Modulating the cytocompatibility of tridimensional carbon nanotube-based scaffolds

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Complete List of Authors:	Nardecchia, Stefania; Instituto de Ciencia de Materiales de Madrid (ICMM) Consejo Superior de Investigaciones Científicas (CSIC), Biomateriales y Materiales Bioinspirados Serrano, Maria; Instituto de Ciencia de Materiales de Madrid (ICMM) Consejo Superior de Investigaciones Científicas (CSIC), Biomateriales y Materiales Bioinspirados Gutierrez, Maria; Instituto de Ciencia de Materiales de Madrid (ICMM) Consejo Superior de Investigaciones Científicas (CSIC), Biomateriales y Materiales Bioinspirados Ferrer, Maria; Instituto de Ciencia de Materiales de Madrid (ICMM) Consejo Superior de Investigaciones Científicas (CSIC), Biomateriales y Materiales Bioinspirados del Monte, Francisco; Instituto de Ciencia de Materiales de Madrid (ICMM) Consejo Superior de Investigaciones Científicas (CSIC), Biomateriales y Materiales Bioinspirados

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Modulating the cytocompatibility of tridimensional carbon nanotube-based scaffolds

Stefania Nardecchia,^a María Concepción Serrano,^{*a} María Concepción Gutiérrez,^a María Luisa Ferrer,^a and Francisco del Monte^{*a}

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Carbon nanotubes (CNTs) have lately attracted significant attention in the field of biomedicine. Although a wide repertoire of CNT-based composites has been explored as substrates for cell growth, the fabrication of 3D scaffolds has been more rarely accomplished. Additionally, concerns referred to CNT biocompatibility make their use in biomaterials still controversial. Herein we explore the interaction of three types of CNT-based 3D scaffolds –prepared with multi-walled CNTs and processed to show different architectural and morphological features at the microscale by using three different polymers (i.e., chitosan, chondroitin sulphate and gelatin) – with three types of mammalian cells displaying different size and adhesion pattern. Cell-material interaction has been assessed by studying cell viability, adhesion, morphology, and apoptosis. By means of time-lapse confocal laser scanning microscopy, we investigate, for the first time in CNT-based scaffolds, cell migration processes in real time. Scaffolds displaying both a pore size in range with that of cells and lower surface roughness reveal the highest viability values. On the contrary, those with a smaller pore size and higher surface roughness account for the lowest cytocompatibility. Results from these studies benefit the fabrication of optimized biomaterials by varying scaffold-dependent parameters in accordance to those of target cells.

1. Introduction

Carbon nanotubes (CNTs) are defined as hollow cylinders exclusively composed of graphitic carbon sheets generally rolled as either a single-walled structure (SWCNTs) or a multi-walled structure (MWCNTs). Since their discovery by Iijima and others,^{1, 2} an extensive progress has been done in an attempt to use CNTs as brick elements for the development of advanced materials with remarkable electrical, thermal and mechanical properties. In this sense, the fabrication of diverse CNT-based composites has opened their use in a wide repertoire of applications, from flexible electrodes in displays to high-performance composites for aircraft and automotive industries.^{3, 4} CNTs have also attracted significant attention in the emerging field of nanobiotechnology, thus being already exploited for the preparation of biosensors,⁵ molecular transporters for drug, gene and protein delivery,⁶ and fuel-powered artificial muscles.⁷ In the area of tissue engineering,⁸⁻¹¹ they have been extensively explored for bone regeneration¹² and nerve tissue repair.¹³ In this context, a wide repertoire of methodologies has been explored and optimized for the preparation of 2D CNT-based composites (e.g., films and fibers).³ However, the fabrication of 3D architectures has been more rarely accomplished, with some approaches including chitosan-based matrices,¹⁴⁻¹⁸ gelatin and/or methacrylate hydrogels^{19, 20} or collagen scaffolds.²¹ Furthermore, diverse polymeric systems have been exploited for CNT

dispersion, such as poly(lactic-co-glycolic acid)²² or poly(methyl methacrylates).²³

