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Properties of carbon films and their biocompatibility using in-vitro tests

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Abstract

In this paper we report the results of a comparative study of the biological response of amorphous carbon coated stainless steel. Films of amorphous carbon (a-C), amorphous carbon nitride (a-CN) and hydrogenated amorphous carbon (a-C:H) were deposited on stainless steel substrates (AISI 316L) using a dc magnetron sputtering system. In-vitro studies were carried out on the coated samples using human osteoblasts cell culture lines and fibroblasts. Preliminary biocompatibility was assessed by cell adhesion and proliferation, as determined by a spectroscopic technique. Comparison of the optical absorbance results between control uncoated disks and the test cultures provided a semi-quantitative analysis of the cytotoxic effect of the different carbon compounds. Osteoblasts cells were grown on uncoated steel, a-C, a-CN and Ti coated steel samples. The degree of fibroblast adhesion measured at 24 h is very similar for all the test samples, however, osteoblasts adhesion was higher for a-C films. Similarly, cellular proliferation at 7 days showed an outstanding increase of osteoblasts cells for a-C and Ti in contrast with uncoated steel. The physical film properties, such as, roughness measured by atomic force microscopy, surface composition determined by both Rutherford Backscattering and Auger Spectroscopy and the electro-optic properties of the films were also determined. The relation between film properties and cellular response is discussed.

Keywords: Amorphous carbon; Carbon nitride; Biomaterials; Sputtering

1. Introduction

The search for better medical implants has received much interest and funding during the last decades. This research has shown that two main requirements must be considered: the compatibility of the solid in the biological environment and the suitability of its mechanical, electrical or other properties according to the implant application. These two conditions are rarely found in one material, since the biocompatibility is mainly controlled by the surface characteristics of the solid, whereas the functional properties are largely determined by the bulk. Fortunately, nature itself gives us the proper solution for this problem, the use of hybrid systems, such us the skeletal bone, which consists of at least three different layers. Therefore, the trend for future medical implants is the development of implants made of a recognized and stable engineering material and a thin coating. Such a coating must not only be biocompatible but also bioactive in order to promote adequate tissue-coating interactions depending on the implant application. For example, it should promote scaffolding for bone in-growth, such as, hydroxyapatite coatings on metallic implants that facilitate joining between the prosthesis and the osseous tissue, increasing the long-term stability and integrity of the implant [1].

Amorphous carbon films deposited on metals have been studied as possible candidates for biomedical applications mainly because of their chemical inertness and the presence of this element in the human body [2,3]. Amorphous carbon films are also known as diamond-like carbon (DLC) [4], however, it is important to notice that this name includes a wide range of film properties dependent on the fraction of sp^3 bonding and H content [4]. Different deposition methods have been developed to produce films extending from 100% sp^2 bonding to approximately 85% sp^3 bonding, with diverse names used to distinguish among the groups [4]. Graphitic carbon films or 'a-C' have a disordered graphitic ordering presenting extremely low hardness (3 GPa) and an optical gap close to zero. Carbon films with a

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high degree of sp^3 bonding are called tetrahedral amorphous carbon, 'ta-C'. These can have a high mechanical hardness (80 GPa) and are wide band gap semiconductors. Hydrogenated carbon films produced by plasma-enhanced chemical vapor deposition (PECVD) methods are called 'a-C:H'. In this case, when the sp^3 fraction is increased, with a fixed H content, a high fraction of C–C tetrahedral bonding is obtained leading to the acronym of 'ta-CH' films. However, a high H% and a high sp^3 fraction is characteristic of 'polymeric-like a-CH' films or 'soft a-CH'. Polymeric-like carbon films have a wide band-gap but the hardness is as much as 8 times lower than in ta-C [4].

Reported work on carbon films biocompatibility has mainly dealt with a-CH, ta-C films and carbon nitride (CN). Biological tests on a-CH films have shown that it is a material with a good bio-tolerance, is efficient against corrosion and metalosis in the biological fluids [5,6], is not cytotoxic [7], is suitable for blood interface applications since it inhibits thrombus formation [8], and it shows a good bio-integration in the oral cavity [9]. However, the main interest in ta-C is because it has very good tribological properties [10] making it a good candidate for applications in severe environments such as artificial knees, hip replacement [11] and femoral heads [12]. The studies have shown an improvement in the wear and corrosion resistance when compared with other bare metallic implants [11]. Tissue and blood compatibility also gave encouraging results [13]. Similarly, for a-CN films deposited on orthopedic substrates very good tribological properties have been obtained [14]. Morphological studies of osteoblasts attachment onto DLC and CN films show good bone attachment and spreading without apparent impairment of cell physiology [13,15].

