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In vitro cytotoxicity of amorphous carbon films

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Abstract. Amorphous carbon (a-C), carbon nitride (a-CN) and titanium films were deposited on stainless steel substrates (SS) using a dc magnetron sputtering system attached to a high vacuum chamber. Films were deposited using a base pressure of 1.3×10^{-4} Pa. For the carbon films a pure graphite target was eroded in an Argon plasma. For the case of the a-CN films, the Ar flux was substituted by 100% N_2 gas. Titanium films were deposited in a different chamber, using a pure Ti target and an argon plasma. *In vitro* studies were carried out on the coated samples using human osteoblasts cells. Cytotoxicity of carbon films was assessed by cellular adhesion and proliferation, as determined by direct cellular counting using a spectroscopic technique and a well-defined standard curve. Osteoblasts cells were also grown on uncoated steel and prepared Petri dishes for comparison. The percentage of osteoblasts adhesion measured at 24 hrs attained maximum values for the a-C films. Similarly, cellular proliferation evaluated at three, five and seven days showed an outstanding increase of osteoblasts cells for the a-C and Ti coatings in contrast to the uncoated steel. The cell functionality was evaluated by the MTT test after incubation periods of 3, 5 and 7 days. The absorbance values obtained for a-C, a-CN and Ti surfaces resulted significantly higher with respect to the positive control, indicating that the surface did not induce any toxic effect. Preliminary bio-mineralization was evaluated by measuring the elemental composition of the mineral grown on the substrates after periods up to 14 days.

1. Introduction

Carbon is a very versatile element that can form both ordered structures, such as diamond, graphite, fullerenes or nanotubes as well as disordered material. Disordered carbon materials are commonly generated as thin films [1]. The advantage of amorphous carbons (a-Cs) over its crystalline forms is the ability to prepare films at room temperature. It is also possible to have films with a great diversity of physical properties due to the different bonding configurations and different degrees of disorder. Bonding in carbon can be: (a) sp^2 like in graphite, in which three of the four carbon valence electrons are assigned to strong σ planar bonds. The fourth electron lies in a $p\pi$ orbital normal to the bonding plane. (b) sp^3 as in diamond that consist of four σ bonds at 109.5° to each other forming a tetrahedron. (c) Linear sp^1 as in the acetylene molecule, compose of two linear σ orbitals, and two $p\pi$ orbitals in perpendicular directions ($CCsp^1$ bonds are rarely seen in amorphous carbons). The energetic of the σ bonds depends only on their bond angles and bond lengths, but the π bonds are different. A π orbital usually interacts with π states on more than one atom to form conjugated systems as in benzene. This medium-range order of the π bonding induces clustering of the sp^2 sites. It has been shown [2] that in amorphous carbons the total

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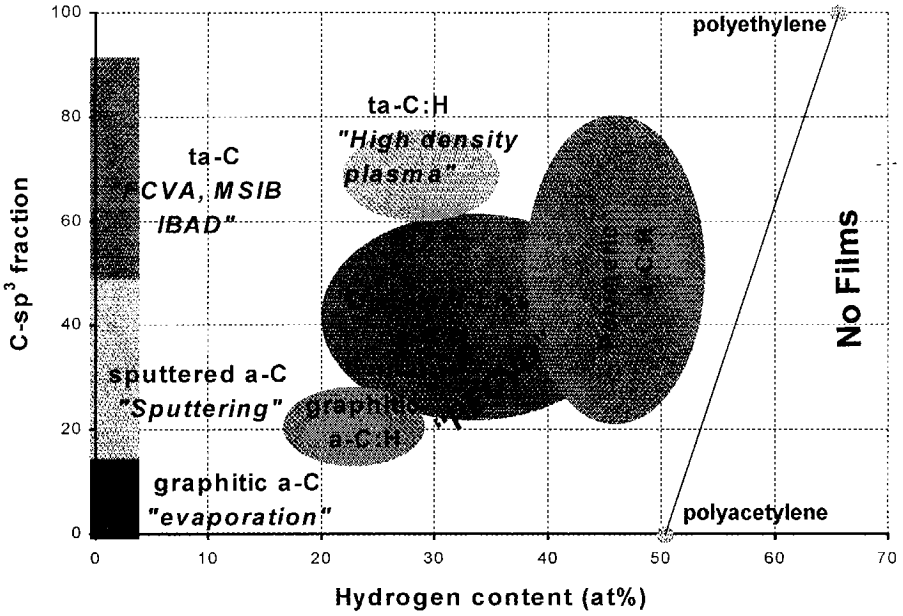


Fig. 1. Diagram of the different types of amorphous carbon films, according to their C-sp^3 and hydrogen content.

