# Bioactive Gyroid Scaffolds Formed by Sacrificial Templating of Nanocellulose and Nanochitin Hydrogels as Instructive Platforms for Biomimetic Tissue Engineering

# Supporting Information

## Bioactive Gyroid Scaffolds Formed by Sacrificial Templating of Nanocellulose and Nanochitin Hydrogels as Instructive Platforms for Biomimetic Tissue Engineering

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### Experimental Section

*Materials:* Unless otherwise stated, all chemicals were of ACS grade, purchased from Sigma-Aldrich and used as supplied. Orasol and Irgacure 819 were donated by BASF SE. Reagents for cell culture were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. Human mesenchymal stem cells (HMSCs) were purchased from Lonza Cologne GmbH (Cologne, Germany). L929 mouse fibroblasts were obtained from Leibniz Institute DSMZ. The MTS-cell titer proliferation assay was purchased from Promega, the MTT from Sigma-Aldrich, and the tissue culture polystyrene (TCPS) plates from Greiner. High glucose Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Biochrom AG (Berlin, Germany).

*Preparation of CNF suspension:* A 1.0 wt% suspension of CNF was prepared according to Isogai et al.[1] by TEMPO-mediated oxidation under alkaline conditions (pH = 10.5) for 30 min of a softwood sulfite pulp and subsequent homogenization at pH 8.5 with a pressure microfluidizer MRT model CR5, applying 3 passes at 1400 bar. The resulting apparent viscosity degree of polymerization ($DP_v$) is 376 and the content of carboxyl groups is 0.54 mmol $g^{-1}$.

*Preparation ChNF suspension:* Based on the procedure reported by Ifuku et al.,[2, 3] a 1.3 wt% suspension of ChNF with deacetylated surface was prepared by first putting chitin powder into a NaOH aqueous solution bath and refluxing for 6 h. The resulting product was washed several times by centrifugation and subjected to homogenization in a stone grinder MKCA6-3 (Masuko Sangyo Co., Ltd.) at a speed rotation of 1500 rpm using -1.5 as gap height. Acetic acid was added

to facilitate disintegration. The resulting suspension has a pH of 4 and it was diluted to 1 wt% with deionized water by shaking. In one of our previous publications,[4] we determined that the degree of deacetylation of the underlying chitin is 10.6 mol %.

*Atomic Force Microscopy (AFM):* Diluted samples of CNF and ChNF (0.005 wt%) in deionized water were deposited on freshly cleaved mica and imaged in tapping mode on a NanoScope V (Digital Instruments Veeco Instruments Santa Barbara, CA).

*Field-Emission Scanning Electron Microscopy (FE-SEM):* Surfaces of supercritically dried scaffolds of CNF and ChNF were observed by SEM using a Hitachi S4800 instrument. All samples were sputtered with a thin layer of Au/Pd.

*Preparation of sacrificial templates:* Unit cells (6 x 6 x 6) were created from the gyroid minimal surface approximation of $\cos(x) \cdot \sin(y) + \cos(y) \cdot \sin(z) + \cos(z) \cdot \sin(x) = 0.60$ in K3Dsurf software by evolving in the boundary conditions x, y, z = [-6π, +6π] and applying boolean operations in Rhinoceros software. Subsequently, we scaled the gyroid scaffolds to a cube with edge lengths of 10 and 15 mm. A liquid photopolymerizable resin (40 wt% *N,N*-dimethyl-acrylamide, 40 wt% methacrylic acid, 10 wt% poly(vinyl pyrrolidone), 7 wt% methacrylic anhydride, 3 wt% Irgacure 819, 0.1 wt% *2,6*-dihydroxyanthraquinone and 0.05 wt% Orasol Orange dye) was subjected to photolithographic exposure in a commercially available Digital Light Processing Mini Multilenses system from Envisiontec equipped with a 180 W Hg vapour lamp, reflected off a 30 × 30 µm micromirror array, illuminating 1400 × 1050 pixels using a brightness of 2.3 mW $cm^{-2}$.