Nanomaterial-induced cytotoxicity has been related to size/mass, shape, surface charge, and functionalization,²⁴ with both size-dependent²⁵ and composition-dependent²⁶ toxicity mechanisms. In the particular case of CNTs, an increasing number of studies have focused on their use as “brick” elements of 2D and 3D materials envisioned for biomedical applications.²⁷⁻³¹ Nevertheless, the toxicity of CNTs remains controversial.³² In order to bring some insights into the still uncertain interaction of 3D structures composed of CNTs with mammalian cells, we explored herein the interaction of three cell types (i.e., murine fibroblasts, human bone sarcoma cells and porcine endothelial progenitor cells) with MWCNT-based 3D scaffolds that showed different architectural and morphological features at the microscale. The introduction of MWCNTs in these structures not only allowed for the acquisition of 3D architectures, but also conferred superior mechanical properties and electrical conductivity to the resulting scaffolds. These matrices were obtained by the application of the ice segregation induced self-assembly (ISISA) process into MWCNT aqueous suspensions containing different polymers (i.e., chitosan, chondroitin sulphate and gelatin), that acted as dispersant agents.³³ The chemical nature of every polymer contributed to the formation of a particular patterned porous structure.³⁴⁻³⁶ Besides the use of different polymers, the scaffold structure was modified by the incorporation of two different types of functionalized MWCNTs

(i.e., long MWCNTs and short MWCNTs; abbreviated as LN and SN, respectively), as well as by the application of two different temperatures during the ISISA process (i.e., -196 and -65 °C). Cell-material interaction was assessed by studying cell viability, adhesion, morphology, and apoptosis. Time-lapse confocal laser scanning microscopy was also used to explore, for the first time in CNT-based scaffolds, cell migration processes in real time.

2. Results and Discussion

2.1. Scaffold characterization

CNT-based scaffolds displaying different architectural and morphological features were obtained by the application of the ISISA process, a simple and versatile bottom-up technique based on the unidirectional immersion of an aqueous solution, a colloidal suspension or even a hydrogel into a liquid nitrogen bath and subsequent freeze-drying. This process has demonstrated its suitability for the preparation of inorganic, organic and hybrid macroporous monoliths and fibers.³⁷⁻³⁹ In this particular case, we applied the ISISA process to MWCNT aqueous suspensions that were prepared using different polymers as dispersant agents. The polymers of choice were chitosan,⁴⁰ chondroitin sulphate^{41, 42} and gelatin,⁴³ all well-known biocompatible polymers. For scaffold crosslinking, we used chemical agents that have been also extensively explored for the fabrication of biocompatible materials (e.g., glutaraldehyde and hexamethylene diisocyanate).^{44, 45} Besides the use of different polymers, the scaffold morphology was modified by the incorporation of two different types of MWCNTs. The first type, defined as LN, was obtained upon acid treatment with HNO₃ that resulted in MWCNT functionalization with carboxylic groups without significant modification of their original length (i.e., 5-9 µm). For the second type (i.e., SN), MWCNTs were exposed to a more aggressive acid treatment with a mixture of HNO₃ and H₂SO₄ that not only promoted the functionalization with carboxylic groups, but also shortened their original length to ca. 3 µm. The resulting six scaffolds (i.e., LNCHI, SNCHI, LNCHO, SNCHO, LNGEL, and SNGEL) exhibited a homogeneous structure throughout the entire monolith that differed depending on the particular polymer and MWCNTs used (Figure 1-SI). Thus, the use of CHI and CHO promoted the formation of scaffolds with a well-patterned cross-section structure, whereas those scaffolds prepared from GEL suspensions exhibited a more disordered patterned-like one (Figure 1 and 2). We also prepared an additional type of LNCHI scaffolds where both the pore diameter and the width of the walls that support the porous structure were modified by the application of the ISISA process at -65 °C (rather than -196 °C) (Figure 3).⁴⁶ These scaffolds were abbreviated as LNCHI-65.

The morphology of the different scaffolds also differed in terms of porosity and surface roughness (Table 1). In regard to porosity, we analyzed from SEM micrographs of cross-sectioned scaffolds the pore area per cross-sectioned area (A_p) and the averaged pore width (W_p). The most significant differences were found between LNCHI-65 and the rest of scaffolds because of the well-known influence that the freezing temperature has on scaffold morphology.⁴⁶ Nonetheless, A_p and W_p of scaffolds prepared at -196 °C from different polymers also experienced a noticeable change, both parameters decreasing from CHI- to

CHO- up to GEL-based scaffolds. Finally, SN-type scaffolds also exhibited significant differences in both parameters as compared to their respective LN-type analogues. Interestingly, A_p increased for every SN-type scaffold whereas W_p increased for SNCHI and SNCHO, but decreased for SNGEL.