In this work we have studied the in-vitro biomedical response of well-characterized carbon and carbon nitride films deposited by a dc magnetron sputtering system in order to obtain a better understanding of the relation between film properties and tissue-coating interaction. Here we reported on the interaction of a-C and a-CN coatings with osteoblast-like cells, by assessment of cell attachment and proliferation and by comparison with Ti and stainless steel uncoated substrates.

2. Experimental

2.1. Film deposition

Films were deposited simultaneously on stainless steel (AISI316L) squares (1 cm×1 cm), low-conductivity

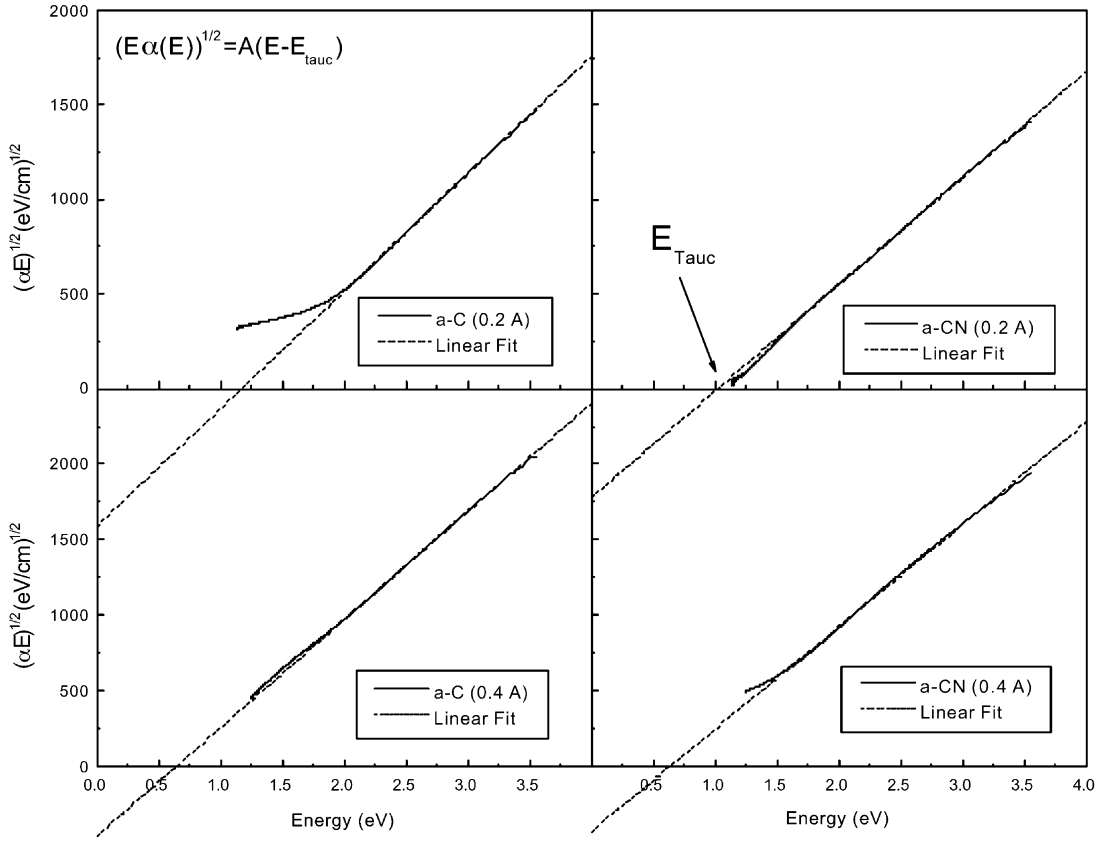

Fig. 1. Tauc optical gap of some representative a-C and a-CN samples deposited at different currents (0.2 and 0.4 A) in the Magnetron sputtering system.

