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SUPPORTING INFORMATION

Modulating the cytocompatibility of tridimensional carbon nanotube-based scaffolds

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Materials and Methods (extended version)

1. Materials

Chemical reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. For scaffold preparation, low molecular weight chitosan (Batch#06513AE, Brookfield viscosity 20000 cps), chondroitin sulphate (Batch#STBB3576, bovine trachea), gelatin (Batch#1189632-52205137, porcine skin), and MWCNTs (reference number 659258, 110-170 nm in diameter, 5-9 μm in length, according to manufacturer specifications) were used. Cell culture media and supplements were purchased from Lonza.

2. Purification and chemical functionalization of MWCNTs

MWCNTs were first purified and functionalized by acidic treatment with HNO_3. Typically, concentrated HNO_3 (40 ml, 14 M) were added to MWCNTs (800 mg) in a round-bottom glass flask. The suspension was stirred overnight to guarantee homogenous dispersion of the nanotubes and then heated at 130 ºC for 5 h. After cooling at room temperature, it was filtered under vacuum by using cellulose filters with 0.2 μm pore size and several washes in distilled water in order to eliminate any acidic residues. Once the pH in the washes was stabilized at 5.5, MWCNTs were freeze-dried for 24 h and stored until used. To simplify, the so obtained MWCNTs will be named as *long* MWCNTs (LN). The *short* MWCNTs (SN) were obtained by simple exposure of LN to a stronger acidic treatment. Particularly, 200 mg of LN were sonicated for 4 h

(DT102H, Bandelin Sondez Digitex) in a concentrated solution containing H_2SO_4 and HNO_3 (12 ml, 3:1). The temperature in the bath was maintained below 40 ºC along the process. After treatment, SN were allowed to cool down at room temperature, then repeatedly washed in distilled water and finally freeze-dried as described above.

3. Preparation of cross-linked CNT-based scaffolds

Chitosan/MWCNT cross-linked scaffolds were prepared by using the ISISA process as previously described.[15, 35] Briefly, chitosan solutions (1 wt %) were made by dissolving chitosan (CHI, 0.1 g) into an aqueous solution of acetic acid (10 g, 0.15 M, pH 4.5). Either LN or SN MWCNTs (300 mg) were then dispersed in the chitosan solution (5 g, 1 wt %) by stirring at room temperature for 24 h and finally sonicated for 4 min. The suspension was then collected into insulin syringes (1 ml) and two different freezing ISISA methodologies were applied in order to modify the scaffold channel dimensions. Some of the samples were dipped at a constant rate of 5.9 mm min^{-1} into a cold bath maintained at a constant temperature of -196 °C (liquid nitrogen). In the other case, the suspensions were dipped at a constant rate of 0.9 mm min^{-1} into an ethanol bath at –65 ºC (dry ice). After this unidirectional freezing procedure, samples were freeze-dried using a thermoSavant Micromodulyo freeze-drier. The resulting monoliths were additionally exposed to glutaraldehyde vapors (GA, 50 wt % in distilled water) at room temperature for 24 h in order to obtain structure reinforcement by chitosan crosslinking. Finally, the so obtained scaffolds (LNCHI and SNCHI, containing either long or short MWCNTs, respectively) were aerated for 24 h. A similar methodology was used for the fabrication of CNT-based scaffolds made of either chondroitin sulphate (CHO) or gelatin (GEL). Both polymers were dissolved in distilled water (1 wt %) prior to MWCNT addition. The resulting scaffolds were crosslinked by exposure to

hexamethylene diisocyanate (HMDI) vapors at 37 ºC for 7 days. The so obtained scaffolds (LNCHO and SNCHO for chondroitin sulphate; LNGEL and SNGEL for gelatin) were also aerated for further 24 h before use. In all cases, singular scaffolds were obtained from freeze-dried monoliths by a similar procedure of cryo-fracture. Differences found between scaffolds are, therefore, attributed to the intrinsic scaffold composition and not to manipulation of the monolith.

Prior to the fabrication of CNT-based scaffolds, GEL was initially dissolved in distilled water at 50 ºC for 3 h under stirring. After cooling at 4 ºC, the resulting hydrogel was dialyzed in distilled water and further dissolved at 50 ºC. The final solution was then freeze-dried and stored at 4 ºC until used. As an additional precaution, nanotube dispersion and syringe preparation for GEL-containing scaffolds were performed at 37 ºC to avoid undesired gelation.

4. Preparation of polymer- and MWCNT/polymer-coated glass coverslips

Solutions of CHI (2 % w/v in acetic acid), CHO (6 % w/v in distilled water) and GEL (2 % w/v in distilled water), as well as MWCNT suspensions (6 % w/v LN or SN) in each polymer solution (CHI 1 wt % in acetic acid, CHO 1 wt % and GEL 1 wt % in distilled water), were prepared and used to homogeneously coat circular glass coverslips by spin-coating at 300 (for CHI) or 450 rpm (for CHO and GEL). Further crosslinking of the coated coverslips was achieved by exposure to either GA or HMDI vapors for 30 min or 7 days, respectively.

5. Scaffold characterization

Details of scaffold architecture, cross-section morphology and pore size were examined by using a DSM-950 scanning electron microscope (SEM, Zeiss). Scaffold

porosity was measured from SEM images by using the UTHSCSA ImageTool software, version 3.00. Briefly, pores were delimited in each scaffold image and their area converted to μm^2 by calibration with the scale bars. Porosity was then expressed as pore area per μm^2 (A_P) and pore width (W_P). Additional SEM micrographs were acquired in a Hitachi S-4700 scanning electron microscope equipped with the MeX software package (version 5.0.1, Alicona) that allowed for 3D data acquisition and object reconstruction using stereo-photogrammetry. Specifically, the software identified common points at different focus depths on a block of three overlapped 2D images that were taken at different tilt angles (5, 0 and -5º). Finally, the software allocated every point from the original 2D images into a 3D mapping that allowed object reconstruction. The root mean square (RMS) roughness (*Rq*) was obtained from the reconstructed 3D model of the different specimens for a path lenght of 15 μm.

