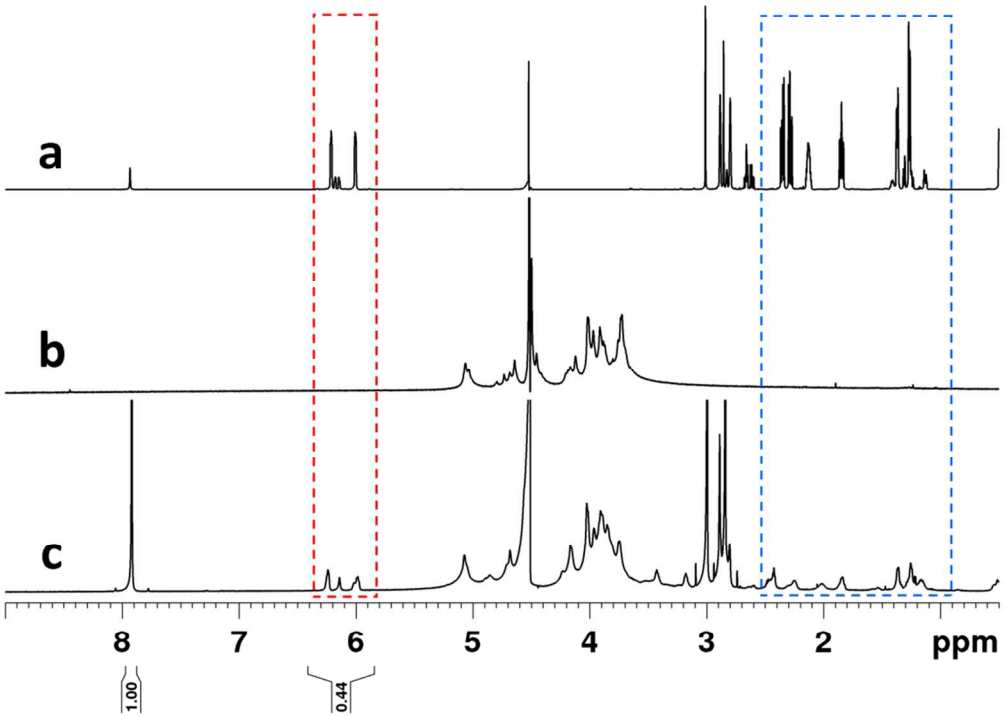


Figure S3. ^{1}H NMR spectra of a) Norbornene methylamine (with 2.6 mM DMF), b) Alginate, and b) Alg-norb (with 2.6 mM of dimethylformamide as internal standard) in D_2O. The appearance of the double bonds of the norbornene groups after the reaction (highlighted in red box at 6.2 ppm) and norbornyl protons (highlighted in blue box) were observed.

Based on the spectrum, the double bonds were integrated to be 0.44 when the aldehyde proton derived from DMF was set to 1.0. This sample comprised of 2.2 mg of Alg-norb dissolved in 0.5 mL of D_2O in the presence of 10.4 mM of DMF. The number of moles of norbornene groups could then be calculated and the % mol of functionalized –COOH groups were estimated to be approximately 11 mol %.

Calculations for estimation of degree of functionalization of norbornene with ^{1}H NMR using DMF as an internal standard:

Mass of NMR sample	2.2	mg
NMR sample	500	uL
[DMF] in sample	1.04×10^{-2}	M
Integration of double bonds	0.4353	
Integration for 1 H	0.218	
[Norbornene]	0.00227	M
No. of mol of norbornene	1.14×10^{-6}	mol
MW of norbornene	123.2	
Mass of norbornene	0.140	mg
Mass of alginate (mass of sample – mass of norbornene)	2.06	mg
MW of alginate	197.9	g/mol
No. of mol of alginate	1.04×10^{-5}	mol
% functionalization	**10.91**	%

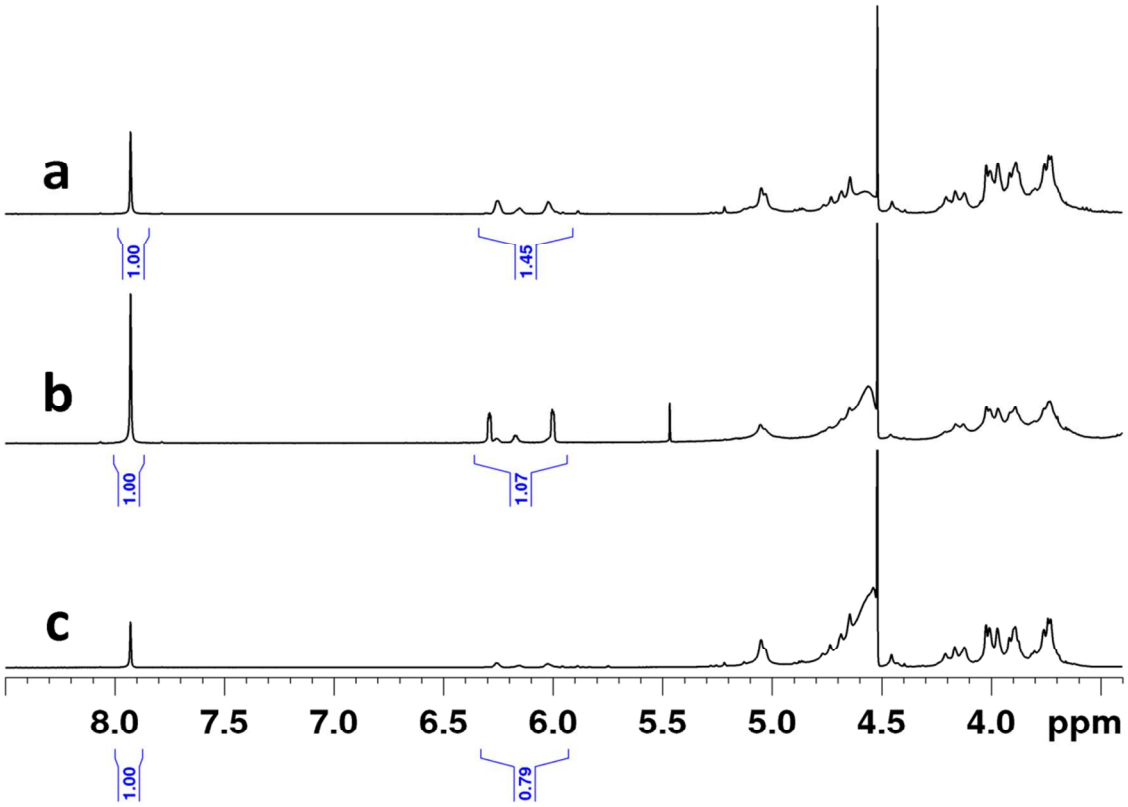
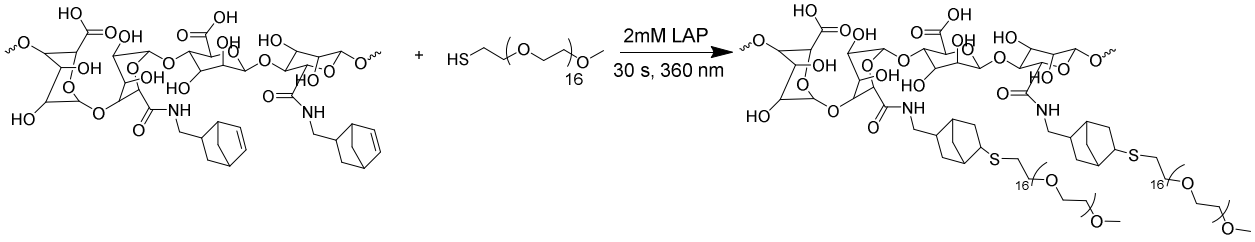


Figure S4. ^{1}H NMR spectra of Alg-norb with different percentage of norbornene functionalization, a) 12 mol %, b) 7.1 mol %, and c) 4.2 mol %. Functionalization was estimated from the double bonds of the norbornene groups at 6.2 ppm (with 2.6 mM of dimethylformamide as internal standard) in D_2O. Further details on samples included in Table 1.