energy of the system is minimized if the π states form clusters of aligned π states, or six-fold aromatics rings, which are embedded in a sp^3 matrix. Depending on the deposition parameters various sp^3/sp^2 ratios and different degrees of ordering of the sp^2 phase are formed and therefore different film properties are obtained. If a hydrocarbon gas is used to grow a-C films, a quantity of hydrogen is incorporated into the microstructure, forming hydrogenated carbon films (a-CH or ta-CH) having a important percentage of CH sp^3, CH sp^2 and/or CH sp^1 sites. The amount of hydrogen incorporated depends on the gas source and ion energy, substrate temperature, etc. used for the deposition [1]. Amorphous carbon films are commonly called diamond-like carbon (DLC) films referring to the similarity of their properties to those of diamond. However, strictly speaking this term is only valid for films without hydrogen and with a high CCsp^3 fraction [3]. Figure 1 shows a detailed classification of the a-C films according the their sp^3 fraction and hydrogen content. In order to promote the less stable CCsp^3 bonding it is necessary to use high-density plasmas or energetic ion beams. Then ions are implanted in the film promoting the formation of the sp^3 bonds. Thus, different deposition methods have been developed to promote the specific properties desired. For example, cathodic arcs (FCVA) and ion beams (IBAD, MSIB) sources are preferentially used to deposit tetrahedral amorphous carbon; ta-C films with a high fraction of CC sp^3 bonding (60–85%) [4] or high density plasmas (PBS or ECWR) to deposit the hydrogenated counterpart, ta-CH [5]. Plasma enhanced chemical vapor deposition (PECVD) is used to deposit a-CH films in which the properties are controlled by using an adequate substrate bias [1]. Another common technique is sputtering used to produce non-hydrogenated a-C films with lower CCsp^3 fractions [6]. In these processes a noble gas is used to sputtered carbon atoms from a graphite target, which are then deposited on a substrate that can be biased to enhance ion bombardment.

Carbon nitride films are an important part of the family of amorphous carbon materials. The introduction of nitrogen is done by working at a certain nitrogen partial pressure or by using nitrogen ion beams. There is also a range of film properties and stoichiometries that lead to the formation of carbon nitride (a-CN) films with interesting and useful properties [7].

Reported work on the biocompatibility of carbon films includes mainly a-CH, ta-C films and more recently a-CN films. Graphitic carbon [8,9] is a well-known implant material, particularly in relation to heart valves. Therefore the particular properties of amorphous carbons in relation to its chemical inertness, corrosion and wear resistance, low friction coefficient, low roughness and surface energy are of special interest in this field. Possible applications of carbon films include heart devices [10], DLC-coated dialysis membranes [11], orthopedic applications [12] as well as medical and surgical instruments [13]. Some of the biomedical tests and their results are reviewed in Table 1 [14–28].

In this work we have studied the *in vitro* cytotoxicity of carbon and carbon nitride films deposited using a dc magnetron sputtering system. We choose magnetron sputtered samples for two main reason: there is little information about the biocompatibility of pure a-C films with a high fraction of sp^2 bonding, such as those obtained in the sputtering system, even though for a long time there has been interest on the bio-compatibility of graphite [8]. The other reason is that the sputtering system is very versatile and it can be easily used for industrial applications.

Nitrogenated carbon films were also included because the chemical interactions between the CN surface and the cells might be completely different than the response to a-C films having similar physical properties.

Since we are mainly interested in orthopedic or dental implants in bone, we choose human osteoblasts cells derived form alveolar bone, i.e., the human cells that actually surround the implant. We evaluated the cytocompatibility of surface-modified biomaterials using primary cells, in realation to the physiological mechanism of interest: bone-forming activity. The bone-forming activity depends primarily on the cell adhesion and proliferation, evaluated in this work by a colorimetric technique and MTT assay, and later on the bone matrix synthesis and bone matrix mineralization. Bio-mineralization was evaluated by the physical observation of the mineral formed.

2. Experimental

2.1. Film deposition

Films were deposited on stainless steel (AISI316L) squares (1 cm × 1 cm). The substrates were ultrasonically cleaned in acetone for 30 min, followed by ultrasonic rinsing in isopropanol (30 min), then air-dried and argon-plasma cleaning (10 min). Carbon and carbon nitride films were produced in a dc-magnetron sputtering system from a high purity hollow cathode graphite target within a high vacuum chamber with a base pressure of 1.3×10^{-4} Pa. The films were deposited at 4 Pa using 0.4 Amps and 20 sccm gas flow-rate; argon for the a-C and nitrogen for the a-CN films. The Ti films were deposited in a similar dc magnetron sputtering system, but using a high purity planar Ti target and an argon plasma at 0.2 Amps and 0.5 Pa.

2.2. Cell preparation

Human alveolar bone-derived cells (HABDCs) were obtained by a conventional explant technique [29,30]. 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 and penicillin 100 U/ml, 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.

