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Cell Viability and Adhesion on as Grown Vertically Aligned Carbon Nanotubes√

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ABSTRACT

This work studies cell viability and cell adhesion on as grown dense films of vertically aligned carbon nanotubes (MWCNT). The MWCNTs were produced by a microwave plasma chemical vapor deposition (2,45GHz) on silicon (Si) and titanium (Ti) surfaces, using a nickel and iron catalyst, respectively. The citotoxicity tests (MTT assay and cellular adhesion) were evaluated by various incubations times with Fibroblast L929 mouse cells. The results show very high cell viability and many layers of cells adhered on the surface formed by the nanotubes tips at films grown on Si surfaces. The MWCNT grown on Ti surfaces presented lower cell viability and a reduced number of cells on the surface formed by the nanotubes tips. The different behavior is most probably related to excess iron contamination present in the case of titanium substrate, while nickel catalyst is probably enclosed by the nanotubes.

INTRODUCTION

Recently, carbon nanotubes (CNTs), have received enormous attention in biomedical applications, as drug delivery agents, biosensors, bone scaffolds material, and even as substrate for neuronal growth [1], due to their specific structure/texture and properties [2], therefore, an evaluation of biocompatibility and toxicity of this nanomaterial is crucial. The biocompatibility and citotoxicity studies have been performed in recent years [3-14]. The citotoxicity studies in vitro have been realized in recent years, with CNTs dispersed in the cell culture [4-8] and CNTs held in some structure in contact with the cell culture [9-12]. Many studies suggest a low biocompatibility, mainly for the ones with CNTs dispersed in the cell culture [3, 4], but other studies show an obvious preference of cell growth on a CNT surface [11, 12]. Clearly, the studies that obtained good cell viability present some kind of purification (to remove metallic particles dispersed around CNTs and amorphous carbon) or functionalization of the CNT [8, 12, 13]. It is still early to establish a general toxicological profile for this type of material and more systematic in vitro and in vivo investigations using biologically compatible CNT are necessary to achieve that [14]. The present study shows the citotoxicity tests (MTT assays and cellular adhesion) of MWCNTs grown on Si and Ti surfaces, with nickel and iron catalysts, respectively, in cultures of L920 mouse fibroblast cells. The comparison shows different cell behavior even though the MWCNT presented very similar quality, with the modification only of the catalyst nanoparticles.

EXPERIMENTAL DETAILS

MWCNT Deposititon

Both substrates, Si and Ti, were squares pieces (1cm^2). The Si surfaces were covered by a 50 nm layer of SiO_2 and by a thin nickel layer (5nm), both deposited by e-beam evaporator. The Ti substrate were heated in air to a temperature of 200ºC for the formation of TiN layer (diffusion barrier) and were dip coated in a $Fe(NO_3)_3 \cdot 9H_2O$ solution in ethanol at a concentration of 37mmol/l.

The MWCNTs were produced in thin film form on Si and Ti surfaces using metallic catalyst (Ni and Fe, respectively), in a microwave plasma chamber (2,45GHz). A pre-treatment, for metallic nanoclusters formation, was carried out during 5 min in plasma of N_2/H_2 (10/90sccm), at a substrate temperature around 760ºC. During the film deposition, CH_4 (14 sccm) was inserted as carbon source during 2 min, at a substrate temperature of 800ºC. The reactor was kept at a pressure of 30 Torr during the whole process. External heating with a Ni-Cr resistance under the substrate holder was necessary to keep the substrate temperature [16].

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were used to observe the structure of the MWCNTs.

MTT Assays

The citotoxicity assay was evaluated according to ISO 10993-5 ‘‘Biological evaluation of medical devices—Test for cytotoxicity: in vitro methods’’ (or EN 30993-5), using direct contact.