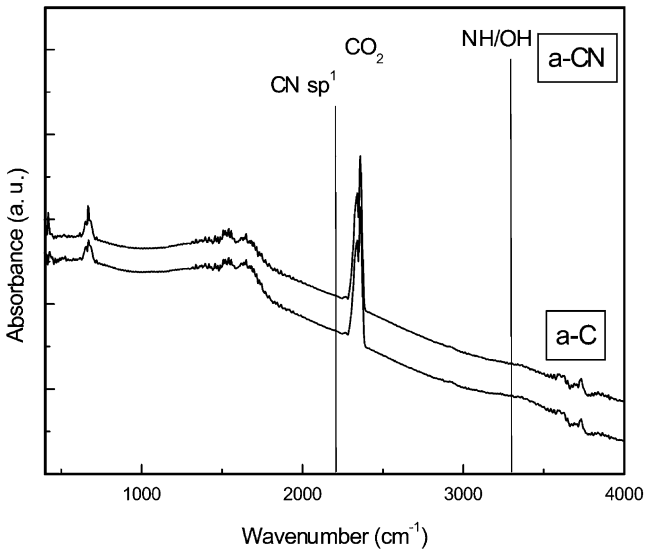

Fig. 2. Infrared spectra for a-C and a-CN samples deposited on silicon.

silicon and glass substrates. The substrates were ultrasonically cleaned using acetone for 30 min, followed by ultrasonic rinsing with isopropanol for another 30 min and then air-dried. Films deposited on silicon and glass were used for characterization of physical properties, while the samples deposited on stainless steel (SS) were used for the biomedical tests.

The carbon and carbon nitride films were produced by a dc magnetron sputtering system attached to a high vacuum chamber (base pressure 1×10^{-6} Torr), using a 4-inch diameter high purity graphite cathode. Prior to sputtering, the substrates were cleaned using an argon, (purity 99.999%) plasma for 10 min, with a shutter to prevent deposition. For the pure carbon films, the shutter was removed and the deposition was carried out for the required time. In the case of CN films, after the cleaning with the Ar this was substituted by 100% N_2 (purity 99.999%) gas. Films were deposited at 30 mtorr, 20 sccm of Ar or N_2 and a dc current of 0.2–0.4 A.

The Ti films were deposited on SS in a similar dc magnetron sputtering system, using a high purity Ti target (99.999%) and Argon plasma at 0.2 A and 4 mtorr.

2.2. Film characterization

The surface roughness of the SS substrates was determined by profilometry and at the microscopic level by atomic force microscopy (AFM). The film thickness was determined by fixed wavelength ellipsometry at 632 nm.

The film composition was obtained by Rutherford backscattering spectroscopy (RBS) using alpha particles at 3.8 MeV. The surface composition and relative C/N ratios by Auger spectroscopy using a Multilab VG system.

Optical absorption in the ultraviolet-visible range was obtained for the samples deposited on glass using an UV-VIS UNICAM spectrometer in the 350–1100 nm range.

The nature of chemical bonding was determined using a FTIR spectrometer (NICOLET205-FTIR) in the 400–4000 cm^{-1} range for the samples deposited on low-conductivity silicon substrates.

2.3. Cell preparation

Human alveolar bone-derived cells (HABDC or osteoblasts) and human periodontal ligament cells (HPLC or fibroblasts) were obtained by the conventional explant technique as reported elsewhere [16,17]. The cells were cultured in 75-cm^2 cell culture flasks in a medium composed of: Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (Streptomycin 100 μg ml^{-1} and penicillin 100 units ml^{-1}, Sigma Chem Co.). The cells were incubated in a 100% humidified environment at 37 °C in an atmosphere of 95% air and 5% CO_2.

2.4. Adhesion and proliferation test

The different carbon films deposited on SS, an uncoated SS substrate, Ti covered SS and a control substrate, were placed in 24-well culture plates. The positive controls were 1-cm^2 squares made of plastic Petri dishes treated with poly-lysine for tissue culture. All samples were sterilized by exposure to UV-light. The HABDCs were plated at an initial density of 1×10^4 cm^{-2} and left to adhere for 5 h. After this time, 600 μl of medium, with the same composition used for the cell preparation, was added. Cellular adhesion was evaluated after 24 h, and for the proliferation tests the cells were left on the culture plates for 1, 3, 5 and 7 days. The experimental and control cultures were treated every other day with fresh media. After incubation, the unattached cells were removed using a phosphate buffered saline (PBS) solution and the attached cells were fixed with 3.5% paraformaldehyde. Evaluation of cell attachment was performed according to Hyman et al. [18] with some modifications. Briefly, fixed cells were incubated with 0.1% Toluidine Blue for 3 h. The dye was extracted with sodium dodecyl sulfate (SDS) and the optical absorption read with enzyme linked immunosorbance assay (ELISA) at 605 nm. The number of cells was then determined by a standard curve. Cellular adhesion assays were performed on 15 different samples, while the proliferation assays were performed in triplicate.