Table 1

Film	Deposition system	Biocompatibility test	Results
a-C:H	PECVD [14]	Cultures of mouse peritoneal macrophages and fibroblasts	No toxic or inflammatory response
a-C:H	CVD [15,16]	– Tribology: orthopedic coated screws – Corrosion in tyroid solution – Biotolerance: *in vivo*	– No degradation of the screws after 100 screwing into animal bone. – No traces of surface degradation at different temperatures. – No phagocytic reactions and no products of corrosion were found after 52 weeks.
a-C:H with Si buffer layer	PECVD [17]	Lactate dehydrogenase assay: murine and two human cell lines	No evidence of cytotoxicity for any cell culture. Normal cellular morphology.
a-C:H	CVD [18]	Effect of biological fluids on the adhesion of DLC to metallic substrates	Distilled water has no apparent effect, whereas PBS caused localized de-bonding and cracking of the films.
ta-C	FCVA [19,20]	– Wear – Corrosion in 10% HCl	Improvement of the wear and corrosion resistance compared to the uncoated CoCrMo alloy, stainless steel (316L and 420) Ti6Al4V and alumina.
ta-C CN	IBAD [21]	Morphological study of the response of osteoblasts	Cells attached, spread and proliferated without any apparent impairment of cell physiology.
a-C:H	Ion plating process [22]	– Protein adsorption: albumin and fibrinogen – Platelet attachment	No activation of platelets on DLC coatings: no thrombus formation. Higher ratio of albumin to fibrinogen proteins.
ta-C CN	IBAD [23]	– Human fibroblasts and osteoblasts – LDH – Blood compatibility	All cells adhere well to DLC and CN. Good haemocompatibility: no thrombogenic.
a-C:H	PECVD [24]	– *In vitro*: LDH release, osteoblasts markers – *In vivo*: intramuscular and transcortical sites	– Human cell lines attached and proliferated on coated polysterene substrates. Cells maintain an osteoblastic phenotype with expression of alkaline phosphatase, osteocalcin and type I collagen. – *In vivo* no inflammatory reactions or cellular necrosis were observed after 12 weeks.
a-CN	IBAD [25]	Macrophage and fibroblasts attachment	CN films exhibited low macrophage attachment. Normal cellular growth of the fibroblasts.
a-C:H	PECVD [26]	Human monocytes	No significant activation of macrophages
a-C:H	Saddle field [27]	– Murine fibroblasts – Mutagenicity – *In vivo*: cortical bone and muscle	– Good adhesion and spreading: low level of cytotoxicity. – No mutagenic. – No macroscopic adverse effect after 4 weeks.
a-C:H	Ion beam [28]	Blood compatibility	Blood compatibility is influenced by the sp^3/sp^2 ratio.

2.3. Adhesion and proliferation test

All samples were sterilized by exposure to UV-light. Three pieces of a-C, a-CN films, bare SS substrates and the Ti films were placed in 24-well culture plates. The positive controls were 1 cm^2 squares of plastic Petri dishes treated for tissue culture with poly-lysine. The HABDCs were plated at an initial density of 1×10^4/well and left to adhere for 3 hours. After this time, 600 μl of medium (DMEM supplemented with 10% FBS and antibiotic solution) were added. For the adhesion assay the cells were left for 24 hours and for the proliferation tests cells were left in the culture plates for 1, 3 and 7 days. The experimental and control cultures were treated every other day with fresh media. After incubation, the unattached cells were removed with phosphate buffered saline (PBS) and the attached cells were fixed with 3.5% paraformaldehyde. Evaluation of cell attachment was performed according to Hayman et al. [31]. Briefly, fixed cells were incubated with 0.1% toluidine blue for 3 hours. The dye was extracted with 0.1% of sodium dodecyl sulfate (SDS) and the optical absorption read with an ELISA (Enzyme Linked Immune Sorbent Assay) micro-plate reader at 600 nm. The number of cells was then determined by correlating the absorbance of the experimental samples with the number of cells in a previously determined standard curve. The cell numbers were statistically compared using the *t*-Student test.

2.4. MTT

Cell viability was checked by the MTT assay. This assay is based on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue (MTT), a tetrazolium salt (3-[4,5-dimethylthiazolyl-2-y]-2,5-diphenyltetrazolium bromide), to an insoluble blue formazan product. Cells were plated at 1×10^4 per well in triplicate and were incubated for 3, 5 and 7 days. After each term, 10 μl of MTT was added and incubated for 3 hours. Then, the supernatant was removed and 600 μl of dimethyl sulfoxide (DMSO) was added to each well. After 60 minutes of slow shaking the absorbance was read at 570 nm.

2.5. Biomineralization

For the biomineralization assay, the samples (a-C, a-CN, Ti, SS and positive control) were treated in the same way as for the proliferation assay but with the addition of 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10^{-7} dexamethasone in the medium. The media was changed every 2 days. Biomineralization was evaluated after 3, 7 and 14 days. Following cultivation the samples were prepared for observation in the scanning electron microscopy: cell cultures were fixed with 4% formaldehyde in 0.1 M phosphate buffer solution (pH 7.3), then dehydrated in graded ethanol and sputter-coated with carbon. The microscope was a JEOL JSM 5600LV SEM equipped with an X-ray microanalysis (EDX) capability. Evaluation of the biomineralization consists in the morphological and compositional examination of the mineral deposited.