The mechanical properties of the different scaffolds were measured at room temperature by using a Perkin-Elmer DMA7 equipment, as previously described.[60] Briefly, the bending strength, σ_f, was determined by a three-point bending test and the Young's modulus calculated from the slopes of the load-deflection curves.

Scaffold conductivity was measured by using a four-point method.[61] Particularly, a constant current (1 mA) was applied between contact points made of silver electrodes by using a digital multimeter Fluke 8840. Two of the contact points were placed at the edges of the scaffold cylinder in the same longitudinal plane (1 cm in length) with a pair of micromanipulators. The voltage drop in the scaffold surface was then measured by using another two silver electrodes located at the scaffold surface by slightly pressuring the scaffold with another pair of micromanipulators. The accuracy of the voltage measurement was approximately 1 μV. Conductivity was then calculated by using the following equation: $\sigma = [L/(\pi r^2 \cdot R)]$, where L is the distance between the electrodes on

the upper part of the scaffold, *R* is the measurement recorded by the multimeter and *r* the diameter of the scaffold cylinder.

6. Cell culture

Prior to cell culture, scaffolds (*ca.* 4.5 mm in diameter, *ca.* 3 mm in thickness) were sterilized under UV radiation for 20 min per side and preconditioned in culture medium for 24 h to eliminate any toxic residues from the synthesis. Three different cell types were tested: murine L929 fibroblasts, human osteoblast-like Saos-2 cells and porcine endothelial cells derived from peripheral blood progenitors (EC_{PC}), obtained as previously described.[62] A total of 10^5 cells was seeded on the top part of the scaffolds in a small fraction of media (typically 30 μl) and allowed to attach for 20 min. Immediately after, scaffolds were completely covered with complete media as follows: DMEM supplemented with fetal bovine serum (10 %), streptomycin (100 UI ml^{-1}), penicillin (100 UI ml^{-1}), and L-glutamine (1 mM) for fibroblasts and osteoblasts, and EGM-2 media for EC_{PC} cells. Cultures were maintained in static conditions at 37 ºC in a sterile incubator under a CO_2 (5 %) atmosphere for different times. Tissue culture polystyrene (TCP) was used as a control surface. Culture media was replaced every other day. Cell cultures were visualized by using an Axiovert CFL-40 optical microscope with an Axiocam ICC-1 digital camera coupled (Zeiss) when possible.

For cell size comparison, and assuming cells as entities with an ellipsoidal shape for simplification, cell dimensions were expressed as *l/w* ratios, where *l* is the cell length (*i.e.*, the longest cell dimension) and *w* the cell width (*i.e.*, the shortest dimension). In this sense, the higher the value of the *l/w* ratio is, the more elongated the cell morphology is; whereas a *l/w* ratio close to the unitary value correlates with a more square cell shape. According to this, cell size was measured and averaged from at least 25 cells from each cell type either right after trypsin treatment for cell detachment (cells

in suspension before seeding) or after culture on TCP for 48 h under standard culture conditions (adhered in culture).

7. Viability and apoptosis studies

To test cell viability, cells cultured on the different substrates were analyzed by using a Live/Dead® Viability kit (Invitrogen) according to manufacturer's instructions. This kit is based on the use of two probes: calcein and ethidium homodimer-1 (EthD-1). Calcein is a non-fluorescent cell-permeable dye that gets converted into a strongly green-light-emitting compound after contact with intracellular esterases and then retained inside live cells. On the contrary, EthD-1 is a DNA-intercalating agent that penetrates cell membranes in dead cells and emits orange/red fluorescence when inserted into the DNA double helix. After staining, samples were visualized by using a Leica SP5 confocal laser scanning microscope. The fluorescence of both probes was excited by an Argon laser tuning to 488 nm. After excitation, emitted fluorescence was separated by using a triple dicroic filter 488/561/633 and measured at 505-570 nm for green fluorescence (calcein) and 630-750 nm for red fluorescence (EthD-1). Physical reflexion from the scaffolds after excitation at 488 nm was also recorded and used to visualize the scaffold structure and the relative cellular location. To quantify cell viability, at least 6 square fields were randomly distributed in 20x confocal images and the number of live (green), dead (red) and apoptotic (green and red) cells counted per square and averaged.

Apoptotic cells in the cultures were specifically detected by using an annexin V-FITC apoptosis detection kit (Sigma). Briefly, Saos-2 osteoblasts and EC_{PC} cells were cultured at a density of $5 \cdot 10^4$ cells per scaffold. Twenty-four hours after seeding, cell cultures were washed with PBS (twice) and incubated with annexin V-FITC conjugate (5 μl in 500 μl of PBS) for 10 min protected from light. Simultaneous staining with the

probe EthD-1 allowed for the discrimination among early apoptotic (green fluorescence), late apoptotic (green and red) and dead cells (red). Stained cells were then visualized by using a Leica DMI 6000B fluorescence microscope. As a result of the externalization of phosphatidylserine, apoptotic cells were detected by their green fluorescence emission. Additionally, some cells were exposed to H_2O_2 (1 mM in PBS) for 90 min prior to annexin V incubation to serve as positive control of apoptosis induction. Cell viability in these cultures was tested in parallel as previously described.