Table 1. Scaffold characterization including porosity, surface roughness, mechanical properties, and electrical conductivity.

Scaffold	Pore area per μm^2 (A_p)	Pore width (W_p) (μm)	RMS roughness (R_q) (nm)	Young's Modulus (MPa)	Conductivity (S cm^{-1})
LNCHI	0.60 ± 0.14	13.8 ± 2.8	220	5.2	1.70
LNCHI-65	0.69 ± 0.05	62.8 ± 43.9	190	1.2	0.70
SNCHI	0.69 ± 0.04	21.3 ± 4.4	120	4.0	0.32
LNCHO	0.51 ± 0.03	8.9 ± 2.7	220	7.0	1.57
SNCHO	0.58 ± 0.03	9.4 ± 3.0	115	5.3	0.13
LNGEL	0.45 ± 0.13	7.4 ± 3.6	380	8.4	0.05
SNGEL	0.51 ± 0.13	5.8 ± 1.8	280	12.1	n/a

Figure 1. SEM images of LNCHI (A) and SNCHI (B) scaffolds, along with their 3D reconstructed images (C and D, respectively). Scale bars: 10 µm (A) and 20 µm (B).

Figure 2. SEM images of LNCHO (A), SNCHO (B), LNGEL (C), and SNGEL (D). 3D reconstructed image from LNGEL (E). Scale bars: 20 µm (A, B) and 10 µm (C, D).

When analyzing the roughness of the walls that define the cross-sectioned structures on which cells were cultured (Figure 1, 2 and 3, and Table 1), the root mean square (RMS) roughness increased from scaffolds with a well-patterned cross-section

structure (*i.e.*, CHI and CHO) to those with a disordered one (*i.e.*, GEL). This behavior was ascribed to both the dimensions of MWCNTs and their rigid nature, which determined a better accommodation of MWCNTs within the walls of LNCHI and LNCHO rather than on LNGEL scaffolds. The relevance of the dimensions of MWCNTs and their rigid nature on this parameter was corroborated on SN-type scaffolds. In this case, RMS roughness experienced a significant decrease in every scaffold because the use of shorter MWCNTs was obviously facilitating their accommodation and the subsequent formation of more compact walls. Additionally, the dimensions of the walls also played a role as lower RMS roughness values were found in the thick walls of LNCHI-65 scaffolds than in the thinner ones of LNCHI.

Figure 3. SEM images showing the macroporous structure of LNCHI-65 (A) and a detail of MWCNTs accommodating within scaffold walls (B). 3D reconstructed images from (A) and (B) are shown (C, D respectively). Scale bars: 40 μm (A) and 5 μm (B).

Finally, the mechanical properties and conductivity of the different scaffolds were found strongly influenced by the above-mentioned differences in architecture and morphology (Table 1). For instance, GEL-based scaffolds showed the highest Young modulus and the lowest conductivity likely because of a more deficient connection between MWCNTs in their poorly compacted walls.

2.2. Cell type rationale and size considerations

We first used murine L929 fibroblasts (Figure 4A-B) as a reference cell line for preliminary biocompatibility studies *in vitro*.⁴⁷ They are considered slow-moving cells with a typically elongated morphology in culture. Human Saos-2 osteoblasts were selected as a mature cell type displaying a more polygonal morphology in culture (Figure 4C-D) and previously used in the cytocompatibility assessment of CNT-based materials.^{28, 29} Finally, we explored the behavior of porcine endothelial cells derived from peripheral blood progenitors (EC_{PC} cells), as previous studies have reported a higher susceptibility of some stem cells to CNT-induced cellular damage.⁴⁸ Since their discovery by Asahara et al. in 1997,⁴⁹ EC_{PC} cells have already demonstrated an excellent therapeutic potential for diverse

applications, such as vascular repair and bone healing.^{50, 51} Contrary to L929 fibroblasts and Saos-2 osteoblasts, EC_{PC} cells are larger (both in length and width) and tend to spread on the substrate acquiring a pavement-like morphology to imitate the vascular endothelial lining (Figure 4E-F). Table 2 summarizes dimensions of the different cells used in this study. For comparative purposes, data were represented as the ratio between cell length and width (*l/w*), so that elongated cells were characterized by higher *l/w* ratios than rounded cells and vice versa. We assumed that the ability of the three different cell types to initially colonize the scaffold was similar, as cells showed a comparable size when trypsinized.