3. Results and discussion

Evaluation of cellular adhesion to biomaterials is the first step to assess the compatibility of a surface with tissue. The adhesion of osteoblasts cells to a-C, a-CN and the metallic surfaces (Ti and SS) was measured at 24 h after plating. Figure 2 shows the results expressed as the percentage of adherent cells with respect to the positive control. Adhesion of osteoblasts is favored in both a-C and a-CN films,

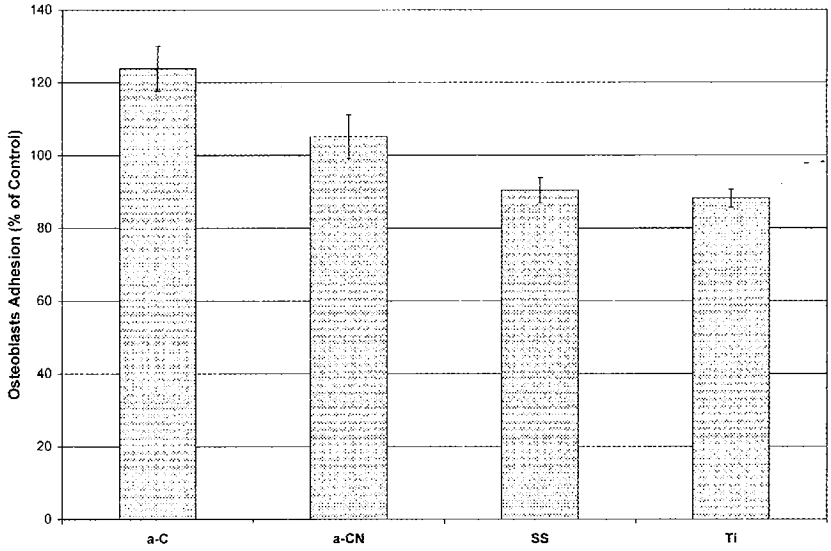


Fig. 2. Osteoblast adhesion after 24 hours expressed as the percentage of cells in comparison with the positive control.

exceeding 100% attachment, whereas the values are much lower for the metallic substrates. Nevertheless, the good attachment to the metallic substrates suggested a lack of toxicity for this surface as well.

Figure 3 shows the histogram of the proliferation assay carried out after 1, 3 and 7 days for the two carbon-based films and the metallic substrates. Positive control cell numbers are also shown for comparison. In the inset, the cell number as a function of the incubation period shows that the initial number of cells after one day was almost double on a-C and a-CN films (from 1×10^4 planted cells to 1.7×10^4 cells). For the metallic surfaces the cell number only reaches 1.3×10^4 in the same period. After longer periods the proliferation of osteoblasts cells shows a saturation for a-C while for Ti there is a linear increase during the time studied, and after 7 days the number of cells is similar in both surfaces. The results indicate that, the particular mechanisms of osteoblasts proliferation are different for Ti and a-C, showing early proliferation and saturation in a-C and constant growth rate in Ti. Saturation in the a-C film might be related to the moment at which the cells reach confluence on the 1 cm^2 area [32]. On the other hand, the rapid cell growth in a-CN during the firsts days is not followed in subsequent days. A negative growth is observed after one week, reaching cell numbers similar to those of SS substrates and below the control. A possible cause of this trend is explained later. On the SS the osteoblasts showed moderate proliferation as compared to control; the number of cells remained approximately constant (1.2×10^4 cells) during the whole period.

Implant materials that can support proliferation are more likely to promote good osteoblasts differentiation and subsequent bone formation. Therefore, the observed good adhesion, spreading, cellular growth rate and proliferation results indicate a good bone-tissue compatibility for the a-C and a-CN coatings. This is in good agreement with the morphological study of osteoblasts behavior by Du et al. [21] on DLC and CN coatings deposited on silicon substrates.

The cell viability was assessed by the MTT assay. The MTT is used as a marker of either cell enzymatic activity or cell growth [33]. However, since nearly apoptotic cells maintain membrane integrity

