Supporting information

The potential of a carbohydrate supramolecular hydrogel for long-term 3D culture of primary fibroblasts

Nadia Kasmi[a], Laetitia Pieruccioni[b], Eve Pitot[c], Isabelle Fourquaux[d], Alexandre Wodrinski[a], Laure Gibot[a], Juliette Fitremann[a]

(a) Laboratoire Softmat, Université de Toulouse, CNRS UMR 5623, Université Toulouse III – Paul Sabatier, Toulouse, France
(b) RESTORE Research Center, Université de Toulouse, INSERM 1301, CNRS 5070, EFS, ENVT, Toulouse, France
(c) Cytometry and Imaging Core facility, Institute of Pharmacology and Structural Biology (IPBS), University of Toulouse, CNRS, Toulouse, France
(d) Centre de Microscopie Electronique Appliquée à la Biologie (CMEAB), Faculté de Médecine Rangueil, Université de Toulouse III - Paul Sabatier, Toulouse, France

SI-1. HFIP monitoring by ^{19}F NMR

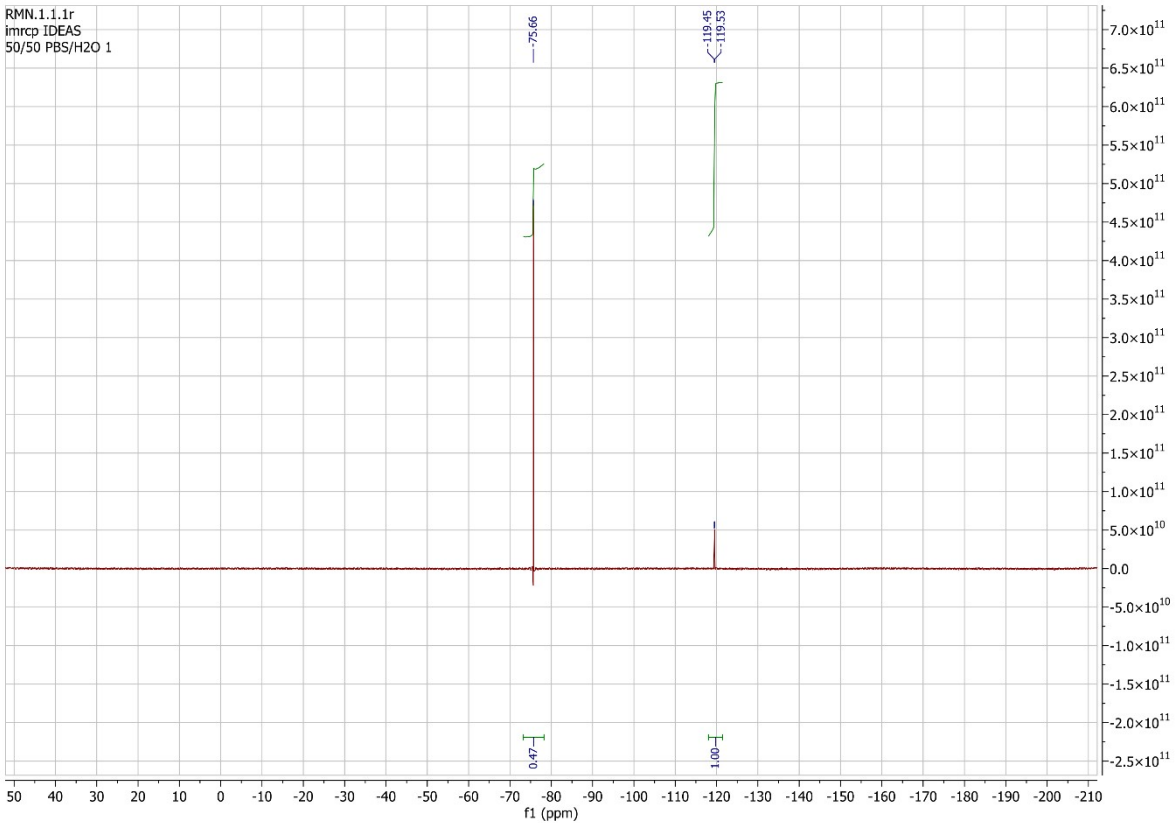


Fig SI-1.1. ^{19}F NMR spectrum of the PBS/H_2O 50/50 supernatant after the first step of washing in 500 mL of PBS/H_2O 50/50. The chemical shift of 1,1,1,2,2,2-hexafluoro-2-propanol, HFIP in ^{19}F NMR is -75.6 ppm. The one of caesium fluoride (CsF) is -119.5 ppm.

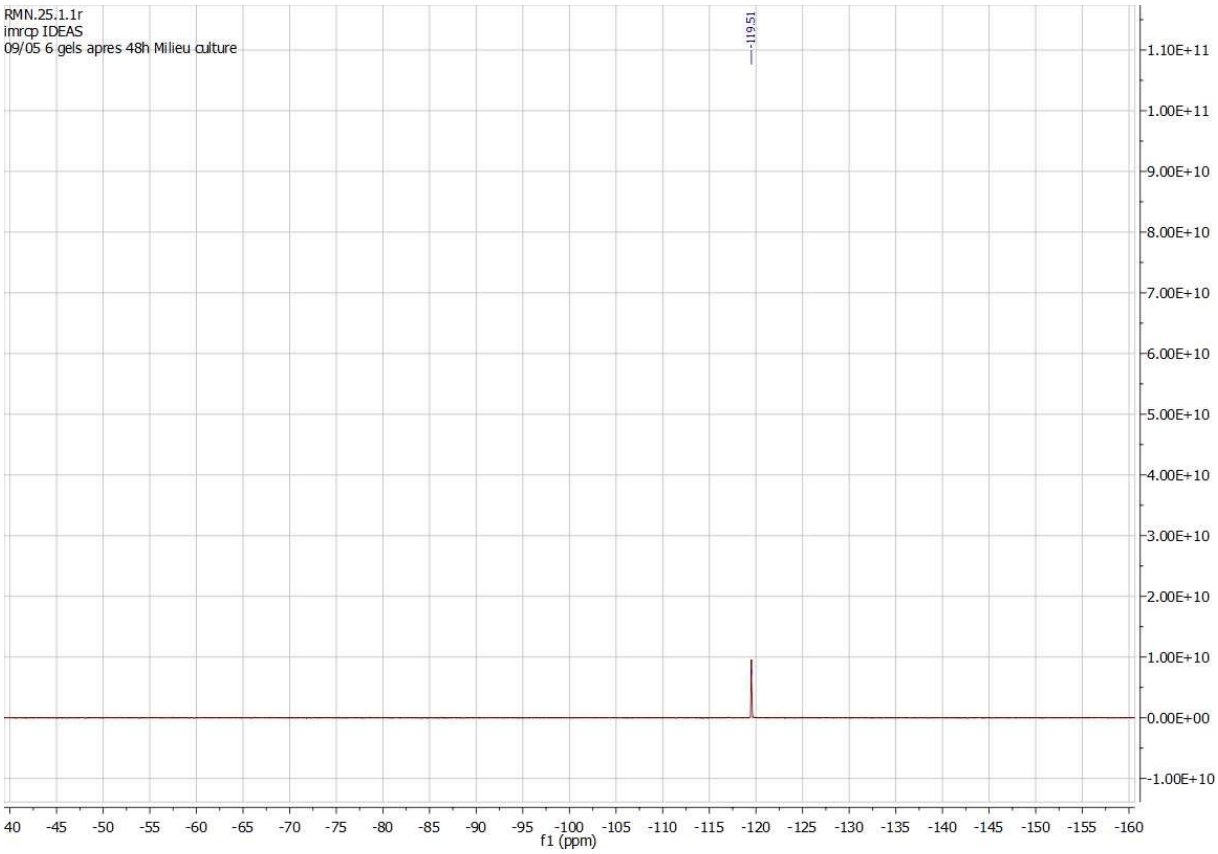


Fig SI-1.2. ^{19}F NMR spectrum of the culture medium supernatant just before seeding. (HFIP: -75 ppm. CsF: -120 ppm). HFIP is no longer detected.

