Modifications of Carbon Nanowalls Using Low Pressure Plasma to Enhance the Fibroblast Attachment

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Abstract. Layers of carbon nanowalls (CNW) were exposed to cold plasma to improve the attachment of fibroblast cells on surface. Plasma was generated at low pressure by a radio-frequency (RF) parallel-plate discharge in Ar/O2 mixture. 10 min of exposure allowed the insertion of oxygen-containing groups and considerable enhancement in surface hydrophilicity while the surface morphology was not significantly changed. The cells were cultivated on CNW samples and the analyses were performed after 24 h culture. Significant differences in density and morphology of cell attached on tested surfaces were observed. The number of viable cells increased after plasma treatment.

Key words: vertically oriented graphene, carbon nanowalls, plasma functionalization, nanostructures, in vitro assay.

1. INTRODUCTION

The material biocompatibility is to a large extent governed by its surface properties. Cold plasma treatment is one method frequently used to treat the material surface properties for improving cell-surface interaction. A special interest is given to chemically reactive functionalities incorporated on modified surfaces, amenable for cell colonization or covalent immobilization of biologically active molecules [1, 2]. The resulted surfaces are further tailored to develop implants, biosensors, tissue culture labware, etc. A major limitation of plasma generated surfaces is the variety of reactive groups produced by the multitudes of chemical reactions that make the cell studies complex and often not clear which functional group is the key factor in influencing cell behaviour (e.g. cell attachment).

In our previous study, we have observed increased fibroblast attachment on oxygen-containing CNW when compared to nitrogen-containing surfaces. This effect is primarily due to the surface chemistry as surface roughness is comparable regardless of the treatment conditions [3]. The experiments were performed in Ar/N2
and Ar/NH₃ plasma mixtures leading to insertion on CNW surfaces of nitrogen-containing groups as well as various oxygen-containing groups by post-plasma oxidation processes. In this work, Ar/O₂ plasma treatment is presented as a tool to insert oxygen-containing functionalities on CNW. RF low pressure plasma has been used and differences in surface chemistry have been obtained while preserving the roughness. Surface chemistry effects, in particular oxygen-containing groups, on cell-substrate interaction were studied with fibroblast cells and described in detail.

Understanding how to engineer material surfaces by plasma [4] with the appropriate properties to present tailored cues to fibroblasts will improve the performance of tissue engineered skin substitutes by tissue restoration and remodelling after injury [5]. CNW possessing selective physical and chemical properties have shown promise in a variety of material science applications, such as super-capacitor [6], cold cathode [7] or green energy [8], as well in biomedical field for biosensors [9, 10] or scaffolds [11, 12].

2. EXPERIMENTAL

2.1. SYNTHESIS AND TREATMENT OF CNW

The procedures of CNW synthesis and treatment are detailed described in [13, 14]. The synthesis of CNW layers was realized by PECVD technique using a RF low pressure Ar plasma jet where C₂H₂ and H₂ gases were injected. Heated oxidized Si substrates were used for CNW grown. The CNW were obtained at gas flow ratio Ar/H₂/C₂H₂ of 1050/25/1 sccm, pressure 120 Pa, substrate temperature 700°C, RF power 300 W, distance between the substrate and the acetylene injection ring 5 cm, and deposition time 30 min. The modification of CNW was performed with low pressure plasma, generated in Ar/O₂ mixture. The substrates were placed on the grounded electrode of a RF parallel-plate discharge. The experimental conditions were: gas flow ratio 10/25, pressure 20 Pa, RF power 25 W, treatment time 10 min.

2.2. SURFACE ANALYSIS

Surface morphology. Scanning electron microscopy (SEM) was performed with an Inspect S Electron Scanning Microscope from FEI Company operating at 20 kV, with a maximum resolution of 3 nm. The CNW morphology was inspected in high vacuum, at a pressure around 10⁻⁴ Pa.

Surface composition. X-ray photoelectron spectroscopy (XPS) was performed with an Axis Ultra (GB) instrument equipped with a monochromatic Al Kα irradiation at 1486 eV (150 W). Charge neutralization was implemented. Spectra
were recorded at pass energy of 80 eV for the estimation of the chemical elemental composition and of 10 eV for highly resolved C 1s peak. The peak fitting procedure was performed with CasaXPS software.

**Surface hydrophobicity.** Static contact angle measurements were performed at room temperature using a CAM101 digital goniometer (KSV instruments). Distilled water, ethylene glycol and diiodomethane drops of 0.5 µl volume were deposited on CNW surface. The CA values were determined with SCA20 imaging software.

