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In vitro studies of the biomineralization in amorphous carbon films

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Abstract

Films of amorphous carbon (a-C) and amorphous carbon nitride (a-CN) were deposited on stainless steel substrates (AISI 316L) using a d.c. magnetron sputtering system. In vitro studies were carried out on the coated samples using human osteoblasts cells. Preliminary bio-activity of carbon films to promote bone growth was assessed by cellular adhesion and proliferation, as determined by a cellular count spectroscopic technique using a well-defined standard curve. Osteoblasts cells were also grown on uncoated steel and Ti coated steel samples for comparison. The degree of osteoblasts adhesion measured at 24 h attained maximum values for a-C films. Similarly, cellular proliferation evaluated at 1, 3 and 7 days showed an outstanding increase of osteoblasts cells for a-C and Ti coatings in contrast with uncoated steel. Bio-mineralization or mineral bone growth was evaluated by measuring the elemental composition and the substrate coverage of the mineral grown on the substrates after periods up to 14 days. Elemental composition analyses were performed by X-ray energy dispersion analysis (EDX) in a scanning electron microscope.

Keywords: Amorphous carbon films; Biocompatibility; Biomineralization

1. Introduction

Biomineralization is the ability of living organisms to mineralize in their cells inorganic compounds from solutions [1]. In the human body this process regulates biological events, such as, bone and tooth development. Another important process controlled by the biomineralization is the osseo-integration. Osseo-integration is the phenomena in which a direct structural and functional connection is established between living bone and the surface of a biomedical implant [2,3]. The surface is colonized by bone cells, where they spread and create a very close bone–metal contact. During the osseo-integration, like in any other biological repair mechanism, cellular adhesion is a crucial phase, which for implants depends on the cellular response to the prostheses surface [4]. This response is modulated by surface properties, such as, chemical composition, surface energy, conductivity, roughness, etc. [4–6]. Similarly, the quantity and quality of the mineral or bone formed depend on the cellular–implant interactions [7–9]. In vitro, osseo-integration is studied in terms of the ability of the osteoblasts cells to spread in the biomedical implant, synthesize cellular matrix, express proteins related to bone-growth or with the direct mineralization of hidroxyapatite or calcium-phosphate mineral [10–13].

The requirements for candidate orthopedic or dental implants constitute an extensive list of properties. Therefore, different kinds of materials and material combinations have been developed and tried as biocompatible implants. None of them fulfill all the requirements under conditions of use, so there is still a continuous research about new and better biomedical materials. The most common metallic implants are stainless steel (AISI 316L) and titanium alloys (Ti6Al4V). In recent years, surfaces modifications of these basic materials have proved to be an efficient way to provoke a better biological response, depending on the specific application [14–17]. Our specific interest concerns biomedical implants in direct contact with bone, in which a good osseo-integration is necessary. We study the effect of surface modification of stainless steel substrates by deposition of an amorphous carbon (a-C) thin film. Amorphous carbon films (also called diamond-like car-

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bon, DLC) or nitrogenated carbon films (a-CN) have passed all first stage biocompatibility tests [18–22]. Films of a-C and a-CN were found chemical inert, showing a good resistance to chemical attack [23], no cytotoxic effect to different cell lines have been observed [24,25], they are not trombogenic [26], good adhesion to substrate can be obtained [27,28] and studies demonstrated a good biointegration in the oral cavity [29]. Amorphous carbon films are currently used as coatings for surgical instruments, heart valves and coronary stents [30,31].

The other interest in DLC and its compounds is the diversity of film properties that can be obtained depending on the main carbon hybridization (sp^2 or sp^3) and the hydrogen or nitrogen content [4]. Film properties vary from very hard, wear and corrosion resistant semiconductor to a semi-metallic softer material. In general, carbon films present good biomedical, mechanic and tribological properties [32], suitable for implant applications.

In this paper we concentrate on the osteoblasts response to sputtered a-C and a-CN films as possible surface modifications for standard stainless steel implants. The biological response is compared to sputtered Ti coatings, bare stainless steel (SS) and control plastic dishes. Osteoblasts response is quantified by evaluating the degree of cell attachment, cell growth and biomineralization.