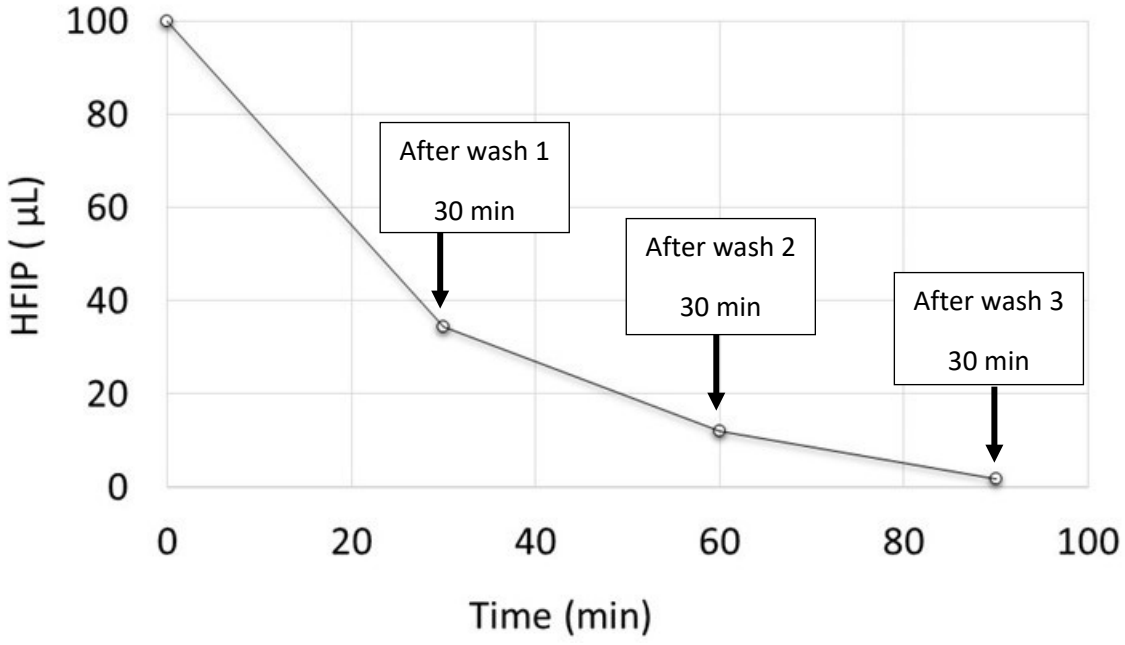


Fig SI-1.3. Example of HFIP decrease with successive washings (example with 1 gel of 500 µL prepared in 400 µL H_2O + 100 µL HFIP and washed in 10 mL of PBS/H_2O 50/50 once, then twice in 10 mL of PBS 100%). 400 µL are taken off the PBS solution and measured by ^{19}F NMR with CsF as an internal standard.

SI-2. Permeation test through the gel by diffusion of a colored indicator

In order to determine the order of magnitude of time needed for rinsing the gels of GalC9 prepared in water/HFIP 80/20, a permeation test with phenol red has been carried out (see Exp. Part section 6). A complete coloration of the gel is observed after 165 minutes (2h45). The rate of diffusion is around $7.5.10^{-5}$ cm/s ($4.5.10^{-3}$ cm/min). This test showed that rinsing steps of several hours should be used for rinsing the hydrogels thoroughly.

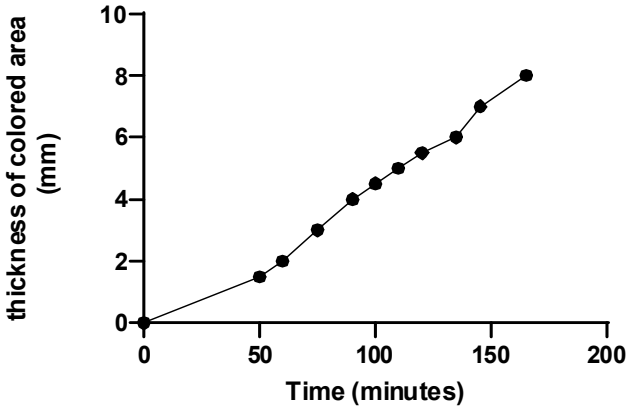


Fig SI-2. time of permeation of phenol red in a gel of GalC9 prepared in water/HFIP 80/20

SI-3. Microstructure of gels prepared in H_2O/HFIP

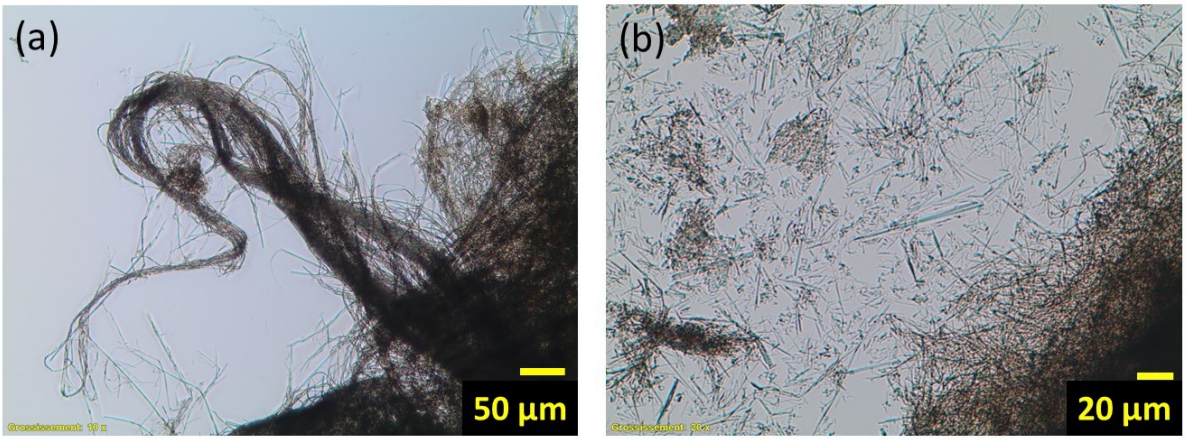


Fig. SI-3.1. Optical microscopy of a GalC9 hydrogel (5 mg/mL, 5wt%) prepared by heating cooling in a mixture H_2O/HFIP (a) H_2O/HFIP: 80/20 ; (b) H_2O/HFIP: 90/10

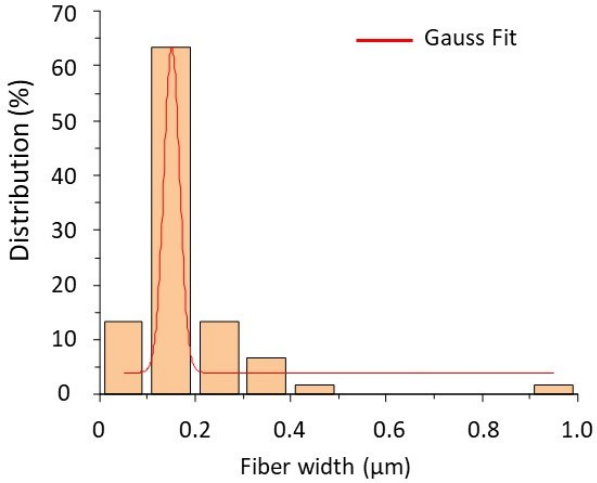


Fig. SI-3.2. Distribution of fibers width observed at the bottom of the gel (gel-polystyrene well interface), by cryo-SEM of unprocessed hydrogels (measured on images acquired with the same method as the ones of Fig. 2a-b).