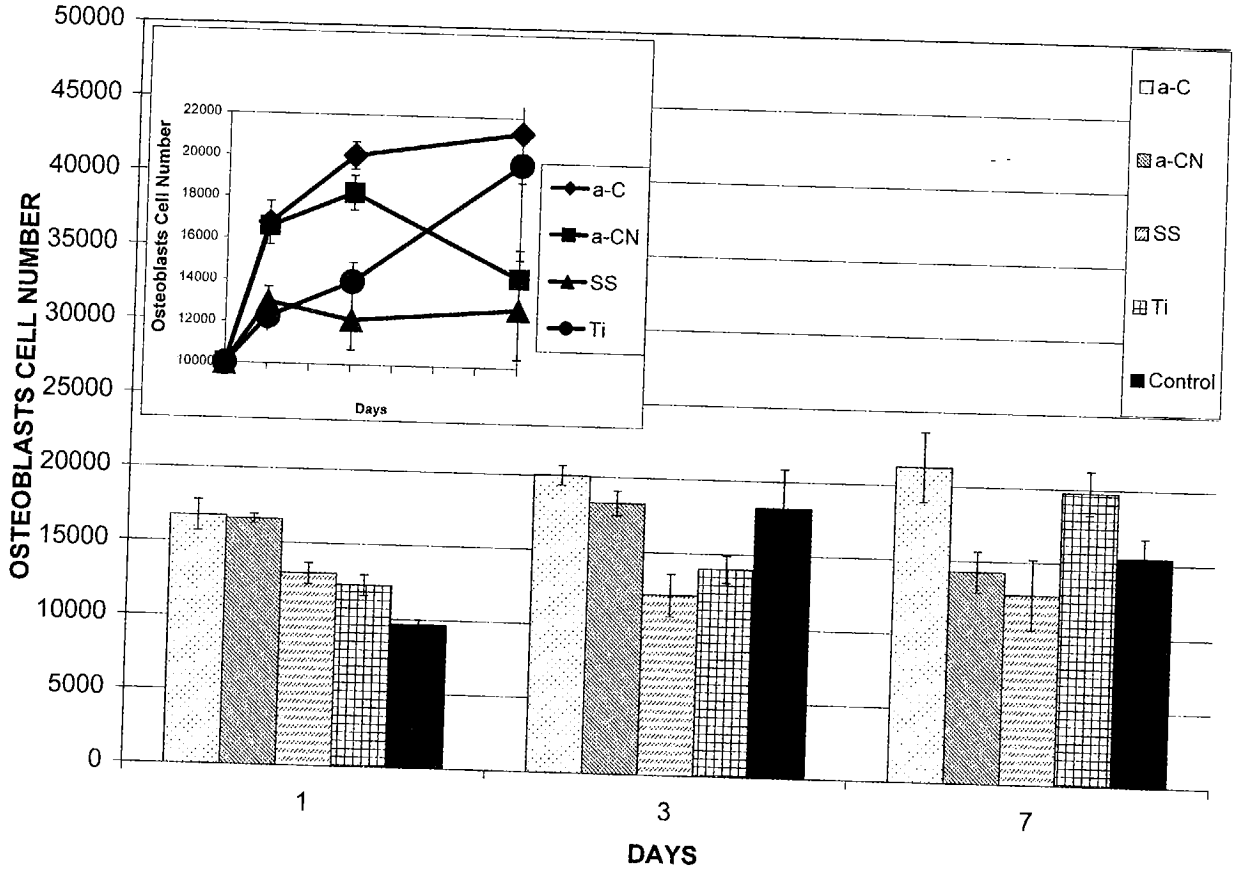


Fig. 3. Osteoblast cell proliferation for 1, 3 and 7 days of incubation. The initial plated cells was 1×10^4 per substrate. The inset shows the cell growth rate.

and mitochondrial functions, the MTT results are not normally in very good agreement with proliferation tests [33]. Figure 4 shows the absorbance at 570 nm, which is directly proportional to the metabolic activity of the cell and inversely proportional to the toxicity of the material. For all samples, there is a positive response attaining values above the positive control, indicating that none of the surfaces is toxic for human osteoblasts cells. The enzymatic activity increased during the first five days for all surfaces, except for a-CN. At 7 days a saturation in cell activity was observed, which is in good agreement with proliferation results for a-C films. In the case of the a-CN samples the trend of the proliferation assays and MTT results is opposite. This discrepancy might be related to film de-bonding and cracking due to the interaction with the biological fluid as was observed by examination of the film before and after the tests with an optical microscope [34]. This is a negative response for the a-CN film, which not necessarily indicates a negative biological response of the film, but a non-stability of the film that needs to be improved prior to further studies.

Following the proliferative phase, osteoblasts begin the deposition of a highly organized matrix and then the process of mineralization of the surrounding extracellular matrix [35–37]. Bio-mineralization is essential for the optimal performance of any orthopedic or dental implant in which a direct structural and functional connection between the living bone and the surface of the implant must be establish. This fusion depends on the possibility of bone formation at the interface and is called osseo-integration.