8. Cell adhesion and morphology studies by scanning electron microscopy (SEM)

Cells cultured on the scaffolds for 48 h were rinsed in PBS twice and fixed with glutaraldehyde 2.5 % in PBS for 30 min. After washing in distilled water, dehydration was performed by slow water replacement using series of ethanol solutions (30 %, 50 %, 70 %, and 90 %) for 15 min twice and a final dehydration in absolute ethanol for 30 min. Samples were then allowed to dry at room temperature for 24 h, and then mounted in stubs and coated in vacuum with gold. Cell cultures on scaffolds were examined with a DSM-950 scanning electron microscope (SEM, Zeiss).

9. Time-lapse confocal laser scanning microscopy (CLSM) recording

Cell migration on the different scaffolds was investigated by using time-lapse CLSM recording. Briefly, Saos-2 cells were seeded at a density of $2.5 \cdot 10^4$ cells per scaffold and permitted to adhere on the scaffold surface for 20 min. Due to the opacity of CNT-based scaffolds, cells were labeled to allow their visualization with alexa fluor® 594-conjugated wheat germ agglutinin (WGA, 5 μg ml^{-1} in PBS, Invitrogen), a fluorescent lectin that selectively binds to N-acetylglucosamine and N-acetylneuramic acid residues present in cell glycoproteins. After 5 minutes of incubation at room

temperature, cell movements were recorded for at least 3 hours by using a Leica SP5 confocal laser scanning microscope with an acclimatized chamber (37 ºC, 5 % CO_2). Simultaneous laser reflection on the scaffold surface permitted a perfect visualization of the scaffold architecture. Cells on TCP were visualized without WGA staining as the transparency of the substrate allowed for their direct observation.

10. Statistics

Values were expressed as mean ± standard deviation. Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS), version 17.0. Comparisons among three or more groups were done by analysis of variance (ANOVA), followed by either Scheffé or Games-Howell *post hoc* tests depending on variance homogeneity among groups. For comparisons between two groups, t Student test was performed. In all statistical evaluations, $p < 0.05$ was considered statistically significant.

Table 1-SI. Cell viability on 2D control substrates containing either LN or SN and the corresponding polymers (i.e., CHI, CHO and GEL).

Cell type	Substrate	Viable cells (green) (%)	Dead cells (red) (%)	Apoptotic cells (green+red) (%)
L929	*LNCHI 2D*	98 ± 2	2 ± 1	0 ± 0
	SNCHI 2D	99 ± 1	1 ± 0	0 ± 0
	LNCHO 2D	61 ± 2	17 ± 2	22 ± 4
	SNCHO 2D	95 ± 3	1 ± 2	3 ± 5
	LNGEL 2D	97 ± 2	2 ± 2	1 ± 1
	SNGEL 2D	99 ± 0	1 ± 0	0 ± 0
Saos-2	*LNCHI 2D*	98 ± 3	2 ± 2	0 ± 0
	SNCHI 2D	99 ± 0	1 ± 0	0 ± 0
	LNCHO 2D	78 ± 0	0 ± 0	22 ± 0
	SNCHO 2D	77 ± 10	18 ± 5	5 ± 6
	LNGEL 2D	89 ± 4	11 ± 4	0 ± 0
	SNGEL 2D	94 ± 2	5 ± 2	1 ± 1
EC_{PC}	*LNCHI 2D*	62 ± 6	18 ± 0	20 ± 5
	SNCHI 2D	82 ± 3	11 ± 4	7 ± 3
	LNCHO 2D	44 ± 3	8 ± 4	47 ± 7
	SNCHO 2D	94 ± 1	3 ± 1	3 ± 0
	LNGEL 2D	97 ± 1	3 ± 1	0 ± 0
	SNGEL 2D	94 ± 5	6 ± 5	0 ± 0

