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Analysis of cellular adhesion on superhydrophobic and superhydrophilic vertically aligned carbon nanotube scaffolds

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ABSTRACT

We analyzed GFP cells after 24 h cultivated on superhydrophilic vertically aligned carbon nanotube scaffolds. We produced two different densities of VACNT scaffolds on Ti using Ni or Fe catalysts. A simple and fast oxygen plasma treatment promoted the superhydrophilicity of them. We used five different substrates, such as: as-grown VACNT produced using Ni as catalyst (Ni), as-grown VACNT produced using Fe as catalyst (Fe), VACNT-O produced using Ni as catalyst (Ni – O), VACNT-O produced using Fe as catalyst (Fe – O) and Ti (control). The 4′,6-diamidino-2-phenylindole reagent nuclei stained the adherent cells cultivated on five different analyzed scaffolds. We used fluorescence microscopy for image collect, ImageJ® to count adhered cell and GraphPad Prism 5® for statistical analysis. We demonstrated in crescent order: Fe, Ni, Ni – O, Fe – O and Ti scaffolds that had an improved cellular adhesion. Oxygen treatment associated to high VACNT density (group Fe – O) presented significantly superior cell adhesion up to 24 h. However, they do not show significant differences compared with Ti substrates (control). We demonstrated that all the analyzed substrates were nontoxic. Also, we proposed that the density and hydrophilicity influenced the cell adhesion behavior.

1. Introduction

Biomedical industries of implants use for several decades carbon structures (pyrolytic carbon) for production of prosthetic heart valves [1]. Chemical vapor deposition (CVD) produced numerous carbon structures, especially in the form of nanosized tubes encouraging their application on biomedicine [2]. Carbon nanotubes (CNTs) presented a great potential for biomedical applications because of their versatile properties, such as high electrical conductivity, chemical stability and mechanical strength. These characteristics may facilitate their interaction with cells and living tissues [3,4]. Vertically-aligned CNT (VACNT) grown on Ti has a similar nanoarchitecture with physiological components of the extracellular matrix (ECM). Besides this, it plays a fundamental role in the survival, proliferation, and cell spreading, making them potentially attractive for use in biomaterials [4,5].

Generally, as-grown CNT presents superhydrophobic behavior, limiting it as a biomaterial. Despite some evidence of cytotoxicity, studies support the idea of the biocompatibility of CNT applied with scaffolds to grown cell such as neuronal cells, osteoblasts, fibroblasts, antibodies, immune system, DNA and drug delivery, among others [6]. To increase this success, the authors suggested covalent or non-covalent treatments to improve affinity of CNTs with biological media. It was applied to break the strong hydrophobic character, making them more hydrophilic. Functionalization promoted better solubility and biocompatibility of CNT films and powders, respectively [4,7,8].

Biomaterial application studies showed a strong correlation between surface nanotopography and cellular adhesion. These demonstrated that the nanotopography can affect positively or negatively the response of cell adhesion. All these characteristics influenced in correlation between cell and substrate surfaces besides of spatial domains, structural composition, and mechanical forces in micro and nano-scale [9–11]. The physical and physicochemical modification of biomaterial surface properties can improve its interaction with cells [12]. Therefore, our group showed a simple and fast method to promote a superhydrophilicity of CNT using oxygen plasma treatment. It occurs because of the surface change that promotes the inclusion of highly polar carboxylic groups (COH, OH, C═O, and COOH) on them [13,14]. Superhydrophilic biomaterials possess a great interest in biomedical applications because of cellular affinity. Their surface plays several biological phenomena that can significantly affect events at the sub-cellular and cellular levels, such as: protein adsorption, cell adhesion and cell spreading [5].

Cellular behavior, viability, proliferation, and programmed cell death are strong dependent of the first contact of the biomaterial surface. Through this control, the interpretation of cellular biocompatibility and potential application of CNT films as biomaterial will be possible [5]. Here, we presented a comparative study of cellular adhesion using

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five different substrates containing as-grown VACNT, superhydrophilic VACNT (VACNT-O) and Ti (control). We evaluated the superhydrophilicity of VACNT produced by oxygen plasma treatment, VACNT densities and topographies. The presented results open possibilities for a better understanding and potential application of the VACNT scaffold as biomaterials.

2. Materials and methods

2.1. Production of vertically aligned multiwalled carbon nanotubes (VACNTs) on Ti

The vertically aligned multiwalled carbon nanotubes were produced as thin films using a microwave plasma chamber equipped with a 2.45 GHz microwave generator (MWCVD). The substrates were 10 mm titanium (Ti) squares (10 mm^2) covered by a thin Fe and Ni layers (10 nm), respectively, deposited by an e-beam evaporator. We pre-treated the Fe and Ni layers to promote nanocluster formation, which forms the catalyst for VACNT film growth. We carried out the pre-treatment for 5 min in plasma atmosphere of N_2/H_2 (10/90 sccm, standard cubic centimeters per minute) with a substrate temperature of around 760 °C. After pre-treatment, we inserted CH_4 (14 sccm) into the chamber at a substrate temperature of 800 °C for 2 min. We kept the reactor at a pressure of 30 Torr during the entire process [13–15].

2.2. VACNT functionalized by polar groups (VACNT-O)

We performed the functionalization of the VACNT tips (VACNT-O) by the incorporation of oxygen-containing groups in a pulsed-direct current plasma reactor with an oxygen flow rate of 1 sccm, at a pressure of 85 mTorr, − 700 V, and with a frequency of 20 kHz. The total time of the plasma etching was 120 s. Detailed descriptions of the superhydrophilic properties to produced VACNT-O are given elsewhere [13].