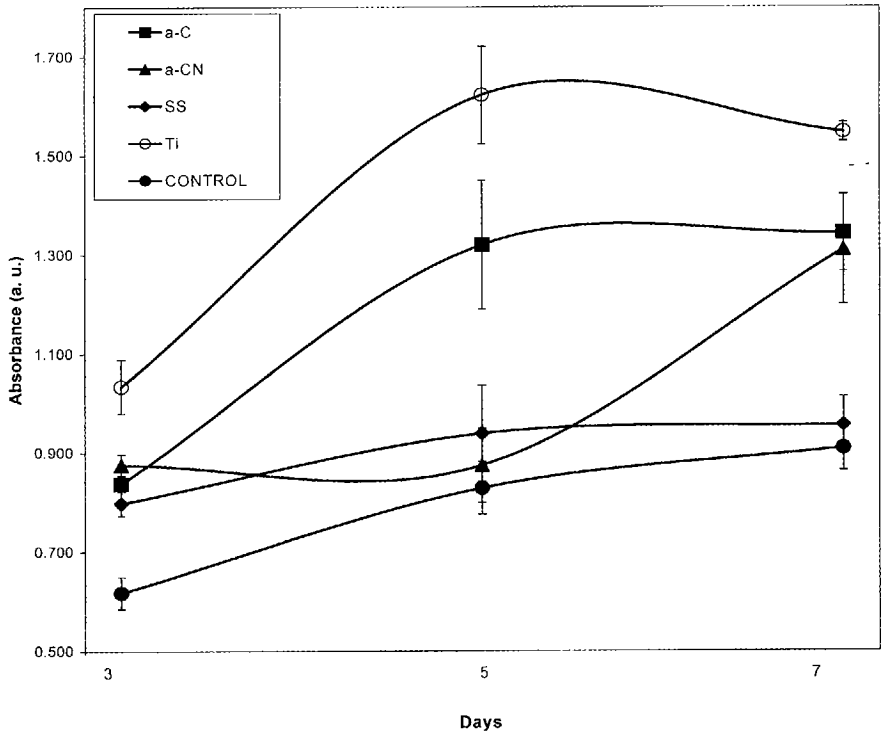
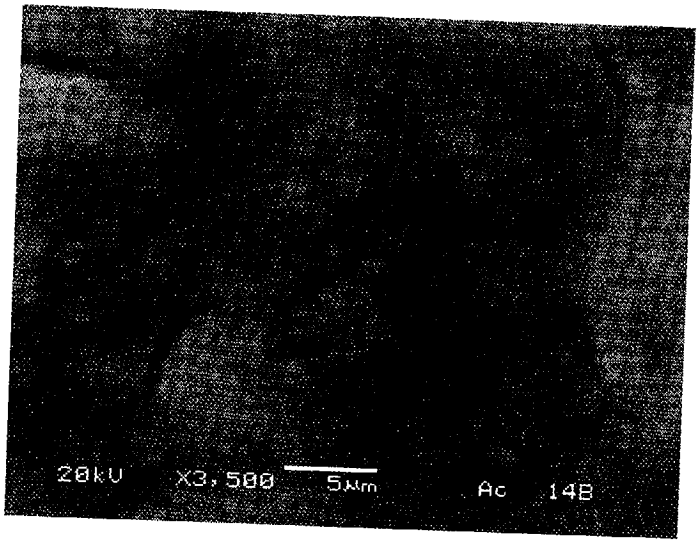


Fig. 4. MTT results expressed as the absorbance at 570 nm.

Osseo-integration is a complex process that involves osteoblasts adhesion, proliferation and mineralization. The ability of osteoblasts to produce mineralized bone-like nodules was assessed by using a low vacuum scanning electron microscopy in combination with energy dispersive spectroscopy. Figures 5, 6 and 7 shows representative backscattered images of the calcium phosphate minerals deposited on the three substrates, a-C, Ti and SS, respectively. (a-CN was not evaluated due to the adhesive failure.) The minerals consist of clusters of globular deposits or bone-like nodules distributed randomly on the whole surface. The composition of the nodules consists of Ca, P and traces of Na, Mg and S. The Ca/P composition ratio was sub-stoichiometry after three days of incubation, but after one and two weeks the general values range between 1.6 and 1.8, i.e., close to the stoichiometry of human bone (1.66) [38]. These nodules appear to be similar to those reported in other studies [39–41]. However, we are not certain about the crystallinity since X-ray diffraction (XRD) performed on the whole substrate gave no signal from any calcium phosphate mineral. This is almost certainly because the percentage of the Ca–P nodules is too small to be detected by XRD, compelling us to prepare samples for the transmission electron microscope in the future. Nevertheless, this study has shown that the a-C surface has the capacity to promote strong osteoblastic mineralization *in vitro*.

4. Conclusions

In the present work, we have studied the biological response of human osteoblasts plated on experimental carbon and carbon nitride surfaces and compared the results with those for conventional implant