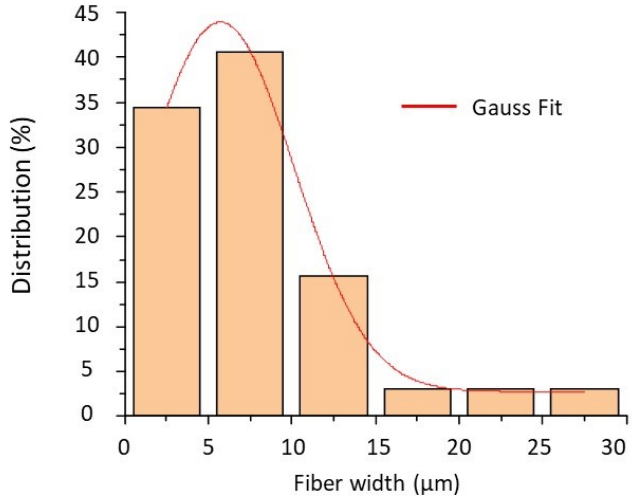


Fig. SI-3.3. Distribution of the width of the very large and short fibers (kind of plates) observed at the top of the gel (gel-air interface), by cryo-SEM of unprocessed hydrogels (measured on images acquired with the same method as the ones of Fig. 2a-b).

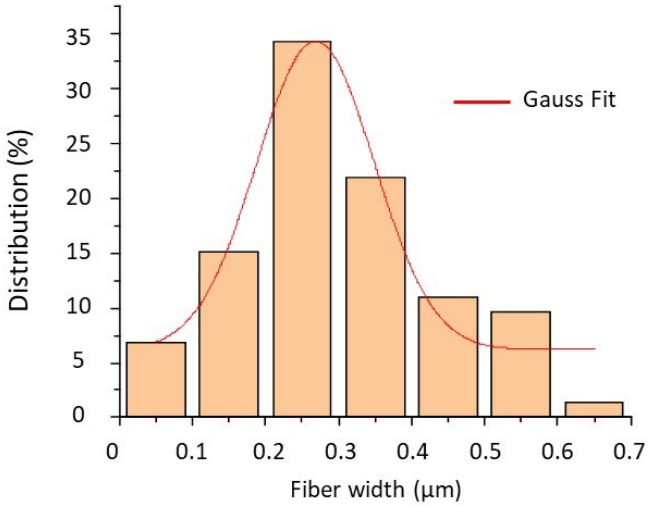


Fig. SI-3.4. Width Distribution of width of the long and thin fibers observed at the top of the gel (gel-air interface), by cryo-SEM of unprocessed hydrogels (measured on images acquired with the same method as the ones of Fig. 2a-b).

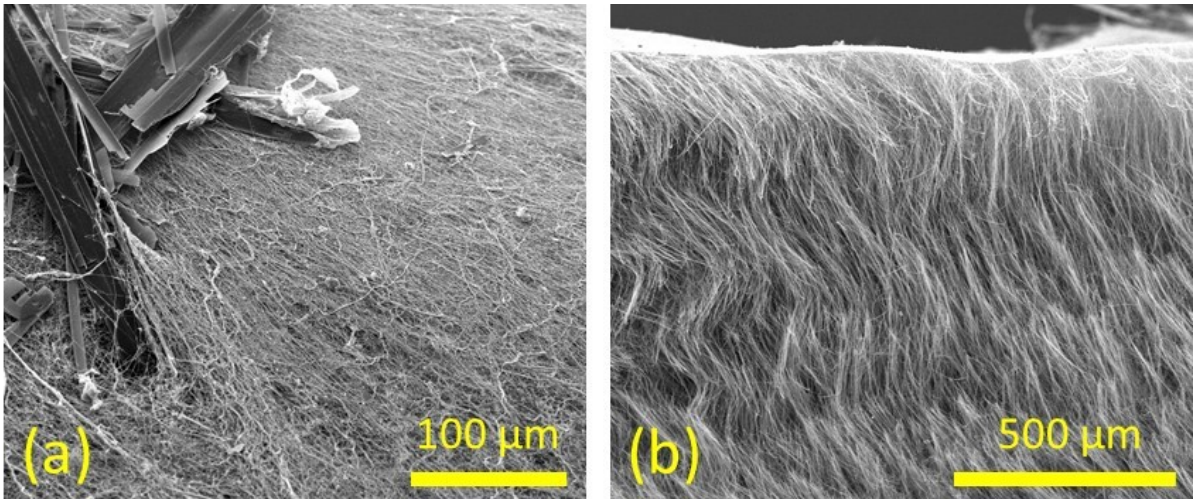


Fig. SI-3.5. (a) Surface of a GalC9 hydrogel without cells prepared from H20-HFIP 80/20, soaked in PBS, and prepared for SEM observation according to protocol 23. The same very large flat fibers are observed by biphoton microscopy with second harmonic generation mode (see SI-9.1). (b) Section of the same hydrogel. The section is made with a blade. Fibers are well aligned in this section, which is not observed for all the sections, but only for some of them.

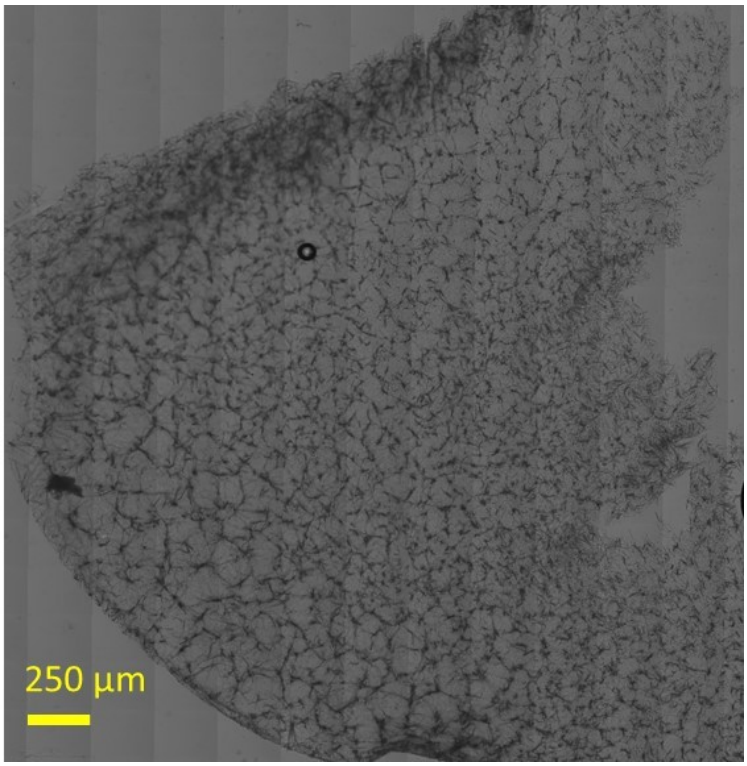


Fig. SI-3.6. Scanning optical microscopy of a horizontal section of the gel without cells (in PBS, gel embedded in agarose, 200 µm thick, white light.