Table 2. Size comparison among the different cell types used in these studies. Cell size was measured and averaged after either trypsin treatment (in suspension) or culture for 48 h on TCP (adhered in culture). The ratio *l/w* was calculated as cell length divided by cell width. Statistics: *** *p* < 0.005.

	L929 fibroblasts	Saos-2 osteoblasts	EC _{PC} cells
<i>In suspension</i>			
Diameter (μm)	5.2 ± 0.69	4.7 ± 0.76	5.5 ± 1.01 ***
<i>Adhered in culture</i>			
Length (μm)	16.0 ± 3.87	13.5 ± 4.04	24.6 ± 7.12 ***
Width (μm)	3.8 ± 0.86	4.7 ± 1.37	7.6 ± 2.35 ***
<i>l/w ratio</i>	4.2	2.9	3.2

Figure 4. Light optical microscopy images of L929 fibroblasts (A, B), Saos-2 osteoblasts (C, D) and EC_{PC} cells (E, F). Cell size was estimated from cell length (*l*) and width (*w*), as indicated in representative cells (white arrows). Scale bars: 20 μm.

2.3. Cell viability and apoptosis studies

Cell viability was investigated on every scaffold for the three different cell types mentioned above. For this purpose, two different probes were used simultaneously: calcein, for alive cells, and EthD-1, for dead cells. Initial studies with L929 fibroblasts revealed that cultures on LNCHI and LNCHO scaffolds exhibited superior viabilities than those on LNGEL (Figure 5 and Table 3). This behaviour could be ascribed to either the presence of polymers with different chemical functionalities or to the different scaffold morphology. To discard the first hypothesis, control cultures were carried out on glass coverslips homogeneously coated with a thin crosslinked polymer layer of CHI, CHO or GEL (Figure 2-SI and Table 3). Cell viability values on the three polymers were comparable to those obtained on tissue culture polystyrene (TCP, control surface) for the three cell types, thus confirming the absence of a relevant role played by polymer chemistry on this parameter. In consequence, we further focused on scaffold architecture and morphology as

key responsible parameters for cell behavior. Interestingly, previous studies by Giannona et al. evidenced a significant influence of CNT organization on cell morphology, orientation and growth of osteoblast-like cells.⁵² For this purpose, L929 fibroblasts were seeded on LNCHI-65 scaffolds, which displayed significant architectural and morphological differences with respect to the rest of scaffolds. Cells on LNCHI were mostly suspended in the air occupying the pore space (Figure 5B), whereas those on LNCHI-65 spread on the thick walls that characterize its structure (Figure 5D). However, despite the architectural differences between scaffolds, similar viability values were obtained.

Table 3. Cell viability on the different substrates. Statistically significant differences ($p < 0.05$): (a) respect to TCP for a particular cell type, (b) between LN and SN for a particular scaffold and cell type and (c) among different cell types for a particular scaffold.

Cell type	Scaffold	Viable cells (green) (%)	Dead cells (red) (%)	Apoptotic cells (green+red) (%)
L929	TCP	99 ± 1	1 ± 0	0 ± 0
	CHI	99 ± 1	1 ± 0	0 ± 0
	LNCHI	88 ± 2 ^a	10 ± 2 ^a	2 ± 1 ^a
	LNCHI-65	80 ± 4 ^a	20 ± 4 ^a	0 ± 0
	CHO	99 ± 1	1 ± 0	0 ± 0
	LNCHO	96 ± 2	4 ± 2	0 ± 0
	GEL	99 ± 1	1 ± 0	0 ± 0
	LNGEL	72 ± 8 ^a	25 ± 9 ^a	3 ± 1 ^a
Saos-2	TCP	99 ± 1	1 ± 0	0 ± 0
	CHI	99 ± 1	1 ± 1	0 ± 0
	LNCHI	76 ± 5 ^a	19 ± 5 ^{a,c}	5 ± 2
	SNCHI	76 ± 2 ^a	23 ± 2 ^a	1 ± 1
	CHO	99 ± 1	1 ± 0	0 ± 0
	LNCHO	82 ± 8	14 ± 9	4 ± 2
	SNCHO	83 ± 2 ^a	15 ± 2 ^a	2 ± 1
	GEL	98 ± 1	2 ± 1	0 ± 0
	LNGEL	61 ± 15 ^a	36 ± 17 ^a	3 ± 3
	SNLNGEL	94 ± 1 ^b	5 ± 2 ^b	0 ± 0
EC _{PC}	TCP	98 ± 1	2 ± 2	0 ± 0
	CHI	99 ± 1	1 ± 1	0 ± 0
	LNCHI	50 ± 15 ^{a,c}	11 ± 5	39 ± 10 ^{a,c}
	SNCHI	58 ± 14	14 ± 3 ^{a,c}	28 ± 13 ^c
	CHO	97 ± 2	2 ± 2	1 ± 1
	LNCHO	73 ± 13	4 ± 6	23 ± 13 ^c
	SNCHO	90 ± 3 ^c	5 ± 1 ^c	5 ± 2 ^{b,c}
	GEL	97 ± 2	2 ± 1	1 ± 1
	LNGEL	0 ± 0 ^{a,c}	46 ± 25 ^a	54 ± 25 ^{a,c}
	SNLNGEL	16 ± 7 ^{a,b,c}	5 ± 5 ^b	79 ± 8 ^{a,c}