Conjugation of O-(2-Mercaptoethyl)-O'-methylpolyethylene glycol to Alg-norb



Alg-norb (0.0068 g, 12 % functionalized, 3.44x 10^{-6} mol of norbornene) was weighed into a glass vial and 0.68 mL of water was added (1 w/v % solution) and the solution was stirred until alg-norb was dissolved completely. LAP (0.600 mg, 2.04 x 10^{-6} mol, 2 mM) and O-(2-Mercaptoethyl)-O′-methylpolyethylene glycol (800 Da, 1.37 mg, 1.71 x 10^{-6} mol) were added to the Alg-norb solution. The mixture was then irradiated with a UV LED (365 nm, 10 mW/cm^2) for 30 s. The reaction solution was transferred to a 10 kDa MWCO tube and dialyzed against deionized water for 2 days with change of dialysate every 10 to 18 hours. Purified polymer solution was then lyophilized to yield PEG-Alg-norb as a white solid (0.0060 g, 90 %).

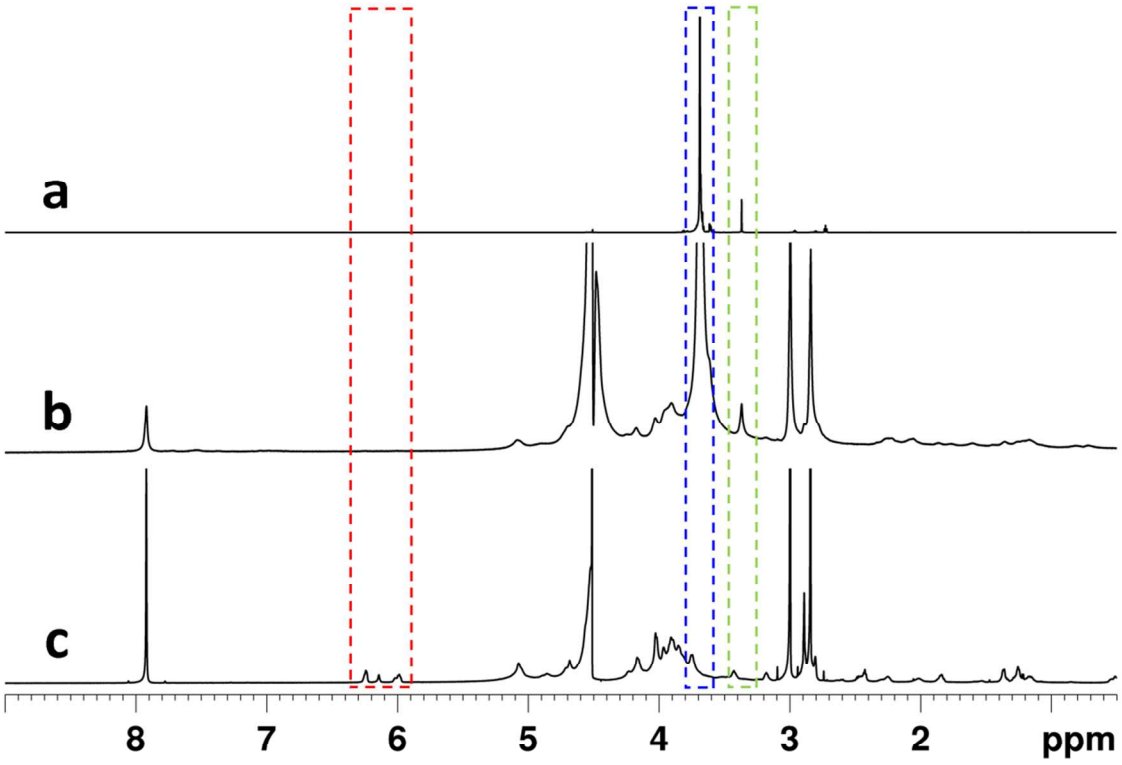


Figure S5. ^{1}H NMR spectra of a) PEG-OMe, b) Alg-norb reacted with PEG-OMe, and c) Alg-norb in D_2O with 2.6 mM of dimethylformamide as internal standard. The disappearance of the double bonds of the norbornene groups after the reaction (highlighted in red box at 6.2 ppm) and the appearance of the methylene protons from the PEG backbone (highlighted in blue box at 3.7 ppm) and the methyl protons on PEG-OMe (highlighted in green box at 3.4 ppm) were observed.

Rheological properties of hydrogels

Determination of viscosity of alginate solutions. Dynamic rheological measurements were performed on a MCR 702 TwinDrive (Anton Paar) rheometer with parallel plate geometry (50 mm bottom plate and 12 mm top plate) at rt. The samples were loaded directly onto the bottom plate and then the gap (0.9 mm for the Alg-norb 2 w/v% and 0.8 mm for the Alginate 2 w/v%) was adjusted to fill the volume between the plates and to obtain a proper meniscus. Preliminary frequency sweeps (0.1 Hz to 10 Hz) were performed to evaluate the optimal shear strain and frequency range. These were found to be 10 % for the Alg-norb 2 w/v%, 5 % for the Alginate 2 w/v%, respectively. The change in dynamic properties with frequency was then followed by using the mentioned parameters.

Both solutions clearly showed shear-thinning behavior, i.e. decreasing viscosity with increasing frequency. This behavior is in fact typical of polymer solutions. The viscosity values are in the order of 0.1-1 Pa*s for the measured frequency range indicating that these are low viscous solutions.

The Cox-Merz rule, stating the equivalence of dynamic and steady shear viscosities, was applied to obtain viscosity data with respect to shear rate, so that linear regression could be applied to fit a flow model with the TA Trios software. With R^2 equal to 0.98, the Alg-norb solution was found to follow the Sisko model:

$$\eta = \eta_\infty + k\dot{\gamma}^{n-1}$$

with $\eta_\infty = 0.16\ Pa * s$ (viscosity at high shear rates), $k = 0.23\ s$ (consistency index) and $n = 0.6$ (power law index).

The Alginate 2 w/v% solution was best fitted via a Carreau-Yasuda flow model, with R^2 equal to 0.99:

$$\frac{\eta - \eta_\infty}{\eta_0 - \eta_\infty} = [1 + (k\dot{\gamma})^a]^{\frac{n-1}{a}}$$

With $\eta_\infty = 4.3\ Pa * s$, $\eta_0 = 3.7\ Pa * s$ (viscosity at low shear rates), $k = 10^{-14}s$, $a = 0.07$ (rate index) and power law index $n = 2$.

Both fits are shown in Figure S6.

