ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Elastomeric Fibrous Hybrid Scaffold Supports In Vitro and In Vivo Tissue Formation

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Supporting information:

GelMa Synthesis:

Briefly, type-A porcine skin gelatin (Sigma–Aldrich) was dissolved in Dulbecco's phosphate buffered saline (DPBS) (GIBCO) at 60 ° C to make a uniform gelatin solution (10% (w/w)). Methacrylic anhydride (MA) (Sigma–Aldrich) was added to the gelatin solution at a rate of 0.5 mL/min under stirring conditions. Final concentrations of MA of 1, 5 and 10% (v/v) were used (referred to herein as 1M, 5M, and 10M GelMa). The mixture was allowed to react for 3 h at 50 ° C. After a 5-times dilution with additional warm DPBS, the GelMa solution was dialyzed against deionized water using 12–14 kDa cut-off dialysis tubes (Spectrum Laboratories) for 7 d at 50 ° C to remove unreacted MA and additional by-products. The dialyzed GelMa solutions were frozen at − 80°C, lyophilized, and stored at room temperature.

MSCs Isolation and EPCs Isolation:

Bone marrow samples were obtained from sheep femurs in ARCH (Protocol No. 13-10-2531R). Prior to the isolation process the samples were preserved in isolation buffer (ACD (Acid-citrate-dextrose) solution and heparin sulfate (American Pharmaceutical Partners)) on ice. 15 ml of Ficoll-Paque Plus (Amersham Pharmacia) was added to each 50 ml Accuspin tube (Sigma-Aldrich, A2055) and spun for 1 min (1200 rpm) to sediment the Ficoll-Paque. The mononuclear cell layer was collected with a syringe and transferred into 50 ml conical tubes on ice. Every 10 ml of collected cells were mixed with 5 ml isolation buffer. The cell pellet was obtained following two sequential spinning and resuspension cycles in isolation buffer. The cells were then ready for cultivation and further harvest.

For EPC isolation blood was derived from sheep donor. Blood was aspirated in the heparinized syringe (20-40ml blood drawn from the right femoral vein using a 19-guage needle). The blood was collected in a 50ml tube including 10 ml isolation buffer (9.9g Sodium Citrate in 640 ml DI water, 3.6g Citric Acid, 11.02g Dextrose [D-(+)-Glucose], 750ml water; filtered). 15 ml Ficoll-Paque plus (GE Healthcare Life Sciences, Product Code: 17-1440-02) is added to 50ml Accuspin tubes and then spun at 1200rpm for 1 min to sediment the Ficoll-Paque below the filter. 30ml of blood/isolation buffer is then added on top of each Accuspin tube and spun at 2700rpm for 15 min at room temperature. Following the centrifuging the cell layer is collected with a pipette and transferred to a new 50ml tube. We again add 5ml of isolation buffer to every 10 ml of collected cell layer. The samples are then spun at 2700 rpm for 5 min. Following removal of the supernatant, the cell pellets are resuspended in 10 ml isolation buffer and spun at 1200 rpm for 10 min. The pellets are resuspended again in 2ml isolation buffer and 6ml ammonium chloride (Sigma Aldrich, Catalog Number: 09685) is added to the suspension to lyse erythrocytes. The solutions are then incubated on ice for 5-10 min. 5ml Isolation buffer is added in the last step and the solution is centrifuged for 5min in 1200rpm. Of note, if pellet still has a red color, the previous steps should be repeated until all color has been removed. Plate the mononuclear cell solutions in 100 mm tissue culture treated plates with EPC medium and place them in an incubator (37°C). 2 hr after the plating, the unbound cell fractions are aspirated and the bound cell fractions are fed with EPC medium regularly.

Cell Seeding & Encapsulation of MSCs in GelMa Hydrogels:

In preparation for cell seeding, P4HB scaffolds were first sterilized by soaking in 70% ethanol for 30 min, followed by high intensity UV exposure (800 mW) for 3 min. The scaffolds were then soaked in culture medium prior to the cell encapsulation. The MSCs were suspended in the GelMa solution (500mg GelMa, 10ml PBS with 5% photo initiator dissolved in the PBS). MSCs were suspended at $1 \times 10^6/cm^2$ within the scaffold in 80 µl of the GelMa solution. The solution was added on top of the scaffolds as shown in the schematic. Photocrosslinking was achieved by exposing the cell-pre-polymer mixture to UV light (360 – 480 nm) for 15 seconds. Thereafter, cell-laden hydrogels encapsulated in fibrous scaffolds were cultured in DMEM for a week in static culture. The scaffold samples for bioreactor were been placed between rubber bands prior to sterilization and then soaked in GelMa and exposed to UV light. Following 1 week static seeding, 8 scaffolds were placed in the bioreactor for further culturing in a flexure and stretch condition. For comparison, 8 more samples were kept for further study in the static condition.