Additional Figures

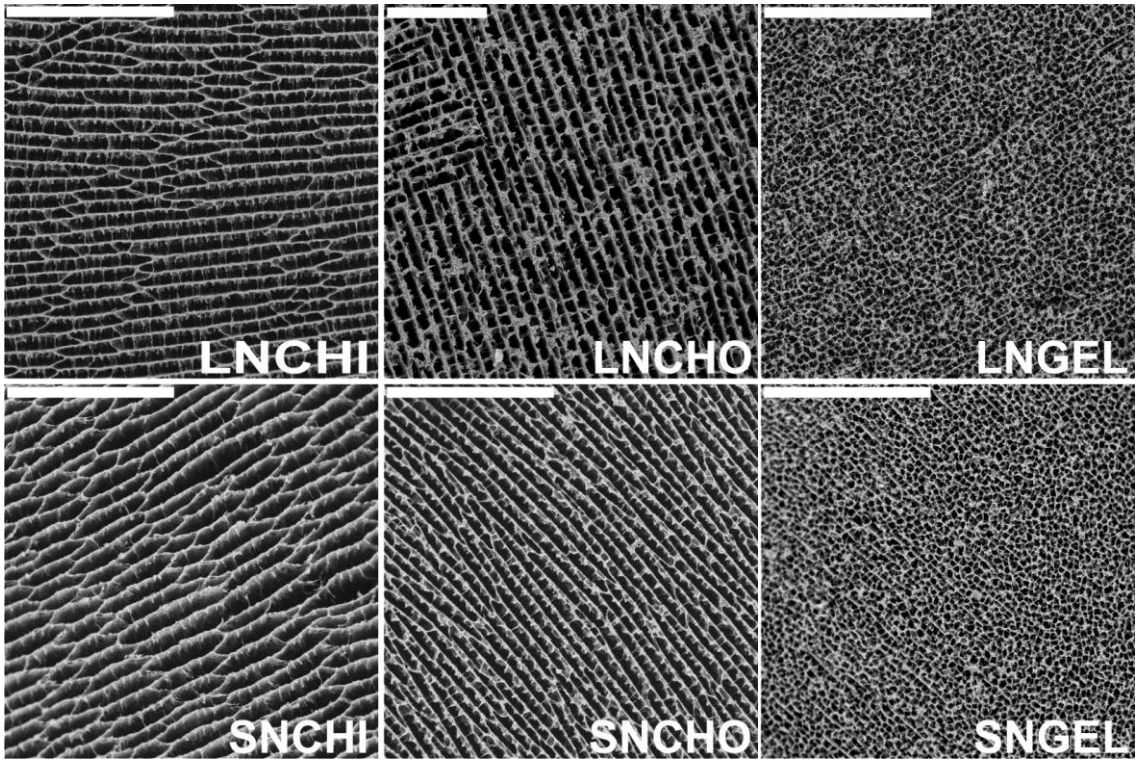

Figure 1-SI. Panoramic SEM micrographs of CHI-, CHO- and GEL-based scaffolds containing either long (LN) or short (SN) MWCNTs to appreciate the particular and homogeneous structure in each case. Scale bars represent 100 μm (LNCHO) or 200 μm (all the rest).

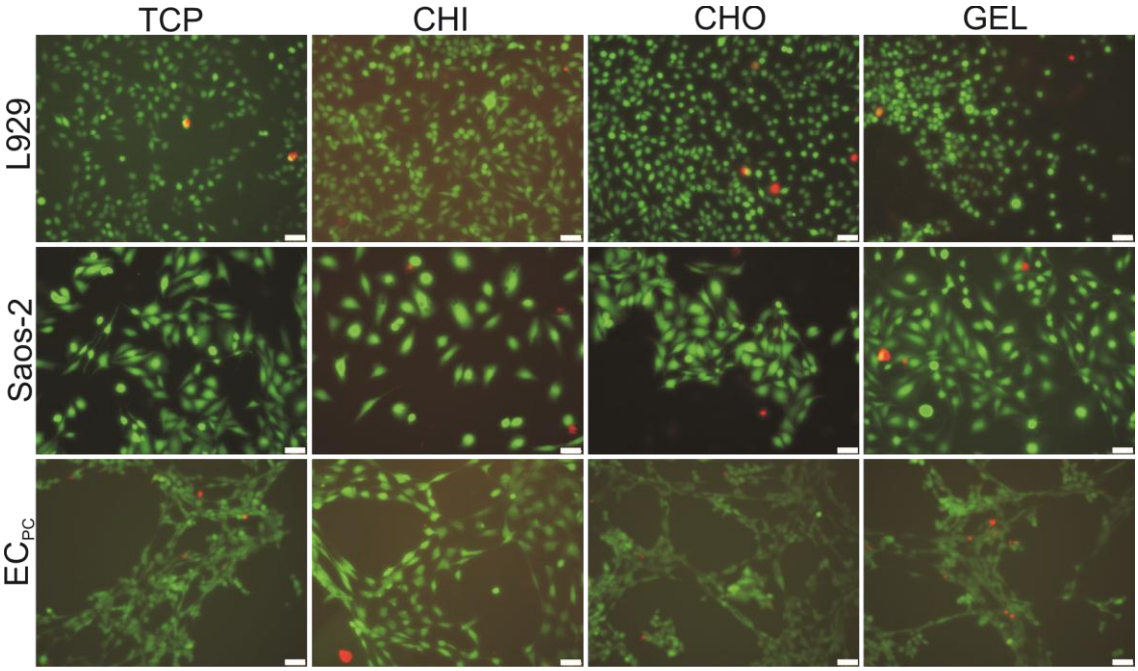

Figure 2-SI. Cell viability of L929 fibroblasts, Saos-2 osteoblasts and EC_{PC} cells on CHI-, CHO- and GEL-coated glass coverslips. Dead cells appear stained in red, while live cells stain in bright green. Representative CLSM images of cultures at 48 h are shown. Scale bars represent 50 μm in all images.