*Preparation of CNF and ChNF based scaffolds:* CNF and ChNF suspensions (both 1 wt%) were separately infiltrated in the sacrificial templates by centrifugation at a speed of 2500 rpm for 10 min. Afterwards, the templates, embedded in the respective suspension, were carefully removed from the centrifuge tube and put in a 1 wt% NaOH aqueous solution. After dissolution of the template, the resulting scaffolds were washed extensively and typically stored in ethanol.

*Supercritical drying (SC):* Supercritical drying on both types of scaffolds was carried out using a E3000 Critical Point Dryer and a Balzers Union CPD apparatus using ethanol as initial solvent. Liquid $CO_2$ was used to replace the ethanol. After heating and pressurization of the samples, slow decompression of the $CO_2$ to atmospheric pressure was carried out as the final step.

*X-ray microcomputed tomography (µCT):* Scaffolds containing microscopic particles of $BaSO_4$ (1 wt%, average diameter 3 µm) were scanned in a small animal dual energy µCT (Tomoscope DUO, CT-Imaging, Erlangen, Germany). Both tubes were operated at voltage 65 kV with current

of 0.5 mA. Each flat panel detector acquired 720 projections containing 1032 x 1012 pixels during one full gantry rotation with duration of 90 s. Volumetric images were reconstructed at voxel size 35 µm, using a modified Feldkamp algorithm with a smooth reconstruction kernel. Image analysis and visualization was performed using ImageJ and Imalytics Preclinical software version 2.0.

*Wide-Angle X-ray Diffraction (XRD)*: XRD was performed using an Empyrean setup from PANalytical. An Empyrean Cu x-ray tube LFF HR (line source of 12×0.04 $mm^2$) provided CuK $\alpha$ radiation with $\lambda$ =1.542 Å at 40 kV voltage and 40 mA current. Bragg Brentano parallel-beam geometry was used.

*Confocal fluorescence microscopy:* Before microscopy experiments, a 5 mg $mL^{-1}$ solution of FITC in DMSO (4 mL) was added to a vial containing a ChNF scaffold in 8 mL of water (pH = 7.5). The solution was carefully mixed and kept overnight. The next day, unbound stain was removed by putting the scaffold in a large water container. Confocal fluorescence images and stacks were obtained on a Leica TCS SP8 confocal laser scanning microscope using a 488 nm Argon laser. During measurement the stained scaffold was placed in glycerol for refractive index matching.

*Gelatin coating of CNF and ChNF materials:* Dithiobis(propionic hydrazide)-functionalized gelatin was synthesized following a protocol as described by Prestwich et al.[5] Dithiobis(propionic hydrazide)-functionalized gelatin (200 mg) was reduced with dithiothreitol (DTT, 1 g) in water adjusted to pH 8.5 using an aqueous solution of sodium hydroxide (1 M). After 24 hours, the solution was adjusted to pH 3.5 with an aqueous solution of hydrogen chloride (1 M) and dialyzed exhaustively at this pH. The solution was quickly adjusted to pH 7 with an aqueous solution of sodium hydroxide (1 M), phosphate-buffered saline (PBS, 10x) was added to buffer the solution at pH 7.4, and the solution was finally diluted to a concentration of 1 mg/mL of thiolated gelatin using PBS (1x). CNF and ChNF materials were immersed in these solutions for 1 hour, removed, rinsed with PBS (1x) and then dried in air facilitating disulfide crosslinking of the thiolated gelatin derivative.

*Biomineralization of films of CNF and ChNF with calcium phosphate:* CNF and ChNF substrates (with or without gelatine coatings) were incubated in an aqueous solution (1 mL) of calcium chloride (200 mM) for 20 minutes, after which the solution was removed and the samples were washed with water (3 x 1 mL). Thereafter, samples were incubated in an aqueous solution (1 mL) of sodium phosphate (120 mM) for 20 minutes, after which the solution was removed and the

samples were washed with water (3 x 1 mL). The cycle of incubation with calcium chloride and sodium phosphate was repeated a further six times, after which the samples were incubated in ethanol/water (70 % ethanol, 30% water) for 30 minutes and allowed to dry in a sterile fume hood overnight.