SI-4. Microstructure of GalC9 hydrogels prepared in H_2O only, without HFIP

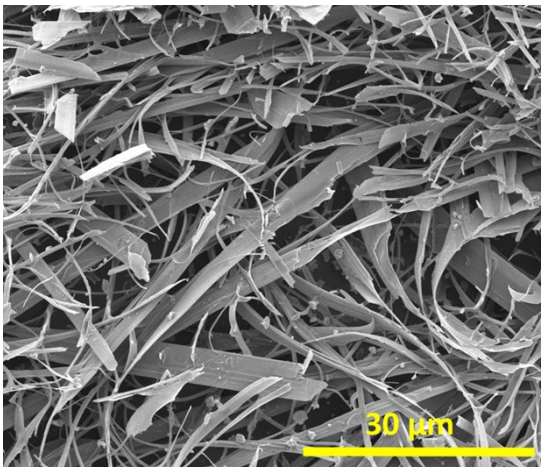


Fig. SI-4.1. Microstructure of GalC9 hydrogels prepared in H2O without HFIP. Cryo-electron microscopy of unprocessed hydrogel.

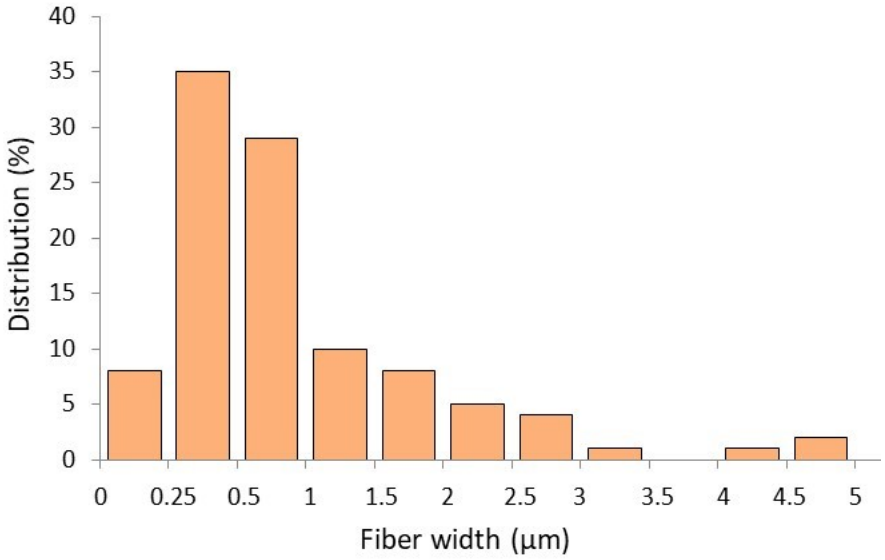


Fig. SI-4.2. Distribution of the fiber width of GalC9 in hydrogels prepared in H2O without HFIP (cryo-SEM of unprocessed hydrogels)

SI-5. Uniaxial compression tests

Compression modulus

Compression rate (mm/s)	Modulus (kPa)	Mean Modulus (kPa)
0,001	47	
0,001	78	
0,001	52	59
0,01	20	
0,01	80	
0,01	36	45
0,1	51	
0,1	43	
0,1	52	49
All rates together	51	51

Table SI-5. Modulus of cylinders of GalC9 hydrogels prepared with H_2O/HFIP (protocol 7 in Exp. Part.) measured by uniaxial unconfined compression tests.

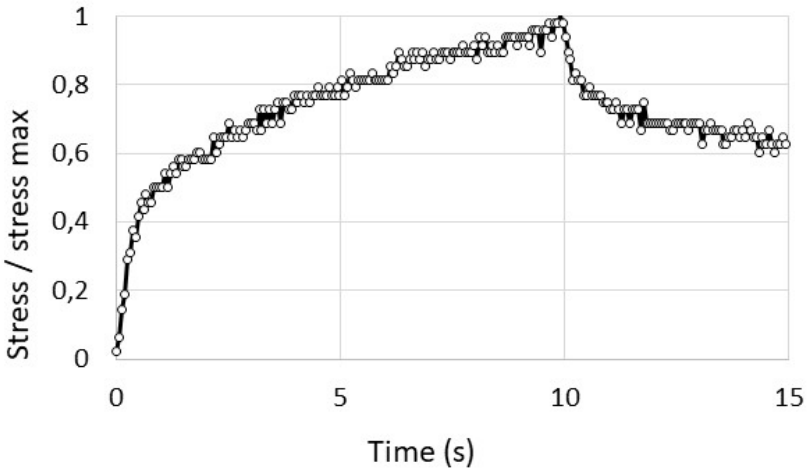


Fig. SI-5. Example of a relaxation curve of a GalC9 hydrogel cylinder prepared with H_2O/HFIP (protocol 7 in Exp. Part.) measured by uniaxial unconfined compression tests (compression to 1 mm at 0.1 mm/s, duration 10 s).