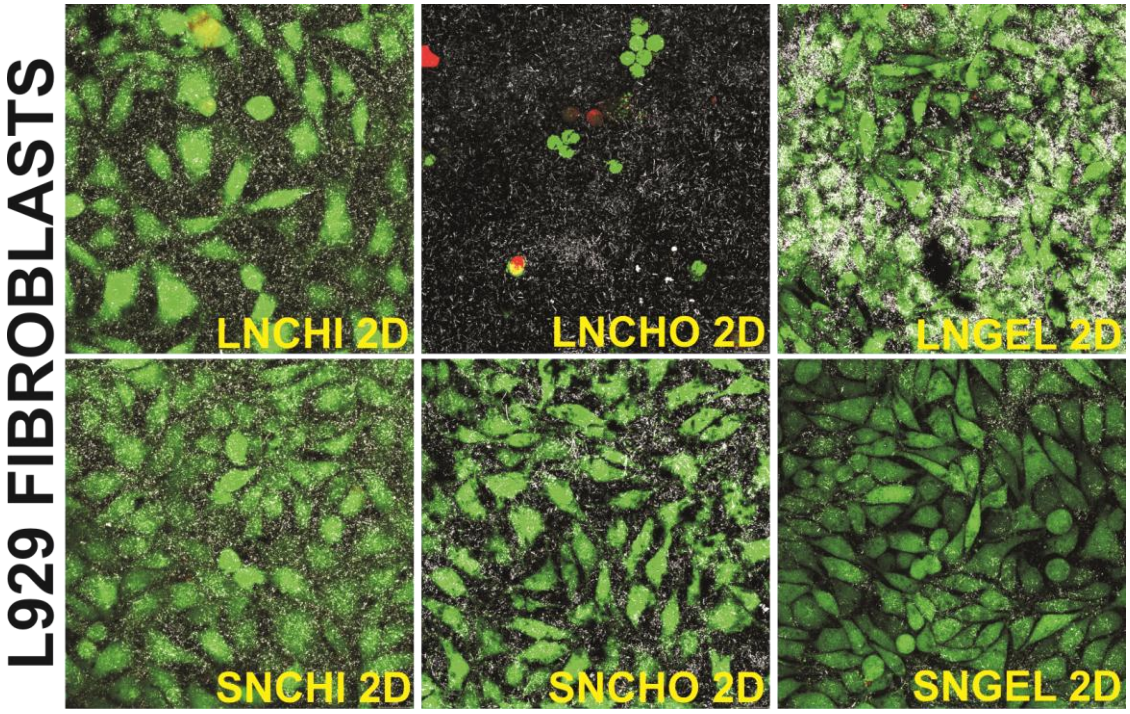

Figure 3-SI. Cell viability of L929 fibroblasts on 2D substrates composed of MWCNTs and either CHI, CHO or GEL. Representative CLSM images of cultures at 48 h are shown.

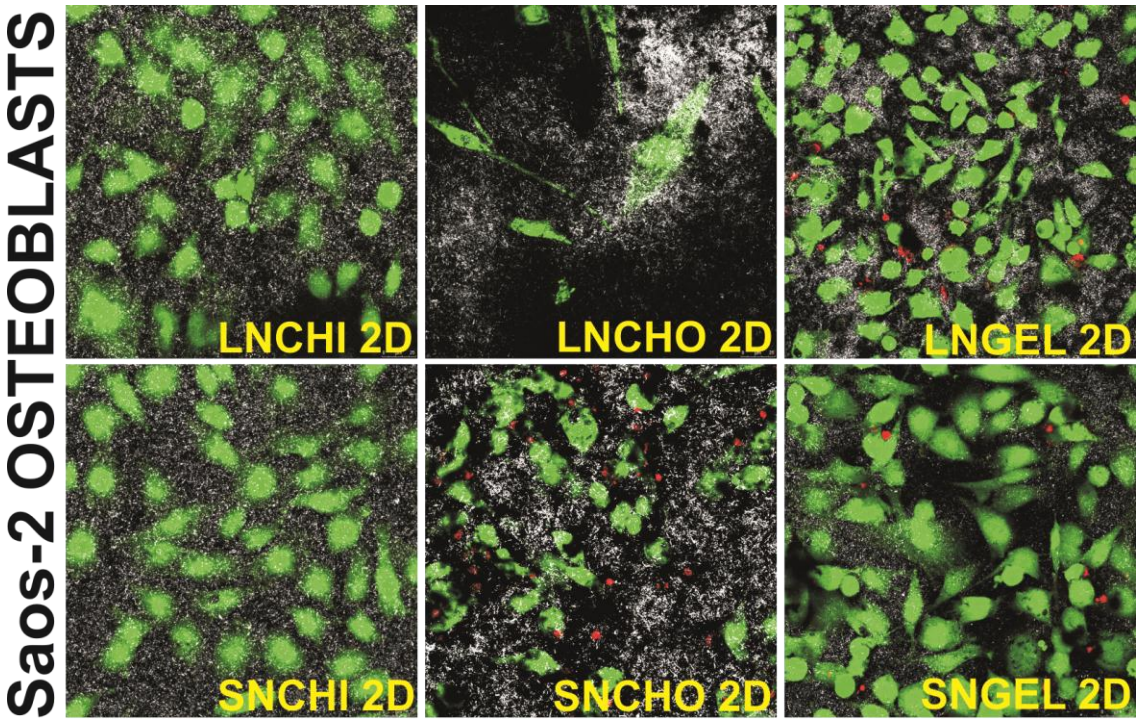

Figure 4-SI. Cell viability of Saos-2 osteoblasts on 2D substrates composed of MWCNTs and either CHI, CHO or GEL. Representative CLSM images of cultures at 48 h are shown.