2. Experimental

2.1. Films deposition

Films were deposited simultaneously on stainless steel (AISI 316L) squares of 1 cm^2. The substrates were ultrasonically cleaned using acetone for 30 min, followed by ultrasonic rinsing with isopropanol for another 30 min and then air-dried. The films were produced by a hollow-cathode d.c. magnetron sputtering system attached to a high vacuum chamber (base pressure 1×10^{-2} Pa), 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 carbon nitride films, after the cleaning with argon, the gas was substituted by pure N_2 (purity 99.999%) gas. Films were deposited at 4 Pa (30 mTorr), 20 sccm of argon or N_2 and a d.c. current of 0.4 A.

The Ti films were deposited on stainless steel in a similar d.c. magnetron sputtering system, using a high purity Ti target (99.999%) and argon plasma at 0.2 A and 0.5 Pa (4 mTorr).

2.2. Cell culture

Human alveolar bone derived cells (osteoblasts) were obtained by a conventional explant technique [33,34]. 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 FSB and antibiotic solution streptomicyn (100 μg/ml) and penicillin (100 units/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.

2.3. Cell attachment and proliferation tests

Samples of 1 cm^2 stainless steel coated with a-C, a-CN and Ti films were placed in 24-well culture plates. The positive and negative controls were 1-cm^2 squares of plastic petri dishes treated with poly-lysine for tissue culture and bare SS, respectively. All samples were sterilized by exposure to UV light. The osteoblasts were plated at an initial density of 1×10^4 cells/cm^2 and left to adhere for 3 h. After this time, 500 μl of medium, with the same composition used for the cell culture, were added. Cellular attachment was evaluated after 24 h, and for the proliferation test the cells were left on the culture plates for 1, 3 and 7 days. The media was replaced every 2 days with fresh medium. After incubation, the unattached cells were removed using a phosphate buffered saline solution (PBS) and the attached cells were fixed with 3.5% paraformaldheyde. The assays were performed at least by triplicate.

2.4. Cell number assay

Cellular counting was performed according to a spectra-photometric technique: fixed cells were stained 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 previously obtained standard curve relating the absorbance with cell number.

2.5. Biomineralization assay

For the biomineralization assay the same samples (a-C, a-CN, Ti and SS) were placed, after sterilization, in 24-well culture plates. Human osteoblasts cells were plated at a density of 1×10^4/cm^2 and left to adhere for 3 h. After this time, we added 500 μl of medium composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotic solution (streptomycin 100 μg/ml and penicillin 100 units/ml), 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10^{-7} dexamethasone.

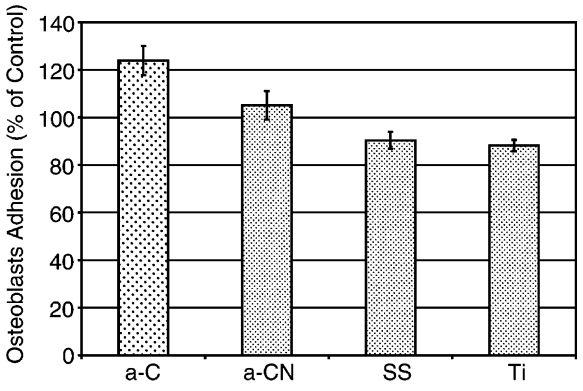

Fig. 1. Adhesion of osteoblasts-like cells on the a-C, a-CN, Ti and bare stainless steel substrates after 1 day.

Biomineralization was evaluated after 3, 7 and 14 days. The media was changed every 2 days. After 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, sputter-coated with carbon and analyzed in a JEOL JSM 5600LV SEM equipped with an X-ray microanalysis capability. Evaluation of biomineralization consists in the morphological and compositional examination of the mineral formed, performed by EDX.

3. Results

3.1. Cell adhesion

Cell attachment is expressed as the percentage of adhere cells in relation to the positive control (Petri dish prepared with poly-lisine). The results after 24 h of cultivation are shown in Fig. 1. The average of 15 samples shows that the number of cells in both a-C and a-CN surfaces exceeds the positive control, whereas SS and Ti surfaces are below 100%. However, the percentages, considering the error bars, were no statistically different when compared with Student's *t*-test at $p < 0.05$.