Mechanical Testing:

Scaffolds were tested by uniaxial mechanical Instron machine (Model 5542, Norwood, MA) to characterize the scaffolds' and tissues' mechanical properties. Samples were cut into 15 mm by 5 mm rectangular strips. Geometric data was imported into the Blue Hill mechanical testing software and samples were stretched to failure using a 10 N load cell to measure the reaction force. The samples were loaded at a 7 mm/min extension rate.

We measured the initial modulus (0-15% strain region; equivalent to the Young's modulus for a linear elastic material for scaffolds). The ultimate tensile strength (UTS) and the strain-to-failure for the scaffolds were also measured.

Pore size and fiber size measurements:

The fiber sizes and pore sizes of the fibrous scaffolds was measured using the image J software. Using the line measurement tool, we were able to draw a line across the diameter of fibers and measure range of fibers in several images obtained from the scaffolds. For pore sizes, we used the tool to measure the pores diameter via drawing a circle around the area and measure the diameter with the software. An average of the range of these measurements was reported as pore sizes.

DNA, Collagen and GAG Assays:

Samples (~2.5 by 2.5 mm) were cut from the cell-seeded scaffolds and weighed prior to the extraction of the ECM. The Sircol™ collagen assay kit (Biocolor LTd., United Kingdom) was used as per the manufacturer's protocol to quantify the collagen content that was synthesized following the 2- and 4- week cultivations. In order to extract the collagen, samples were placed in centrifuge tubes in 100 µL of extraction solution (0.5 M acetic acid and 1 mg/ml pepsin A in water) overnight on an orbital rocker at room temperature. GAGs were extracted utilizing the Sircol™ GAG assay kit (Biocolor LTd., United Kingdom). Briefly, the samples were soaked in a 1 ml solution of 4 M guanidine-HCl and 0.5 M sodium acetate overnight at 2-8^{0}C. Following the extraction steps, ECM proteins (collagen and GAG content) were measured according to the protocol provided with the Sircol™ assay kits using a Genesys 20 spectrophotometer (Thermo Spectronic, Rochester, NY).

DNA content was quantified on fibrous, microfabricated and tri-layered scaffolds at each specific time point by using a PicoGreen dsDNA quantification kit (Invitrogen) per manufacturer's instructions using a Spectramax Gemini XS plate reader (Molecular Devices, Inc., Sunnyvale, CA)[23,31]. Samples (~2 mm by 2 mm) were first cut from the cell-seeded scaffolds and weighed. The samples were then incubated in microcentrifuge tubes with 1 ml of buffered 0.125 mg/ml papain solution (DNA extraction solution) for 16 hr in a 60°C water bath before performing the PicoGreen assay.

Histology and Immunostaining:

Samples were first fixed in 4% paraformaldehyde (PFA) for 30 min, then rinsed in PBS, after which they were stored in 30% sucrose solution at 4°C overnight. Then samples were rinsed with PBS and embedded in OCT (optimum critical temperature) medium (Finetek). Cryosections of 10 µm were cut and stored at -20°C. Sections were thawed for 30 min before performing hematoxylin and eosin (H&E) staining for general morphology. To visualize myofibroblast-like differentiation, cell-seeded scaffold sections were stained for alpha smooth muscle actin (a-SMA, mouse monoclonal 1A4, Dako) using immunofluorescence. Normal horse serum (4%) was used as blocking solution. AlexaFluor 488 labeled secondary goat-anti mouse (Invitrogen) served as the secondary antibody. Sections were coverslipped with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)-containing Vectashield mounting media to counterstain the nuclei. Images were taken with a Nikon iEclipse microscope equipped with a digital camera (Nikon Instruments, Melville, NY).