2.3. CELL CULTURE

**Cell culture.** NCTC fibroblast-like cell line purchased from the American Type Culture Collection was cultured in Minimum Essential Medium Eagle-Sigma Aldrich supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin–neomycin antibiotic mixture in a humidified incubator at 37°C, 95% humidity and 5% CO₂ until it reached confluence. CNW samples were sterilized for 6 h using an UV lamp, without affecting the their properties. Cells were seeded in 24-well plates containing corresponding samples at a density of 3 × 10⁴ cells/cm², incubated at 37°C in a CO₂ controlled humidified incubator and allowed to adhere for 24 h.

**Cell morphology, spreading and density.** SEM investigations were performed after fixing the cells with cacodylate-buffer and glutaraldehyde, desiccating four times at different ethyl alcohol concentrations (50%, 70%, 96% and 100%) and coating the samples with a thin gold film using magnetron sputtering. The cell density was calculated using ImageJ open-source image analysis software [15].

**Cell viability.** The cell viability was quantified by neutral red (NR) assay. After 24 h of cell cultivation, the culture medium was replaced with NR solution (50 µg/ml) in MEM. The cells were incubated at 37°C for 3 h. Cells were fixed with a formaldehyde- calcium chloride solution, and the retained NR was dissolved in 1% (v/v) acetic acid in 50% (v/v) ethanol. The plates were incubated on a shaker for 15 min and the absorbance was measured at 540 nm using a Tecan Sunrise (Austria) microplate reader.

**Cell cycle.** After 24 h of cell cultivation, the cells were trypsinized, washed twice with cold PBS, transferred in cold ethanol (70%), while vortexing to prevent the formation of cell aggregates and incubated 16 h at 4°C. The fixed cells were washed twice with PBS and incubated 30 min with 50 μg/mL RN-ase (Promega) at 37°C. After two more washes in PBS the cells were stained with 100 μg/mL propidium iodide (BD) at 4°C for 30 min. Cells were analysed with a LSR II flow cytometer (USA) and the cell cycle distribution was determined using ModFit™ LT software (USA).
3. RESULTS AND DISCUSSION

3.1. CNW SURFACE MORPHOLOGY, CHEMISTRY AND HYDROPHOBICITY

A typical morphology of CNW is presented in Fig. 1a. CNW appear as interconnected lamellar sheets perpendicular on substrate, with thickness of tens nm and length in the micron scale. Previous studies confirmed that these sheets are made from vertically graphene layers overlapped one to another, as an interconnected network of lamellar structures [16, 17, 18].

The effects of Ar/O2 plasma treatment on CNW morphology can be observed in Fig. 1b. The treatment led to a slight erosion of walls and edges and to an enhancement of morphological details of connections between CNW. The integrity of CNW structure is preserved while a large amount of oxygen containing radicals was expected to be adsorbed by this material. It is known that the adsorption of oxygen alters the highly periodic π-bond networks of graphitic structures, which results in changes in their electrical, chemical and wetting properties [19].

An overview of CNW elemental compositions is given by XPS surveys in Fig. 2. The untreated CNW contain mostly carbon (92.5%) and small amounts of nitrogen (2.0%) and oxygen (5.5%). After exposure to plasma, the amount of carbon decreases, while 53.5% of oxygen and 7.9% of nitrogen were incorporated into surface. The O/C ratio increased from 5.9% to 137.4%. High-resolution data in C1s region were acquired to identify the presence and distribution of carbon-based functional groups.

The C1s peak of the CNW (Fig. 3a) with no treatment has been resolved into six components positioned at binding energies (BE) of ~ 284.6, 285.8, 286.6, 288.4, 290.1 and 291.3 eV assigned to C = C (sp2), C – C (sp3) / C – H, C – O / O – C – O / C – N, C = O, O = C – O and π – π* shake-up, respectively.
A decrease of C = C and π − π* shake-up peaks intensities is observed after treatment (Fig. 3b). Moreover, the modified CNW exhibit C1s components characteristics of single and doubly bonded C-O functionalities as well as carboxyl groups with a relative concentration that is higher than those of untreated one. The change of content of each chemical component is given in Table 1.