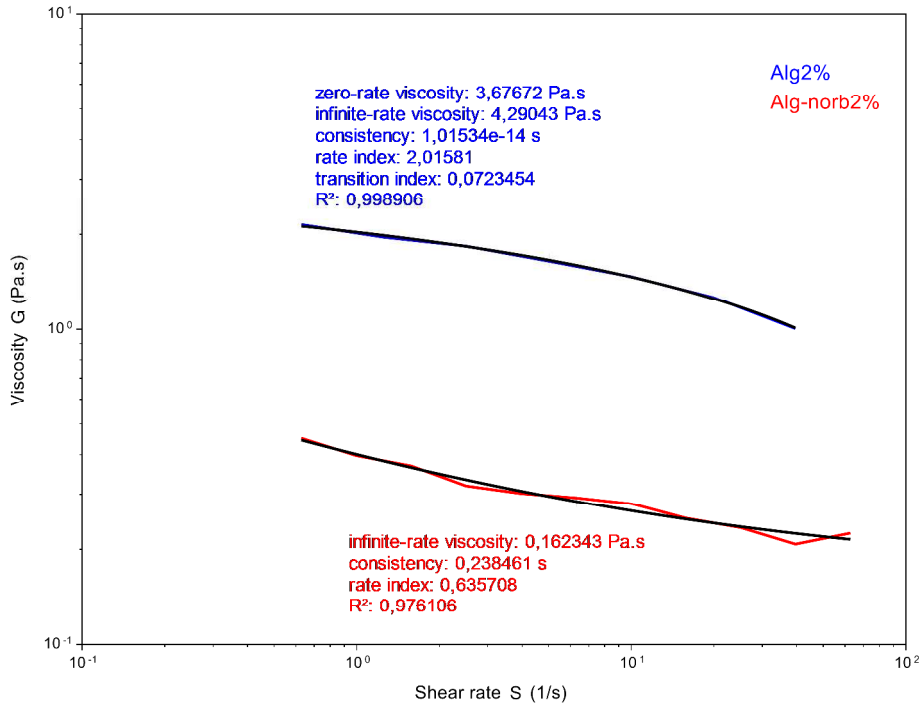


Figure S6. Complex viscosity of the two solutions (Alg-norb 2 w/v% = red, Alginate 2 w/v% = blue) under evaluation. Next to each curve, the parameters of the best fitting flow model. In black, the result of the fitting.

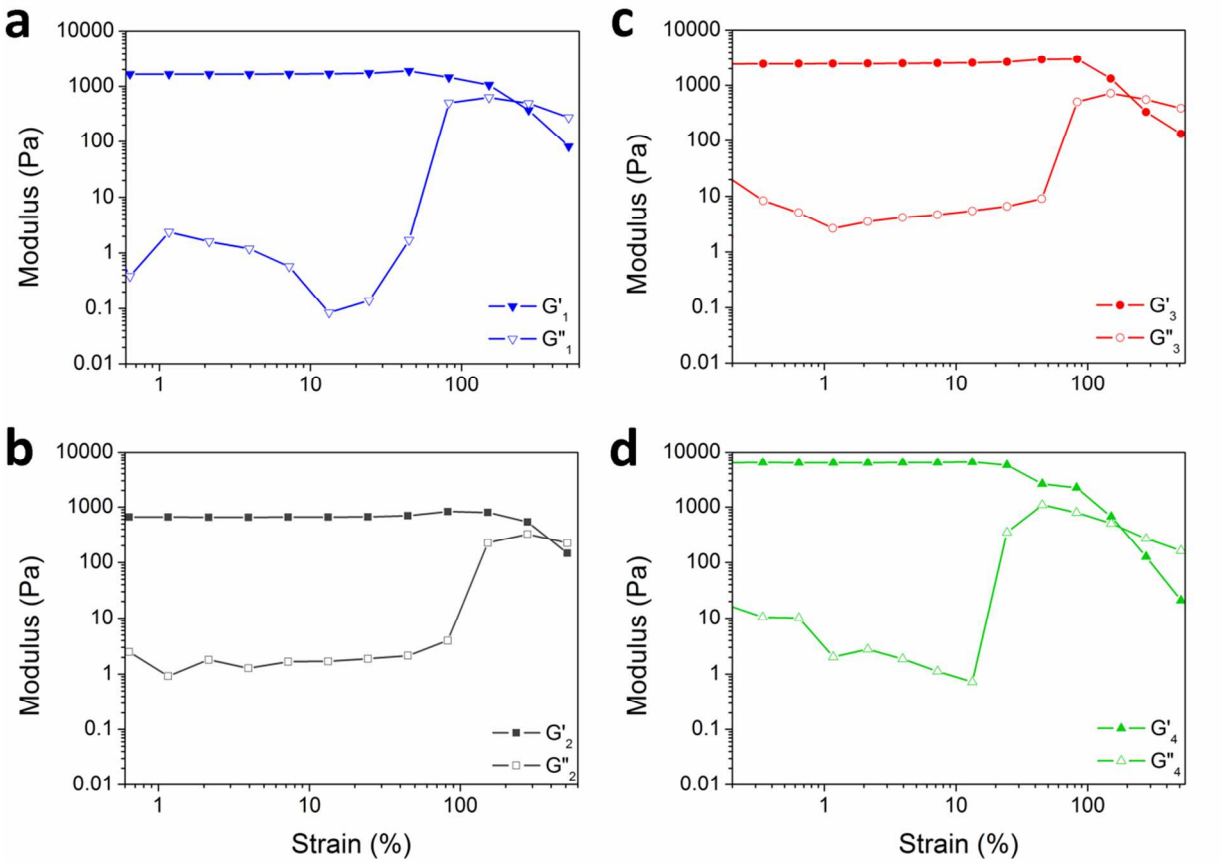


Figure S7. Strain sweep curves of alg-norb (11 mol % functionalized) hydrogel crosslinked with different PEG crosslinkers (**a**, 1 = Alg-norb10-1; **b,** 2 = Alg-norb10-2; **c**, 3 = Alg-norb10-3; **d,** 4 = Alg-norb10-4) in PBS. It should be noted that we have found within these samples with a large tan δ (the ratio of G' to G"), that often the G" approaches the limits of the rheometer and becomes noisy.[3] Measurements carried out in duplo – values reported is one example.

