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Research Article

Nanocrystalline Diamond as a Coating for Joint Implants: Cytotoxicity and Biocompatibility Assessment

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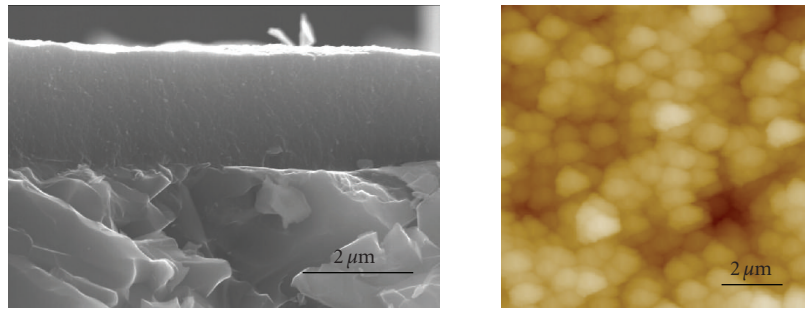
Nanocrystalline diamond (NCD) coatings combine a very low surface roughness with the outstanding diamond properties, such as superlative hardness, low self-friction coefficient, high wear and corrosion resistance, and biotolerance, which are ideal features for applications in medicine (knee and hip replacement) and surgical tools. The present work presents a comprehensive study of the cytotoxicity and biocompatibility of NCD films grown by hot-filament chemical vapour deposition (HFCVD) technique, aiming such future applications. Cytotoxicity was evaluated in vitro by seeding human gingival fibroblasts on the NCD surface for 14 days, while specific biocompatibility was assessed on samples seeded with human bone marrow-derived osteoblasts during 21 days. The NCD coatings proved to be noncytotoxic in the preliminary human gingival fibroblast cell cultures, as denoted by a notable sequence of cell attachment, spreading, and proliferation events. In the specific biocompatibility assay envisaging bone tissue applications, NCD coatings induced human osteoblast proliferation and the stimulation of differentiation markers, compared to standard polystyrene tissue culture plates.

1. INTRODUCTION

Diamond has been one of the most desired and investigated materials in the past years. From an extensive list of superlative properties, the high hardness, the chemical inertness, the high thermal conductivity, and the high optical transparency are just a few examples of its remarkable nature. Applications such as cutting tools, abrasives, structural components, heat sinks, bearings, and optical windows (X-ray, IR, and laser windows) are the proof that diamond has a wide-ranging impact in several fields. Diamond coatings with nanosized crystallites, the so-called nanocrystalline diamond (NCD), present a great potential to become a serious challenging technology in new application areas like biomedicine and biotechnology. NCD combines surface smoothness with high corrosion resistance and biotolerance, which are ideal features for applications in medicine onto surgical tools and medical implants. For example, joint implants coated with NCD can take benefit of its protective character. The NCD coating acts as a selective protective barrier between the implant and the human environment, preventing the release of metallic ions into the body [1]. NCD presents the highest resistance to bacterial colonization when compared to medical steel and titanium [2]. This property is very important since infection due to microbial colonization of the implant surface may lead to implant rejection. In addition, the high wear resistance and the low coefficient of friction of NCD allow the reduction of the amount of wear debris generated during the joint functioning, increasing the life of the prosthesis [3, 4]. Even more, in this case,



(a) (b)

Figure 1: NCD as grown: SEM cross-sectional (a) and AFM scan (b) views.

the residues formed due to wear are diamond particles, which are completely harmless initiating little or no adverse reactions from human monocytes and polymorphonuclear leukocytes [5–7]. In fact, recently, it was shown that diamond particles appear to possess high bioactivity at the molecular level, presenting antioxidant and anticarcinogenic properties [8].

Nowadays, a new generation of biomaterials, able to control the biological response with precision, is arisen. NCD is also included in this recent group of materials and can be used as a template for the immobilization of active molecules for biological applications or for biosensoric applications [9–14]. One example is the functionalization of NCD with bone morphogenetic protein-2 (BMP-2) creating a biomimetic coating that results in improved osseointegration, which is a powerful strategy in tissue engineering and in the context of bone tissue regeneration [15]. The NCD surface can also be modified with the linking of an antibody, human immunoglobulin G (IgG), which provides biomolecular recognition capability, turning out a biologically sensitive field-effect transistor (Bio-Fet) [14].