The cell-seeded scaffolds were prepared for nuclei and F-actin visualization. Samples were first rinsed in *Hank's Balanced Salt Solution* (HBSS) and then fixed in 10% neutral buffered formalin (Sigma) for 20 min. The samples were then allowed to incubate at room temperature for 2 hr in 0.2% (v/v) Triton X-100 (Sigma) in HBSS. The samples were then rinsed 3 times for 5 min each in 0.05% (v/v) Triton X-100 in HBSS and then blocked in 1% (w/v) bovine serum albumin (Sigma) and 0.05% (v/v) Triton X-100 in HBSS for 2 hr. Once the blocking was complete, samples were incubated for 3 hr in Alexa Fluor 488-phalloidin (1:40 (v/v) dilution of stock solution in 1% (w/v) bovine serum albumin and 0.05% (v/v) Triton X-100 in HBSS); Invitrogen). The scaffolds were then rinsed 5 times for 5 min each in HBBS and stored in the refrigerator overnight. The samples were then placed on glass slides and coverslipped with a drop of Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) to counterstain cell nuclei.

Thrombogenicity assay:

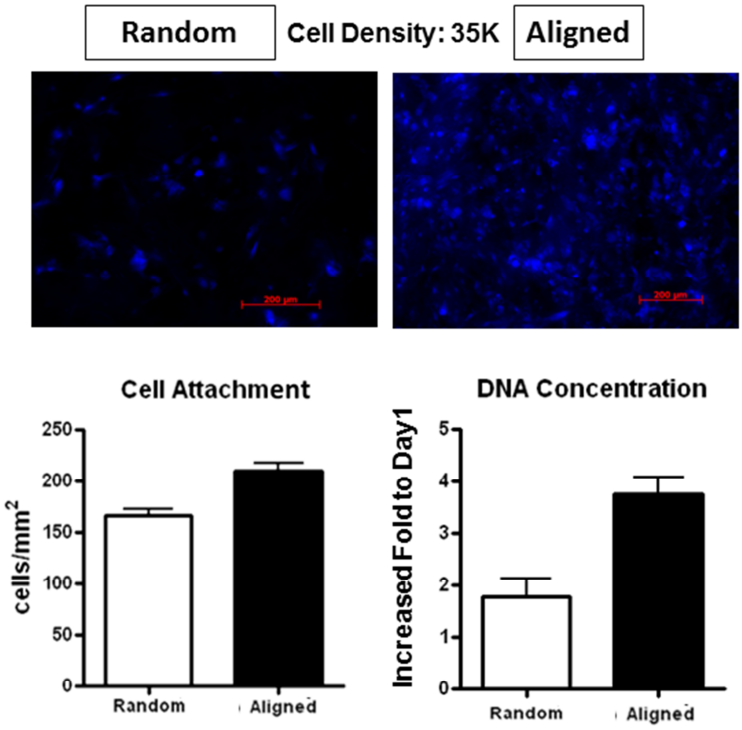
Human platelet rich plasma concentrates with approximately 1,000,000 platelets/ml were obtained from ZenBio. Inc. NC. The platelets were spun down in 50 ml tubes (2700 rpm for 5 min). The pellet was resuspended in 500 µl of media which led to a concentration of roughly 100,000,000 platelets/ml. Scaffolds were washed with PBS and placed in 12 well plates. Samples were submerged in 400 µl of the platelet solution for 1 hr on a rocker in an incubator. Following the soaking process, samples were washed with PBS, fixed in 10% formalin for 20 min and immunohistology was conducted as described above using anti-human CD41 (Invitrogen Carlsbad, CA) (1:200 for 1 hr at 37°C) as a primary antibody and anti-mouse Alexa568 (1:40 for 1 hr at room temperature) as a secondary antibody. Samples were stained with mouse anti-human CD41 (Invitrogen, Carlsbad, CA) (1:200 for 1 hr at room temperature). The samples were then

washed and soaked in a solution of Alexaflour 568 anti-mouse (1:40 for 1 hr at room temperature).