SI-6. Fluorescent macroscope at D1 and D22

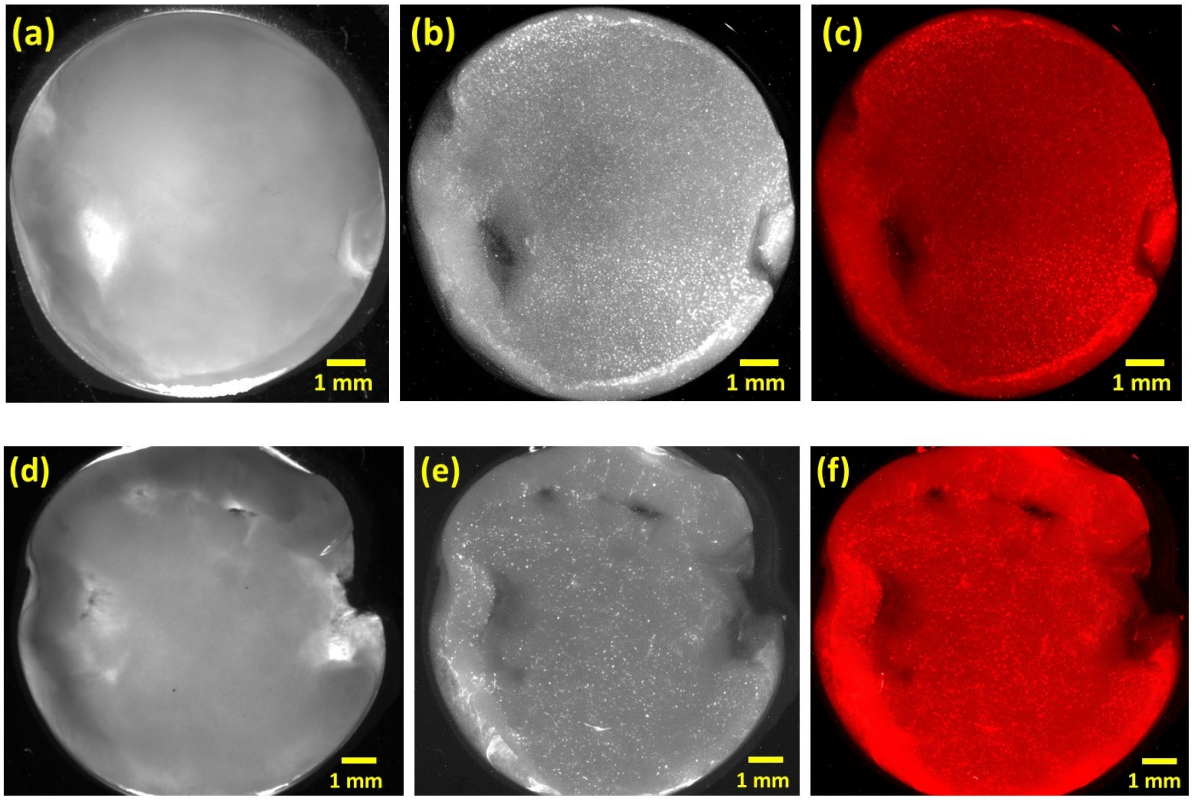


Fig. SI-6.1. Overview of the cells grown at the surface of the gel (top view) at low magnification (MacroFluo). (a-b-c) = 24h (D1) post seeding. (d-e-f)= 22 days post-seeding (D22). (a,d) bright field; (b,e) fluorescence signal; (c,f) same as (b,e) with fluorescence channel set in red. Cell staining: DyLight™594-phalloidine (F-actin, red) (and Hoechst for staining the nucleus, blue, but not visible at this magnification). After 24h (a-c), cells are distributed on the whole surface, but the density of cells is higher on the edge of the gel (close to the interface gel- wall of the well). This tendency has been often observed. After 22 days, clusters are visible and are distributed more uniformly throughout the surface of the gel.

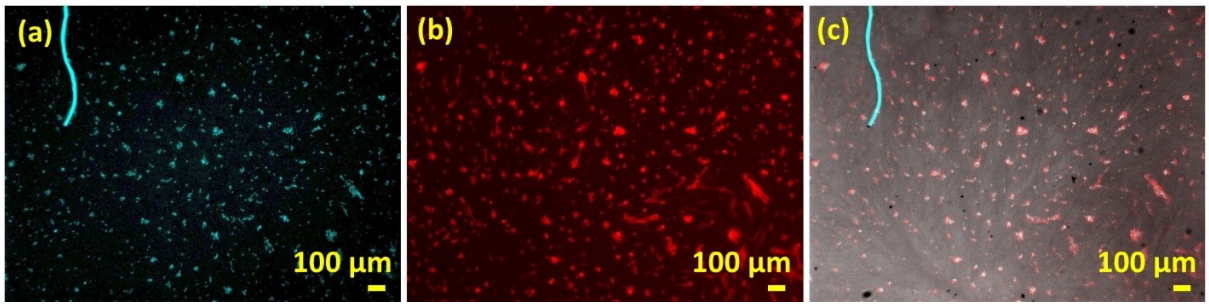
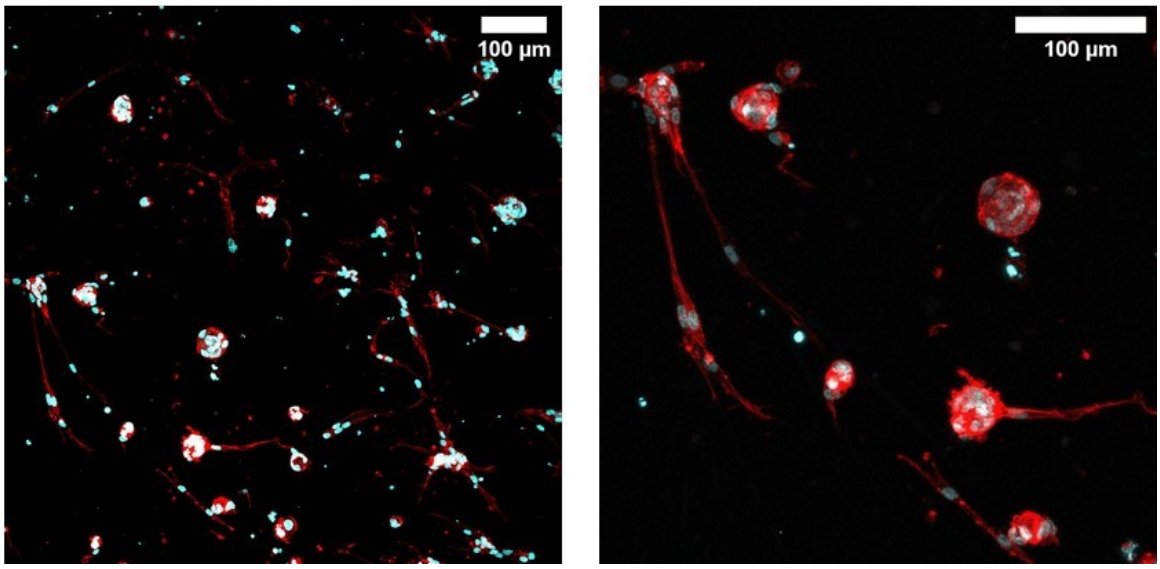


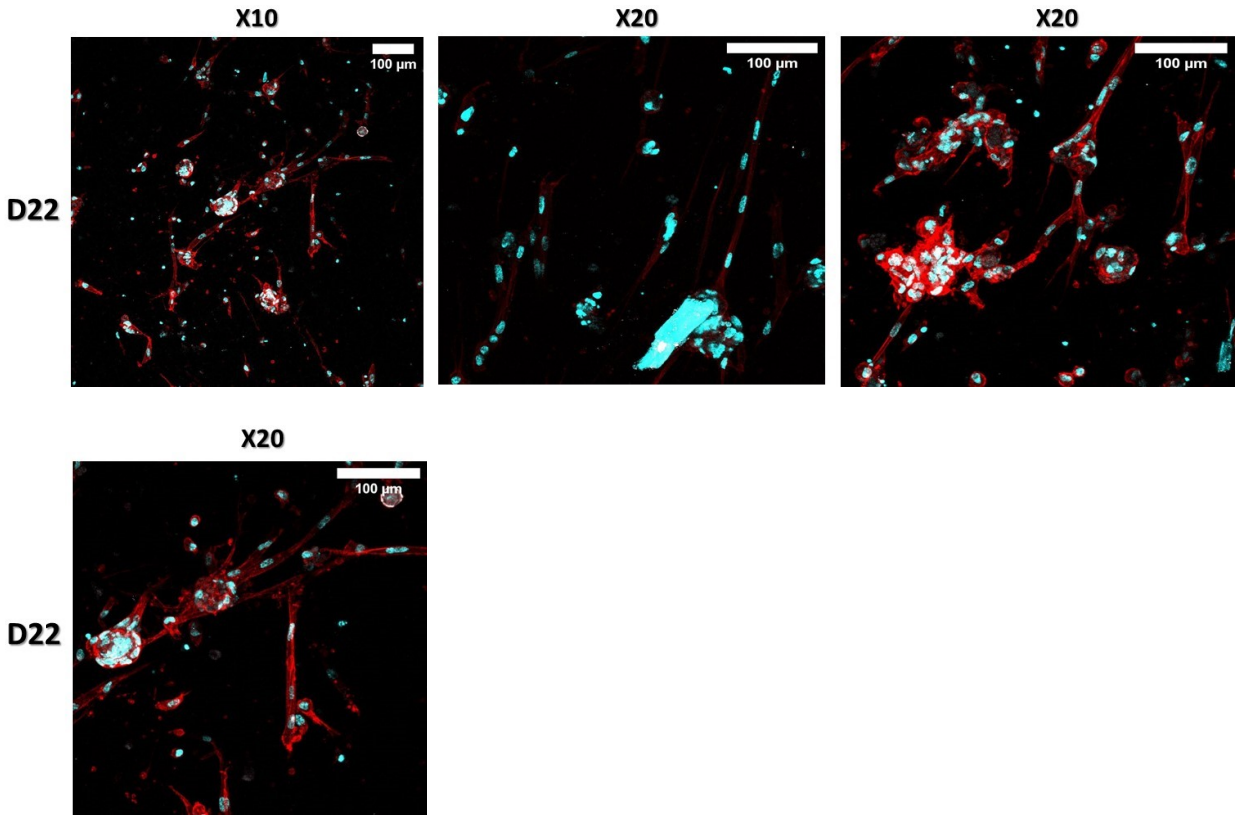
Fig.SI-6.2. Overview of the cells grown at the surface of the gel (top view) at intermediate magnification and 24h (D1) post seeding (MacroFluo). (a) UV: detection of nucleus stained with Hoechst; (b) red emission: detection of actin stained with DyLight™594-phalloidine; (c) bright field: detection of the largest supramolecular fibers.