3.2. Proliferation assay

Cell proliferation was evaluated after 1, 3 and 7 days for all the substrates. Fig. 2 shows the average number of cells (3 samples) for each substrate as a function of incubation days. The number of cells after 1 day increases rapidly on a-C and a-CN from 10 000 plated cells to 17 000 cells. However, for the metallic surfaces the cell number only reaches 13 000 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 whole time. Nevertheless, at 7 days the number of cells is similar in both surfaces. The rapid cell growth in a-CN during the first day is not followed in subsequent days decreasing to values similar than those of SS substrates after one week. For the SS surface the number of cells remains approximately constant (12 000 cells) during the whole period. The error associated with the quantification makes it difficult to draw any conclusive difference between a-C, a-CN and Ti films. Only after 7 days stainless steel substrate cell-number was significantly less than a-C films ($p < 0.05$).

The strong cell-number decrease observed for a-CN films is most probably a consequence of surface modification of the film. We observed these samples with the optical microscope and found that a-CN films suffer an adhesive failure during the incubation period. Fig. 3a shows typical signature of adhesive failure for thin films. Adhesive failure was most likely due to interaction of the CN surface with the liquid media, because samples stored in dry atmosphere for long periods have not failed, as shown in Fig. 3b. This also might explain the similar cell-numbers obtained for a-CN and SS after 7 days of study.

3.3. Biomineralization

Figs. 4 and 5 show low-vacuum SEM images of mineral deposits in a-C and Ti samples after 7 days and 14 days of incubation, respectively. Under conditions of low-vacuum the image is formed by backscattered electrons and consist of a mixture of topographical and compositional information. The figures show clusters of globular or spherules minerals with similar composition on top of the crystalline grains of the stainless steel substrate. The formation of mineral deposits was observed after the first 3 days of incubation. The density

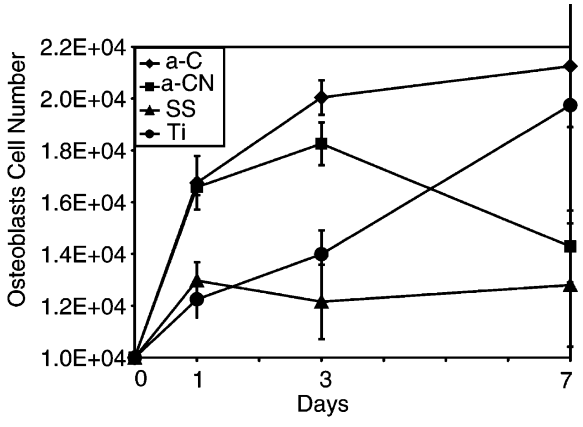

Fig. 2. Cell number growth as a function of the incubation period.