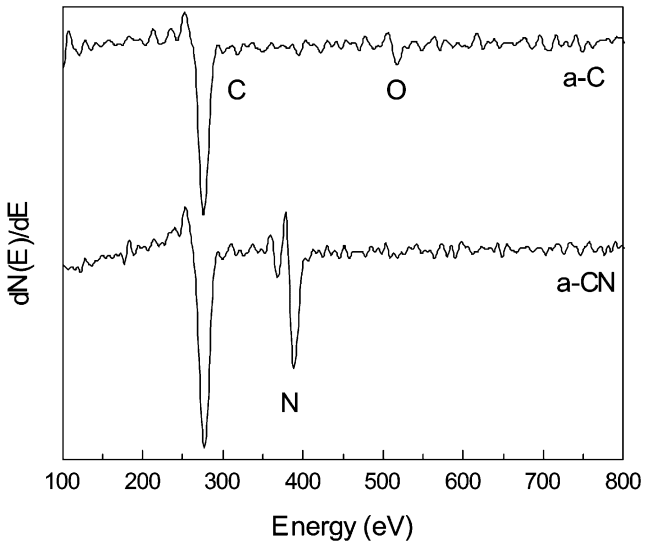

Fig. 3. Derivative Auger spectra for a-C and a-CN samples deposited on SS substrates.

3. Results

3.1. Physical properties

The RBS results measured for two CN samples gave nitrogen to carbon composition ratio close to 1. Auger C/N ($\sim$1.2) composition ratio was also constant among different samples, however, no quantitative values were obtained because of the absence of a proper standard.

The SS substrates were not polished, therefore the roughness of the films at the macroscopic scale, as measured with a profilometer lead to values between 50 and 100 nm depending on the direction of the scan. Atomic force microscopy (AFM) in the contact mode was used to study the microscopic surface morphology. The average roughness, R_a, in 300×300 nm area was 3 and 2.5 nm for C and CN films, respectively. In larger areas the R_a values were much higher, reflecting the substrate roughness.

UV-visible light transmission (T) for the samples deposited on glass showed that both, a-C and a-CN, have a small band gap (0.5–1 eV). These rough values were obtained using Beer's Law ($T=e^{-\alpha d}$), where α is the absorption coefficient and d is the film thickness. There are two common definitions of the optical gap in amorphous semiconductors, namely the Tauc Gap and the E_{04} gap [19]. The Tauc gap assumes that the absorption, α, near the gap can be estimated from the joint density of states. If parabolic bands are assumed in the relevant absorption range (approx. 10^4 cm^{-1}), this can be expressed as:

$$E\alpha(E)=A(E-E_{\text{Tauc}})^{1/2} \quad (1)$$

where E is the energy of the incident light and E_{Tauc} is the estimate of the optical band gap and A is a constant. Thus, by plotting the variation of $\sqrt{\alpha E}$ against E and then performing a linear extrapolation to the x-axis, E_{Tauc} can be found, as shown in Fig. 1.

The IR spectra of a-C and a-CN samples can be seen in Fig. 2. Unfortunately, interpretation of the IR spectra of C and CN films is still controversial. For pure carbon films, there is only one broad band centered at 1500 cm^{-1}, this is ascribed to C=C vibrations that become allowed in the IR due to the disorder, since such vibrations are not allowed in crystalline carbon. For the carbon nitride films, a similar band is also observed but of much higher intensity. This has been ascribed to Raman bands which become IR active due to the presence of nitrogen [20], polarizable IR-active C–N and C=N bonds [21] or C=C polarized bonds due to the presence of CN sp^1 bonds in the neighborhood [22]. However, our interpretation [23] is that it is due to the presence of conjugated CC/CN bonds which creates a high number of delocalized π electrons, increasing the effective dipolar moment in the conjugated chains or rings. For the CN films there are also two other absorption bands, one in the 2200 cm^{-1} range due to CN sp^1 bonds and a broad band at approximately 3200 cm^{-1}, indicating the presence of hydrogenated groups; either NH or OH bonds. This is common in CN films, since they are known to promptly absorb water from the environment [24].