Despite all the referenced works report the study of NCD bioproperties, the biocompatibility of this coating is not yet full assessed. NCD films can differ to some extent in their surface and bulk properties depending on the deposition method and parameters applied [9]. Besides, biocompatibility refers to the ability of a material to execute a specific application with an appropriate host response [16]. Therefore, for each type of NCD and respective application, the biocompatibility must be assessed considering adequate in vitro and in vivo biological systems to evaluate specific cell and tissue response.

The present work reports the in vitro study of the citotoxicity and biocompatibility of NCD films grown on Si_3N_4 substrates by hot-filament chemical vapour deposition (HFCVD) technique, using Ar-CH_4-H_2 gas mixtures, considering their future application as a coating for joint implants (knee and hip replacement). Si_3N_4 ceramics are used as substrates because they are not cytotoxic [17–19] and provide an excellent film adhesion due to a small thermal expansion coefficient mismatch with diamond. The HFCVD is a simple and low-cost method, able to coat complex shapes and internal surfaces, like the femoral head and acetabular cup of hip prosthesis. Biotribological tests for checking the mechanical suitability of NCD as a coating for joint implants have already been made by the present group [3, 4].

In this work, cytotoxicity was evaluated by seeding NCD coatings with human gingival fibroblasts while specific biocompatibility was assessed by the characterization of the seeded samples with human bone marrow-derived osteoblasts.

2. MATERIALS AND EXPERIMENTS

2.1. NCD-coated samples

NCD coatings were produced by the HFCVD technique. Dense Si_3N_4 ceramics substrates were prepared according to a processing route that can be found elsewhere [20]. Prior to deposition, the disc-shaped samples (diameter = 10 mm, thickness = 3 mm) were ground and polished with 15 μm diamond slurry and submitted to a 1-hour ultrasonic bath in 1 μm diamond powder suspension in n-hexane (1 g/100 mL), followed by ultrasonic cleaning with ethanol for 10 minutes.

The NCD deposition conditions were as follows: T_s = 750°C (substrate temperature), T_f = 2300°C (tungsten filament temperature), Ar/H_2 = 0.1, CH_4/H_2 = 0.04, P = 50 mbar (total gas pressure), F = 50 mL·min^{-1} (total gas flow), t_d = 2 hours (deposition time). The HFCVD deposition method enabled an NCD coating of 2 μm in thickness, that is, a growth rate of about 1 μm·hr^{-1}. Figure 1(a) gives a representative cross-section of the film/substrate interface as taken by scanning electron microscopy (SEM, Hitachi S4100). The typical surface microstructure of the NCD coatings is depicted in the atomic force micrograph (AFM, Digital Instrument Multimode IIIa) in Figure 1(b). The diamond nanocrystals, with an average size of 28 nm [21], are agglomerated as micrometric clusters, leading to a root-mean-square roughness (RMS) value of 68 ± 5 nm (from AFM 100 × 100 μm scans). A previous work demonstrated that the NCD coating supported a normal load of 400N without spalling-off and presented an interfacial crack resistance of 6.8 N mm^{-1} [22]. Before being seeded, the

NCD-coated Si_3N_4 samples were washed with ethanol in an ultrasonic cleaner and sterilized by autoclaving.

2.2. Cell cultures

2.2.1. Human gingival fibroblast cells

Primary cultures were obtained by culturing explants of gingiva from a patient undergoing a third molar extraction for orthodontic reasons. Informed consent to use this biological tissue that would be otherwise discarded was obtained. The tissue was washed in phosphate buffer saline (PBS), cut into small pieces, and cultured in α-Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum, $50\,\mu g \cdot mL^{-1}$ ascorbic acid, $50\,\mu g \cdot mL^{-1}$ gentamicin, and $2.5\,\mu g \cdot mL^{-1}$ fungizone at 37°C, in a humidified atmosphere of 5% CO_2 in air. Cell outgrowth from the tissue explants was observed 1 to 2 weeks after the beginning of the incubation. Cultures showed a high proliferation rate and reached confluence in approximately 1 week, with cells oriented along parallel lines, a typical feature of this culture system [23]. Primary cultures were subcultured (0.05% trypsin, 0.25% EDTA) at 70–80% confluence. First-passage human gingival (HG) cells were cultured (10^4 cells$\cdot cm^{-2}$) for 14 days in standard plastic culture plates and on the surface of the NCD films. The medium was changed every 2-3 days. Cultures were characterized at 3, 7, and 14 days for cell viability/proliferation (MTT assay). In addition, cultures were observed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) to assess cell morphology during cell adhesion to the substratum (first 24 hours) and throughout the culture time.