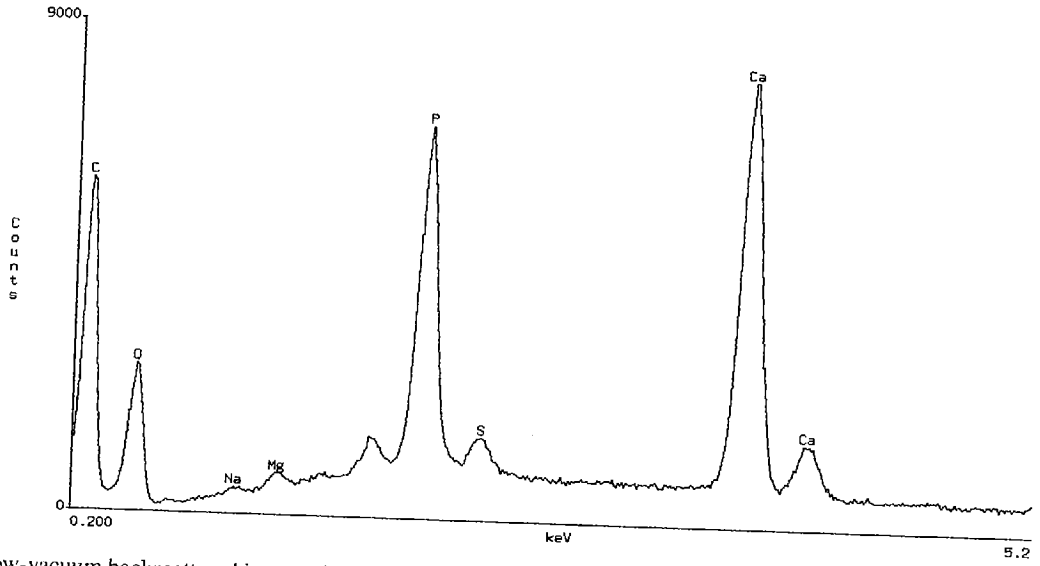
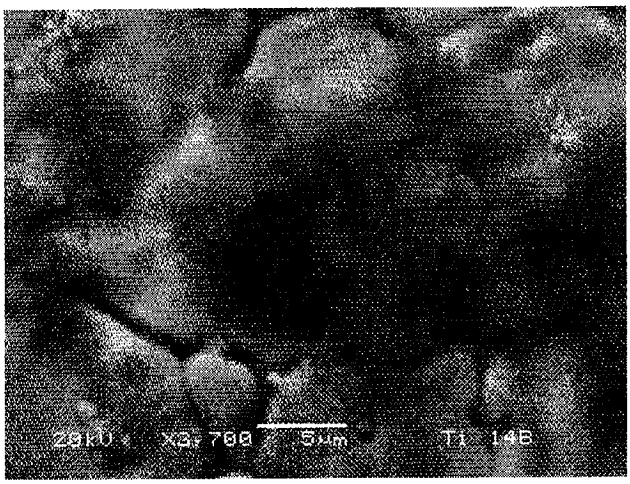


Fig. 5. Low-vacuum backscattered image of the bone-like nodules found in the a-C film after 14 days of incubation. The spectra shows the X-ray energy spectrum (EDX) of the mineralized areas. The Ca/P ratio of this point was 1.9.

materials, stainless steel (AISI 316L) and titanium. The results of cell attachment studies indicate that osteoblasts attached slightly better to a-C and a-CN than to the metallic substrates. This good attachment was followed by a high early proliferation as compared to SS, Ti and the control. However, the adhesion between a-CN sample and the metallic substrate was affected during the incubation in the biological medium. This is one of the main concerns regarding the use of biomedical coatings. However, it is possible to improve the mechanical properties of the substrate–film interface and this will be done in a future study.

Cell viability tests confirmed the high enzymatic activity of the osteoblasts cells in agreement with the high cellular growth rates. Mineralization, considered as the strongest indicator of osteoblasts dif-



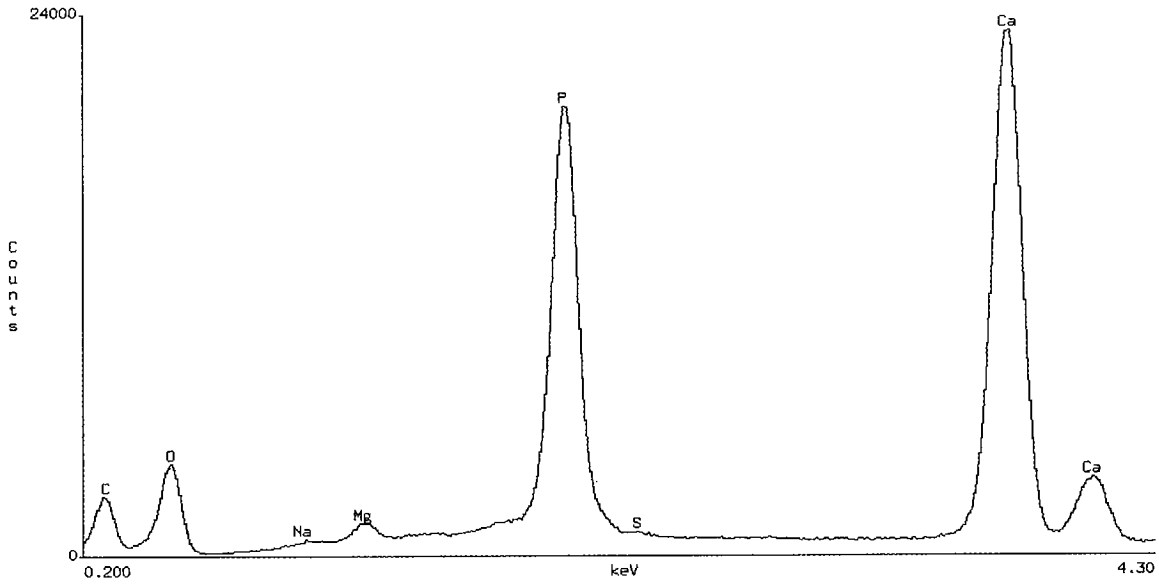


Fig. 6. Low-vacuum backscattered image and EDX spectra of the bone-like nodules found in the Ti film after 14 days of incubation. The Ca/P ratio is 1.66.