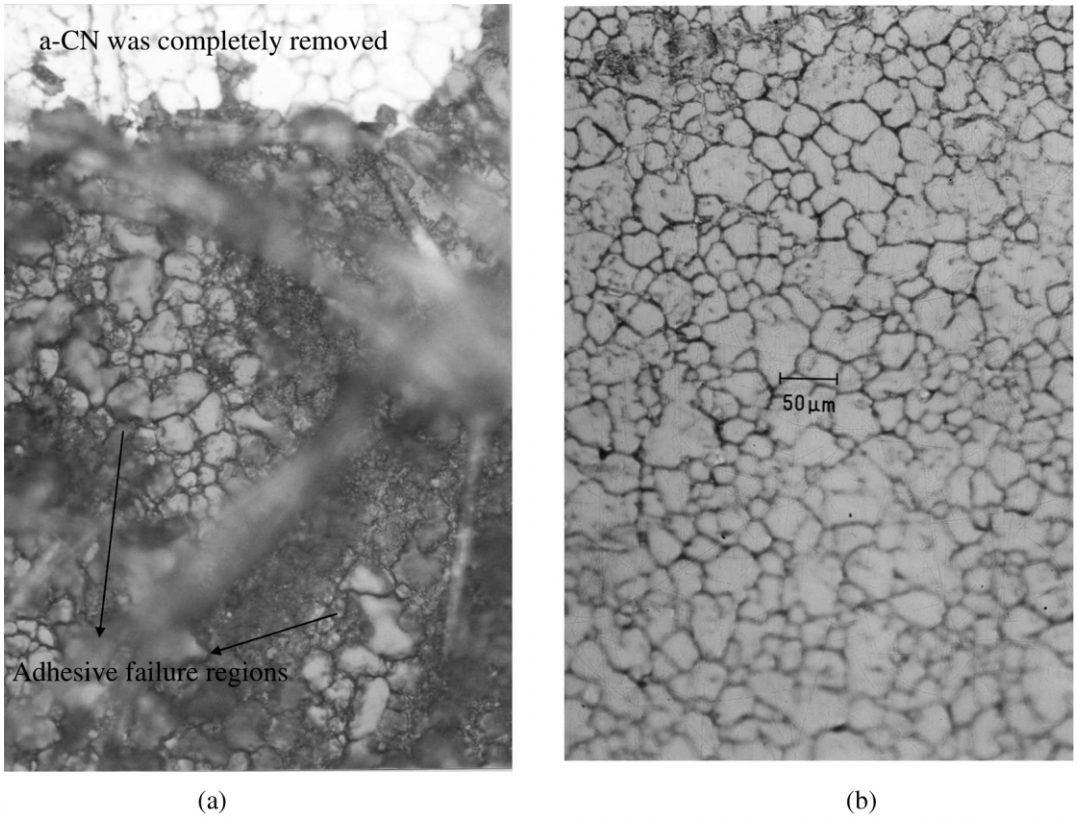

Fig. 3. Optical photography of a-CN films showing signals of adhesive failure after the proliferation assay (a) and a-CN sample stored in a dry atmosphere (b) showing good substrate adherence.

of the globular mineral deposits and the size of the clusters increase with time for both a-C and Ti. In both cases, X-ray microanalysis of the globular deposits showed the presence of calcium and phosphorus, with minor traces of sodium, magnesium and sulfur, as shown in Fig. 6. The composition was evaluated in different regions for each sample. From this information, we can conclude that after 3 days some nodules are Ca deficient, presenting Ca/P ratios lower than one. However, after 7 days the stoichiometry of most of the nodules range