*MTS Proliferation assay.* To test the biocompatibity of nanocellulose and nanochitin surfaces, a proliferation assay was performed over the time course of three days. Wells of a 96-well TCPS plate were coated with nanocellulose (150 µL of 1 wt%) and nanochitin (150 µL of 1 wt%) (n = 3). The coating was dried overnight under sterile conditions, sterilized with 70% ethanol, and washed twice with 1X phosphate buffered saline (1X PBS). Mouse fibroblasts (L292) were seeded at a concentration of 100,000 cells/mL, 10,000 cells per well. Cells were seeded in a non-coated TCPS well as a control. An MTS proliferation assay was performed after 1, 2, 3, and 6 days. The absorption was measured at 490 nm. This experiment was performed twice with similar results.

*Proliferation in scaffolds using MTT staining.* To test the ability of mouse fibroblasts to adhere to the 3D gyroid scaffolds, 700,000 cells were added to each scaffold in consecutive steps while letting them incubate for approximately 2 hours before transfer to a TCPS well containing 5 mL of RPMI media. At 3 days, an MTT stain was performed to visualize the amount of live cells inside the scaffold.

*HSMCs Cell Culture:* Commercially available tissue-culture treated Corning® Costar® tissue culture plates were used for control experiments. CNF and ChNF materials were sterilized by incubation in 70 % ethanol solution followed by exposure to UV for 60 minutes. After sterilization, the samples were incubated for 30 minutes under 3 mm of HMSC growth medium. HMSC growth medium was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 440 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), and 2 ng $mL^{-1}$ basic fibroblast growth factor. Medium was aspirated and replaced prior to HMSC seeding. Cell viability before starting the experiment was determined by the Trypan Blue exclusion method, and the measured viability exceeded 95 % in all cases. HMSCs were seeded at 10,000 cells per $cm^2$ under 3 mm of medium, and incubated at 37 °C, 95 % humidity, and a $CO_2$ content of 5 %. After 3 days the medium was aspirated, the materials were washed gently with PBS and replaced with osteogenic medium. Osteogenic medium was composed of: high glucose Dulbecco's Modified Eagle Medium (DMEM, 425 mL); fetal bovine serum (50 mL); antibiotic-antimycotic (5 mL); non-essential amino acids (5 mL), dexamethasone

(100 nM), β-glycerol phosphate (10 mM) and ascorbic acid (50 μM). Thereafter the osteogenic medium was aspirated and replaced every 2 days until the samples were analysed.

*Nucleus and Cytoskeleton Staining:* Cells were fixed with 4 % paraformaldehyde in PBS for 15 min, permeabilized with 0.1 % Triton X-100 and 2 % bovine serum albumin (BSA) in PBS buffer for 5 min, followed by blocking with 2 % BSA in PBS buffer for 30 min at room temperature. Actin filaments and cell nuclei within cells were stained with Alexa Fluor 488 Phalloidin (Life Technologies GmbH, Darmstadt, Germany) for 30 min and 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The cells were then washed three times with PBS buffer after each stain and stored at 4 °C until images were acquired. Fluorescence images of cells were captured using a camera AxioCam MRm attached to a Zeiss Axio Observer Z1 equipped with an ApoTome unit. Images are representative of 3 samples.

*Alkaline Phosphatase Staining:* Alkaline Phosphatase (ALP) activity was visualized with a Leukocyte Alkaline Phosphatase Kit using the manufacturer's protocol. Images of stained cells were obtained using a camera AxioCam MRm attached to a Zeiss Axio Observer Z1 equipped with an ApoTome unit. Images are representative of 3 samples.

*DNA Quantitation:* DNA was quantified using PicoGreen assay (Life Technologies GmbH, Darmstadt, Germany) using a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments GmbH, Bad Friedrichshall, Germany).