### Table 1

<table>
<thead>
<tr>
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<th>Untreated CNW</th>
<th>Plasma treated CNW</th>
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<tr>
<td></td>
<td>Binding energies (eV)</td>
<td>Relative concentrations (%)</td>
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<tr>
<td>C = C</td>
<td>284.6</td>
<td>72.8</td>
</tr>
<tr>
<td>C − C / C − H</td>
<td>258.8</td>
<td>8.4</td>
</tr>
<tr>
<td>C − O / O − C − O / C − N</td>
<td>286.6</td>
<td>8.3</td>
</tr>
<tr>
<td>C = O</td>
<td>288.4</td>
<td>4.5</td>
</tr>
<tr>
<td>O = C − O</td>
<td>290.1</td>
<td>2.3</td>
</tr>
<tr>
<td>π − π*</td>
<td>291.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>
Contact angles of three liquids on corresponding surfaces are shown in Fig. 4. CNW are almost super hydrophobic, with correspondingly high water contact angle of 125°. The water contact angle is drastically narrowed to 7° after treatment. The ethylene glycol contact angle is also reduced from 31° to 9°. This means that super hydrophilic surface was successfully obtained after exposure of CNW to oxygen-containing plasma treatment. For the non-polar diiodomethane, with plasma treatment, the contact angle is not changed. Hence, diiodomethane wets the CNW without any surface alteration.

The increased hydrophilicity is attributed to the incorporation of oxygen functionalities during and after treatment. The total surface energy (sum of dispersion and polar components, according to the OWRK method [20] and calculated taking in account the contact angles of water and diiodomethane) increased from 55.4 mN/m in the case of untreated CNW to 79.4 mN/m for oxygen plasma treated CNW.

### 3.2. CELL MORPHOLOGY, VIABILITY AND CYCLE DISTRIBUTION

SEM micrographs showed a clear difference in terms of fibroblast morphology when comparing the untreated CNW and plasma treated CNW (Fig. 5). The morphology of fibroblast cells is quite different.

On the untreated surface, cells are small, spherically shaped and separated from each other (Fig. 5a, b). No lamellipodia-like structures were seen developed by cells. A loose cell attachment and only very few filopodia-like structures could be observed.

Cells cultured on treated CNW spread homogenously as presented in Fig. 5c. Cells showed a stable anchorage to the plasma treated surfaces, by showing flat, elongated cell morphology. Lamellipodium-like structures were observed on a significant number of cells (Fig. 5d).
It is already known that early cell attachment process is followed by cell spreading, initiated by sporadic filopodia-substrate contacts, which finally lead to replacement of transient filopodia by a circumferential lamellipodium [21, 22]. The CNW morphology was slightly changed by plasma and therefore the cell behavior is mainly attributed to surface chemistry properties. Oxygen-containing plasma treatment leads mainly to the formation of carboxyl and carbonyl groups, which contribute to hydrophilicity changes. It has been hypothesized that in the absence of carboxyl groups, carbonyl groups do not promote the cell growth [23]. The plasma treated CNW which was found by XPS to contain 6.3% COOH and 8.3% C = O functional groups appears to have supported cell attachment. Cell density was higher when comparing the treated CNW with the untreated one (2.3% COOH and 4.5% C = O).

The cell densities on the corresponding CNW surfaces are given in Fig. 6. The values reported are the number of all the cells, independently of cell morphology (round or flat). Image J analysis showed that CNW promoted fibroblast cell attachment, meaning, 166 cell/mm² after 24 h of culture. Plasma treatment increased with 34% the percent of attached cells in 24 h. The metabolic assay used in the present study revealed a 14% increase of active cells (Fig. 6) on the plasma treated surface when compared to the untreated control.
DNA content analysis was used to assess any major changes in the studied cell’s genome such as polyploidy or DNA fragmentation. Cell cycle distribution was used as an indicator for good/normal cell proliferative rate.

Plasma treatment did not interfere with cell cycle stages. A similar percentage of cells in the mitosis phase was registered on both untreated and treated surface (Fig. 7). The heterogeneity of the fibroblast cells, with varied nuclei sizes, specific for a cell line, can explain the wide G1 and G2 peaks on the cell histograms.

4. CONCLUSIONS

The oxygen-containing plasma at low pressure was introduced as a tool to prepare the surface properties of CNW for cell-substrate study. Plasma processing led to changes in surface chemistry features as the presence of oxygen-containing groups while there was almost no change in surface morphology of the initial nano-porous architecture. The new surface chemistry properties were evaluated as cell activity modulators *in vitro*. In terms of early cell adhesion, an enhanced fibroblast cells attachment was observed on surfaces enriched in carboxyl and
carbonyl groups by plasma treatment. Nevertheless, the percent of metabolically active cells slightly increased suggesting that even the cells manage to remain viable and exhibit a more well spread morphology on modified surfaces, the proliferation mechanism is probably affected.

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