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A cytotoxicity study of silicon oxycarbide nanowires as cell scaffold for biomedical applications

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ABSTRACT

Goal: Nanowires are promising biomaterials in multiple clinical applications. The goal of this study was to investigate the cytotoxicity of carbon-doped silica nanowires (SiO_xC_y NWs) on a fibroblastic cell line in vitro.
Materials and methods: SiO_xC_y NWs were grown on Si substrates by CVD process. Murine L929 fibroblasts were cultured in complete DMEM and indirect and direct cytotoxicity tests were performed in agreement with ISO 19003-5, by quantitating cell viability at MTT and chemiluminescent assay. Cell cultures were investigated at Scanning Electron Microscope (SEM) and immunocytochemistry to observe their morphology and investigate cell-NWs interactions. Furthermore, hemocompatibility with Platelet-rich Plasma was assayed at SEM and by ELISA assay.
Results: SiOxCy NWs proved biocompatible and did not impair cell proliferation at contact assays. L929 were able to attach on NWs and proliferate. Most interestingly, L929 reorganised the NW scaffold by displacing the nanostructure and creating tunnels within the NW network. NWs moreover did not impair platelet activation and behaved similarly to flat SiO_2.
Conclusions: Our data show that SiOxCy NWs did not release cytotoxic species and acted as a viable and adaptable scaffold for fibroblastic cells, thus representing a promising platform for implantable devices.

1. Introduction

Tissue regeneration requires biomaterials capable to provide a scaffold for ingrowing cells and replace the missing extracellular matrix (ECM). The ECM is a complex structure, a controlled microenvironment that coordinates cell activity by providing a vast array of biochemical and mechanical signals [1]. Biomaterials, therefore, should ideally be bioactive, i.e. possess adequate physical and chemical properties to promote wound healing and regeneration, support cell function, and provide cells with direct stimuli to promote cell growth and differentiation and activate metabolic cascades that are conducive to tissue repair. Tissue extracts or derivatives are often used in vitro and in vivo as scaffolding material because they mimic the structure of pristine tissues, in spite of their availability and performance limits [2,3]. Alternative materials, which can be tailored to meet tissue requirements, are therefore a significant need for research and therapy alike. Silicon oxycarbide (SiO_xC_y), which is obtained by carbon monoxide gas as a dopant precursor, has been shown to have higher elastic modulus, bending strength and hardness, higher chemical durability than conventional silicate glasses in aggressive environments and greater stability at high temperatures [4–6]. Furthermore, silicon oxycarbide has been demonstrated to increase platelet aggregation and activation [7], thereby promoting rapid clot formation and the onset of the acute inflammatory process, which is necessary for the creation of an adequate provisional matrix and subsequent wound healing [8,9]. Furthermore, SiO_xC_y can be easily engineered through functionalization and decoration with macro-molecules and nanoparticles [10–12], which makes it an interesting platform for several experimental approaches.

Micro- and nano-structured materials can provide specific topographical and mechanical cues, which are known to affect cell behavior through activation of focal adhesion-mediated intracellular signaling cascades [13–16]. Nanowires (NWs) are one-dimensional structures that can be arranged in three-dimensional (3D) bundles, which strikingly resemble the organization of ECM fibrils and could therefore be a promising candidate for artificial matrices in different clinical situations [17].

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The aim of the present works was to further investigate carbon-doped SiO_xC_y nanowires grown by CVD process [18] as a viable scaffold candidate for the regeneration of connective tissue by assessing the responses of a fibroblastic cell line to NWs-coated substrates and the effects of such NW coating on platelet activation.

2. Materials and methods

2.1. Nanowire production

SiO_xC_y NWs were grown on Si substrates by an established CVD process, lowering the growth temperature to 1070 °C. In this process, carbon monoxide acts as dopant precursor, so that carbon-doped understoichiometric silicon dioxide NWs are obtained [18]. X-Ray Photoemission Spectroscopy (XPS) measurements indicated that carbon content was 13.4 at.% (silicon 35.8 at.%, oxygen 50.8 at.%) at the NW surface. The typical SiO_xC_y NW length was several tens of ♣m whereas the average diameter was slightly larger, approximately 100 nm.

The samples were analyzed by a Field-Emission SUPRA40 Zeiss Scanning Electron Microscope (SEM) equipped with a GEMINI FESEM detection column. These observations proved that the substrate was entirely covered by a highly dense NW network (between 5 and 10 exposed NWs per square micron), and that the morphology was comparable for all the NWs. The NW structure and core size and crystallinity were determined by Transmission Electron Microscopy (TEM) analyses, performed on single NWs in a Field–Emission JEOL JEM-2200FS TEM microscope operated at 200 kV, either in conventional or scanning (STEM) mode.