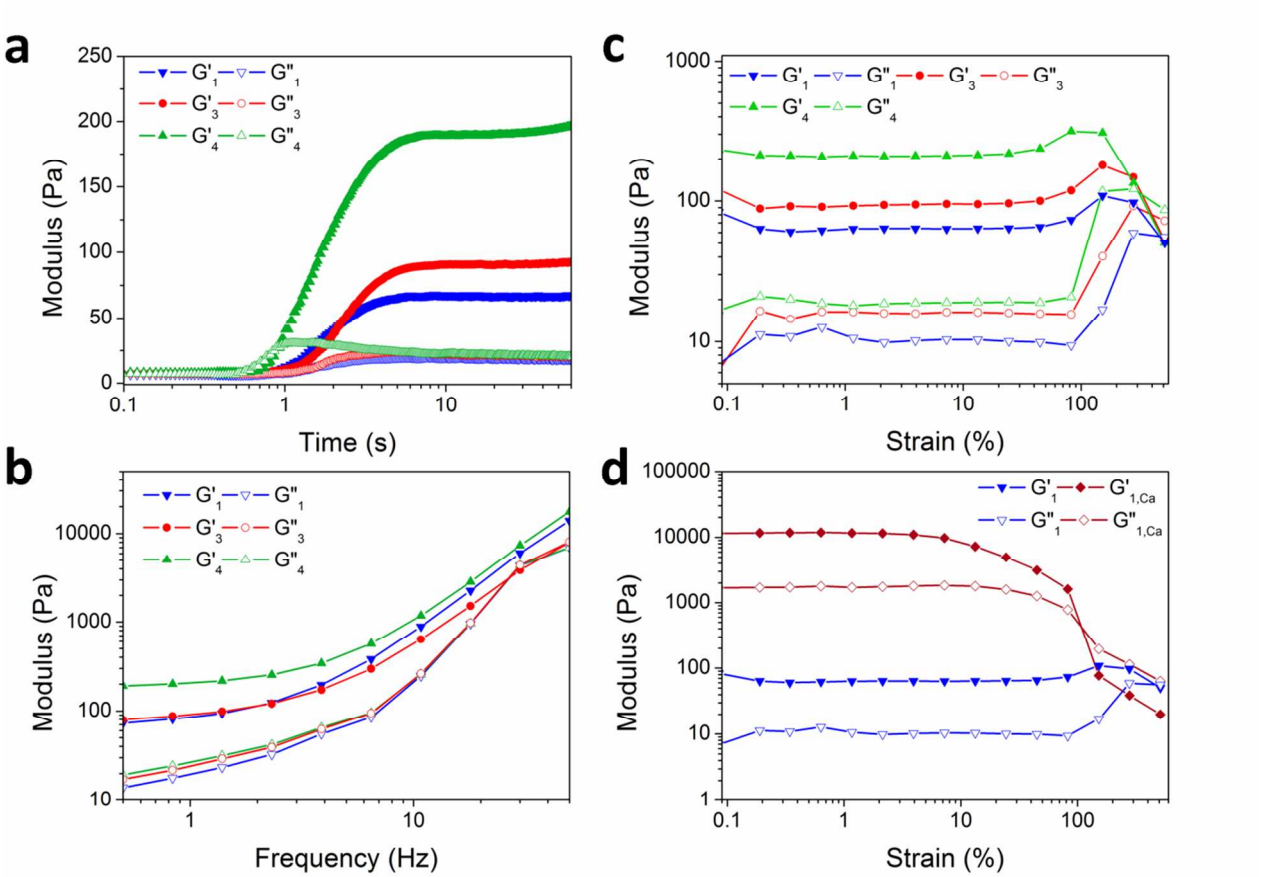


Figure S8. Rheology data for Alg-norb (0.24 mol % functionalized) with different PEG crosslinkers (1 = PEG dithiol 1500, 3 = PEG dithiol 5000, 4 = 4-arm PEG thiol 5000) in PBS. a) *In situ* photorheometry measurements (1 W/cm^2). b) Strain sweep curves at 1 Hz. c) Frequency sweep curves at 1 % strain. d) Strain sweep of Alg-norb (0.24 mol % functionalized) hydrogel crosslinked with PEG dithiol 1500 before (blue lines) and after addition of 100 mM $CaCl_2$ for 3 minutes (red lines).

Table S1. Estimated average mesh sizes and crosslink density for Alg-norb hydrogels (0.24 and 11 mol % norbornene) with different PEG crosslinkers

Crosslinker	Ratio of SH:norbornene	mol % crosslinker	G' (Pa)	G" (Pa)	G* (Pa)	*ρ_x (mol/m^3)	**ξ_a (nm)
Alg-norb (11 mol %)							
PEG dithiol 1500	0.9:1	10	1628	4	1628	0.67	16.8
PEG dithiol 1500	0.5:1	5	659	5	659	0.27	22.72
PEG dithiol 5000	0.9:1	10	2475	7	2475	1.02	14.61
4arm PEG 5000	0.9:1	10	6384	6	6384	2.62	10.66
PEG dithiol 1500	1.4:1	15	1585	4	1585	0.65	16.96
PEG dithiol 1500 and $CaCl_2$	0.9:1	10	6028	851	6087	2.5	10.83
Alg-norb (0.24 mol %)							
PEG dithiol 1500	42:1	10	85	25	88	0.036	44.3
PEG dithiol 5000	42:1	10	90	15	91	0.037	43.9
4arm PEG 5000	42:1	10	200	20	201	0.083	33.7
PEG dithiol 1500 and $CaCl_2$	42:1	10	11800	1800	11936	4.900	8.7

*Crosslink density (ρ_x) was calculated based on $\rho_x = \frac{G}{R_{gas}T}$ where G is the shear modulus of the hydrogel, R_{gas} is the universal gas constant, and T is the absolute temperature.

**Average mesh size (ξ_a) was calculated based on $\xi_a = \left(\frac{6}{\pi \rho_x N_A}\right)^{\frac{1}{3}}$ where N_A is Avogadro's constant.

Swelling experiment of hydrogel slab in cell culture medium

Stock solutions in PBS of 2.5 wt.% Alg-norb, 169.95 mM LAP, and 100 mM of PEG dithiol 1500 crosslinker were prepared. Alg-norb solutions with the different crosslinkers were then prepared by mixing the stock solutions homogeneously in Eppendorf tubes at appropriate dilution ratios. Final concentrations of Alg-norb and LAP were 2 wt. % and 2 mM, respectively. 80 μL of polymer solution was added to each mold (n = 5) and hydrogels were prepared at 10 mW/cm^2 for 60 s using a 365 nm LED. Masses of gels were measured immediately after exposure to light. Gels were transferred to a 6-well plate and incubated in 2 mL of DMEM at 37 °C. The masses of the gels were measured at different times until constant mass. The masses are recorded in Table S2 below. The mass swelling ratio was estimated as 1.6 derived from the mass ratio of the hydrogels at equilibrium to the gels after exposure to light.

Table S2. Mass of Alg-norb hydrogel crosslinked with PEG dithiol 1500 incubated in DMEM at 37 °C over time

Time (h)	H1	H2	H3	H4	H5
0	50.10	75.00	75.30	76.30	76.90
5	74.70	102.0	109.0	108.0	116.1
24	82.00	117.5	121.4	128.0	125.3
47	82.10	118.0	123.3	127.9	126.4

Bioprinting and biocompatibility of Alg-norb

To determine the suitability of our alginate bioink platform for extrusion based bioprinting, we adopted a number of strategies in attempt to produce stable and well-defined structures. For example, scaffolds were bioprinted on non-treated tissue culture plates in air, into baths of cell culture medium, gelatin or nanocellulose solutions. After numerous attempts with alteration of deposition and pressure parameters, scaffolds bioprinted in air still did not show very promising results as the hydrogels coalesced after extruding and subsequent layers were typically disrupted during deposition due to the lack of contact points with the previous layer. Therefore, addition of modifiers like nanocellulose and high molecular weight polyethylene oxide (900 kDa) to Alg-norb was attempted in efforts to increase the viscosity of the polymer solution and to improve the fibers deposition during bioprinting. While these viscosity modifiers did show better results, the structures produced were not as defined as the ones bioprinted in cell culture medium, gelatin or nanocellulose baths. In these baths, bioprinted constructs showed relatively well-defined pore structures. However, in nanocellulose bath, the nanocellulose fibers were difficult to wash away and could not be separated from the scaffolds without compromising its shape. Gelatin solution could potentially be used as a bioprinting bath, but gelatin is in solid form at ambient temperature and has to be kept warm during the bioprinting process. Examples of bioprinted structures with modifiers or in baths are included in Figure S9 and S10 in the Supporting Information.

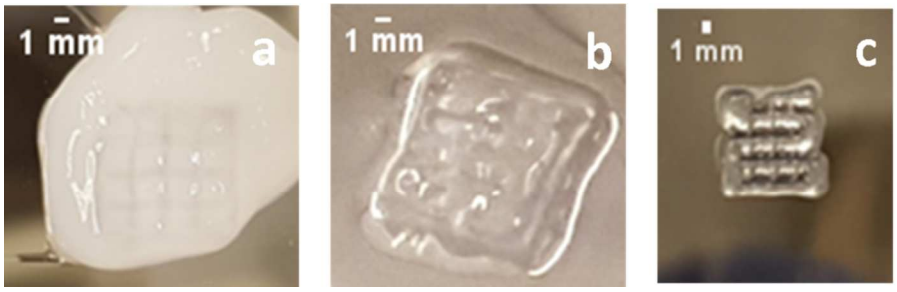


Figure S9. Alg-norb (3 w/v%) crosslinked with PEG dithiol 1500 (10 mol %) in different conditions: a) in nanocellulose solution (2.5 wt. %), b) in gelatin, c) mixed with 2.5 wt. % nanocellulose.