*Alkaline Phosphatase Quantitation:* Films were scraped and broken up in a buffer of 0.2% Triton X-100. ALP activity was measured using an ALP LiquiColor kit (Stanbio, Boerne, TX) in accordance with the manufacturer's protocol. The sample and reagents were incubated in a 96 well plate for 1 h at 37 °C and then read using a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments GmbH, Bad Friedrichshall, Germany). Data were normalized to DNA quantity.

**Table S1.** Geometric parameters of the printed scaffolds

| Structure | Gyroid (10 mm) | Gyroid (15 mm) |
|---|---|---|
| Number of unit cells | 6 × 6 × 6 | 6 × 6 × 6 |
| Total Interfacial area [$mm^2$] | 2161 | 4861 |
| Porosity [%] | 50 | 50 |
| Volume of CAD designs [$mm^3$] | 500 | 1687 |

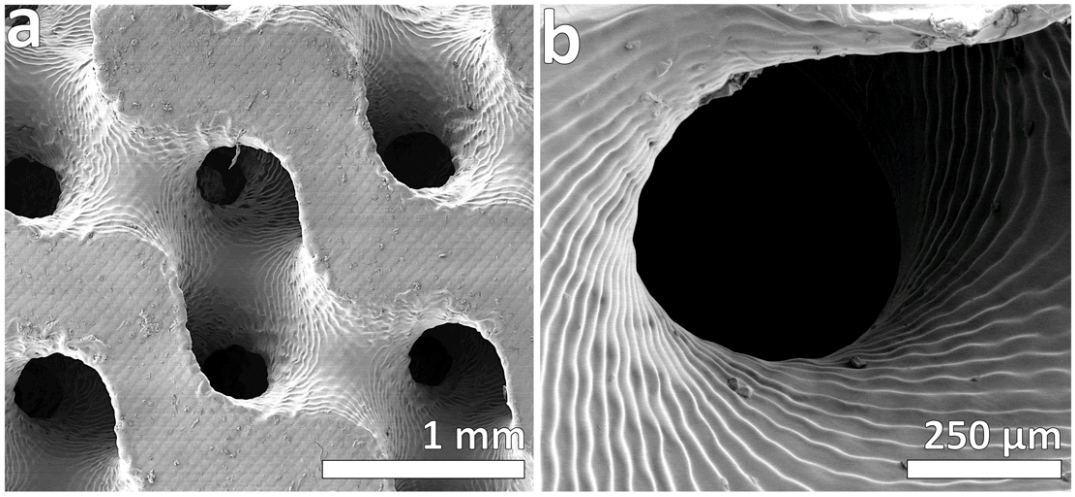


**Figure S1.** SEM of the templates after step-wise lithography revealing the fabrication layers.

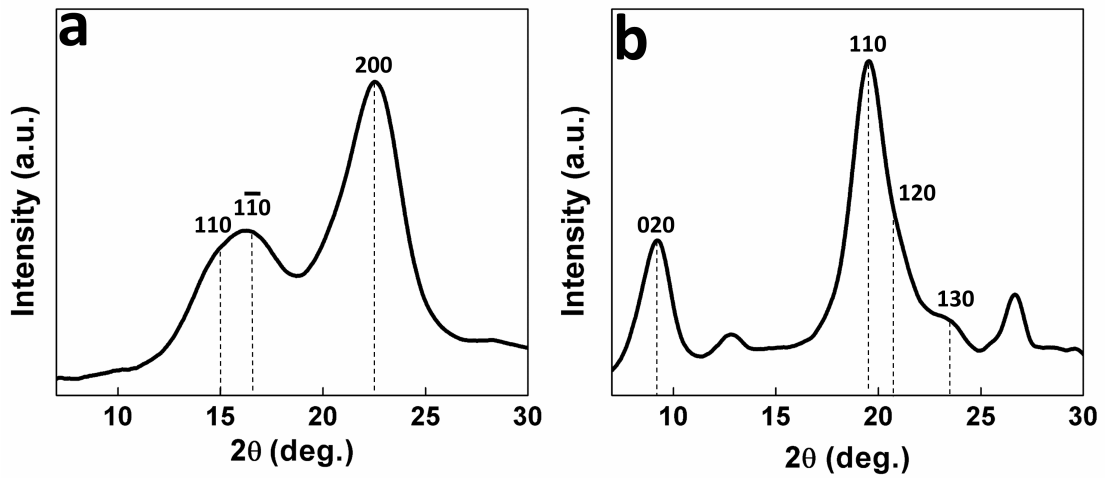


**Figure S2.** X-ray diffraction profiles of supercritically dried (a) CNF scaffold and (b) ChNF scaffolds. Degrees of crystallinity (63% for CNF and 70 % for ChNF) were obtained by applying the deconvolution method to separate amorphous and crystalline peaks. The values are in agreement with previous studies.[7-9]

**Supplementary Note 1**

*Network pore size estimation.* We estimated the average pore size of the 3D networks present in both CNF and ChNF scaffolds using equations 1 and 2, following a model by Fabry et al.[6] They discussed an analytical model for the porosity of 3D fibrillar networks with random orientation in the limiting case of long fibrils. The most probable nearest obstacle distance, $\sigma$, of random points in such a network can be obtained as:

$$\sigma = \frac{1}{\sqrt{2\pi\lambda}} \quad (1)$$

where $\lambda$ is the total line length per unit volume. The total line length, $\lambda$, per unit volume can be calculated based on the known solid content of the hydrogels (1 wt%), density for nanocellulose or nanochitin ($\delta \approx 1.5$ g cm$^{-3}$) and the average diameters determined by statistical height analysis (CNF diameter = 2.5 ± 2 nm, ChNF diameter = 3.2 ± 1.1 nm) assuming infinitely long cylinders.

Following to that, the average pore radius, $r_{pore}$, relates to the most probable nearest obstacle distance, $\sigma$, as follows:

$$r_{pore} \approx 1.86\,\sigma \quad (2)$$

The calculations give average pore radii of 20 nm and 26 nm for 1 wt% CNF and ChNF hydrogels, respectively.

To compare to experimentally accessible values, we performed a pore size distribution analysis on SEM images of supercritically dried CNF scaffolds using ImageJ after binarizing the image data and using the "analyze particle module". All obtained asymmetric areas were recalculated to the radius of a circle of equivalent area. 6000 pores were average. Figure S1 shows the obtained histogram for a CNF image, which has an average of ca. 14 nm.

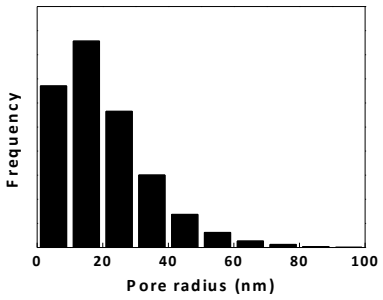


**Figure S3.** Pore radius distribution of networks in CNF scaffolds for 6000 pores.

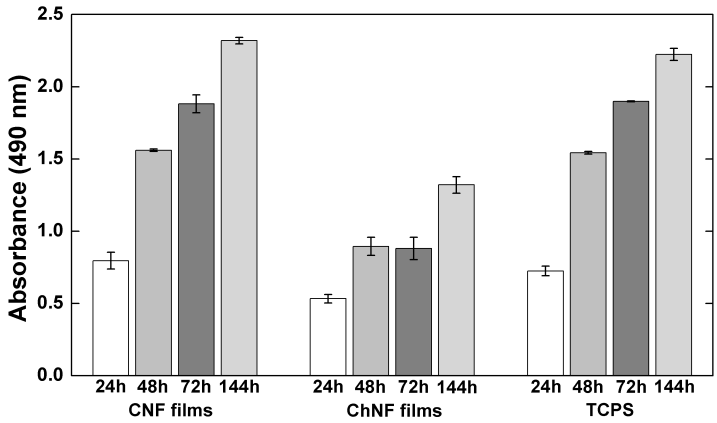


**Figure S4.** Mouse fibroblasts proliferation activity assessed by MTS assay on CNF and ChNF films, and as compared to a standard TCPS plate.