2.2.2. Human bone marrow cells

Human bone marrow, obtained from orthopaedic surgical procedures after patient-informed consent, was cultured in the same experimental conditions as those used in the fibroblast cell cultures. Primary cultures were maintained until near confluence (10–15 days) and, at this stage, adherent cells were enzymatically released (trypsin–EDTA solution) and seeded at a density of 10^4 cells$\cdot cm^{-2}$ in standard polystyrene culture plates and on the surface of the NCD films. Control and seeded material samples were cultured for 21 days in the experimental conditions described above, with the culture medium further supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone. All the experiments were performed in the first subculture since the sequential passage of bone marrow osteoblastic cells fallout in a progressive loss of the osteoblast phenotype [24].

Control cultures and seeded material samples were characterized at days 3, 7, 14, and 21 for cell viability/proliferation (MTT assay), total protein content, alkaline phosphatase (ALP) activity, and observation by SEM and CLSM to evaluate cell morphology and matrix mineralization.

2.3. Biochemical and microscopy assays

2.3.1. Cell viability/proliferation

MTT assay (reduction of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrasodium bromide to a purple formazan reaction product by living cells) was used to estimate cell viability/proliferation. Cultures were incubated with $0.5\,\mu g \cdot mL^{-1}$ of MTT for the last 4 hours of the culture period tested. Subsequently, formazan salts were dissolved with dimethylsulphoxide (the seeded material samples were previously transferred to a new plate) and the absorbance (A) was measured at 600 nm in an ELISA reader. The results were normalised in terms of macroscopic area and expressed as $A \cdot cm^{-2}$.

2.3.2. Total protein content and alkaline phosphatase activity

Culture samples were washed twice in PBS, frozen at −20°C, and evaluated at the end of the culture time. The total amount of protein present in the cultures was assayed by the Lowry's method with bovine serum albumin used as a standard. The results were expressed as $\mu g \cdot cm^{-2}$. ALP activity was determined in cell-layer lysates (obtained by treatment of the cultures with 0.1% triton in water) and assayed by the hydrolysis of *p*-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (*p*-nitrophenol) at 405 nm. Hydrolysis was carried out for 30 minutes at 37°C. Results were expressed as nanomoles of p-nitrophenol produced per minute per μg of protein ($nmol \cdot min^{-1}/\mu g$ Protein).

2.3.3. SEM and CLSM microscopy

For SEM/EDS observation (JEOL JSM 6301F, equipped with an X-ray energy dispersive spectroscopy voyager XRMA system, Noran Instruments), cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (10 minutes, pH 7.3), dehydrated in graded alcohols, critical-point dried, and sputter-coated with gold.

For CLSM assessment, fibroblast and osteoblast cell cultures were fixed in 3.7% paraformaldehyde (15 minutes) followed by incubation in albumin ($10\,mg \cdot mL^{-1}$), in order to reduce nonspecific staining. Cell cytoskeleton filamentous actin (F-actin) was visualised treating the cells with Alexa Fluor 488 Phalloidin (1:20 dilution in PBS, 1 hour) and counterstaining with propidium iodide ($1\,\mu g \cdot mL^{-1}$, 10 minutes) for cell nuclei labelling. In addition, osteoblast cell cultures were also labelled with calcein for the visualization of calcium-containing deposits. Cultures were incubated with calcein ($25\,\mu g \cdot mL^{-1}$) for the last 3 hours of each test-point, at 37°C, washed several times in PBS, and fixed in 3.7% paraformaldehyde (15 minutes). Labelled cultures were mounted in Vectashield and examined with a Leica SP2 AOBS (Leica Microsystems) microscopy.

2.4. Statistical analysis

Three experiments were performed for each period of culture evaluation. The results are shown as the arithmetic mean ± the standard deviation (±SD). Analysis of the results was carried out using the nonparametric Kruskall-Wallis test, with a significance level of $P < .05$.