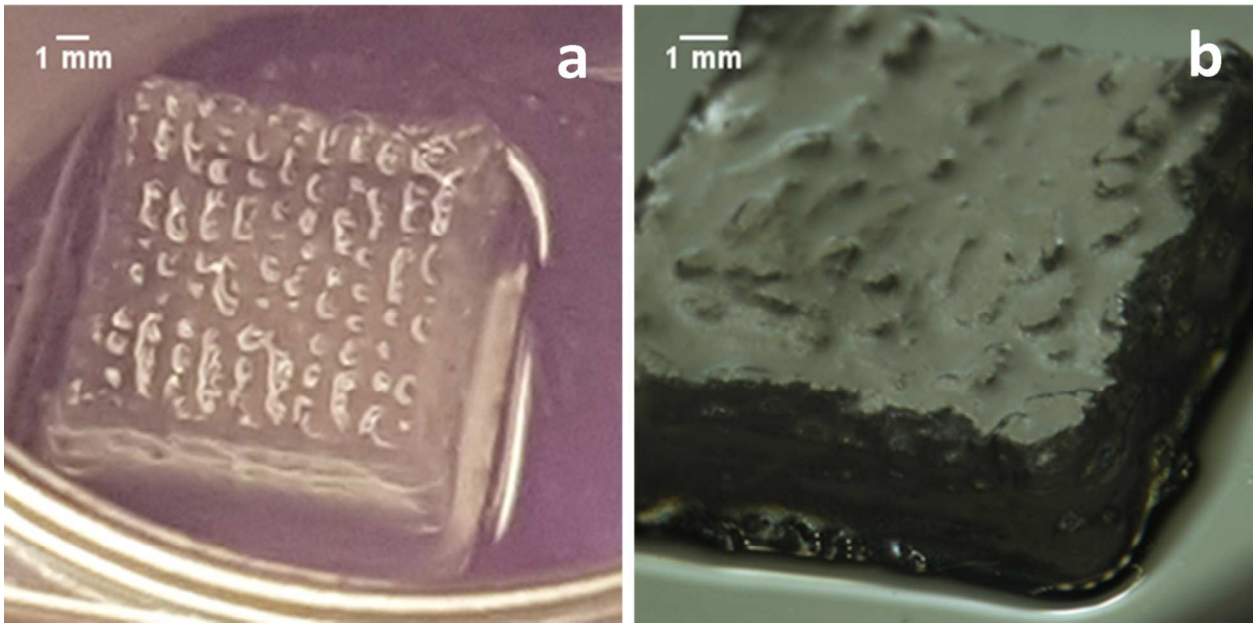


Figure S10. 25 layers of Alg-norb (2 w/v%) crosslinked with PEG dithiol 1500 (10 mol %) bioprinted in DMEM without phenol red at 35 kPa with an extrusion velocity of 5 mm/s. Image of the same scaffold in a) in DMEM and b) air.

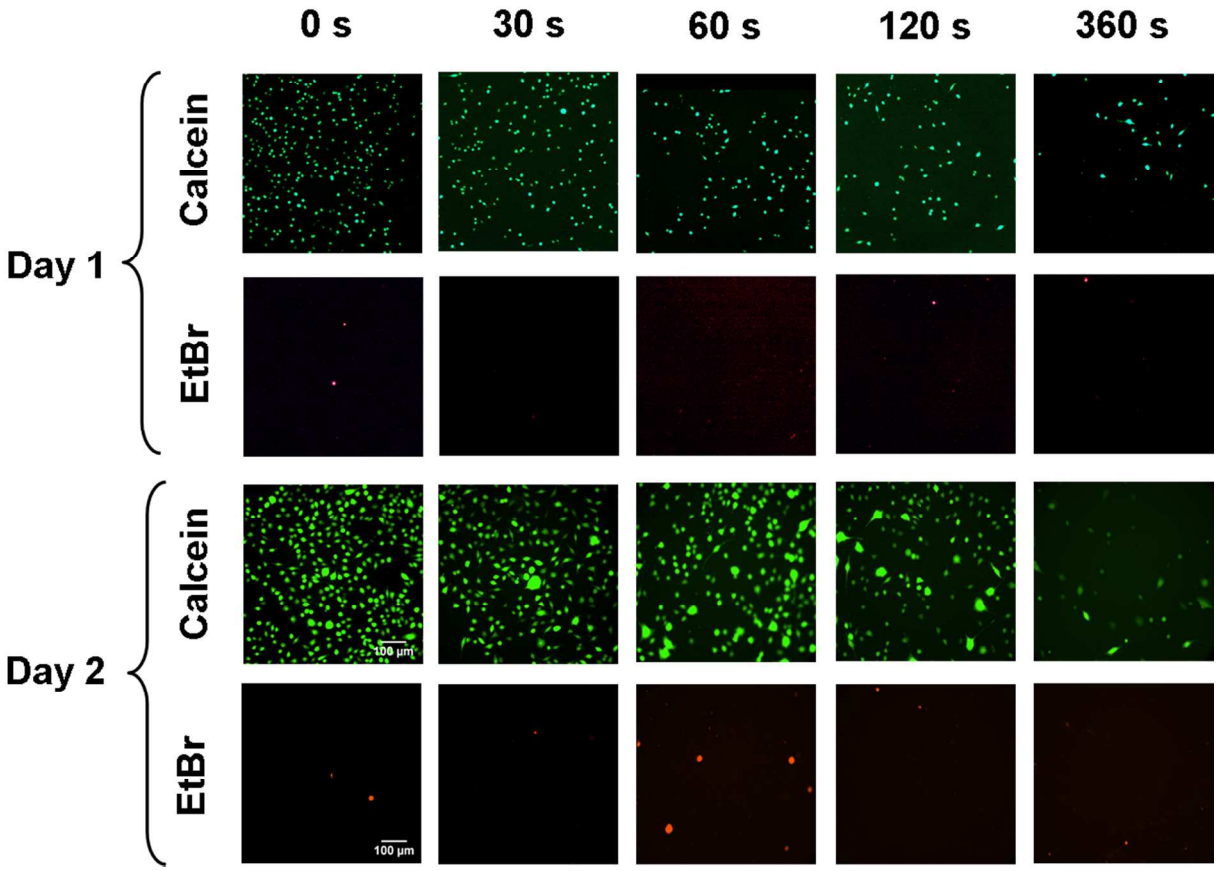


Figure S11. L929 viability assay on tissue culture plates at different irradiation times at 365 nm (10 mW/cm^2) for day 1 and 2. Live cells stained by calcein (green) and dead cells stained by ethidium bromide (red).