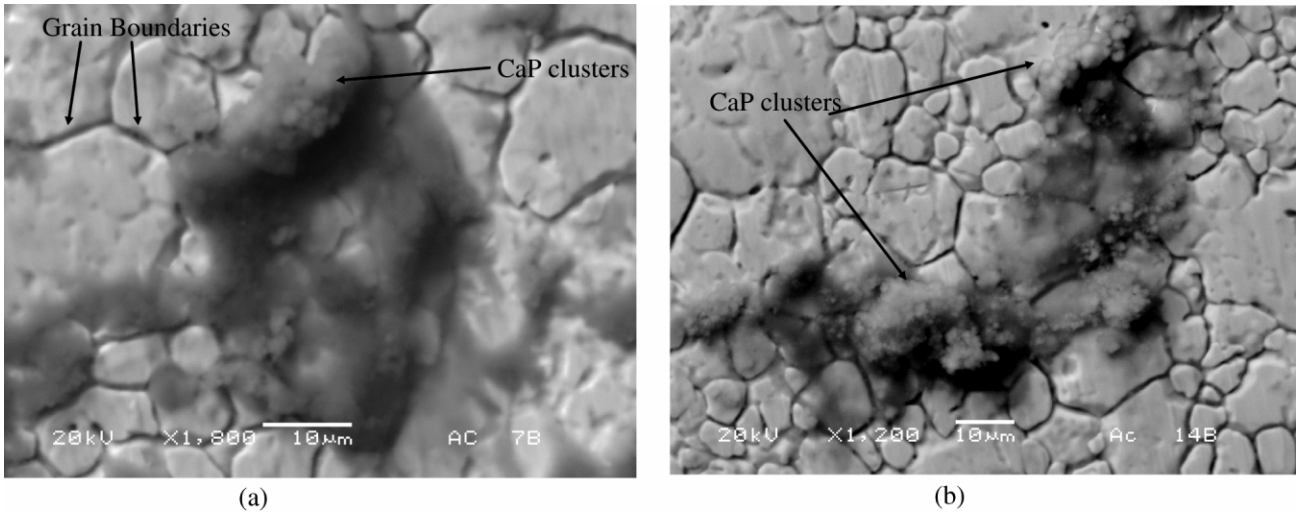

Fig. 4. Low vacuum SEM images of the biomineralization in carbon surfaces after 7 (a) and 14 days (b). The cross-hair in (b) shows the region in which compositional analysis was performed.

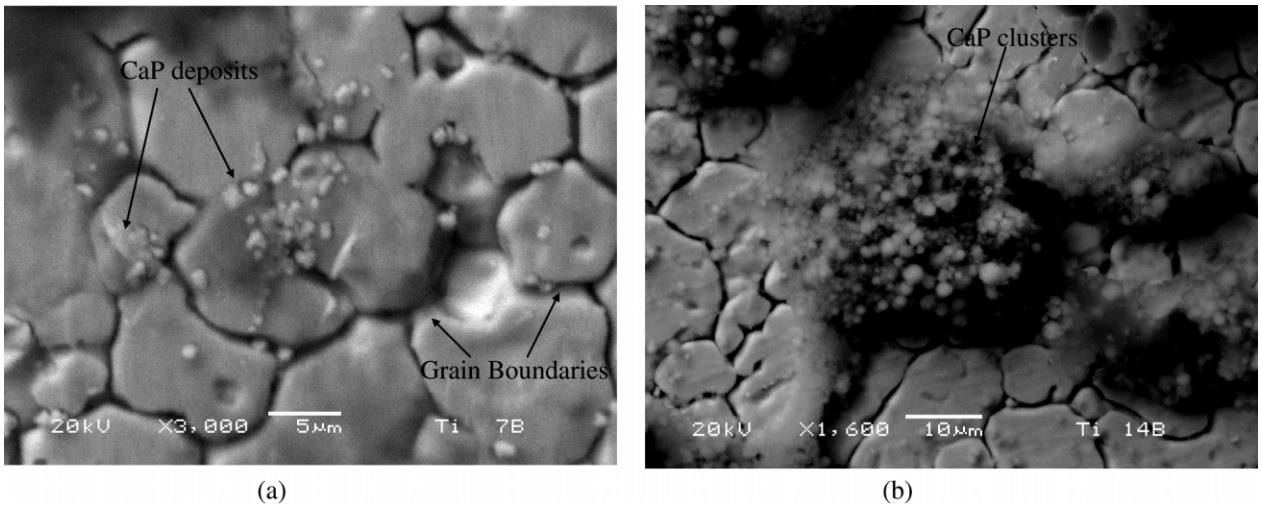

Fig. 5. Low vacuum SEM images of the globular mineral deposits found on Ti surfaces after 7 (a) and 14 days (b).

between 1.5 and 2.3, indicating the formation of calcium phosphate mineral with stoichiometries close to human bone. The corresponding Ca-P nodules and clusters in the SS substrate are smaller than in a-C or Ti. Mineral evaluation was not performed for the a-CN samples since the effect of the adhesive failure could be confusing, although globular deposits were also present.