Figure 5. L929 fibroblast cell viability on LNCHI (A, B), LNCHI-65 (C, D), LNCHO (E, F), and LNGEL (G, H) scaffolds. Dead cells appear stained in red, while live cells stain in bright green. Representative culture images at 48 h are shown. Scale bars: 75 μ m (top) and 25 μ m (bottom).

The lack of conclusive results from L929 fibroblast cultures decided us to investigate the role of morphological scaffold features on the viability of more selective cell types (e.g., Saos-2 and EC_{PC} cells). While some cytocompatibility studies of Saos-2 cells cultured on CNT-based scaffolds exist,^{28, 29} the behavior of

EC_{PC} cultures on this type of materials has not been explored yet. CLSM studies demonstrated a similar trend on the viability of Saos-2 cells than that described for L929 fibroblasts (i.e., LNCHO = LNCHI >> LNGEL). However, the viability of EC_{PC} cells experienced a significant decrease, reaching a dramatic situation for LNGEL scaffolds, where negligible values of viable cells were found (Figure 6, 3-SI and 4-SI, and Table 3). A close inspection of these micrographs revealed a higher percentage of cells with double green/red fluorescence, likely attributed to apoptotic cells, when EC_{PC} cells were cultured on LNGEL. This result is not surprising, as cells respond differently depending on the structure of nanomaterials⁵³ and CNTs have been described to induce apoptosis in several cell types.²⁷ For instance, epidermal keratinocytes, a cell type with a spread adhesion pattern comparable to that in EC_{PC} cells, experienced oxidative stress and reduction in cell viability when exposed to MWCNTs.⁵⁴ To further confirm this aspect, we analyzed the cellular externalization of phosphatidylserine by annexin-V binding on LNGEL (low viability) and LNCHI scaffolds (high viability) (Figure 7). Saos-2 cells were also tested as a cell control with high viability. On LNCHI, some EC_{PC} cells were identified in early and late apoptotic stages (green and green/red fluorescence, respectively), as well as dead cells (red). On LNGEL, however, both EC_{PC} cell death and apoptosis significantly increased, thus confirming the reduction in cell viability previously observed. Contrary to results with EC_{PC} cells, Saos-2 cell cultures showed negligible cell death (either necrotic or apoptotic) on LNCHI scaffolds and a majority of viable cells in LNGEL.

Figure 6. Cell viability of Saos-2 osteoblasts and EC_{PC} cells on LNCHI, LNCHO and LNGEL scaffolds. Representative culture images at 48 h are shown. Scale bars: 25 μ m.

Figure 7. Apoptosis studies on EC_{PC} (A-D) and Saos-2 cells (E-H) cultured on LNCHI (C, G) or LNGEL scaffolds (D, H). Initial viability of the cultures was also confirmed (A, E). Incubation with H₂O₂ for 1 h was used as positive control for apoptosis induction (B, F). Early apoptotic cells expressing annexin-V were stained in green (white arrows), while dead cells were marked in red (*). Cells with double staining were indicative of cells experiencing late apoptosis (#). Scale bars: 75 μm.