Ex-situ Auger (Fig. 3) survey scans on different C and CN samples showed that there was minimum oxygen contamination on the surface, even though the samples were kept for various days in atmospheric conditions.

3.2. Biological response

In order to evaluate the biocompatibility of the films we studied the changes in the number of vital cells (fibroblasts and osteoblasts) by spectrophotometric techniques for different periods of time. This procedure gave us information about the cytotoxicity of the material and the cell functionality at the material surface.

Fig. 4 shows the fibroblast adhesion for the C, CN and bare SS substrates after an incubation period of 24 h. The results are presented as the cellular percentage of attached cells in relation to the positive control (1 cm^2 Petri dishes). Fibroblast adhesion is very similar for both films, but less than the control.

Fig. 5 shows the adhesion of osteoblasts-like cells on the materials after 24 h, including the sputtered Ti films. The adhesion of osteoblasts can be seen to be favored for both the C and CN films, exceeding a 100% attachment, whereas the values are much lower for the metallic substrates. However, there is no statistical difference between samples when compared with Student's t-test at $P<0.05$.

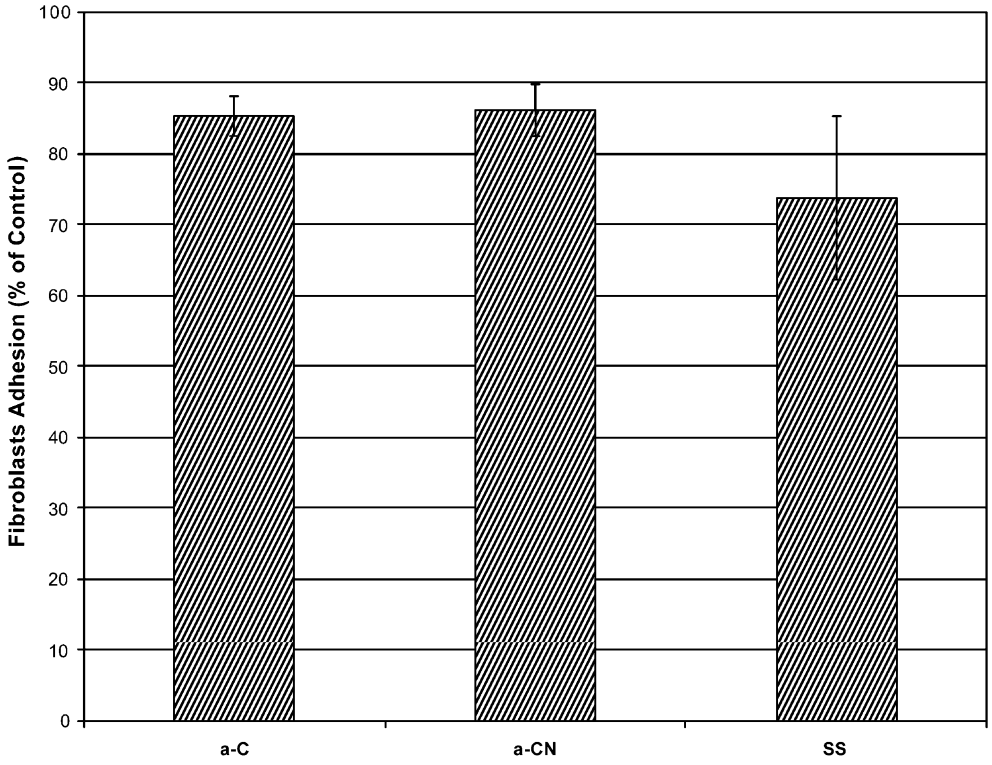

Fig. 4. The fibroblast adhesion for the a-C, a-CN and bare SS substrates after an incubation period of 24 h.