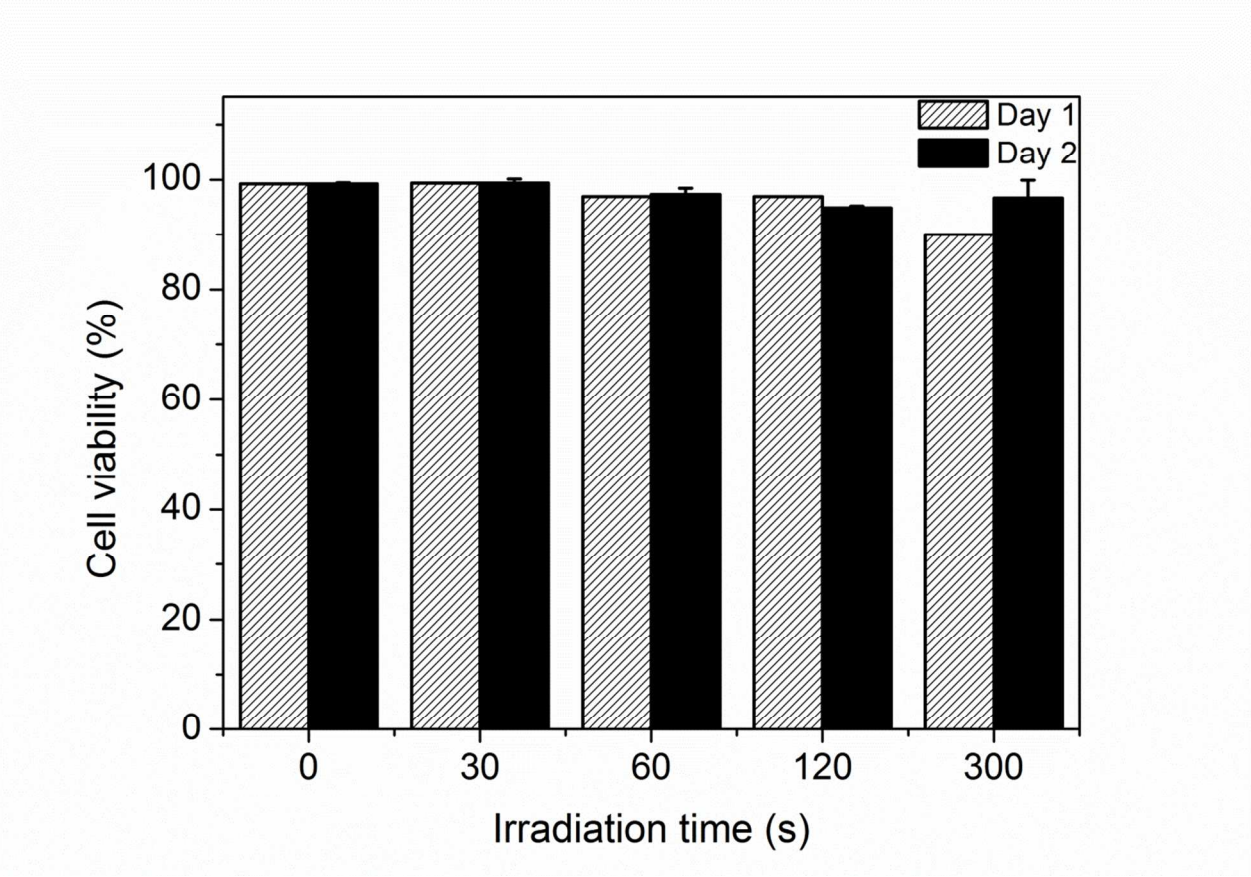


Figure S12. Quantification of viability (number of live cells/total cells) of L929 at on tissue culture plates at different irradiation times at 365 nm (10 mW/cm^2) for day 1 and 2. Day 1 data consists of a single measurement, while Day 2 was measured in triplo. Error bars (standard deviation) are shown for day 2.

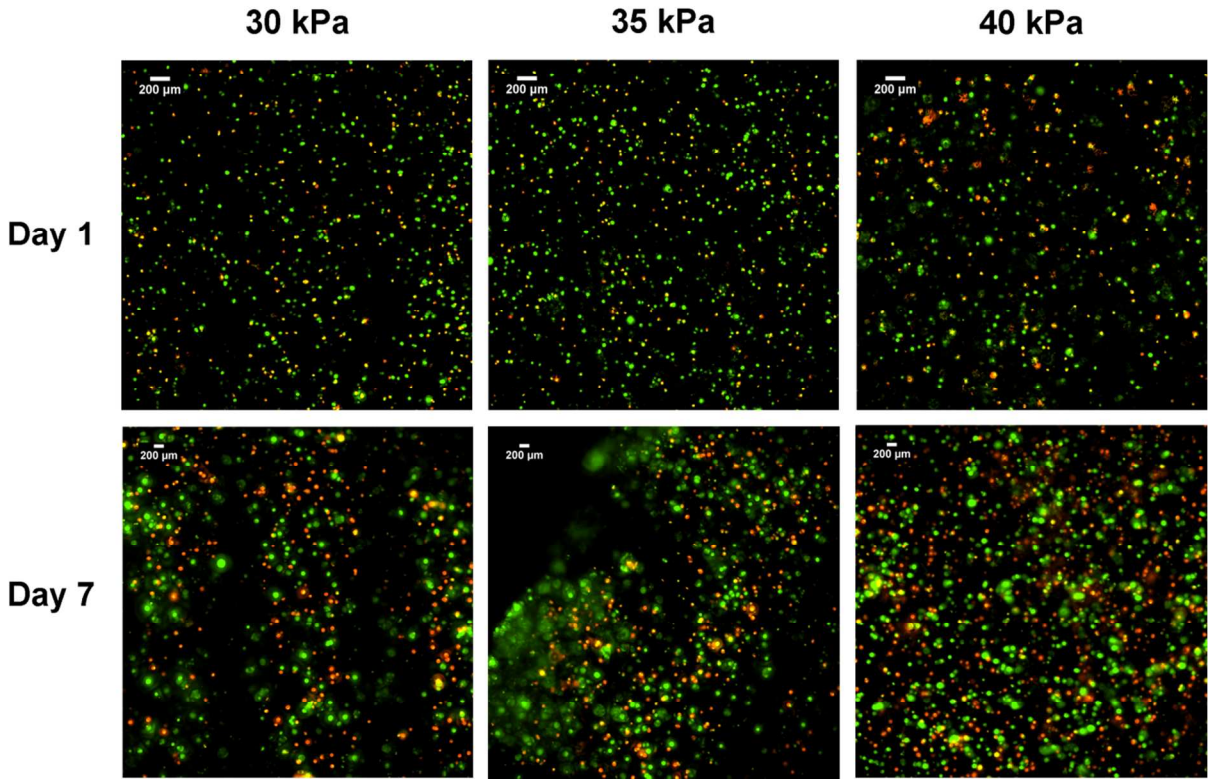


Figure S13. Cell viability of scaffolds produced by 2 w/v % alg-norb L929-laden hydrogels crosslinked with PEG dithiol 1500 (10 %). Scaffolds with 2 layers and extruded at a velocity of 5 mm/s.

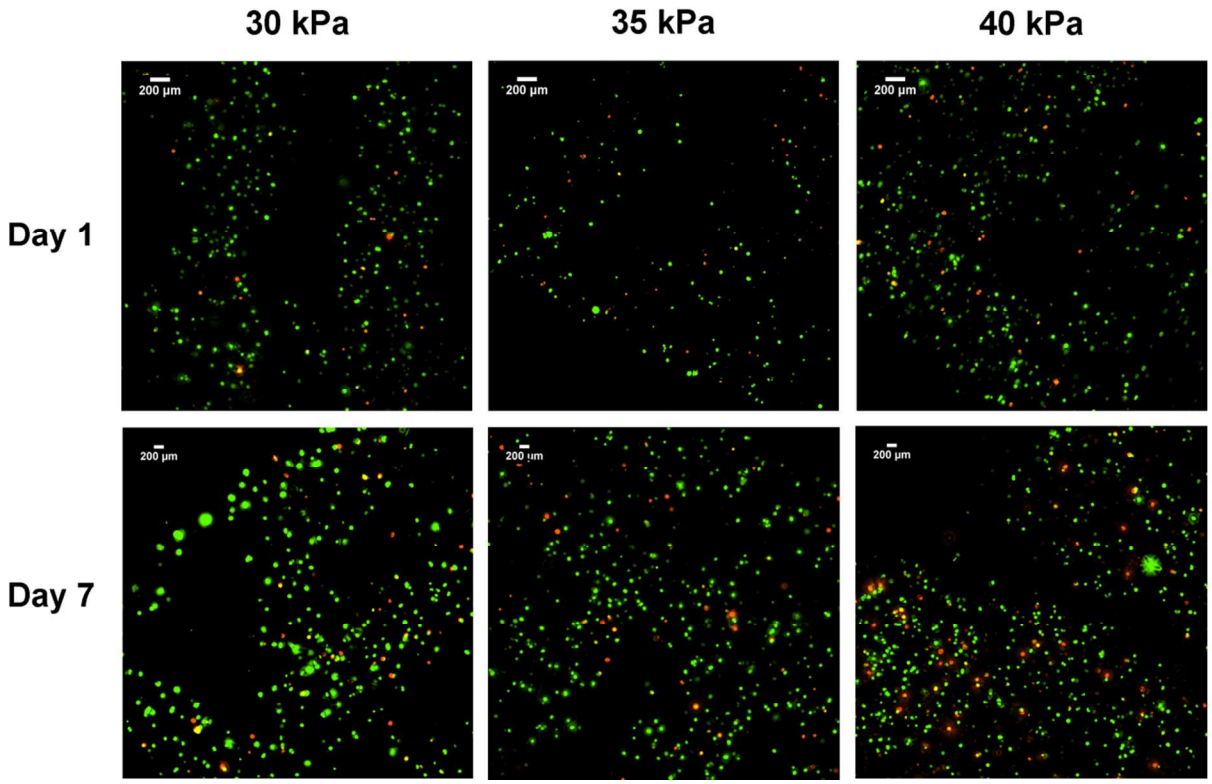


Figure S14. Cell viability of scaffolds produced by 2 w/v % alg-norb ATDC5-laden hydrogels crosslinked with PEG dithiol 1500 (10 mol %). Scaffolds with 2 layers and extruded at a velocity of 5 mm/s.