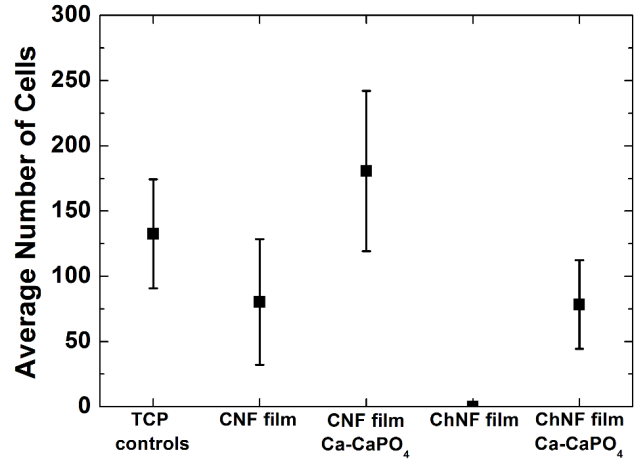


**Figure S5.** Average number of HMSCs per $1.6 \cdot 10^6$ $\mu m^2$ as determined by statistical image analysis and averaging over 3 samples for each type of material.

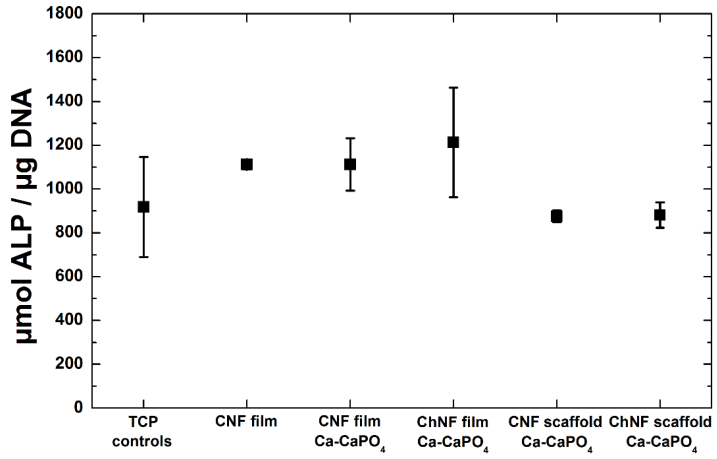


**Figure S6**. ALP activity normalized to the DNA content for the different materials. ALP activity was not determined for ChNF surfaces due to the small numbers of adhering cells.