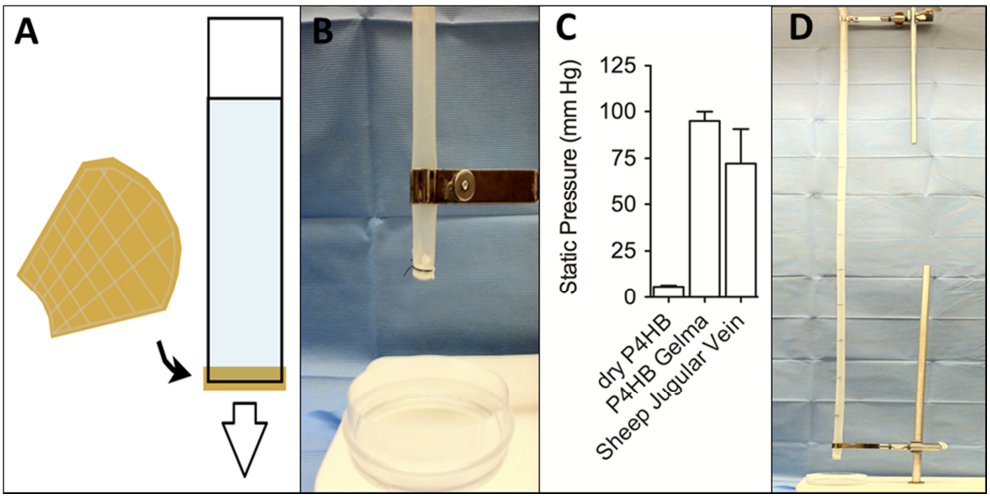
Scanning electron microscopy (SEM) and Confocal Microscopy:
Scaffolds were imaged at different magnifications (e.g., 50x, 100x) using an environmental scanning electron microscope (ESEM), SEMXL30 at low vacuum with a 32 kV accelerating voltage, 11 mm working distance. Immunohistology was visualized using a fluorescence microscope equipped with florescence camera (Axio Cam. MRm) and manufactured ApoTome for depth imaging (Carl Zeiss MicroImaging, Gottingen, Germany).

Surgical Implantation
The animal protocol was approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital (protocol #13-10-2531R). The animal was pre-medicated with atropine 0.04mg/kg IM followed by ketamine 10mg/kg and versed 0.1mg/kg IV. Following this, the animal was intubated with and endotracheal tube, and general Isoflurane anesthesia was administered. A 10French Foley bladder catheter was inserted directly into the urethra and a 6French percutaneous arterial catheter was placed in the right femoral artery for arterial pressure monitoring. A 7 French triple lumen venous catheter was inserted in the right external jugular vein. To control ventilation and allow hemostatic transection of the muscular layers of the chest, cisatracurium was administered to achieve reversible muscular paralysis. Heart rate and blood pressure were monitored to ensure deep anesthesia while the animal was paralyzed. The animal was continuously monitored by the following parameters: arterial blood pressure, central venous pressure, heart rate and rhythm, oxygenation, temperature and urine output. Ancef 20mg/kg IV was additionally given for antimicrobial prophylaxis.
The left thorax was prepared by shearing and painting with Betadine, and was draped using sterile drapes, and an anterolateral left-sided thoracotomy was performed in the 3rd intercostal space. With the lung retracted posteroinferiorly, the pericardium was opened longitudinally to expose the main pulmonary artery. A segment of main pulmonary artery was isolated with a partial occlusion clamp above the sinotubular junction. The pulmonary artery was then incised longitudinally (2cm) and the patch material (P4HB/GelMa) 2cm x 1.5cm was sutured into the incision site as an only patch. After hemostasis was ensured, the partial occlusion clamp was removed, chest tubes were placed (one in the left pleural space and the other behind the base of the heart), and secured to the skin. Intercostal sutures (0-vicryl) were placed to approximate the ribs. An intercostal block was placed using 0.25% sensorcaine 1mg/kg. Soft tissue and skin were closed using PDS (4-0/2-0) and monocryl 4-0, respectively. Dermabond was administered over the wound. Subsequently, the sheep recovered form anesthesia and was returned to housing.