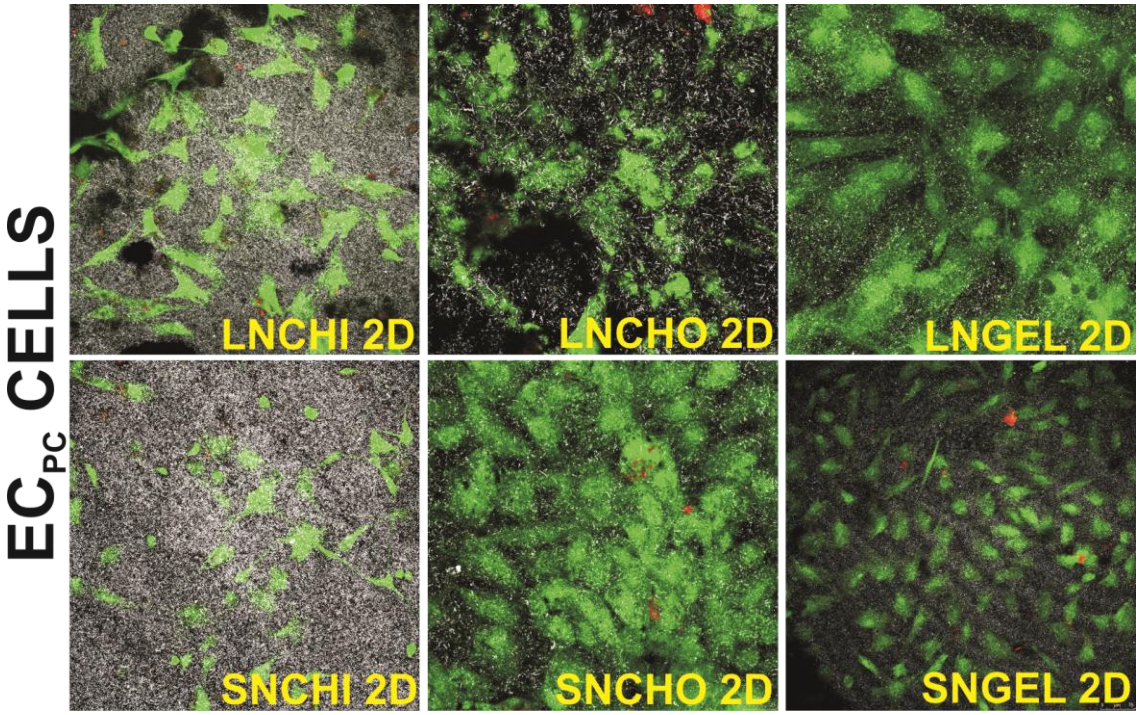

Figure 5-SI. Cell viability of EC_{PC} cells on 2D substrates composed of MWCNTs and either CHI, CHO or GEL. Representative CLSM images of cultures at 48 h are shown.

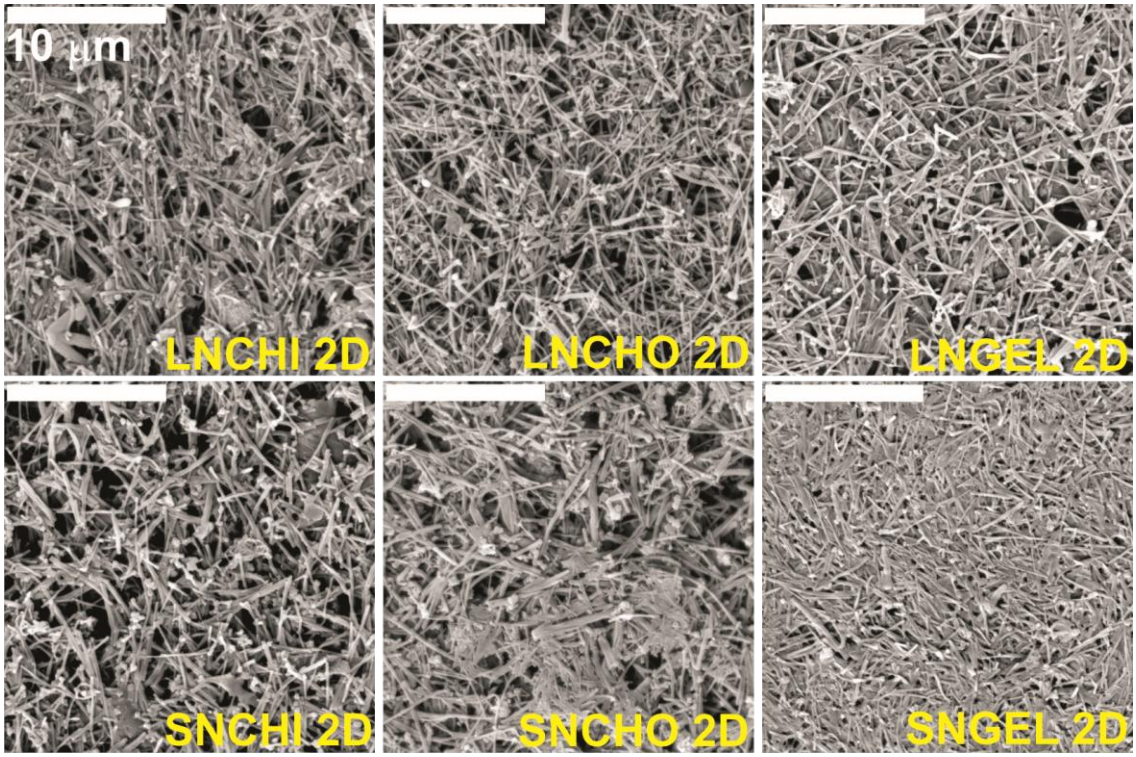

Figure 6-SI. SEM micrographs illustrating the top view of 2D substrates composed of MWCNTs and either CHI, CHO or GEL to appreciate the particular surface roughness in each case. Scale bars represent 10 μm in all cases.