After 22 days, clusters are visible and are distributed more uniformly throughout the surface of the gel.

SI-7. Two-photon microscopy – Additional images of cell culture at D1 and D22



SI-7.1. *Two-photon microscopy of primary human dermal fibroblasts on GalC9 hydrogels at Day 1 and Day 22. Nuclei are stained in cyan (Hoechst) and actin cytoskeleton in red (phalloidin labelling). In these images it is clearly seen that many cellular extensions issued from cell clusters are in fact chains of single cells (they contains chains of single nuclei).*



SI-7.2. Possible effect of transient drying on adhesion: Two-photon microscopy of primary human dermal fibroblasts on GalC9 hydrogels at Day 22. Just after seeding, the cell culture that has been incidentally let without culture medium on its top for 1 hour instead of 10 minutes at 37°C, to favor adhesion.

SI-8. Additional images of cell culture at D22 – Scanning Electron Microscopy of surfaces and sections

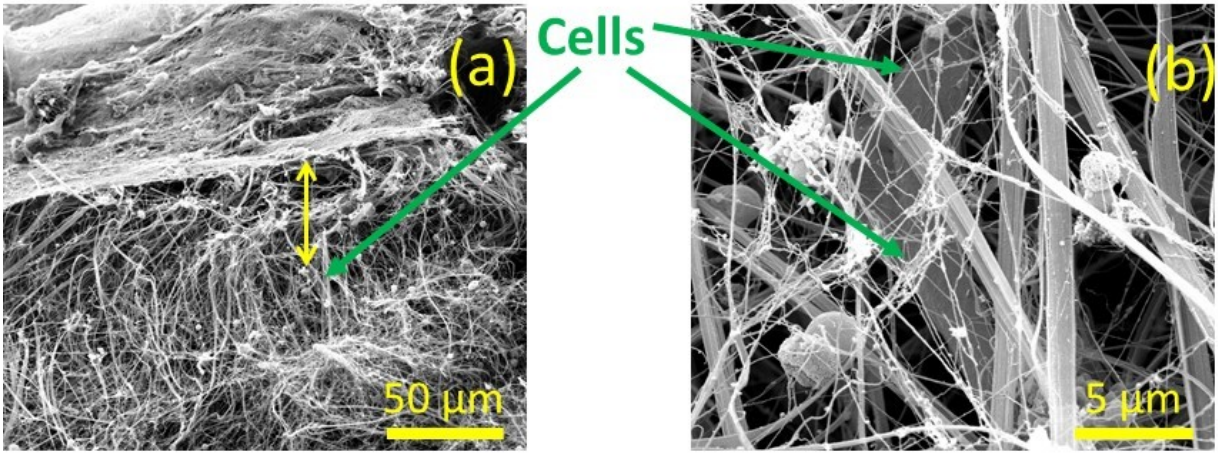


Fig. SI-8.1. Additional images of the upper part of the gel section, showing a cell embedded within the hydrogel fibers at a depth of around 50 µm. (a) overview of the section, where the surface and the section are distinctly observed. (b) magnification on the triangular fibroblast embedded and surrounding vesicles. The cell has a granular surface. The fibers have a grooved surface.

