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Surface plasma functionalization influences macrophage behavior on carbon nanowalls

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ABSTRACT

The surfaces of carbon nanowall samples as scaffolds for tissue engineering applications were treated with oxygen or nitrogen plasma to improve their wettability and to functionalize their surfaces with different functional groups. X-ray photoelectron spectroscopy and water contact angle results illustrated the effective conversion of the carbon nanowall surfaces from hydrophobic to hydrophilic and the incorporation of various amounts of carbon, oxygen and nitrogen functional groups during the treatments. The early inflammatory responses elicited by un-treated and modified carbon nanowall surfaces were investigated by quantifying tumor necrosis factor-alpha and macrophage inflammatory protein-1 alpha released by attached RAW 264.7 macrophage cells. Scanning electron microscopy and fluorescence studies were employed to investigate the changes in macrophage morphology and adhesive properties, while MTT assay was used to quantify cell proliferation. All samples sustained macrophage adhesion and growth. In addition, nitrogen plasma treatment was more beneficial for cell adhesion in comparison with un-modified carbon nanowall surfaces. Instead, oxygen plasma functionalization led to increased macrophage adhesion and spreading suggesting a more activated phenotype, confirmed by elevated cytokine release. Thus, our findings showed that the chemical surface alterations which occur as a result of plasma treatment, independent of surface wettability, affect macrophage response in vitro.

1. Introduction

Graphene has captured tremendous interests in the fields of materials science, physics, chemistry and nanotechnology and proved to be a promising material for many potential applications, such as graphene electronic transistors [1,2], integrated circuits [3], transparent and flexible electronics [4,5], composite materials [6], supercapacitors [7], as well as gas sensors [8,9]. Beyond the broad range of technical applications aforementioned, the biomedical application of graphene is a relative new area with very rapid expansion. So far, investigations have been carried out to explore the use of graphene for drug delivery [10–12], biological sensing and imaging [13–15], antibacterial materials [16] and biocompatible scaffolds for cell culture [17–19]. Much of the biological work regarding graphene has focused on assessing the cytotoxicity, cell adhesion, proliferation, and antibacterial properties of graphene oxide as well as biodistribution, toxicology, and internalization of various suspensions of graphene oxide complexes. Besides graphene oxide and graphene, in the last few years, a lot of research interest has been paid to vertical graphene multilayers, also known as carbon nanowalls (CNWs). These are few tens of nm thick carbon nanosheets, terminated with ultrathin edges having lateral dimension in the micron range, interconnected and vertically oriented on substrate. One example of a biomedical application that has already been accomplished is the biological modification of CNW with DNA strands to be used as a platform for biosensors [20,21]. Due to their high surface-area-to-volume ratio, to the structure that renders numerous interaction points for the cells to adhere to and the proven biocompatibility of graphene sheets, CNW could also be an attractive candidate for tissue engineering applications. To date, the number of published articles about CNW in relation with biological entities is relatively small and the interaction of CNW with cells remains largely unknown. Previous work in our laboratory evaluated the possibility of using CNW as support for fibroblast adhesion [22]. As CNW was found to be very hydrophobic, we observed that the CNW surface was not appropriate for fibroblast adhesion. Therefore, attempts to modify their surfaces in order to make cell culture feasible have been made. Thus, we have found that N_2 plasma treatment enhanced the biological performance of CNW surfaces with fibroblast-like cells [23]. Hori et al. [24] reported on the interaction of HeLa cells with as-deposited and plasma modified CNW of different densities. They showed that density of sub-micron-scaled edges of the carbon nanowalls brought significant difference in

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cell proliferation rates depending on their chemical modification. Moreover, the authors suggest that by proper modifications of CNW, a precise control of cell morphology and cell proliferation could be achieved for tissue engineering applications. More recently, CNW subjected to UV/O_3 treatment has proven to be a suitable substrate for neuronal cell growth [25] and interesting material for biomedical electrodes.

The prospective use of a biomaterial in vivo depends on the absence of a significant pro-inflammatory activity associated with it. The macrophage has been suggested to play a central role in the promotion and resolution of inflammation, as well as in the support of tissue restoration following biomaterial/medical device implantation due to its ability to produce a wide range of biologically active molecules, such as cytokines/chemokines and growth factors that direct the cellular activities of other inflammatory/wound-healing cells. Therefore, understanding macrophage interactions with biomaterial surfaces can provide important insight to the design of biocompatible materials.

The present study was designed to explore the short term interaction of macrophage cells with as-deposited and plasma functionalized CNW and to assess how the surface properties dictate cell behavior. The results were related to plane surfaces of silicon and tissue culture plates (TCPS).