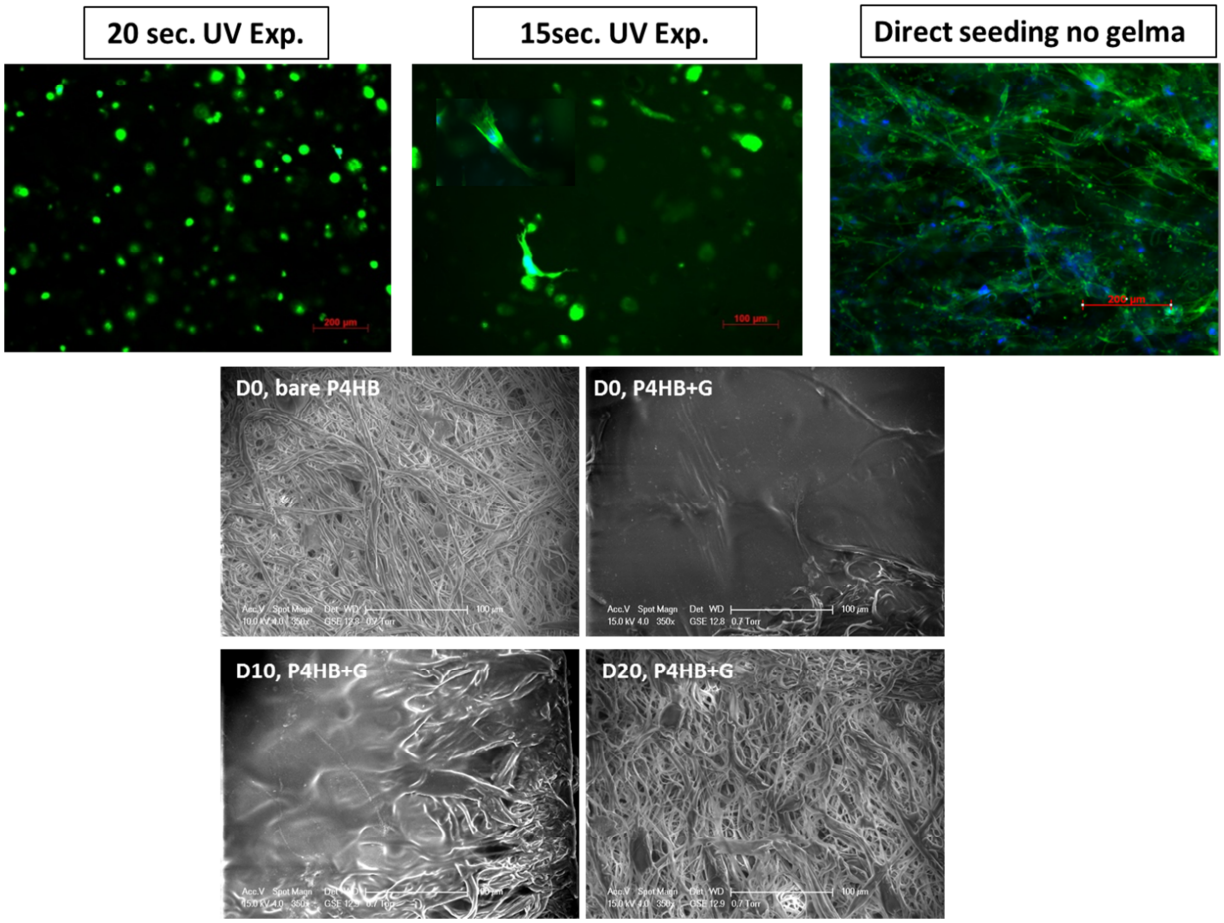
Supplementary Figures:



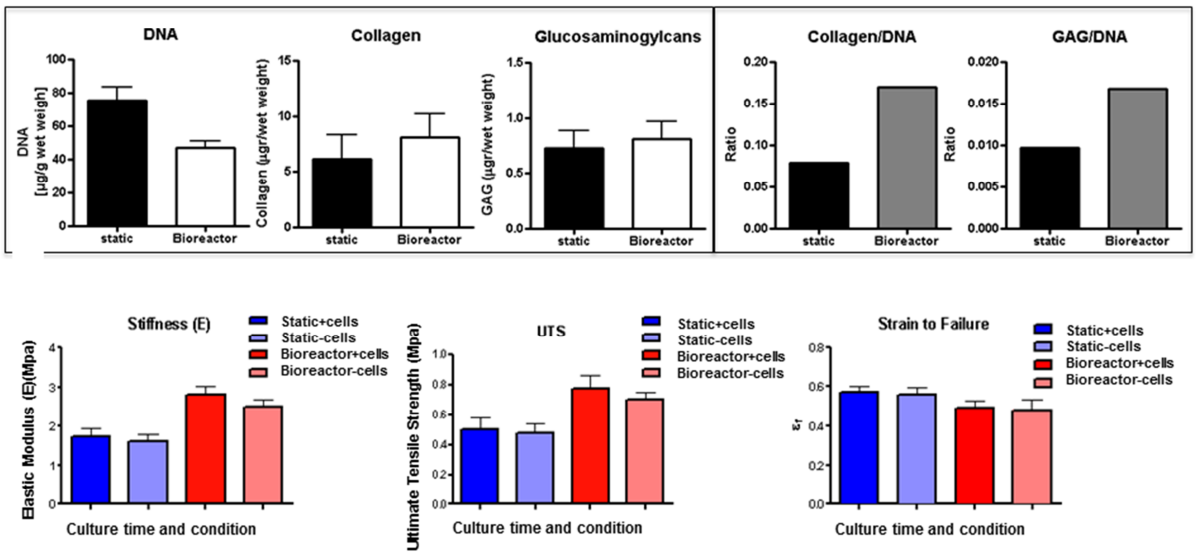
Supplementary Figure 1: Dapi staining of the cell nuclei on the P4HB scaffolds with random and aligned fibrous structures. The attachment analysis (measured from the number of cell nuclei stained on the surface of the scaffolds and DNA Pico green assay) detected higher cell numbers in aligned scaffolds versus scaffolds comprse of random fibers.