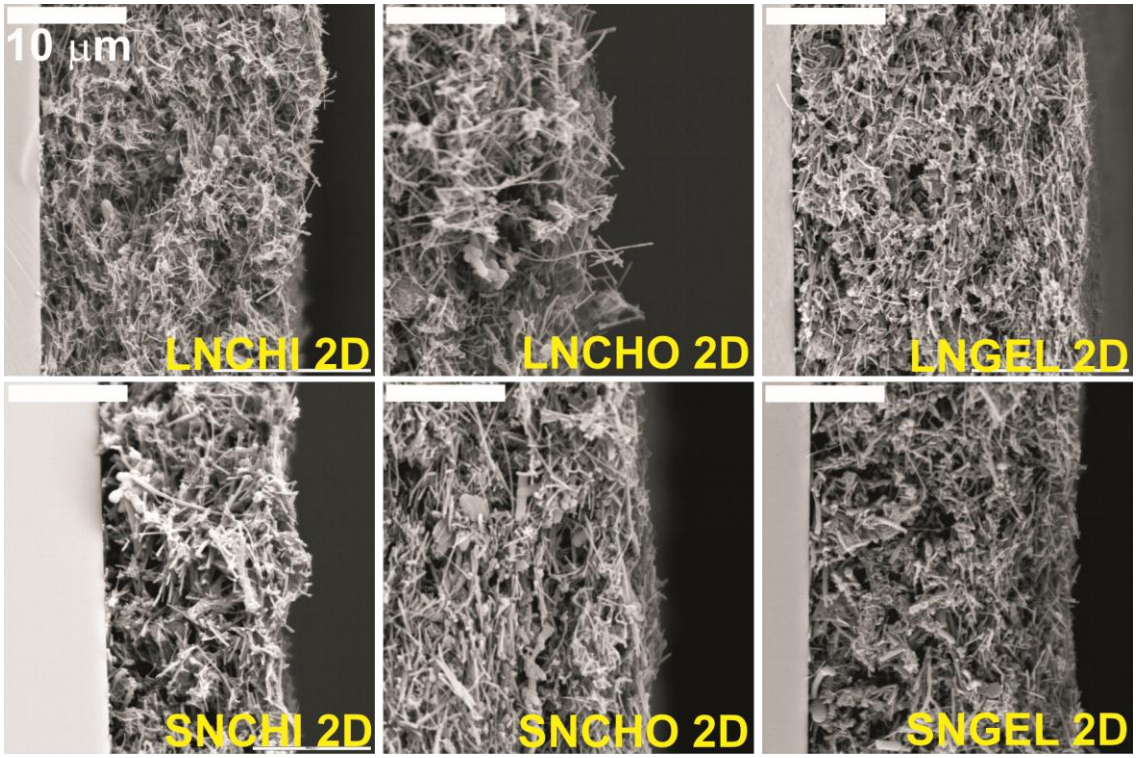

Figure 7-SI. SEM micrographs illustrating the cross-section view of 2D substrates composed of MWCNTs and either CHI, CHO or GEL to appreciate the particular surface roughness in each case. Scale bars represent 10 μm in all cases.

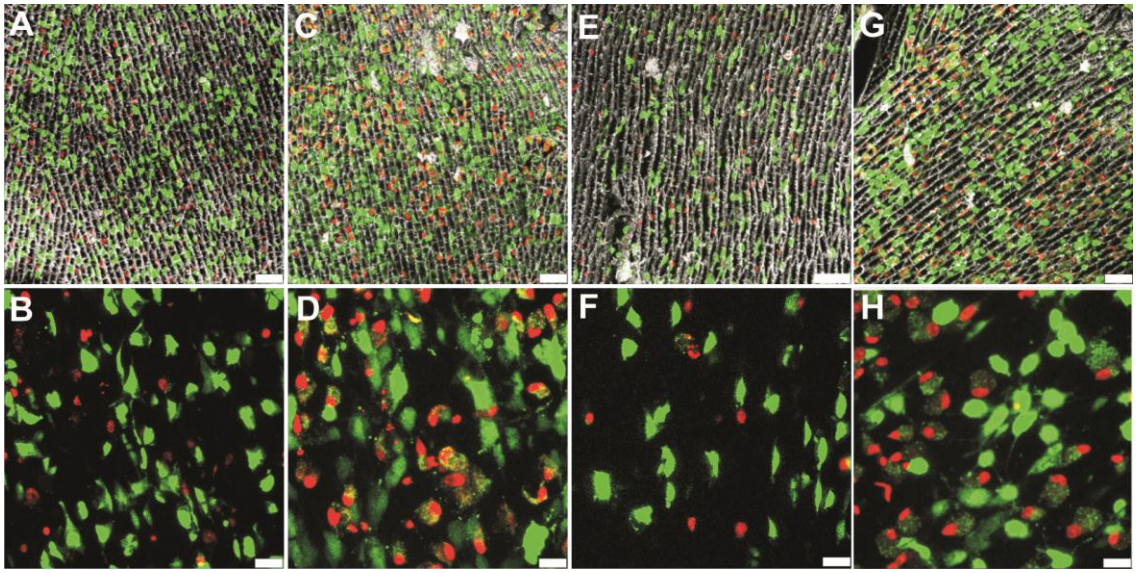

Figure 8-SI. Cell viability on LNCHI (A-D) and SNCHI (E-H) scaffolds. Human Saos-2 osteoblasts (A, B, E, F) and porcine EC_{PC} cells (C, D, G, H) were cultured on the different scaffolds for 48 h. Dead cells appear stained in red, while live cells stain in bright green. Representative CLSM images are shown. Scale bars represent 75 μm (top) or 25 μm (bottom).