The proliferative activity of cultured cells was determined with the MTT colorimetric assay, as described by Mosmann [17]. Latex fragments were used as positive control. Fragments of filter paper of proven non-toxic nature were used as negative control. The dimensions of these fragments were the same as the substrates with MWCNTs. L929 mouse fibroblasts were seeded in each well at concentration of 5×10^5 cells/ml, supplemented with 10% fetal bovine serum (Gibco, BRL). The incubation was performed under a CO_2 (5%) atmosphere and a 37º C for different times. The viability was determined after, 2 h, 24 h, 48 h, 72 h and 96 h. After the incubation time, the substrates with MWCNT and the positive and negative control fragments were removed from the respective wells. Only the cells adhered to the well walls were incubated with a tetrazolium salt solution (MTT), 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, Saint Louis, Missouri, USA) for 4 h at 37°C. After removal of the medium, ethanol-dimethyl sulfoxide (DMSO) (Sigma, Saint Louis, Missouri, USA) solution (1:1) was added to each well. After complete solubilization of the dark-blue crystal of MTT formazan, the absorbance of the content of each well was measured at 570 nm with a 24-well microplate reader on a spectrophotometer Spectra Count (Packard). The blank reference was taken from wells without cells, also incubated with the MTT solution. The cell viability was calculated by the normalization of optical densities (OD) to the negative control.

Cellular adhesion

The capacity of cellular adhesion of the L929 mouse fibroblasts cells on the aligned MWCNT films was tested at incubation periods of 48 h and 7 days. After culturing for a period

of time, the attached cells on the substrate were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h and dehydrated in a graded ethanol solution series (30%, 50%, 70%, 95%, 100%) for 10 min each. The drying stage used a solution of ethanol (50%) with hexamethyldisilazane (HMDS) and HMDS (100%) for a period of 10 minutes, respectively. After this, the samples dried at room temperature for 30 minutes. After the deposition of a thin gold layer the samples were evaluated by SEM.

RESULTS AND DISCUSSION

Figure 2 (a-b) shows the cell viability found for the MWCNT on $Si/SiO_2/Ni$ (a) and Ti/TiN/Fe (b) surfaces, obtained by MTT assays. The cell viability is positively correlated with the degree of MTT reduction [5, 17, 18]. The cell viability in MWCNT grown on $Si/SiO_2/Ni$ samples is higher than 80% from the very beginning and it clearly increases with the incubation time to meet 100% viability. For the positive control samples, the OD decrease rapidly, characterizing cell death. Compared with the positive control, the as grown MWCNT are evidently non-toxic. However, for Ti/TiN/Fe substrates, a different result is observed. The results of tests with the MWCNTs grown on Ti/TiN/Fe surfaces presented lower cell viability, with a decreasing behavior, with values of cellular viability around 60% at 96 h. The OD of negative control is in fact the normalization parameter so that it is shown as 100% viability.

The high cell viability obtained on the aligned MWCNT is compatible with recent ones presented in literature for experiments in which the CNT are held in some structure in contact with the cell culture. The application of MTT assay tests for evaluation of the cell viability is controversial, with a tendency to show lower viability [7] and toxicity [8]. In this case, to avoid methodological doubts, results are correlated only with the cells left adhered to the well surfaces, because the cells adhered to the samples are removed with them. The same procedure is adopted with the removal of the negative and positive control samples. The direct correlation among the OD measurements is assured because in all cases, the samples have the same size and the free area for cell adhesion on well surfaces is always the same. This procedure assures no interference of the MWCNT with MTT test products.

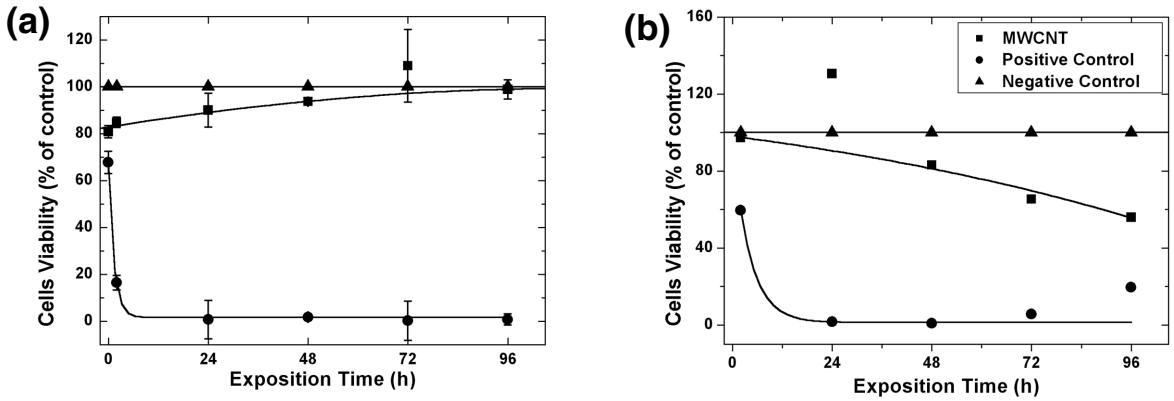

Figure 2. L929 mouse fibroblasts viability curve measured by MTT assay of MWCNT grown on (a) Si and (b) Ti surfaces. The percentage of cell viability was calculated by normalization of optical density (OD) to the negative control.