4. Discussion

In this study, we used an in vitro cell culture system to investigate biomineralization processes in carbon and carbon nitride films. Bio-mineralization is essential for the optimal performance of any orthopedic or dental implant in which a complete fusion between the material's surface and the bone tissue must be established. Osseo-integration is a complex process that involves osteoblasts adhesion, proliferation and mineralization. Osteoblasts adhesion is the initial phase of interaction between cell and implant material and the quality of this phase will influence the cells capabilities to proliferate and differentiate in futures phases. Examination of cell attachment to the carbon-based films and metallic control surfaces, indicated better adhesion in both a-C and a-CN films than in Ti or SS. This enhanced adhesion was reflected in the cell growth results after 1 day, showing greater number of cells in carbon-based films. However, adhesive failure of the nitrogenated films induces cell death or negative growth for longer periods. In contrast, negligible growth was observed for the stainless steel substrate and positive growth for the Ti substrate. These results show that although cell attachment is the first step for cell growth, there are other factors involved in the process. Attachment in the first stage is controlled by the formation of physico–chemical linkages between cells and the materials involving mainly surface-bonding forces. Meanwhile, the longer term adhesion and proliferation are associated to the presence of specific biological molecules and/or proteins. During osteoblasts/material interactions protein expression, synthesis of extra-cellular matrix, and growth factor production are modified according to the surface characteristic of the material. Therefore, in order to give an explanation of the different response observed between a-C and Ti surfaces, assays of protein expres-

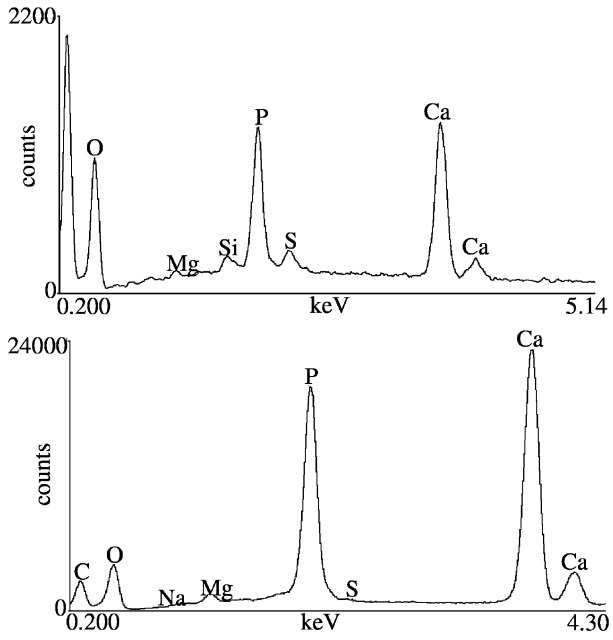

Fig. 6. EDX spectra of the globular deposits in a-C (a) and Ti (b) after 14 days. The Ca/P ratios are 1.64 in a-C and 1.66 in Ti.

sion or alkaline phosphatase (ALP) activity must be performed and correlated with the film's surface properties. Allen et al. [18] reported the response of human osteoblasts culture lines to DLC-coated polystyrene, showing that the cells maintain an osteoblastic phenotype with expression of ALP, osteocalcin and type I collagen. The expression of these three markers was similar to the uncoated polystyrene, which is known as biocompatible. However, no comparison has been made between DLC films and metallic implants.

In vitro, osteoblasts cells grow and spread in the presence of culture medium but to promote mineralization it is necessary to add β-glycerphosphate, dexamethasone and ascorbic acid [35,36]. Biomineralization was studied by observing the samples in the scanning electron microscope. Sample preparation for the SEM is a crucial issue if good image resolution is needed [37]. In this study, we were able to identify mineral deposits and also to determine their composition. However, the morphology or crystallinity degree of the minerals is still unclear, they look as irregular globules similar to those reported in other works [38–40]. The composition, although showing high Ca/P ratios, was also different from nodule to nodule, even in the same sample. This might indicate different stages in the formation of the minerals.

5. Conclusions

Deposition of thin films of amorphous carbon improves the osteoblasts adhesion and proliferation in stainless steel substrates to similar levels than those obtained in titanium substrates. The different biological response between a-C and a-CN, initially attributed to the nature of the CN bonding [41], was shown to be a consequence of the adhesive failure of the a-CN film. Future works to improve the film adhesion to SS substrates must be performed for a competitive comparison between the two materials.

The capacity of mineralization of the osteoblasts cells was not impeded by any of the films. Moreover, Ca/P composition ratios of the mineralized areas were between 1.5 and 2.3 similar to human bone ratios. Thus, carbon-coated stainless steel might function as a good biomedical implant in contact with bone.

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