2.4. Cell adhesion and morphology studies by SEM

With the aim of identifying more precisely the parameter that was governing the difference in viability observed, the adhesion and colonization of the scaffold surface by Saos-2 osteoblasts and EC_{PC} cells were studied by SEM (**Figure 8**). At first glance, the presence of CNTs did not alter cell adhesion and morphology when compared to cultures on TCP (**Figure 4**) and thin polymer films (**Figure 2-SI**), as previously described for other CNT-based materials.⁵⁵ Interestingly, as anticipated by CLSM studies (**Figure 6**), Saos-2 osteoblasts colonized LNCHI and LNCHO scaffolds similarly to L929 fibroblasts, with cells mostly suspended in the air occupying pore spaces between scaffold walls. The comparable size of both cell types with the pore width allowed them to fit into the pores, thus minimizing the contact points between cell and substrate (**Figure 8C**). This was not the situation for EC_{PC} cells, whose larger size made them cover more than a single pore when forming a pavement-like monolayer that resembled native endothelialization of vascular conduits (**Figure 8D and 8E**). As compared to L929 and Saos-2 cells, the innate adhesion pattern of EC_{PC} cells led to increase their contact surface with the scaffold. This situation was stressed for cultures on LNGEL, where its narrower pores (**Figure 2 and Table 1**) forced EC_{PC} cells to spread across a larger number of pores (**Figure 8F and 8I**).

Figure 8. Cell adhesion and morphology of Saos-2 osteoblasts on LNCHI (A-C) and porcine EC_{PC} cells on LNCHI (D, G), LNCHO (E, H) and LNGEL (F, I). Representative 48-h culture images are shown. Scale bars: 50 μm (A) and 20 μm (B-I).

However, the great deal of cell harm caused by LNGEL scaffolds is difficult to justify only in terms of contact surface. At this stage, one should consider that also roughness has to play a role in cell damage. Thus, the reduced and negligible viability found in LNGEL (for Saos-2 and EC_{PC} cells, respectively) could be ascribed not only to the increase in contact surface, but also to its roughness, much higher than in any of the other scaffolds (**Table 1**). This issue was actually corroborated by the viability enhancement observed in all SN-type scaffolds when compared to their LN counterparts (**Figure 9**). Particularly insightful was the case of EC_{PC} cells cultured on SN-type scaffolds where, as compared to LN-type ones, the decrease in RMS roughness was accompanied by a significant increase in the percentage of viable cells (**Figure 9F, Table 3**). Unfortunately, in the particular case of SNGEL scaffolds and while recognizing the achievement of a neat viability improvement, this was not sufficient to support adequate EC_{PC} cell viability and makes questionable the use of these scaffolds for EC_{PC} cell growth. Finally, it is worth noting that SNCHI scaffolds not only exhibited a decrease in RMS roughness, but also an increase in A_p and W_p that might also benefit cell viability. In any case, a similar cell viability improvement was also observed in SNCHO, where changes in A_p and W_p were negligible when compared to LNCHO, thus confirming roughness as a critical parameter for cell damage.

Figure 9. Cell viability of EC_{PC} cells cultured on LNCHO (A), SNCHO (B), LNGEL (C), and SNGEL (D) scaffolds. Details of stained cells are included as insets. Representative 48-h culture images are shown. Scale bars: 25 μm. (E, F) Simultaneous increase in scaffold pore area per μm² (open labels, left Y axis) and cell viability (filled labels, right Y axis) induced by the use of SN in Saos-2 (E) and EC_{PC} (F) cell cultures.

2.5. Cell migration studies by time-lapse CLSM recording

Finally, we hypothesized that the visualization of the early cell-material interaction would provide evidence of cell movements for scaffold colonization and further corroborate the relevance of porosity, roughness and contact surface on material cytocompatibility. Time-lapse recording is a powerful technique recently applied for this purpose.⁵⁶ However, and despite its potential, it has been rarely used for the exploration of cell behavior on 3D matrices^{57, 58} and no reports exist to date on the application of this technique to evaluate cell interactions with CNT-based substrates. Herein, we report for the first time the use of time-lapse CLSM to visualize migration of human Saos-2 cells