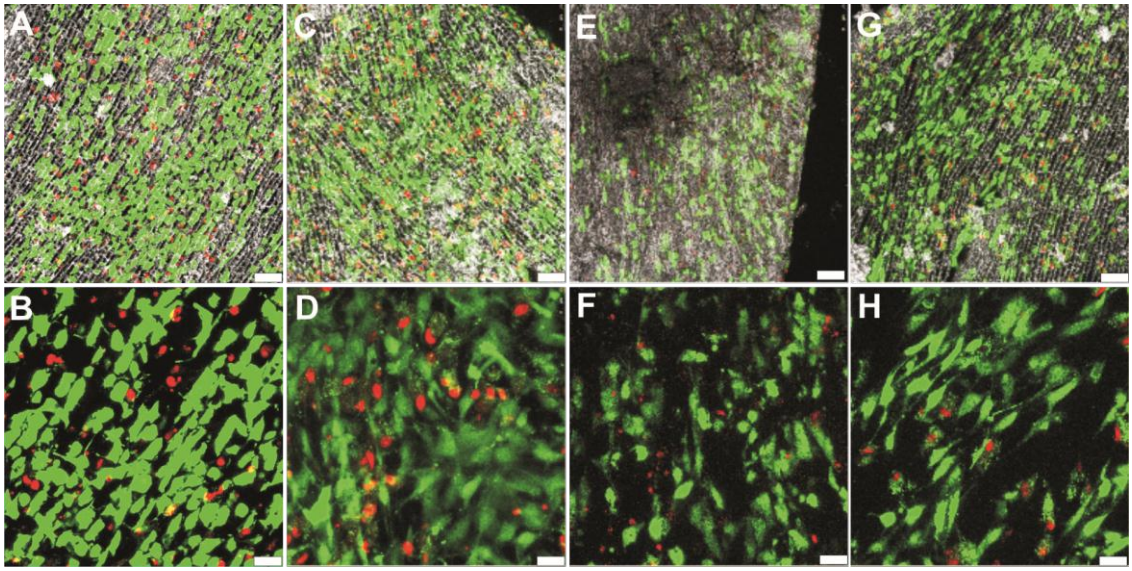

Figure 9-SI. Cell viability on LNCHO (A-D) and SNCHO (E-H) scaffolds. Human Saos-2 osteoblasts (A, B, E, F) and porcine EC_{PC} cells (C, D, G, H) were cultured on the different scaffolds for 48 h. Dead cells appear stained in red, while live cells stain in bright green. Representative CLSM images are shown. Scale bars represent 75 μm (top) or 25 μm (bottom).

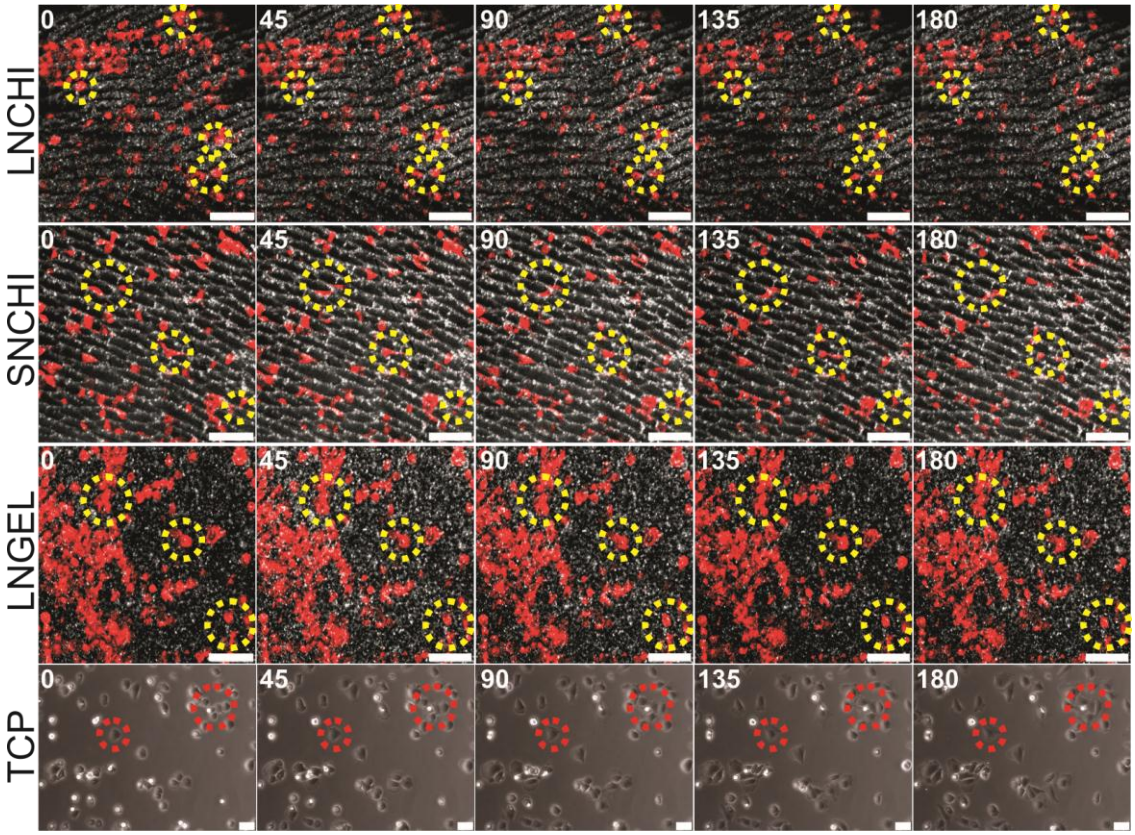

Figure 10-SI. Migration recordings by CLSM of Saos-2 cells cultured on LNCHI, SNCHI and LNGEL scaffolds. Cells were stained with WGA for visualization. Yellow circles are used to facilitate appreciation of cell migration. Numbers on the left top corner indicate time (min). Scale bar represents 75 μm. Saos-2 cells cultured on TCP are also included for reference. Scale bar on TCP represents 50 μm.

Movie 1-SI. Time-lapse CLSM recording of Saos-2 cell migration on LNCHI scaffolds.

Movie 2-SI. Time-lapse CLSM recording of Saos-2 cell migration on SNCHI scaffolds.

Movie 3-SI. Time-lapse CLSM recording of Saos-2 cell migration on LNGEL scaffolds.