In the results shown in Fig. 2 there is no influence of amorphous carbon, since in both cases the carbon nanotubos are of high purity, due to the very reducing atmosphere of the microwave plasma. Probably Ni nanoparticles are not exposed to the cell culture, which explains the higher viability. The increased cell viability observed here indicates that the causes for a lower viability are being blocked with the incubation time.

The result of lower cell viability on the MWCNT grown on the Ti/TiN/Fe surface is most probably related to an excess of iron particles present around. Experimental observations show that the behavior of Ni and Fe particles are different in this system, while all growth conditions are the same in both cases. Nickel catalyst looses its activity after 2 to 3 minutes of growth and MWCNT are completely eroded after that. Iron catalyst does not loose activity and the growth continues for long periods. Some growths were performed up to 30 minutes. It seems that Ni nanoparticles melt and flow inside MWCNT since TEM images frequently show Ni fillets inside individual nanotubes. Fe nanoparticles are rarely observed by TEM inside individual nanotubes. These experiments indicate that Ni nanoparticles are most probably enclosed inside the nanotubes while Fe nanoparticles continue to promote the base growth of the nanotube. The base growth is characterized by the reduction of growth rate with time because of the increased difficulty for the gases to arrive to the Fe nanoparticles through the dense MWCNT film.

EDX spectra for MWCNT grown on $Si/SiO_2/Ni$ and Ti/TiN/Fe show Ni and Fe, respectively, but clearly the amount of Fe is larger. All these considerations indicate that it is very likely to have excess Fe nanoparticles exposed to the cell culture. Many authors [19-23] have shown the need for CNTs purification to increase cell viability. Purification was not performed in this case because the objective was to compare the biocompatibility of the as grown aligned MWCNT. Any purification method would destroy the alignment.

Typical observations of NCTC Clone 929 cells adhered on the aligned MWCNT grown on Si and Ti surfaces, at different magnifications are shown in Figure 4, in two parts. Part I shows SEM images after 48 h incubation. Part II shows SEM images after 7 days incubation. The left side refers to the $Si/SiO_2/Ni$ samples while the right side refers to the Ti/TiN/Fe samples. Clearly the cell behavior on MWCNT film surface is completely different in both cases. While on the $Si/SiO_2/Ni$ sample the first layer of fibroblast spreads flat on surface up to touching neighbor cells, virtually covering the whole surface, on the Ti/TiN/Fe the cells have elongated or triangular form, extending only some projections to encounter neighbor cells, which leaves most MWCNT film uncovered. After 7 days the number of division cells is much larger on $Si/SiO_2/Ni$ sample than on Ti/TiN/Fe. This reflects the result of the cell viability shown in Fig 2.

CONCLUSIONS

In conclusion, this work shows a high level of L929 mouse fibroblast cells biocompatibility with as grown aligned MWCNTs films on $Si/SiO_2/Ni$, probably because Ni nanoparticles are enclosed by the nanotubes and have no contact with the cell culture. This high cell viability is not confirmed on Ti/TiN/Fe samples because Fe nanoparticles are very likely to be exposed to the cell culture. These results evidences that CNT funcionalization is not necessary to obtain or increase biocompatibility. It is enough that the CNT are pure, without metallic or amorphous carbon residues on surface. The particular interaction between the L929 mouse fibroblasts cells and the aligned MWCNTs films is impressive. The spreading of the fibroblast cells flat on surface and to all directions is a key observation that indicates a high and hardly comparable adhesion level. The presence of Fe nanoparticles affects negatively on cell adhesion.