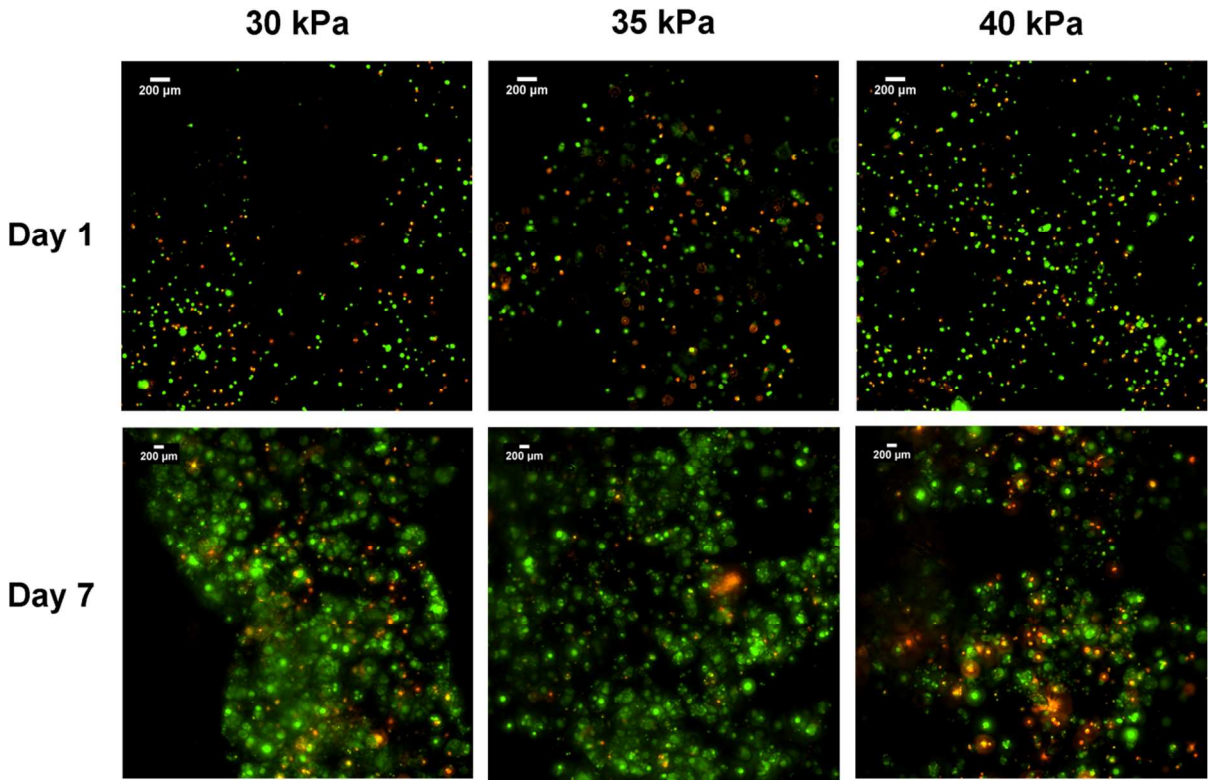


Figure S15. Cell viability of scaffolds produced by 2 w/v % Alg-norb L929-laden hydrogels crosslinked with PEG dithiol 1500 (10 mol %). Scaffolds with 2 layers and extruded at a velocity of 10 mm/s.

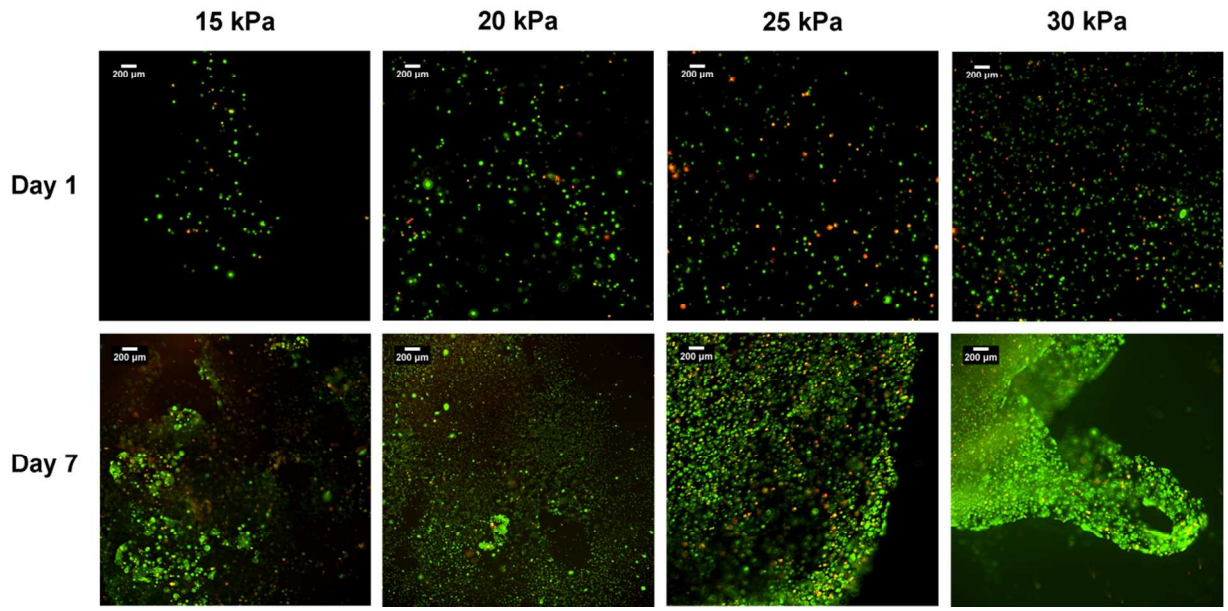


Figure S16. Cell viability of scaffolds produced by 1 % alg-norb L929-laden hydrogels crosslinked with PEG dithiol 1500 (10 mol %). Scaffolds with 2 layers and extruded at a velocity of 10 mm/s.

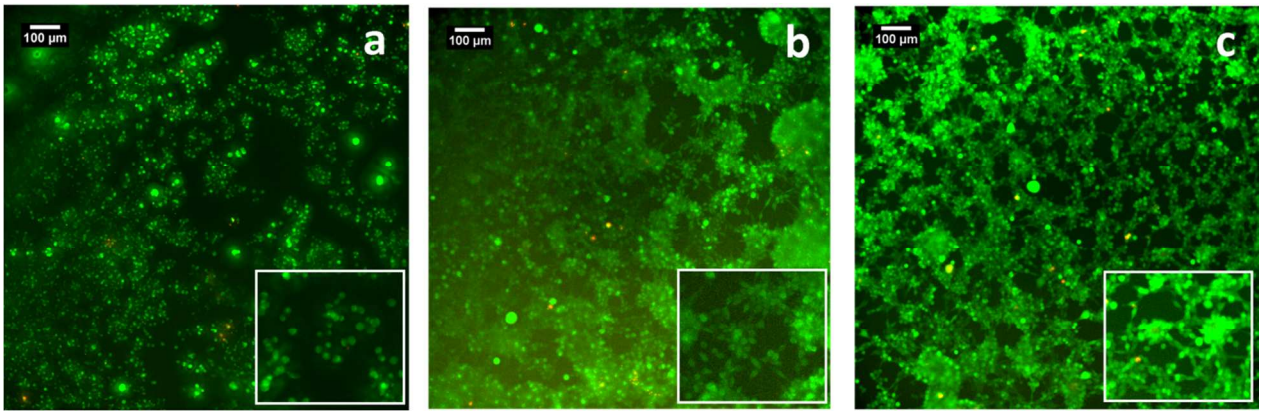


Figure S17. Day 7 of hydrogels slabs crosslinked with PEG dithiol 1500 (10 mol %) with different amounts of RGD: a) 0 mM, b) 0.2 mM, and c) 2 mM. Live/dead assay of L929 seeded on the surfaces of hydrogel slabs.