2.3. Extractions of embryonic fibroblasts from transgenic mice C57BL/6 GFP

This protocol was approved by the Ethics Committee of State University of Campinas — UNICAMP (CEUA—Permit Number 1797-1). We extracted fibroblasts in accordance to the protocol described in "Manipulating the Mouse Embryo, A Laboratory Manual" — 2nd edition, 1994 [16]. We dissected mouse embryos of 12.5–14.5 days of development and placed in Petri dishes containing minimal essential medium (MEM). We removed the limbs, viscera and head. The carcasses were rinsed by stirring two or three times with MEM. Then we cut them into small pieces, subjected to enzyme activity in 10 ml of 0.05% trypsin/0.02% EDTA, and kept under stirring at 37 °C for 10 min. We repeated this procedure by several times until most of the cells let go and only the cartilage remained in the tube. We removed 5 ml (aliquot) in each time of the digestion solution and stored in a 50 ml tube containing an equal volume of MEM plus 10% fetal bovine serum (FBS). We centrifuged the cell suspension obtained for 1500 rpm for 10 min and then suspended in about 50 ml of MEM 10% (v/v) FBS. The cells were distributed in approximately 10 plates of 90 mm diameter and maintained in an oven at 37 °C with 5% CO_2 until confluence. Upon confluence, the cells were frozen in MEM + 10% (v/v) FBS containing 10% (v/v) DMSO and kept in liquid nitrogen. Before use, cells were thawed, expanded, and cultivated until the confluence of the monolayer.

2.4. Analysis of preferential cell adhesion

GFP fibroblasts are cells with green fluorescent protein (GFP) constitutively expressed. They were used because the films are not transparent and do not allow analysis by common microscopy, necessitating the use of a fluorescence microscope. We cultured GFP embryonic fibroblasts on different substrates. We performed the analysis by counting cell nucleic obtained within each substrate in fluorescent microscopy by labeling the nuclei with fluorescent marker DAPI (4′,6-diamidino-2-phenylindole, dilactate), using the program ImageJ®. The samples were observed with a fluorescence microscope model DM-5000B LEICA the CPU: CPR 5000 using excitation filter model BP 490/20 at a wavelength of 490 nm, BP405/10 in lengths of 405 nm.

2.5. Scanning and transmission electron microscopy

We used scanning electron microscopy (SEM), a JEOL JSM 5610VPI, and field emission gun electron (FEG-SEM Jeol JSM-6330F) microscopies to carry out the morphological analysis of VACNT substrates. We used high resolution transmission electron microscopy (HR-TEM JEOL 3010, 300 kV) for characterization of the graphene sheet structures produced after exfoliation of VACNT using oxygen plasma etching treatment to produce VACNT-O. We fixed the attached cells on the substrate with a 3% glutaraldehyde/0.1 M sodium cacodylate buffer for 1 h and dehydrated in a graded ethanol solution series (30%, 50%, 70%, 95%, and 100%) for 10 min each. We used 1:1 solution of ethanol with hexamethyldisilazane at room temperature for the drying stage. The samples were coated by a thin gold film to improve the visualization. We evaporated a thin gold layer before the cellular analyses by SEM (SEM, JEOL JSM 5610 VPI). We performed the electron micrographs using 100–15,000×.

2.6. Contact angle and cell adhesion force analyses

We performed the contact angle (CA) measurements using an Easy Drop contact angle Measuring Instrument model (Easy Drop DSA 100S, KrÜss) by the Sessile Drop Technique with deionized water (2 μl) and diiodomethane (2 μl) in a temperature and pressure controlled atmosphere. We performed measurements immediately after the drop deposition on the surface to avoid disturbances by evaporation or adsorption. The wettability study of the VACNT and VACNT-O surfaces performed by CA measurements, using the liquids cited above, allowed surface energy calculation by the Owens and Wendt method [17]. A complete description of these measurements should be found elsewhere [13]. Five measurements of CA were taken for calculation of standard deviation. The hydrophobic or hydrophilic character can influence the cell adhesion on the biomaterial.

The surface energy composed of polar and dispersive components of the samples was evaluated by the measurement of contact angle. The interfacial tension between two condensed phases can be determined by Young' equation, according to which

$$\cos\theta\gamma_{LV} = \gamma_{SV} - \gamma_{SL} \tag{1}$$

where θ is the measured contact angle between liquid and solid, and γ_{LV}, γ_{SV}, and γ_{SL} are the interfacial energies of the liquid/vapor, solid/vapor and solid/liquid interfaces, respectively. This equation can be rewritten as the Young–Duprè equation:

$$W_a = \gamma_{LV}(1 + \cos\theta) = \gamma_{SV} - \gamma_{SL} \tag{2}$$

where W_a is the adhesion energy per unit area of the solid and liquid surfaces. In the general form, Eqs. (1) and (2) then can be written as:

$$\gamma_{LV}(1 + \cos\theta) = 2\sqrt{\gamma_L^p \gamma_S^p} + 2\sqrt{\gamma_L^D \gamma_S^D} \tag{3}$$

where $\gamma^p{}_L$ and $\gamma^p{}_S$ are the polar components of the surface energy of liquid phase and solid phase, respectively while $\gamma^D{}_L$ and $\gamma^D{}_S$ are the dispersive component of the surface energy of the liquid phase and solid phase, respectively. Since $\gamma^D{}_L$ and $\gamma^p{}_L$ have been published for many liquids, it is possible to approximate $\gamma^D{}_S$ and $\gamma^p{}_S$ from a single measurement of θ by the use of Eq. (3). Therefore, by measuring the contact angles of two different liquids (distilled water and diiodomethane) with well-known polar and dispersive components of surface energy (Table 1).