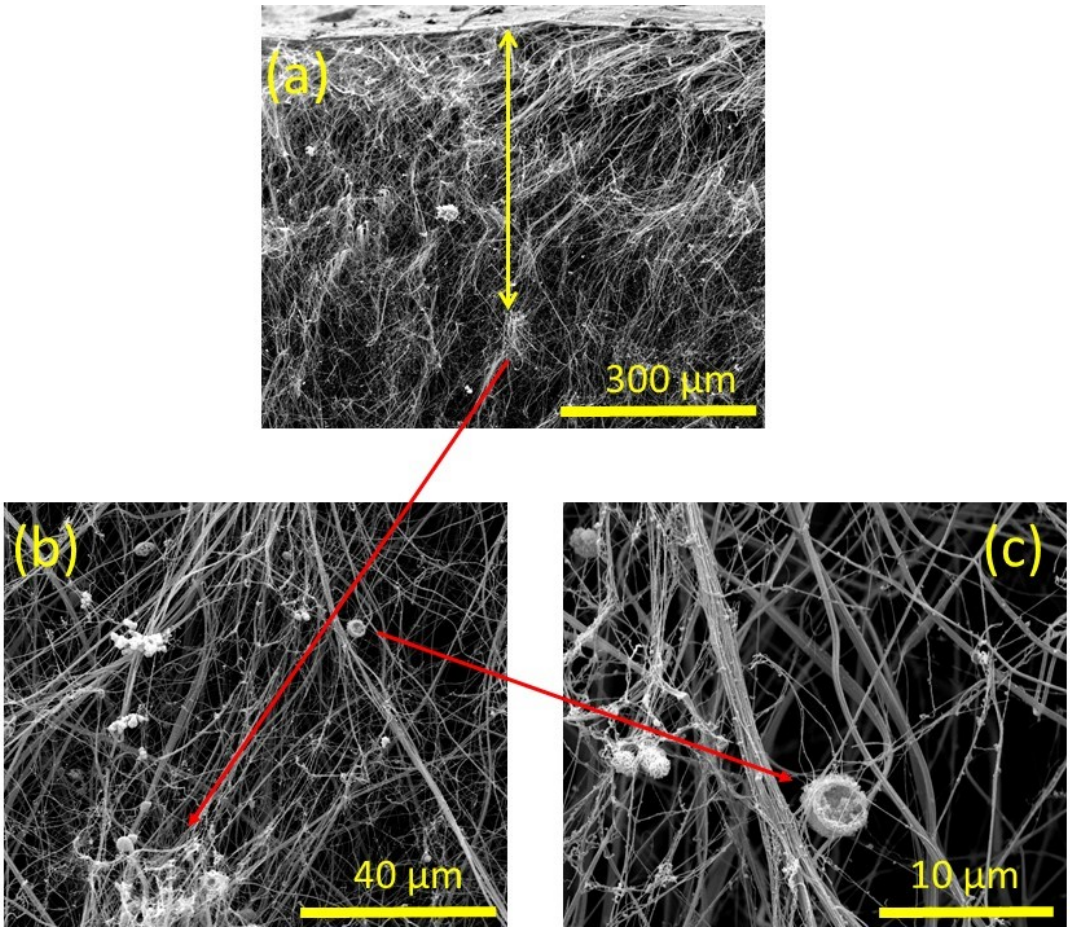


Fig. SI-8.2. Depth of penetration of the vesicles within the hydrogel. (a) overview of the section, where the surface and the section are distinctly observed. (b) magnification of the part at the tip of the yellow arrow. (c) magnification of one of the vesicles found embedded at a depth of around 450 µm.

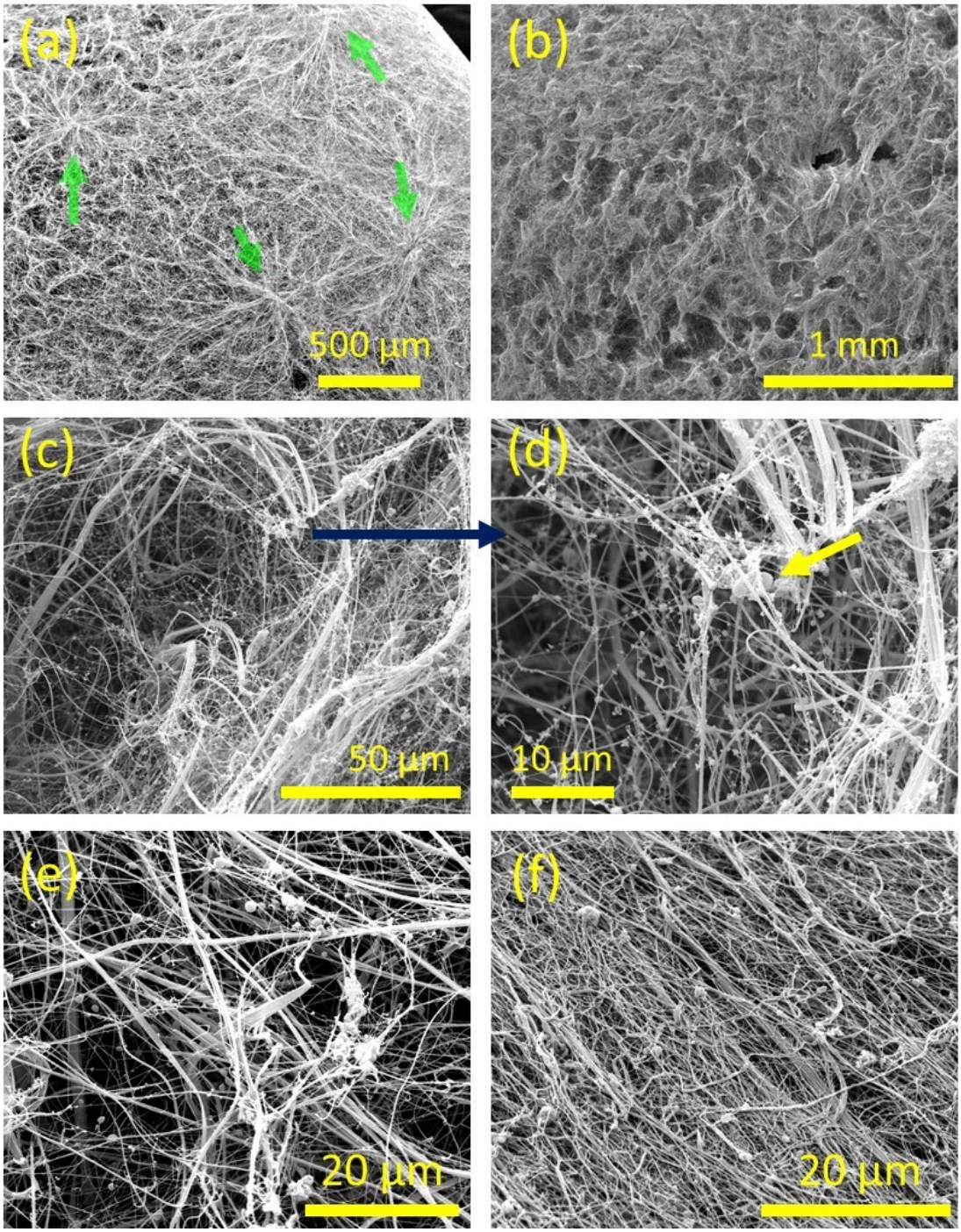


Fig. SI-8.3. Bottom of the gel after 22 days of cell culture. (a,b) overview of the structure of the gel at the bottom (formed at the interface between the gel solution and the culture plate well). Green arrows show the nucleation points. (c,d,e) Magnification of the bottom surface where are observed aggregates of biological material and vesicles (yellow arrow). (f) To make the comparison with (e) at the same scale (20 µm), SEM image of the upper surface of a control gel soaked in culture medium for 24h.

In (a), nucleation points at the origin of the fiber growth and gelation are clearly seen (green arrows). On the contrary of the upper surface of the gel (formed at the air-gel solution interface), there are no clusters of very big fibers and it does not seem to have a crust. Making the cell culture on this bottom surface rather than on the upper surface could be interesting because its structure appears to be more homogeneous and more porous (Fig. (b) and comparison between (c), bottom surface and (f) upper surface).

SI-9. Biphoton microscopy of the gel in second harmonic generation + polylysine-FITC staining

Control gels stained with polylysine-FITC have been observed in biphoton microscopy. The polylysine-FITC adsorbes itself on the fibers (and on the cells when cells are present), enhancing the fiber signal in the green wavelength. The signal of second harmonic generation (SHG) has been also collected. The second harmonic generation strongly enhances the signal of the very big fibers while these big fibers do not give any signal in the green channel with the polylysine-FITC staining. The SHG mode also seems to "select" only some of the thin fibers, not all of them. The images from the two modes therefore give a quite different information on the gel structure.