3. RESULTS AND DISCUSSION

3.1. Biological profile of the substrate, Si_3N_4 ceramic

The biological profile of Si_3N_4 ceramics, used as substrate material for the NCD coating, was addressed in previous in vitro and in vivo studies. Human osteoblast-like cells cultured on polished Si_3N_4 ceramics showed enhanced proliferation and osteocalcin production compared to standard polystyrene culture plates [17]. Also, porous intramedullary Si_3N_4 rods implanted in rabbit femurs supported bone ingrowth [18]. In addition, it was shown that a Si_3N_4-bioglass composite stimulated the proliferation of MG-63 osteoblast-like cells and allowed the osteogenic differentiation of human osteoblastic bone marrow cells, with the formation of a mineralised matrix [19]. Considering these previous studies, in the present work, cultures performed on standard polystyrene culture plates were used as control in the biological assessment of the NCD coating.

3.2. Nonspecific cytotoxicity of NCD coating

Results regarding the behaviour of human fibroblast (HG) cells cultured on NCD coatings are shown in Figures 2 and 3, respectively, for viability/proliferation and cell morphology throughout the 14-day culture time.

MTT assay was used to assess cell viability/proliferation (Figure 2), based on mitochondrial function. Mitochondria are vulnerable targets for toxic injury because of their crucial role in maintaining cellular function via aerobic ATP production. HG cells showed a lag phase during the first week, followed by an exponential cell growth. At day 3, values of MTT reduction were similar in control cultures and seeded NCD films, suggesting an identical number of attached cells. Subsequently, cells growing over the NCD samples presented a slightly higher proliferation rate, but differences did not attain statistical significance.

Figures 3(a)–3(c) shows the process of cell adhesion and cytoplasm spreading occurring during the first hours of culture, with the acquisition of a progressively elongated morphology. At 24 hours, cells showed a spindle-shaped morphology, typical of this cell type [23], and cell-to-cell contact (Figure 3(d)). Displaying the typical phenotype morphology is a key requisite for the following cellular events related to cell proliferation and functional activity [25]. Accordingly, cells growing on the NCD coating showed a high proliferation rate, as it was already suggested by the cell viability/proliferation results. In addition, at long incubation time (14 days, Figures 3(d), 3(e)), cultures presented phenotype features, namely, a flattened sheet of continuous cell multilayers displaying a parallel alignment, which is typical of human fibroblastic cell populations

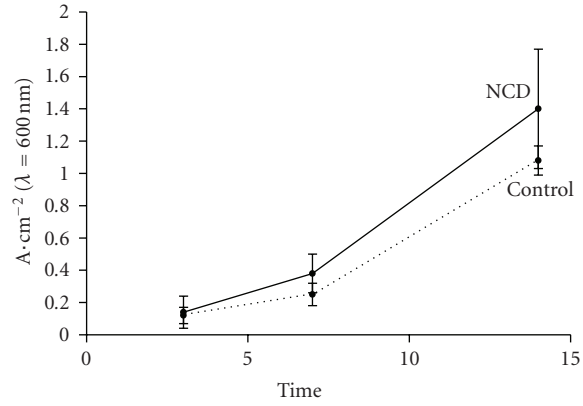


Figure 2: Cell viability/proliferation of human gingival fibroblast cells cultured on NCD-coated Si_3N_4 substrates for 14 days, estimated by the MTT assay. ∗ statistically different from the control.

derived from adult tissues [23, 26]. The morphological events observed during cell adhesion and spreading on NCD coating and the subsequent pattern of cell growth described above were similar to those found in the cultures performed on standard tissue culture plates.

Fibroblast cell cultures established from normal tissues are a useful tool regarding the evaluation of the cytotoxicity profile of medical devices, due to their representative behaviour regarding the fibroblast cell population [27, 28]. Fibroblasts are ubiquitous cells on human tissues that not only function to sustain various organs and tissues as stroma cells but also act directly to regulate adjacent cell behaviour including migration, proliferation, and differentiation [26]. In this way, biological evaluation with fibroblast cell cultures might be regarded as a general bioassay, providing reliable information concerning acute and long-term cytotoxicity or other biological responses [28].