on 3D CNT-based scaffolds. For these studies, LNCHI, SNCHI and LNGEL scaffolds were selected as representative of the different architectures and morphologies investigated in this work (Figure 5-SI and Movie 1, 2 and 3 in supplementary data). TCP was used as a control surface (bottom panel in Figure 5-SI). As shown in the recordings, Saos-2 cells exhibited not only a well spread morphology once adhered to the substrates, but also an evident motility that exploited in the exploration of the scaffold surface. Interestingly, cells on LNCHI and SNCHI scaffolds seemed to move more actively than those on LNGEL or TCP, where cell displacements slightly diminished. Particularly, cell movements were more evidently appreciated in SNCHI. According to this, Aoki et al. also observed a strikingly extended morphology of Saos-2 cells when in culture on CNT-containing polycarbonate scaffolds,²⁹ probably attributed to an enhanced protein adsorption driven by the inherent adsorptive properties of CNTs. The biocompatible polymers used herein for CNT dispersion and scaffold fabrication likely contributed to this favored cell adhesion and motility, as Mwenifumbo et al. observed a rounded morphology in Saos-2 cells when cultured on plain nano-structured MWCNT-based constructs.²⁸ From a molecular perspective, this higher cell motility could be related to CNT-induced effects on the expression of the focal adhesion kinase (FAK), actively implicated in cytoskeleton organization, migration and proliferation,⁵⁹ as previously observed in HeLa cells on non-oriented CNT-based scaffolds.²⁷ We hypothesize that these movements described by the cells in order to find proper adhesion locations further contributed to the induction of cell damage on those scaffolds where contact surface and roughness were enhanced.

3. Experimental

3.1. Materials

Chemical reagents were purchased from Sigma-Aldrich and used as received unless otherwise indicated. For scaffold preparation, low molecular weight chitosan, chondroitin sulphate, gelatin, and MWCNTs (110-170 nm in diameter, 5-9 μm in length) were used. Cell culture media and supplements were purchased from Lonza.

3.2. Purification and chemical functionalization of MWCNTs

MWCNTs were purified and functionalized by acidic treatment with HNO_3 at 130 $^\circ\text{C}$ for 5 h. After cooling at room temperature, the suspension was filtered by using cellulose filters (0.2 μm pore size) and washed in distilled water to eliminate any acidic residues. MWCNTs were then freeze-dried and stored until used. The so obtained MWCNTs were named as *long* MWCNTs (LN). The *short* MWCNTs (SN) were obtained by exposure of LN to a stronger acidic treatment with H_2SO_4 and HNO_3 .

3.3. 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, either LN or SN MWCNTs were dispersed in chitosan solutions (1 wt % in acetic acid 0.15 M, pH 4.5). 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: constant dipping rate of 5.9 mm min^{-1} at -196 $^\circ\text{C}$ (liquid nitrogen) or 0.9 mm min^{-1} at -65 $^\circ\text{C}$ (dry ice). The resulting monoliths were additionally exposed to glutaraldehyde

vapors (GA, 50 wt % in distilled water) at room temperature for 24 h 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) with the exception that scaffold crosslinking was achieved by exposure to hexamethylene diisocyanate (HMDI) vapors at 37 $^\circ\text{C}$ 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. Singular scaffolds were obtained from freeze-dried monoliths by cryo-fracture.

3.4. Preparation of 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) were used to homogeneously coat circular glass coverslips by spin-coating, followed by polymer crosslinking with GA or HMDI.

3.5. 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. Porosity was 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 for 3D data acquisition and object reconstruction using stereo-photogrammetry. The root mean square (RMS) roughness (R_q) was obtained from the reconstructed 3D model of the different specimens for a path length 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 Scaffold conductivity was measured by using a four-point method.⁶¹

3.6. 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. DMEM supplemented with fetal bovine serum (10 %), streptomycin (100 UI ml^{-1}), penicillin (100 UI ml^{-1}), and L-glutamine (1 mM) was used for fibroblasts and osteoblasts and EGM-2 media for EC_{PC} cells. Cultures were maintained in static conditions at 37 $^\circ\text{C}$ in a sterile incubator under a CO_2 (5 %) atmosphere and visualized by using an Axiovert CFL-40 optical microscope when possible. Tissue culture polystyrene (TCP) was used as a control surface.

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 or after culture on TCP.

3.7. Viability and apoptosis studies

To test cell viability, cells cultured on the different substrates were analyzed by using a Live/Dead® Viability kit (Invitrogen), based on the use of two probes: calcein and ethidium homodimer-1 (EthD-1). 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 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 to visualize the scaffold structure and the relative cellular location. To quantify cell viability, the number of live (green), dead (red) and apoptotic (green and red) cells were counted from 20 x images.