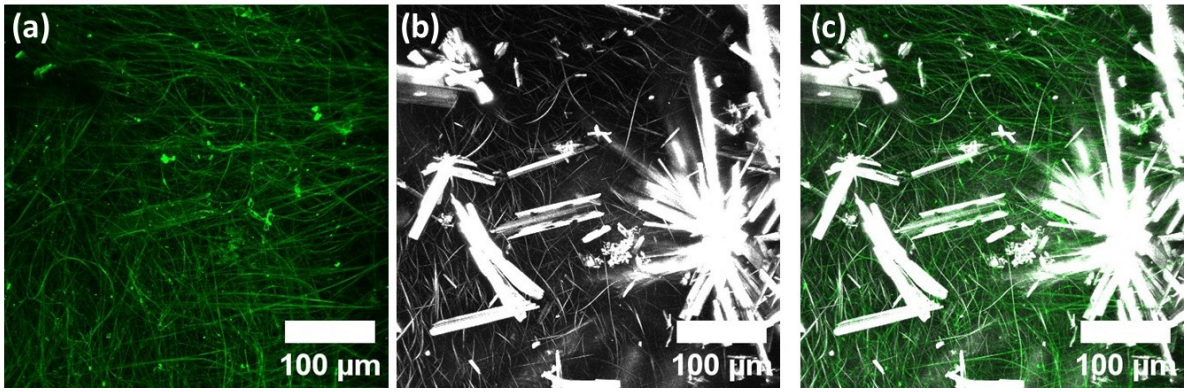


Fig. SI-9.1. Control gels without cells stained with polylysine-FITC (a): green channel: fibers stained with polylysine-FITC; (b) second channel: second harmonic generation (SHG); (c) merged. Conditions of observation: biphoton "configuration 2". Superposition of all the stacks. The upper surface of the gel is at the top.

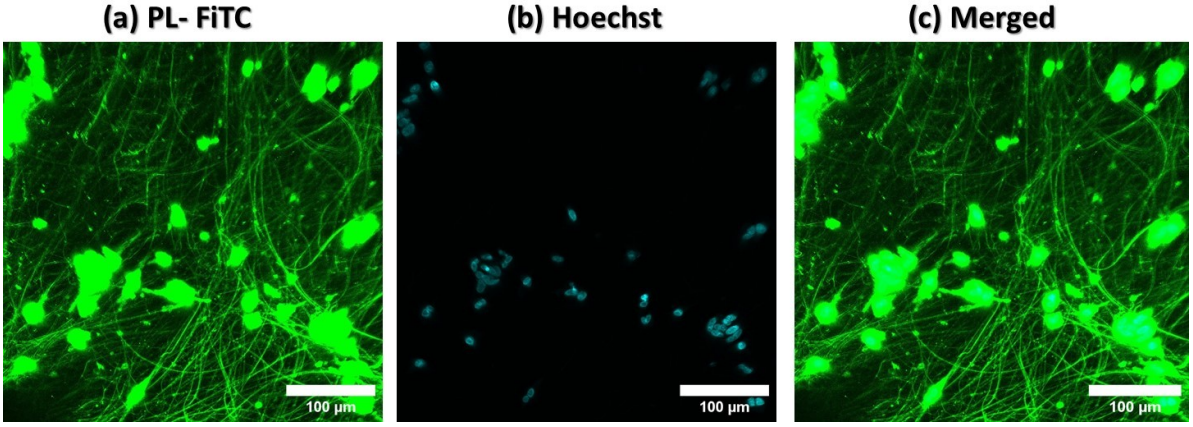


Fig. SI-9.2. Gels seeded without cells, 4 days of culture, stained with polylysine-FITC (a): green channel: fibers and cells are stained with polylysine-FITC; (b) blue channel: Hoechst staining (nuclei); (c) merged. Conditions of observation: biphoton "configuration 2", without SHG. Superposition of all the stacks. The upper surface of the gel is at the top.

SI-10- Images of the cells and gel in bright field and laser reflection mode

Other modes have been tested for the observation of the gels with the confocal monophoton microscopy, using photo counting mode (T-PMT) and laser reflection. The laser reflection, like the SHG mode, enhances the signal of some fibers only, giving again another picture of the gel. It correlates only partly with the T-PMT mode. The laser reflection mode seems to "select" only the straight fibers. Flexible fibers do not give signal.

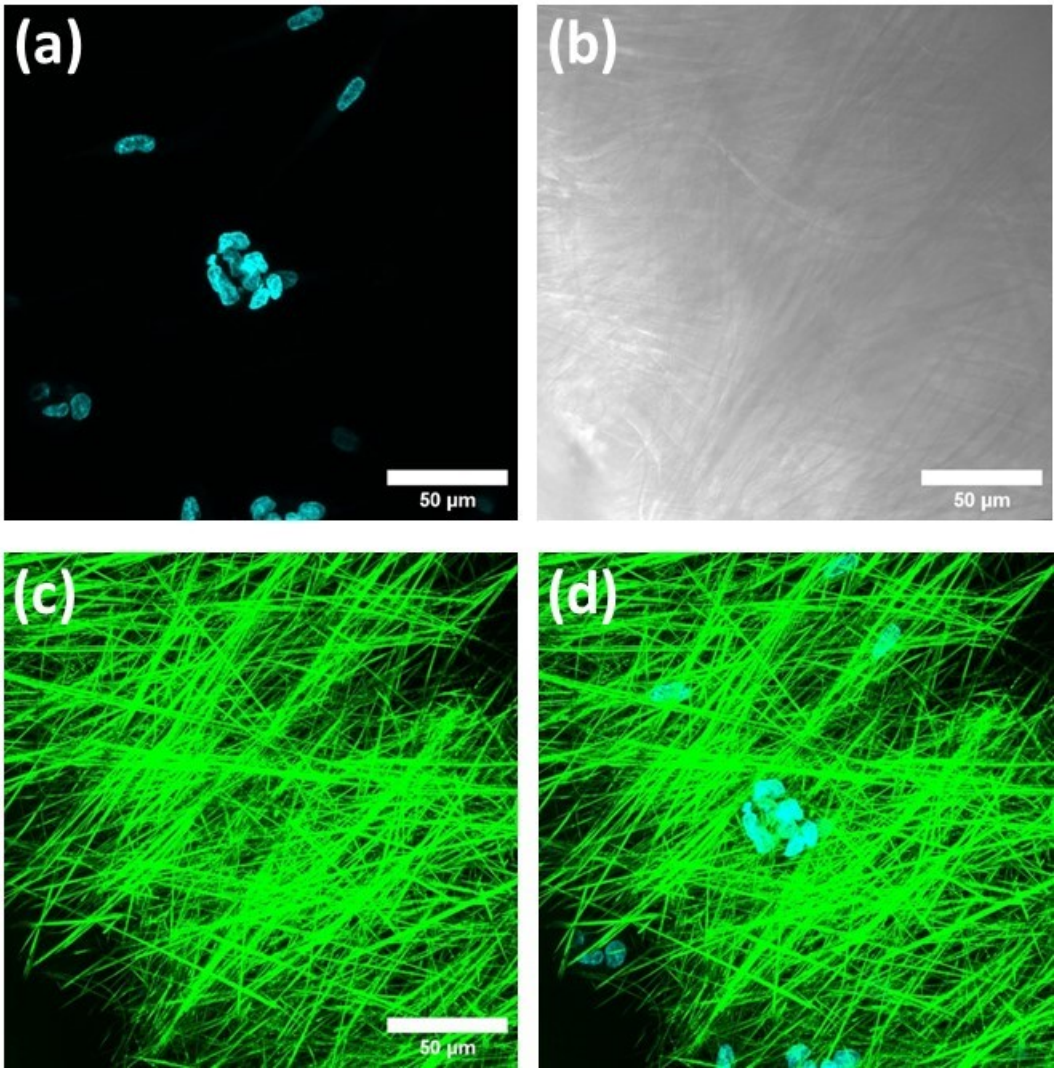


Fig. SI-10. Fibroblasts in the gel at day 4. (a) Hoechst (b) white light with T-PMT mode (photo counting) (c) laser reflection (d)= merge (a)+(c). Conditions of acquisition: Confocal monophoton microscopy (configuration 3)