Results showed that the NCD films allowed the adhesion and proliferation of human fibroblast cells with the reproduction of the typical morphological features and cell growth pattern expected for this cell type [23, 26], suggesting a lack of cytotoxicity of the coating. Only few publications report on the biological response to NCD coatings. These include the contact of blood platelet with NCD-coated medical steel [29], the response of osteoblastic SaOs-2 cells to a composite of NCD and amorphous carbon [30] and also to NCD deposited by MWCVD [31], the cell adhesion of human dermal fibroblasts on photochemically functionalized ultrananocrystalline diamond surfaces [32], and the behaviour of several cell lines on ultrananocrystalline diamond [33]. Most of these studies were very specific, while the present work addresses the basal cytotoxicity profile of NCD coatings regarding ubiquitous cells on human tissues, in agreement with the standard ISO guidelines for in vitro cytotoxicity evaluation of medical devices [28].

3.3. Osteoblastic biocompatibility of NCD coating

The clinical success of a bone tissue implant relies on its osseointegration reflected by a proper bone metabolism

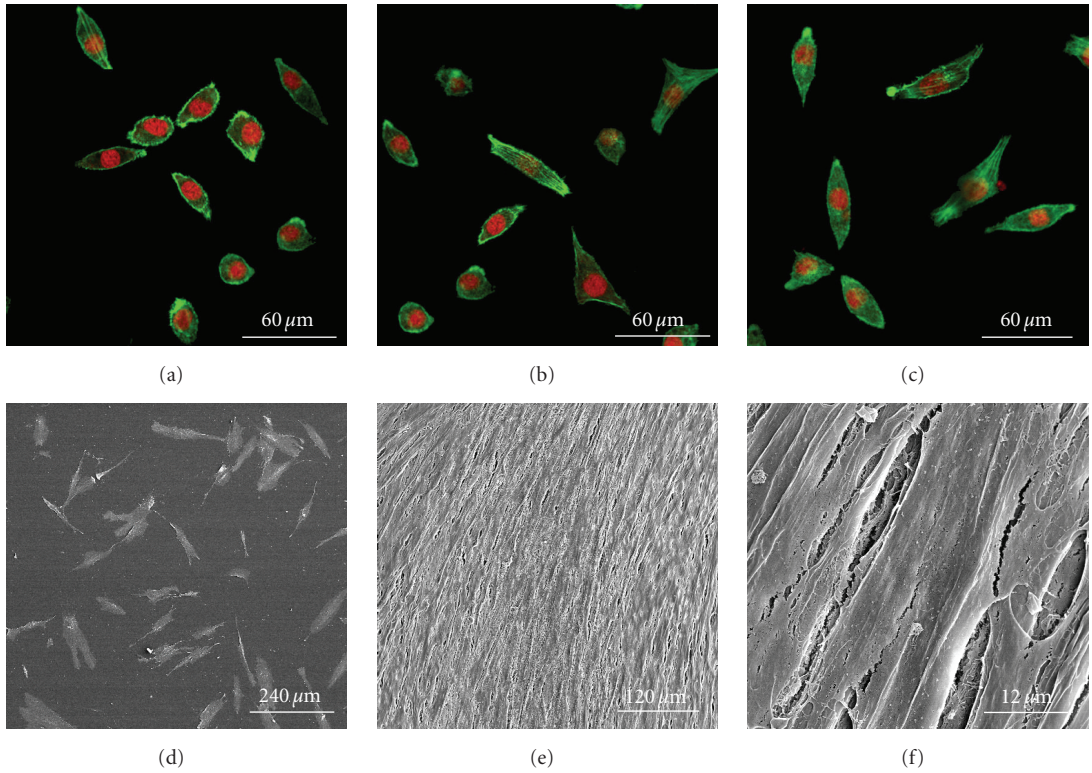


FIGURE 3: Time-course behaviour of human gingival fibroblast cell cultures grown over NCD-coated Si_3N_4 substrates for 14 days. CLSM (phalloidin and propidium iodide labelling): 6 hours (a), 12 hours (b), and 24 hours (c). SEM: 1 day (d) and 14 days (e), (f).

at the bone/implant interface. This represents a dynamic process involving the migration of osteoprogenitor cells from the adjacent bone marrow and the subsequent adhesion to the material surface, and their proliferation and differentiation with the production of a mineralised matrix around the implant material. In this work, NCD coatings were seeded with first-passage human bone marrow (HBM) cells cultured in the presence of ascorbic acid, β-glycerophosphate and dexamethasone for 21 days, conditions that allow the expression of the entire sequence of the osteoblastic differentiation pathway [34, 35]. Results are presented in Figures 4–6.