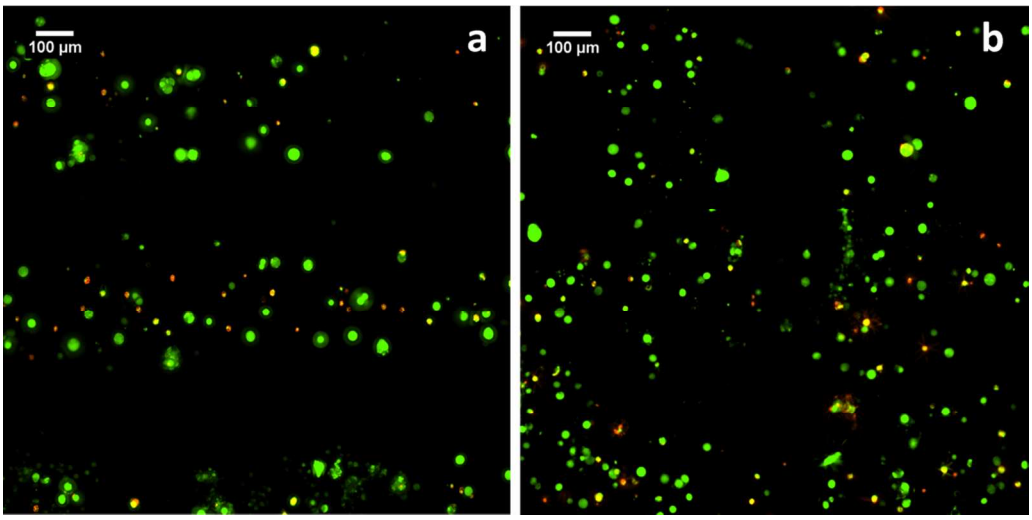


Figure S18. Day 7 of hydrogels crosslinked with PEG dithiol 1500 (10 %) with a) 0 mM and b) 0.2 mM RGD encapsulated with L929 cells.

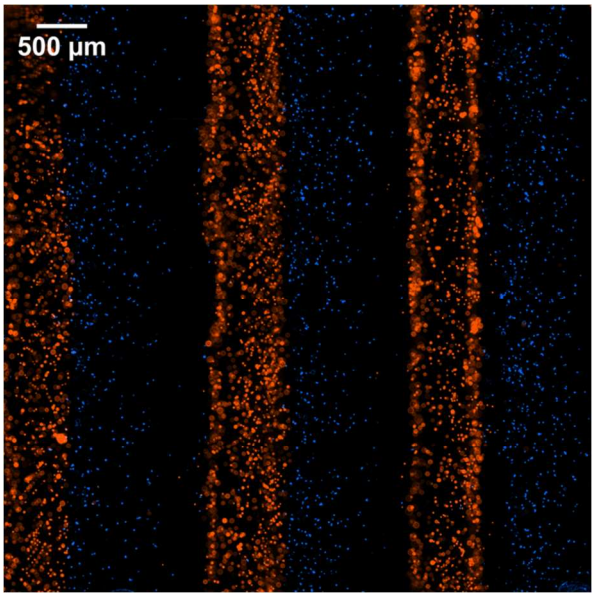


Figure S19. Red cell tracker labeled L929 encapsulated in Alg-norb10-1 and blue cell tracker labeled ATDC5 encapsulated in Alg-norb10-4 bioprinted as alternating fibers in the X-Y plane.

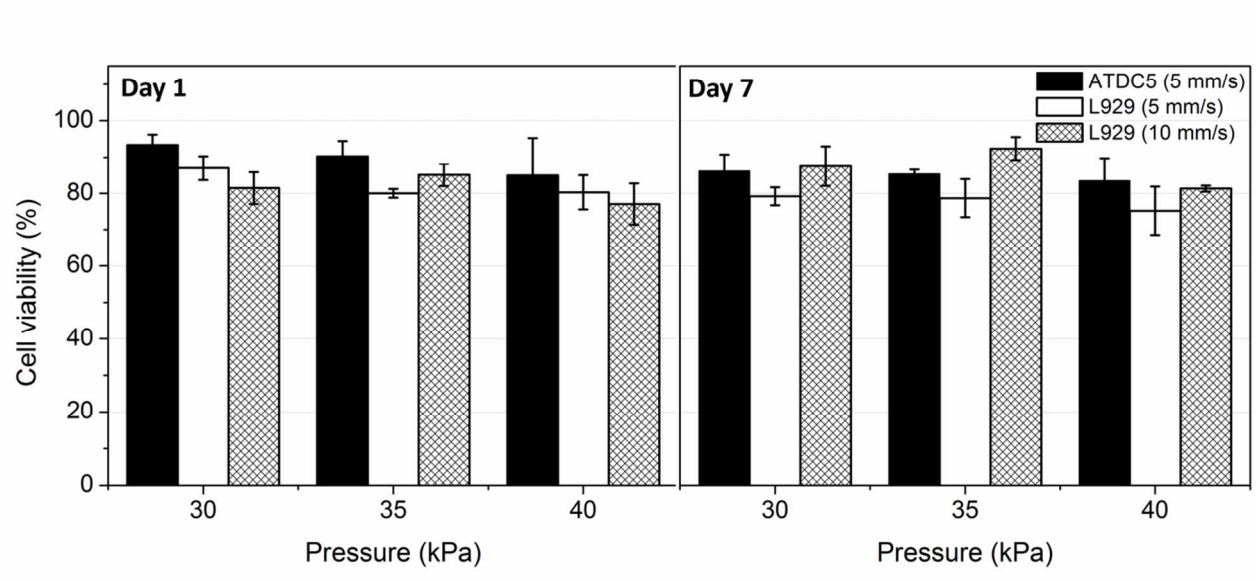


Figure S20. Quantification of viability (number of live cells/total cells) of ATDC5 and L929 in 2 w/v % Alg-norb laden hydrogels crosslinked with PEG dithiol 1500 (10 mol %) at day 1 and 7. Scaffolds were 2 layers extruded at different pressures (30, 35, and 40 kPa). L929-laden hydrogels were bioprinted at 5 mm/s and 10 mm/s (n = 3). These results have been quantified via image analysis of flattened Z-stacks. We advise that the trends be taken as representative and not absolute.

References

(1) Grasdalen, H. High-Field, 1H-N.M.R. Spectroscopy of Alginate: Sequential Structure and Linkage Conformations. *Carbohydr. Res.* **1983**, *118*, 255–260.

(2) Penman, A.; Sanderson, G. R. A Method for the Determination of Uronic Acid Sequence in Alginates. *Carbohydr. Res.* **1972**, *25* (2), 273–282.

(3) Zuidema, J. M.; Rivet, C. J.; Gilbert, R. J.; Morrison, F. A. A Protocol for Rheological Characterization of Hydrogels for Tissue Engineering Strategies. *J. Biomed. Mater. Res. - Part B Appl. Biomater.* **2014**, *102* (5), 1063–1073.