2. Materials and methods

2.1. CNW substrate preparation and functionalization

The CNW layers were synthesized onto silicon wafers (size 2 × 2 cm) heated at 700 °C by plasma enhanced chemical vapor deposition, using an argon radiofrequency plasma jet (RF jet) operated at 300 W, and injected with acetylene and hydrogen. The flow rates of the $Ar/H_2/C_2H_2$ gases were 1400/25/1 sccm, leading to a process pressure of 120 Pa. The layer is formed from carbon radicals and ionic species created by the acetylene decomposition in plasma, and transported at the substrate [26]. More details about the deposition procedure can be found in the reference [27]. Thick films of 4 μm were obtained during 60 min of deposition.

The same argon RF plasma jet was applied to the as-deposited layers for surface functionalization. In this case oxygen or nitrogen was injected in plasma. The treatment settings were: gas flow rates in ratio $Ar/(N_2$ or $O_2)$ of 100/10 sccm, treatment pressure 0.2 mbar. The RF forward power and duration were set for a mild treatment, at only 50 W power and 5 min., in order to avoid the modification of sample morphology by plasma.

Each of the as-deposited CNW and plasma treated CNW samples (2 × 2 cm) were cut in pieces of 1 × 1 cm and submitted to material characterization and further biological studies.

2.2. Material characterization

The morphology of CNW layers and of cells adhered on surface was revealed by scanning electron microscopy (SEM), using an Inspect S Scanning Electron Microscope from FEI Company operating at 20 kV, with a maximum resolution of 3 nm. The samples were investigated in high vacuum, at a pressure around 10^{-4} Pa.

Sample topography was investigated by atomic force microscopy (AFM) and surface reconstruction. A Quesant Nomad AFM instrument with a scanning range of 50 μm was used in the non-contact mode. The surface reconstruction was realized using the top-view SEM images with the MountainMap software trial version (Digital Surf).

The surface chemistry was studied by the X-ray photoelectron spectroscopy (XPS). The Kratos Axis Ultra instrument equipped with an X-ray monochromatized source (source energy radiation Al Mono Kα1, $h\nu = 1486.6$ eV) was used. The C1s line at 284.6 eV was used as reference in order to correct the binding energies for charge energy shift. The overall composition of surface was obtained from survey spectra. The percentage of the chemical functional groups present at surface was obtained by fitting high resolution spectra recorded in the regions of C1s, N1s and O1s binding energies. More details on XPS spectra interpretation can be found in [23].

The surface wettability was studied by water contact angle (WCA) measurements using a KSV 100 instrument. The contact angles were measured in static mode by the sessile drop technique. Five measurements were carried out for each sample, in air, at room temperature, using distilled water as probing liquid. The results for each surface are presented as mean ± standard deviation of these measurements. A detailed description of the contact angle procedure used in this study was presented in Ref. [28].

2.3. Cell culture

Macrophage-like RAW 264.7 cells (American Type Culture Collection), a murine leukemic monocyte cell line, were seeded onto the samples placed in 12-well tissue culture plates and maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS), at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. For cell proliferation and cytokine/chemokine analysis, the macrophages were seeded at a density of 5×10^4 cells·cm^{-2} while for scanning electron microscopy (SEM) and fluorescence microscopy studies, a cell density of 1.5×10^4 cells·cm^{-2} was used. Prior to macrophage seeding, samples were sterilized by exposure to ultraviolet light in a sterile tissue culture hood, for 3 h on each side. Uncoated silicon samples and tissue culture polystyrene were used as control groups for the experiments.

2.4. Cell viability and proliferation assay

The viability of cells maintained in contact with test samples was determined using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, L-3224), which measures the membrane integrity of cells. Viable cells fluoresce green through the reaction of calcein AM with intracellular esterase, whereas non-viable cells fluoresce red due to the diffusion of ethidium homodimer across damaged cell membranes and binding with nucleic acids.

Cell proliferation was further quantified by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] colorimetric assay, as previously reported [29].

2.5. Assessment of cell morphology

RAW 264.7 cell morphology on each sample was examined using SEM. Cells that had been incubated on substrates for 48 h were rinsed twice in phosphate-buffered saline (PBS) and soaked in 2.5% glutaraldehyde in 0.1 M PBS for 1 h at room temperature. After fixation, they were rinsed three times in PBS for 10 min each. Samples were dehydrated in a graded series of ethanol (35, 50, 75, 90 and 100%) for 10 min each and subsequently were air dried. To ensure the appropriate dry, the samples were additionally treated with hexamethyldisilazane. SEM imaging was conducted on a field emission scanning electron microscope (Inspect S Electron Scanning Microscope, FEI Company) after sample surfaces were sputter coated with a gold film.

2.6. Fluorescence staining of actin

After cell seeding and 48 h cultivation, the macrophages grown on test samples were fixed with 4% paraformaldehyde prepared in PBS solution, permeabilized with 0.1% Triton X-100/2% bovine serum albumin for 30 min. and washed with PBS. The samples were then incubated with phalloidin–FITC (fluorescein isothiocyanate) (10 μg/ml; Sigma-Aldrich Co.) for 15 min, washed three times with PBS and examined with an inverted fluorescence microscope Olympus IX71. Images were captured by means of Cell F image acquiring system.