HBM cells grown in control conditions proliferated during the first two weeks presenting a high cell growth rate during the first week, as evident in the MTT assay (Figure 4(a)). Seeded NCD coating showed a similar pattern, but MTT values were significantly higher at days 7, 14, and 21. Similar results were assessed by the total protein content measurements (Figure 4(b)), which are also an indicator of cell proliferation. HBM cells increased the production of ALP during the first 2 weeks, attaining the maximum level by day 14, and decreasing later (Figure 4(c)). ALP is a membrane-linked polypeptide, synthesized by the osteoblasts, that it is thought to promote crystal formation in matrix vesicles by removing nucleation inhibitors, mediating the calcification process [36]. ALP activity, normalised as a function of the protein content, was higher on the cultures grown on NCD surface, which may suggest that NCD is able to stimulate osteoblast metabolic differentiated activities.

CLSM and SEM (Figures 5 and 6) observations of seeded NCD samples confirmed the favourable profile of the NCD coating suggested by the biochemical assays. After 7–14 days (Figures 5(a), 5(b), 5(d), 5(e)), cells were well spread presenting a homogenous distribution on the NCD surface and, by day 21, mineralised globular structures were observed interspersed within the cell layer (Figures 5(c), 5(f)). At low magnification (Figure 6(a)), an elevated number of those mineralised deposits were present. Mineralised structures were intimately associated with the fibrillar matrix (Figure 6(b)), and the X-ray spectrum from EDS analysis (labelled with an asterisk in Figure 6(b)) proves the inorganic nature of these structures by showing strong Ca and P peaks. This sequence of events was similar to that occurring on standard polystyrene culture plates (Figures 5(g)–5(i)). Quantitative assessment of the relative abundance of mineral structures on the cell layer was not evaluated, but SEM qualitative analysis strongly suggested that mineral deposition density was higher on the NCD coating. This might be related with the presence of increased cell number on the coating (as suggested by the MTT assay), presenting an identical activity regarding matrix mineralization. Also, induction of this phenotype feature by the NCD coating might be hypothesized, taking in account the increased ALP activity measured on the coating. However, the results of this

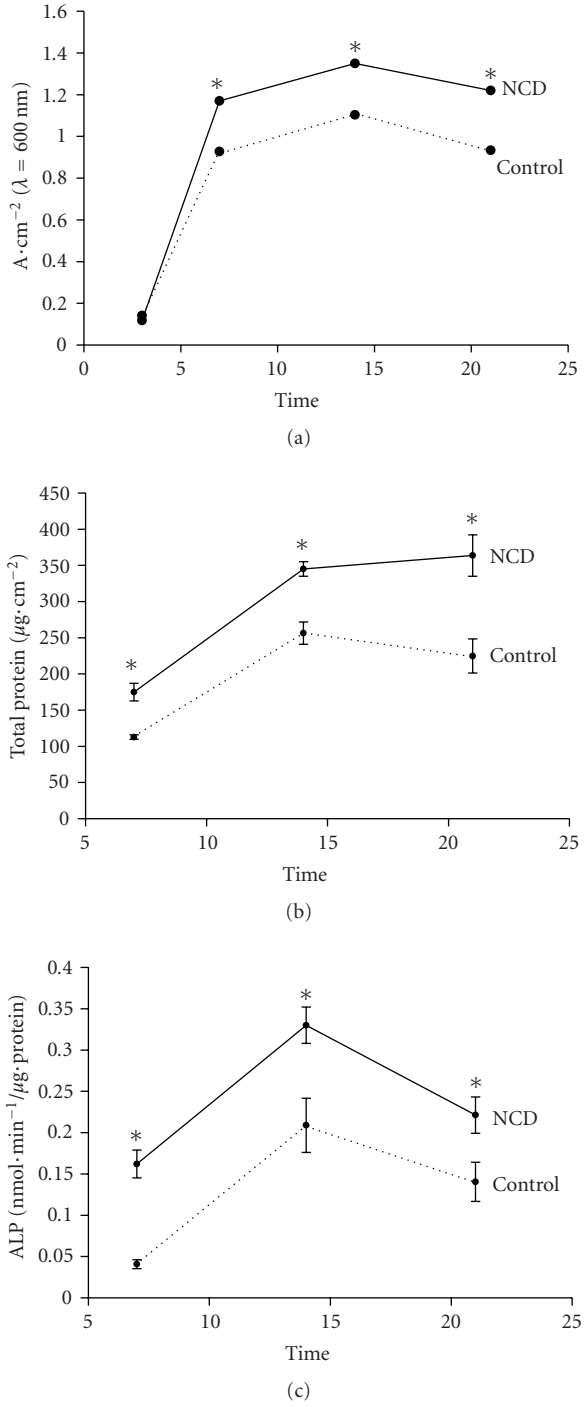


Figure 4: Cell viability/proliferation (a), total protein content (b), and ALP activity (c) of human bone marrow osteoblast cells grown on NCD-coated Si_3N_4 for 21 days. $*$ statistically different from the control.