ferentiation and osteogenesis, was also studied. The results obtained in this work suggest that a-C films promoted the formation of globular deposits with a high degree of mineralization, comparable in quantity and quality to those obtained in Ti and medical grade stainless steel. Indeed, none of the studied surfaces were toxic to osteoblasts cells and they all exhibited good cellular adhesion, proliferation and mineralization. However, the results for a-C were always better than those of stainless steel and very close to the Ti surface, the best implant material. Nonetheless, there are still a number of issues left to examine, such as the density of nodules, the effect of surface roughness as well as additional chemical properties of the surface.

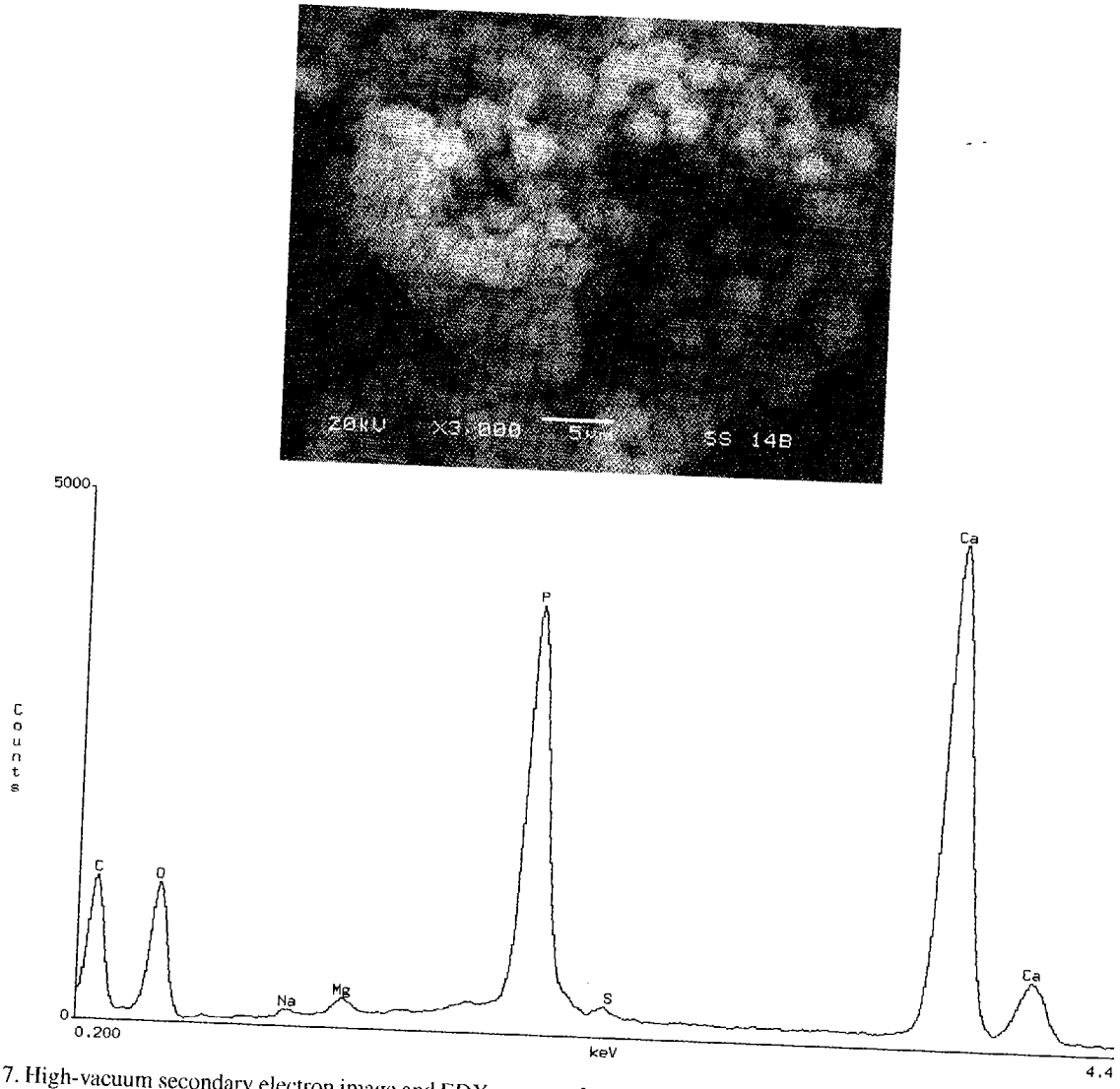


Fig. 7. High-vacuum secondary electron image and EDX spectra of the Ca–P nodules found in the bare stainless steel substrates. The Ca/P ratio is 1.7.

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