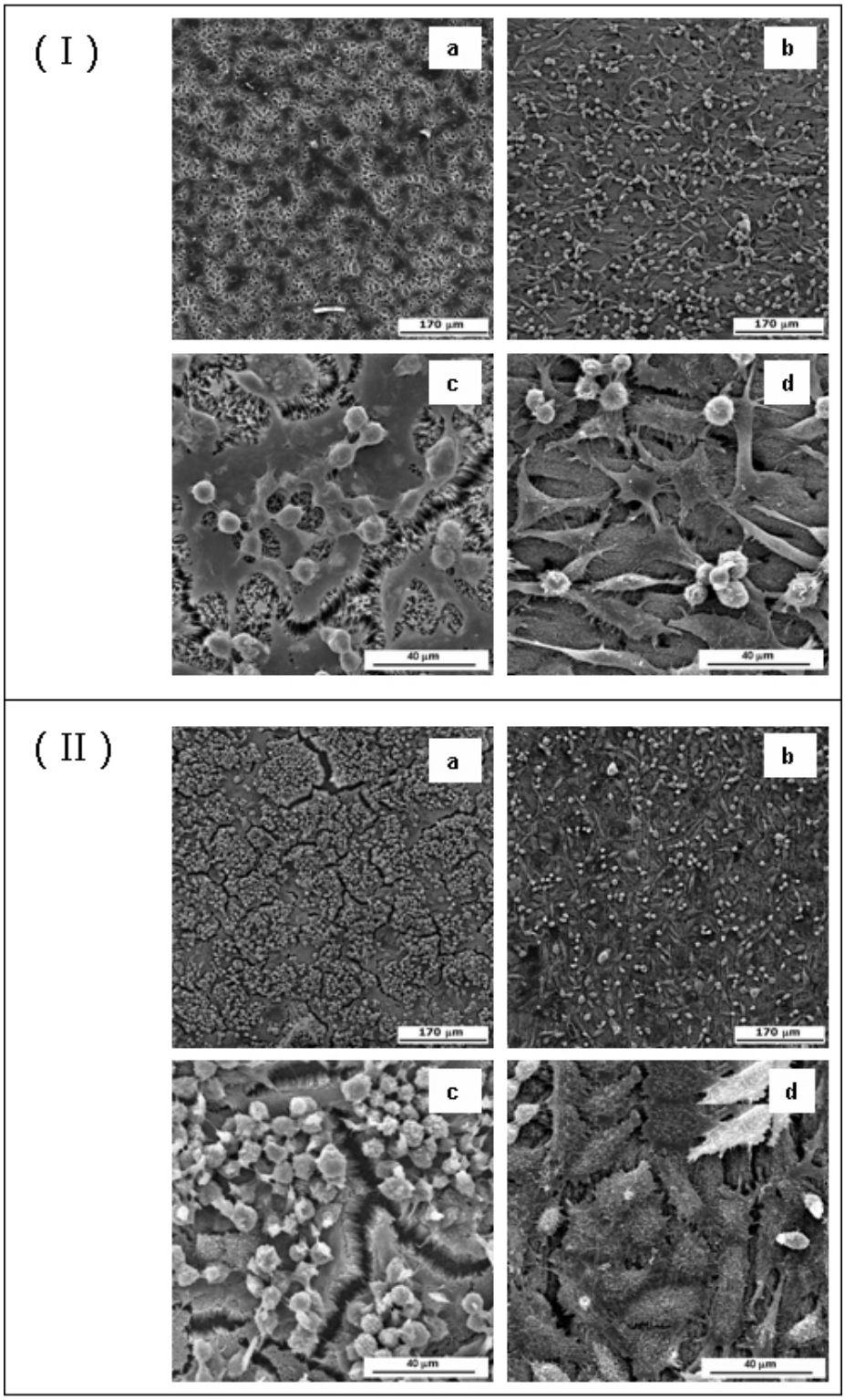

Figure 4. SEM images of interaction between the L929 fibroblasts cells and the aligned MWCNT grown on Si and Ti surfaces. (I) 48h: (a,c) pictures obtained of the MWCNT grown on Si surfaces and (b,d) MWCNT grown on Ti surfaces (II) 7 days: (a,c) pictures obtained of the MWCNT grown on Si surfaces and (b,d) MWCNT grown on Ti surfaces Magnification: a) and c) 200X; b) and d)1000X.

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