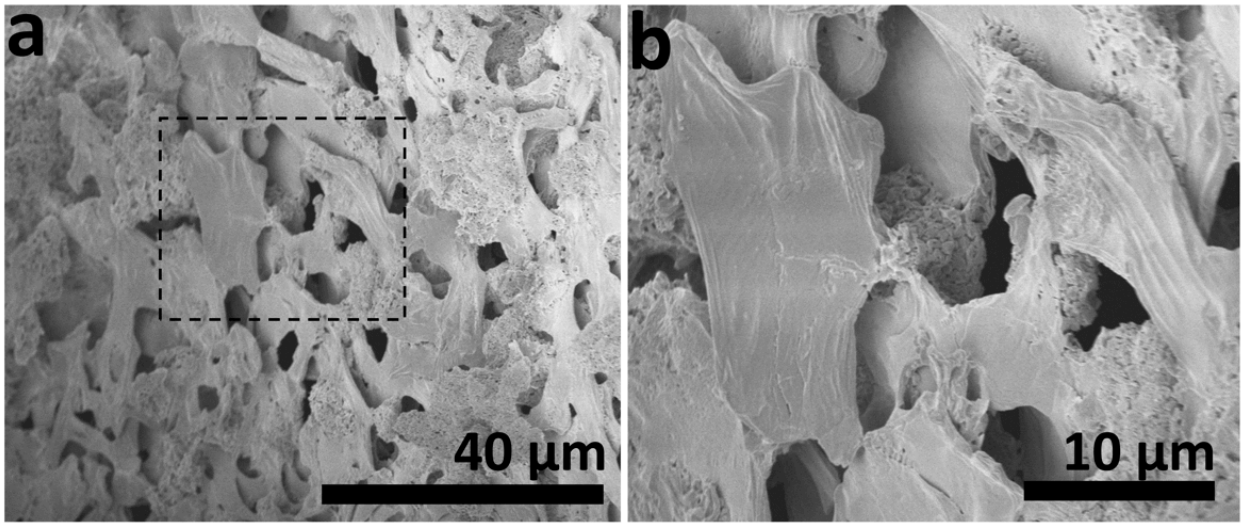


**Figure S7.** SEM images of confluent HMSCs in CNF scaffolds.

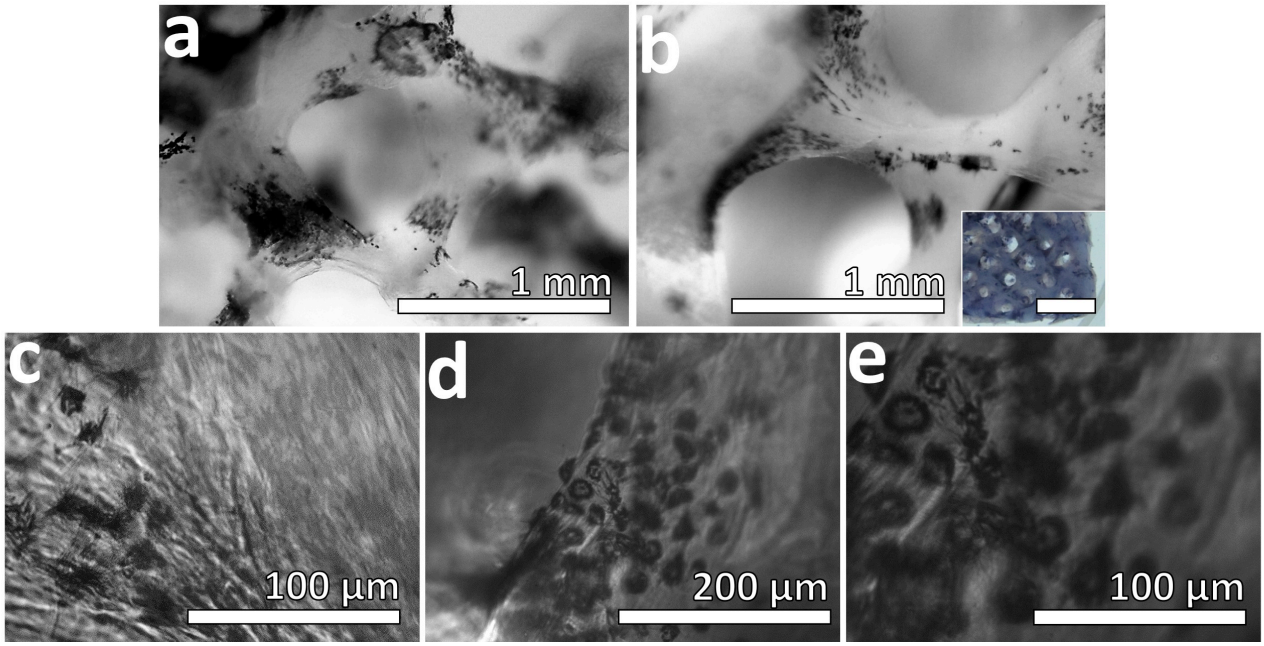


**Figure S8.** MTT stained mouse fibroblasts in the bulk of a (a) CNF scaffold and (b) ChNF scaffold at low magnification (at day 3). The inset in b depicts a photograph of a MTT stained ChNF scaffold populated by fibroblasts (scale bar 2 mm). Higher magnification images localizing fibroblast on the scaffold struts for CNF (c) and ChNF (d-e) scaffolds. Pristine scaffolds remain are not stained by the MTT assay, which was verified by controls that were treated equally (data not shown).

## References


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