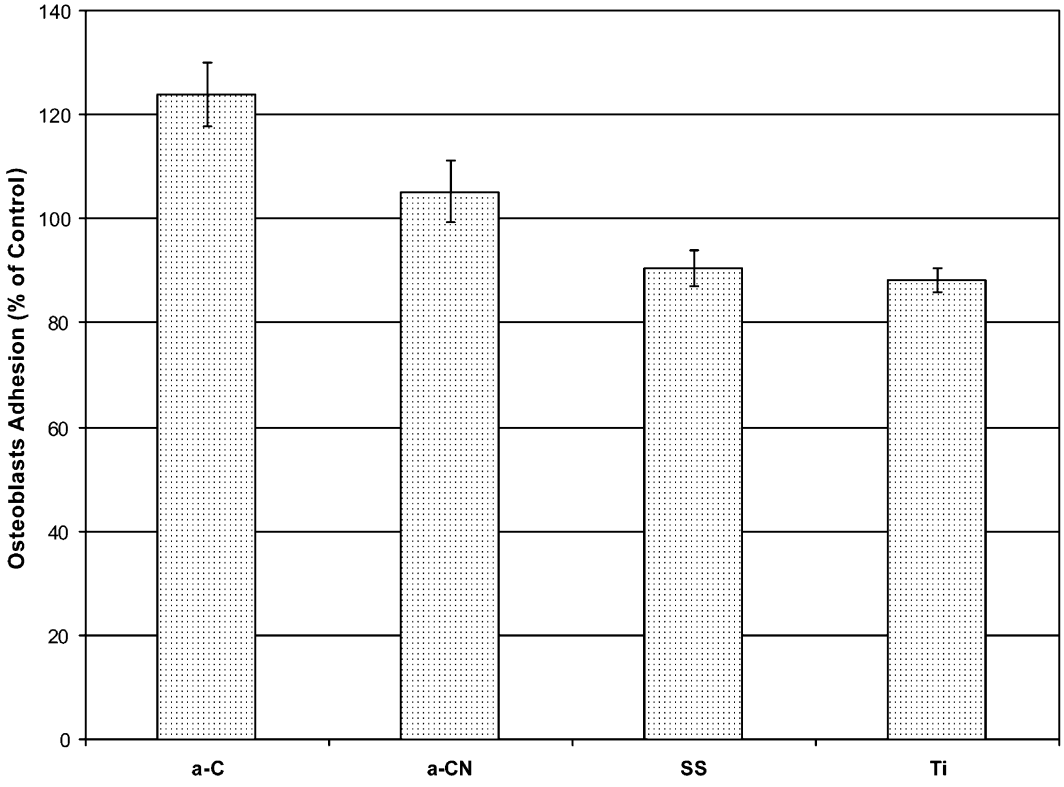

Fig. 5. The adhesion of osteoblasts-like cells on the a-C, a-CN, sputtered Ti and bare SS substrates after an incubation period of 24 h.