Supplementary Figure 2: The pressure test confirmed that P4HB-Gelma scaffolds hold 100mmHg of hydrostatic pressure while fibrous structure leaked due to the prosity in the scaffold.

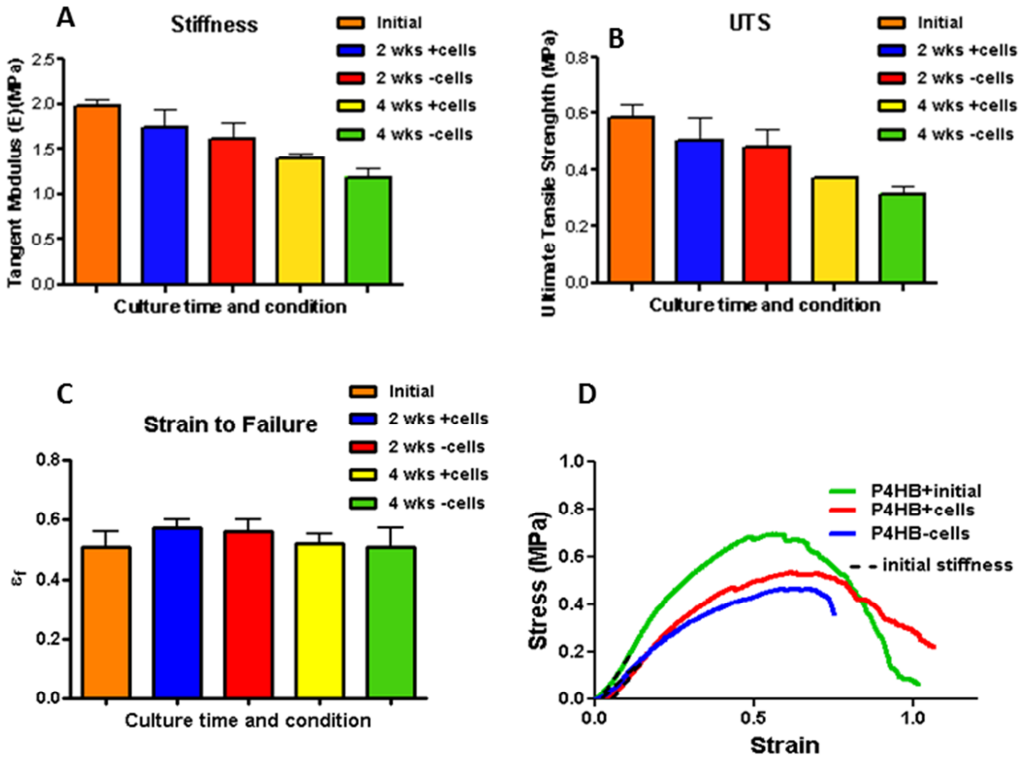


Supplementary figure 3: F-actin images confirm the cell sprouting in the optimum Gelma propreties. Cells stays round shape in stiffer gelma corsslinked wth longer UV exposure. SEM images also confirmed that the Gelma digerades leaving the hybrid scaffolds with only fibers after 20 days.