study do not provide information regarding the subjacent mechanism.

The present observations are in line with recent studies on the in vitro osteoblastic biocompatibility of NCD-coated surfaces that reported favourable results with osteosarcoma SaOS-2 cells. The absence of cytotoxicity of a composite of NCD and amorphous carbon was observed by SEM [30], and NCD deposited by MWCVD showed to provide good conditions for the adhesion and spreading of SaOS-2 cells as well as their viability [31].

According to a variety of studies, osteoblasts recognise differences in nanometer range compared with conventional topographies. Webster et al. found increased functions of osteoblasts on nanophase compared to conventional ceramics, polymers, carbon nanofibers, metals, and composites of these materials [37–40]. Biomolecules such as proteins, nucleic acids, lipids, and carbohydrates possess unique properties determined by the size, folding, and patterns at the nanoscale [41, 42]. Also, hydroxyapatite and collagen type I, the two main components of the bone tissue, present nanofeatures [41, 42] and, in this way, in vivo, osteoblasts interact with surfaces with a large degree of nanometric roughness. Also, the greater surface reactivity of nanophase materials, related to a higher number of atoms at the surface compared to bulk, the greater areas of surface defects and the larger proportions of surface electron delocalizations [43] appear to improve cell performance. The surface properties of nanophase materials have been shown to influence initial protein interactions that control cell adhesion, a determinant event for the subsequent cell proliferation and function [44, 45]. Also, the quantitative increase in the total length of particle boundaries and total number of pores between surface particles of nanophase materials provide a significantly extended surface area for osteoblast proliferation, compared to micron-structured surfaces.

Results obtained under this in vitro study strongly suggest that the prepared NCD coating presented improved features for osteoblast proliferation and function. In vivo experiments in animals, including histological studies and push-out strength measurements, are now imperative for a complete characterization of NCD as a biomaterial.

4. CONCLUSIONS

Hot-filament CVD grown nanocrystalline diamond (NCD) coatings provided a suitable surface for cell attachment, spreading, and proliferation, as assessed by the behaviour of human gingival fibroblast cells in a nonspecific cytotoxicity assessment. In addition, the seeded film allowed the reproduction of the typical morphological features and pattern of cell growth of this system, further suggesting a lack of cytotoxicity. In an osteoblastic biocompatibility assay, NCD coatings elicited an improved human osteoblast proliferation and the stimulation of differentiated markers, like ALP activity and matrix mineralization, compared to standard polystyrene tissue culture plates. These results suggest the potential of NCD as a coating for joint implant applications. The unique mechanical properties and very low surface roughness of NCD support excellent biotribological behaviour at the prosthesis sliding contact. On the other side, by simulating the nanometric features of the bone tissue and by inducing bone growth, NCD coatings are able to play a proper osseointegration role.