Apoptotic cells in the cultures were specifically detected by using an annexin V-FITC apoptosis detection kit (Sigma). 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. Positive controls were obtained after cell treatment with H₂O₂ (1 mM in PBS) for 90 min.

3.8. Cell adhesion and morphology studies by SEM

Cells cultured on the scaffolds for 48 h were examined by using a DSM-950 scanning electron microscope (Zeiss).

3.9. Time-lapse confocal laser scanning microscopy (CLSM)

Cell migration on the different scaffolds was investigated by using time-lapse CLSM recording. 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). 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 °C, 5 % CO₂).

3.10. Statistics

Values were expressed as mean ± standard deviation. Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS). Comparisons were done by analysis of variance (3 or more groups) or t Student (for 2 groups). In all cases, $p < 0.05$ was considered statistically significant.

4. Conclusions

Herein we explored the interaction of three types of mammalian cells with MWCNT-based 3D scaffolds that showed different architectural and morphological features at the microscale. **Figure 10** summarizes the different scenarios reported in this work. Particularly, scaffolds displaying both a pore size in range with that of cells and lower surface roughness revealed the highest viability values (e.g., SNCHO). On the contrary, those with a smaller pore size and higher surface roughness accounted for the lowest cytocompatibility (e.g., LNGEL). These dramatic differences in cell viability found among scaffolds evidenced both material- and cell-dependent parameters as responsible for an adequate cell-material interaction, thus making a case-by-case

study necessary. As a general trend, minimizing both the contact points between cell and substrate and the roughness of this contact surface provides evident benefits on cell viability. In this sense, CHI and CHO scaffolds were presented as more favorable substrates for cell growth. Additionally, we reported for the first time highly viable cultures of EC_{PC} cells on 3D CNT-based scaffolds, thus opening their application in stem cell research as previously evidenced for other types of progenitor cells.^{13, 63} Results from these studies bring novel insights into the still uncertain biological reactivity of CNT-based materials and benefit the fabrication of optimized biomaterials by varying scaffold-dependent parameters in accordance to those of target cells.

Figure 10. Scheme summarizing the correlation between scaffold- and cell-dependent parameters in the overall cytocompatibility of MWCNT-based 3D scaffolds.

Notes and references

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^a Instituto de Ciencia de Materiales de Madrid (ICMM), Consejo Superior de Investigaciones Científicas (CSIC), Calle Sor Juana Inés de la Cruz 3, 28049-Madrid, Spain. Fax: (+34) 91 3720623; Tel: (+34) 91 3349000; E-mail: conchi.serrano@icmm.csic.es, delmonte@icmm.csic.es

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Graphical Abstract

Carbon nanotubes (CNT) are acquiring a tremendous impact as structural and functional components of biomaterials. Herein, we extensively explore the influence of architectural/morphological features and cell-dependent parameters on the cytocompatibility *in vitro* of 3D CNT-based scaffolds to benefit their optimization as cell growth platforms.

SUPPORTING INFORMATION

Modulating the cytocompatibility of tridimensional carbon nanotube-based scaffolds

Stefania Nardecchia, María Concepción Serrano^{*}, María Concepción Gutiérrez, María Luisa Ferrer, and Francisco del Monte F^{*}

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 $^\circ\text{C}$ 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 °C 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 °C (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 °C 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 °C for 3 h under stirring. After cooling at 4 °C, the resulting hydrogel was dialyzed in distilled water and further dissolved at 50 °C. The final solution was then freeze-dried and stored at 4 °C until used. As an additional precaution, nanotube dispersion and syringe preparation for GEL-containing scaffolds were performed at 37 °C to avoid undesired gelation.

4. Preparation of 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) were prepared and used to homogeneously coat circular glass coverslips by spin-coating at 500 (for CHI) or 750 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 -5°). 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 (R_q) was obtained from the reconstructed 3D model of the different specimens for a path length 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 μ l) 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 °C in a sterile incubator under a CO₂ (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 dichroic 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 μ l in 500 μ l 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, $5 \mu\text{g ml}^{-1}$ 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 °C, 5 % CO₂). 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 $\pm$ 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.

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 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 4-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 5-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.