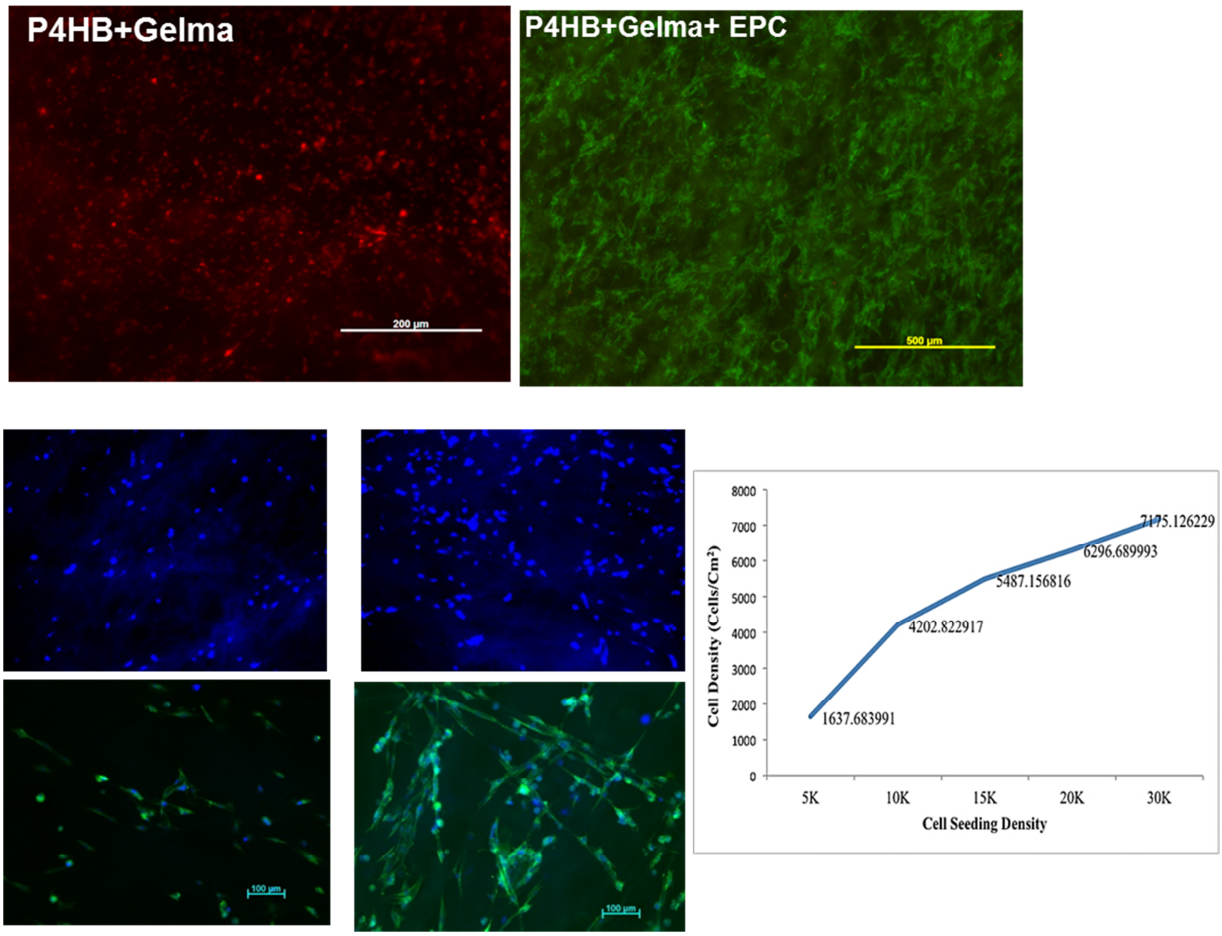


Supplementary figure 4: Bioassays results performed on scaffolds with random fibers also confirmed the resulst obtained with aligned fibrous scaffolds. Culturing the seeded scaffolds in the stretch/flexure bioreactor resulted in the higher production of ECM (i.e., Collagen aand

GAG). The results is in accordance with improved mechanical properteis of the scaffolds after being cultured in the bioreactor. The higher E and UTS fir non seeded scaffolds in the bioreactor is due to random fibers reorienting toward the stress direction and forming a more aligned fibers.



Supplementary figure 5: Mechanical properties of the scaffolds seeded in static cultures for a period of 4 weeks. Lower UTS and E are the clear induication of scaffolds degredation after incubation for a month wihout cells.



Supplementary figure 6: Thrombogenicity assay showed that hybrid scaffolds can attarct blood cells and therefore, scaffolds seeded with endothelial cells (EC) showed no sign of plasma attached to the scaffolds. The EC seeding process was quantified and optimized via staining process.