2.2. Cell cultures

Dulbecco's Modified Eagle Medium (DMEM) was purchased from PAA, GE Healthcare, Uppsala, Sweden, fetal bovine serum (FBS) was purchased from Gibco, ThermoFisher, Waltham, MA, USA, antibiotics for cell cultures and all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA); disposable sterile plastics was purchased from Costar (Cambridge, MA, USA). L929 mouse fibroblast cells (NCTC clone 929 of strain L, derived from a C3H/An male mouse) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA distributed by LGC Standards S.R.L., Sesto S. Giovanni, MI, Italy) and cultured in complete DMEM. All media were supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-Glutamine (2 mM) and cells were maintained at 37 °C and 5% CO_2 in a water saturated atmosphere.

2.3. Indirect contact cytotoxicity test

Three thousand cells/ml were seeded in 96 multiwell plates in DMEM and the plates were maintained at 37 °C and 5% CO_2 in a humidified atmosphere for 24 h. A NW sample was soaked for 24 or 240 h in DMEM at 37 °C and 95% of humidity, and, as a control, the same volume of DMEM was treated in the same condition. To analyze the potential release of cytotoxic agents from the sample, cells were cultured for 24 h. Conditioned medium was added to the pristine medium at different percentages (50%, 70% and 100% of the total DMEM volume; protocol in agreement with ISO 10993-5 guidelines for porous materials) and the cells were cultured for further 24 h. As recommended by ISO guidelines, an MTT colorimetric assay (Roche Applied Science, Penzberg, Germany), based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye, was performed to assess cell viability. To confirm the results, a CellTiter-Glo chemiluminescent cell viability assay (Promega, Madison, WI, USA), a homogeneous method of determining the number of viable cells based on quantitation of the ATP present, was performed.

2.4. Direct contact cytotoxicity test

Seventy thousand cells/ml were seeded on 1 cm × 1 cm NW samples and cultured in complete DMEM as described above. Plates were maintained at 37 °C and 5% CO_2 in a humidified atmosphere for 24, 48 h and 96 h. To evaluate viable and dead cells on NWs, the samples were rinsed with abundant DMEM and phosphate buffer solution (PBS, Sigma-Aldrich), and marked with calcein, a fluorescent dye with excitation/emission wavelengths of 495/515 nm and specific for living cells, while dead cells were labelled with propidium iodide, a fluorescent dye with excitation/emission wavelengths of 560/720 nm. Cell viability was then analyzed via Fluorescence microscopy (Axioscope, Zeiss, Germany). For cell viability quantitation, the area covered by cells was measured with Zen Pro software (Zeiss) and normalized to the control. Fluorescence microscopy Fluorescence microscopy observations were performed at 24, 48, and 96 h.

2.5. Viability test

Twenty-thousand cells/ml were seeded on 1 cm × 1 cm NW samples and cultured in DMEM. Plates were maintained at 37 °C and 5% CO_2 in a humidified atmosphere for 24, 48 and 96 h; the cell viability was then analyzed via CellTiter-Glo assay, as preliminary studies determined that MTT assays were not feasible for the NW samples due to direct chemical interactions with the material that did not reflect the biological response.

2.6. Real time PCR

Total RNA was purified from cell cultures using Trizol (Life Technologies Italy, San Giuliano Milanese, MI, Italy) according to the manufacturer's directions. TaqMan quantitative RT-PCR was performed as previously described using the following primer probe sets from Applied Biosystems (Foster City, CA, USA): Alkaline Phosphatase (Mm00475834_m1); Cyclin D1 (Mm00432359_m1); Collagen 1a1 (Mm00483387_m1). GAPDH (Mm99999915_g1) was used as housekeeping gene.

2.7. SEM imaging of cells on nanowires

For SEM observation, the samples were first washed with PBS at 37 °C and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30′ at RT. After that, samples were dehydrated in ethanol at increasing concentrations. Finally, the samples were critical point dried with liquid carbon dioxide (CPD 030 Baltec, Wallruf, Germany) and the samples were covered by a 8 nm thick gold layer deposited by sputtering using a SCD 040 coating device (Balzer Union, Wallruf, Germany). Cell distribution and morphology on NWs were characterized using a Field-Emission SUPRA40 Zeiss SEM equipped with a GEMINI FESEM detection column, operated at 3 keV.

2.8. Cytoskeleton and focal adhesion analysis

The intracellular cytoskeleton protein vinculin was used as a marker for focal contacts at 24 h of culture. Cells were fixed with 4% paraformaldehyde for 10 min followed by three rinses with PBS. They were then permeabilized with 0.1% Triton-X100 for 5 min followed by three rinses with PBS. Non-specific binding sites were blocked by incubating the samples in 1% bovine serum albumin in PBS for 20 min. Double staining was then performed on each sample. Cells were first stained by a mouse monoclonal anti-vinculin antibody (MAB3574, Chemicon) for 1 h followed by three rinses with PBS. Cells were labelled with FITC-anti-mouse IgG antibody (AP124F, Chemicon) and TRITC-phalloidin for 1 h followed by three rinses with PBS. Nuclear counterstaining was performed by incubation with DAPI (D1306, Molecular Probes, Invitrogen) for 5 min followed by three rinses with PBS. All the steps were carried out inside the culture well at room temperature. The samples were then