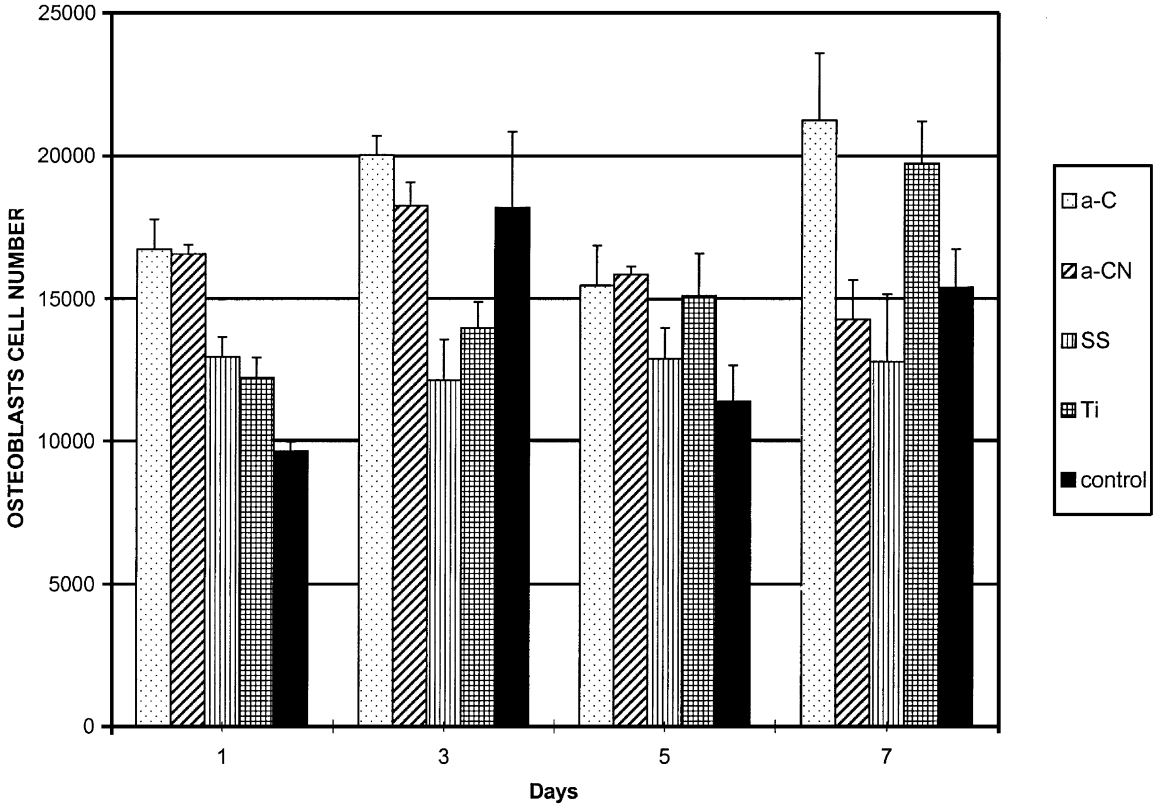

Fig. 6. The results of the proliferation assay carried out after 1, 3, 5 and 7 days for the two carbon films and the metallic substrates (Ti and SS).

Fig. 6 shows the result of the proliferation assay carried out after 1, 3, 5 and 7 days for the two carbon films and the metallic substrates (Ti and SS). The initial number of cells (10 000) is represented by the control and the plot shows that the number of cells increased for a-C, a-CN and Ti. However, the error associated with the quantification makes it difficult to draw any conclusive difference between a-C, a-CN and Ti. Only after 7 days is the proliferation of osteoblasts on the SS substrate significantly less than for the a-C films ($P < 0.05$).

4. Discussion

An important aim of the study was to investigate the correlation between film properties and in-vitro biocompatibility since the cellular adhesion and proliferation at the biomaterial surface depends on the mechanical and physical-chemical characteristics, such as, the surface charge, micro-macrostructure (roughness, crystallinity, etc.) and the presence of contaminants.

In particular, it has been shown that cells are highly sensitive to surface morphology and this interaction affects several cellular functions (cell shape and predominant type, migration, adhesion and tissue organization) [25]. For example, osteoblast-like cells adhere better to rough surfaces with irregular morphologies than on smooth surfaces [26] and, conversely, fibroblasts prefer smooth surfaces than roughened ones [27]. This might explain the different results of the adhesion between fibroblasts and osteoblasts cells (Figs. 4 and 5). As we are interested on looking for a material with good bond bonding properties, in this initial study we used unpolished SS substrates. However, the sample characterization demonstrated that there are two roughness scales, a macroscopic millimeter range with roughness of 50–100 nm and a microscopic nanometric roughness of 2–3 nm. Since the important roughness at the level of cell adhesion is approximately micrometers [28], it is not entirely clear how the two roughness scales might effect the cell adhesion and growth. Certainly, the roughness does probably increase the measurement uncertainty in the present study. Our intention is to examine this aspect in a more controlled manner in the near future.

The longer-term results (cellular proliferation in Fig. 6) suggested a better performance of the a-C films compared to a-CN. This difference might be a consequence of the different bonding as evidenced by IR and Auger spectroscopy. The extra electron of nitrogen probably increases the number of delocalized π electrons and therefore the charge mobility at the surface. However, there may be a difference in the total surface energy and therefore in the surface wettability, which also affects cellular-tissue interactions.

It is also very important to note that the number of osteoblasts cells in a-C films is always very close to those obtained for Ti films. Ti and Ti alloys are conventionally utilized for orthopedic/dental implants because of their good biocompatibility and in particular because of the high osteointegration obtained [29].

5. Conclusions

We have investigated the biocompatibility of sputtered carbon and carbon nitride films confirming the results reported in the literature that the performance of these films is better than uncoated stainless steel substrates and at the level of the most used metal implant (Ti). No cytotoxic effects were evident and cell functionality was good enough to allow population growth in the period investigated. The different biological response between a-C and a-CN films seems to be correlated with the nature of the CN bonding itself, since no significant difference in the surface topography or the electro-optical properties was observed. Further film characterization and control of the substrate roughness are needed to confirm and understand the effects of composition on host tissues.

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