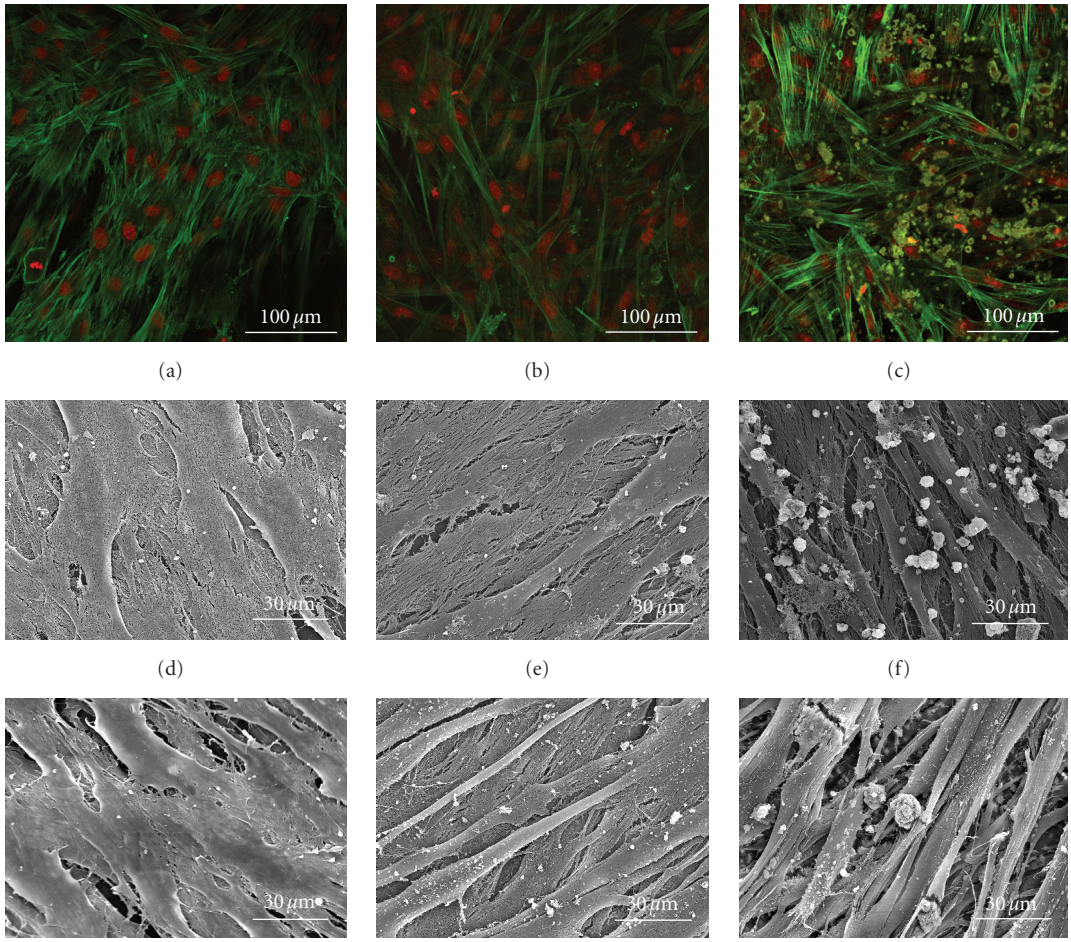


Figure 5: Time-course behaviour of human bone marrow osteoblast cell cultures grown over NCD-coated Si_3N_4 substrates at 7 days ((a), (d)), 14 days (b), (e), and 21 days (c), (f). CLSM: (phalloidin and propidium iodide labelling, (a), (b); plus calcein staining, (c) and SEM (d)–(f) photographs. For comparison, the cell behaviour observed on standard polystyrene culture plates is also shown (SEM): (g), (h), (i), respectively, for 7, 14, and 21 days).

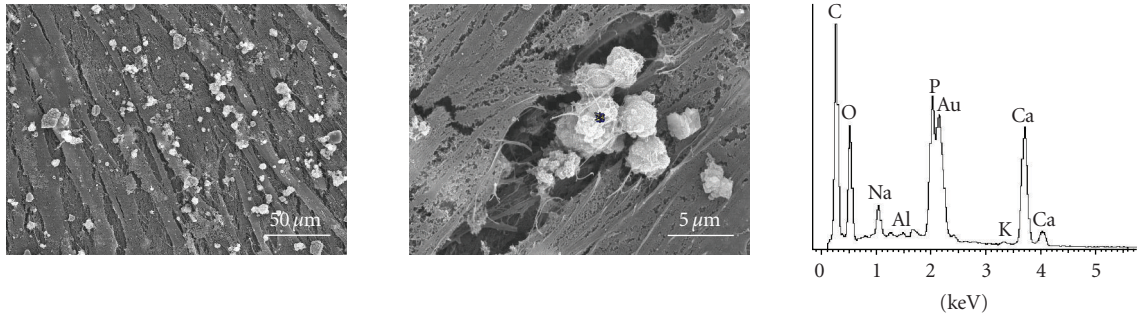


Figure 6: Matrix mineralization by human bone marrow osteoblast cell cultures grown over the NCD films at day 21. SEM: low magnification (a), detail of the mineralised structures (b), EDS spectrum of the mineralised deposits (c).

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