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In Vitro Studies of Osteoblasts Response Onto Zinc Aluminate Ceramic Films

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Zinc based or doped ceramics have shown to be capable of increasing osteoblasts proliferation, biomineralization and bone formation. However, studies regarding the biological applications processes in $ZnAl_2O_4$ ceramic films are very scarce. For this reason, the objective of this in vitro study was to investigate the response of osteoblasts cells cultured onto $ZnAl_2O_4$ films. Our results showed a good biological response related to attachment and viability, with good cell morphology attached to the semi-spherical grains of the ceramic and the analysis of mineral-like tissue showed a high quantity of mineral deposited and organized as tiny spherical-like nodules attached to nanostructure surface of $ZnAl_2O_4$ material films. Based in our results, $ZnAl_2O_4$ films stimulated the bioactivity of osteoblasts cells and provide a microenvironment that favors cell differentiation and mineralization processes, suggesting their potential use as osteoconductive coating onto currently orthopedic and dental implants.

Keywords: *ceramic, zinc aluminate, nanostructure materials, biomineralization, biocompatibility, cell differentiation*

1. Introduction

Ceramics are non-metallic inorganic materials with a broad range of sizes and distributions of grains, porosity and composition, which are used as scaffolds for orthopedic and bone regeneration[1]. Recently, with the progress in the synthesis and characterization of nanostructured ceramics, the applications in bone tissue engineering field has gained much attention for researches who seek to duplicate its enviable mechanical properties at nanoscale level of bone, in which both high strength and fracture toughness can be achieved due to the unique architecture and the way in which it is organized[2,3]. Furthermore, bone tissue engineering has focused on surface topography that is a very important character of the film coating, and it can substantially affect the mechanical, optical, electrical and biological properties. Topography of film coating may be induced by its inherent relaxation, mechanical roughening, chemical patterning and even electric fields. Designing a proper surface topography of the film can control the properties of surface interaction with other materials or cells[4]. For example, in bone tissue engineering the surface topography of the biomaterial scaffolds is a feature that has a decisive influence in osteoblasts cell behavior, ranging from changes in cell adhesion, cell growth, cell proliferation, cell orientation, cytoskeletal condensation and also in cell differentiation[5-8]. These biological responses, of the ceramic implants for bone tissue regeneration, could be up-regulated through optimization of the coating surface properties of the substrates with the use of novel fabrication techniques[9-12].

The spray pyrolysis technique, with ultrasonic generation, is a well established process for depositing films. Spray pyrolysis is relatively simple and probably the least expensive non-vacuum nanofabrication technique suitable for coating or deposition over large areas of nanostructured material from solution-base chemical approaches and it is expected to achieve: a) chemically homogeneous and phase-pure specimens, b) low crystallization and sintering temperatures of the materials, and c) narrow-sized distribution of particles[13]. This deposition technique has been successfully used in the synthesis of zinc aluminate ($ZnAl_2O_4$) films, a well-known spinel ceramic semiconductor with a wide bandgap and also with unique catalytic, mechanical and surface properties when it is produced in the nanometers range[14,15]. This material has been widely used as a high-temperature ceramic material, ultraviolet photoelectronic material, optical and electronic coating; and as catalytic material in chemical and petrochemical industries[16,17]. Furthermore, the coating synthesis, deposition parameters, microstructure, surface morphology and optical luminescent properties of $ZnAl_2O_4$ ceramic films had been previously studied[18-20].

On the other hand, zinc is known to be an essential element in bone formation and mineralization[21,22]. It is a constituent of the enzyme alkaline phosphatase (ALP), which is involved in the mineralization of bone matrix, and is also required for a number of metabolic functions[23]. Zinc has also been determined as a useful antibacterial agent in glass-ionomer-based cements[24] and ceramic coatings[25]. Thus the addition of zinc to various materials and their use in bone tissue engineering may have important implications for the appropriate integration to implant sites with minimal bone infec-

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tion risk, which is a complication often associated with the repair of skeletal defects. In addition, zinc based or doped ceramics are believed to be nontoxic, biocompatible and they have shown to be capable of increasing osteoblasts proliferation, stimulated bone cell differentiation and manifest stimulatory effects on bone formation both in vitro and in vivo, when zinc was incorporated into implanted material[26-29]. Biological applications of $ZnAl_2O_4$ ceramic films has been evaluated previously, based on cell culture tests using a human gingival fibroblasts transfected with CEMP1 gene, which let the cell adhesion maintenance and increasing the expression of bone-related molecules with a good mineralization process onto zinc aluminate nanostructured films[30]. Based on all previous results, it would be of great interest to understand new mechanisms to produce nontoxic and biocompatible zinc based ceramics materials, because almost there is not information about the role of zinc aluminate on the osteoblasts behavior. For this reason, the aim of the present contribution is a further and detailed study to determine the biological activity of human osteoblasts cells in order to evaluate the cellular adhesion, spreading and cell viability as well as its potential to promote mineralization at in vitro cell culture onto zinc aluminate ceramic film, produced by spray pyrolysis.

2. Materials and Methods

Zinc aluminate ($ZnAl_2O_4$) films were deposited by the ultrasonic spray pyrolysis technique, generated from a solution consisted of Zinc acetate [$Zn(CH_3COO)_2 \cdot 2H_2O$] (0.05 M) and Aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) (0.025 M). The mist generated was deposited on corning glass slice pieces at a flow rate of 3 mL/min with a substrate temperature of 550 °C. After this, the material was characterized for crystalline phase by X-ray diffraction (XRD) using a Siemens D-5000 diffractometer with wavelength radiation of 1.5406 Å (Cu K_α); for chemical composition by EDS with a Cambridge-Leica electron microscope Stereoscan 440, equipped with a Beryllium window X-ray detector and surface morphology analyzed by atomic force microscopy using a Jeol JSPM-421 in AC mode with a silicon cantilever CSC15/25 Ultrasharp.

For cell culture, osteoblasts cells were cultured in 75 cm^3 cell culture flasks containing a DMEM media, supplemented with 10% of fetal bovine serum (FBS), and an antibiotic solution (streptomycin 100 $\mu g.mL^{-1}$ and penicillin 100 $U.mL^{-1}$, Sigma Chem. Co). The osteoblasts cells were incubated in a 100 % humidified environment at 37 °C in an atmosphere of 95% air and 5% CO_2. For the biomineralization assay cells were culture in "mineralizing media" (10% FBS, 10 mM β-glycerophosphate and 50 $\mu g.mL^{-1}$ of freshly prepared ascorbic acid). Osteoblasts cells at the 2nd passage were used for all the experimental procedures.

The in vitro cell adhesion of osteoblasts cells cultured onto $ZnAl_2O_4$ films was carried at individual wells of 12 well culture plates at initial concentration of 1.5×10^3 cells and incubated for 12 and 24 hours in DMEM culture medium at 37 °C. After prescribed time, the substrates were rinsed with phosphate buffered saline (PBS) to remove non-adherent cells. The adherent cells were fixed with 4% paraformaldehyde for 10 minutes and fixed cells were incubated with 0.1% Toluidine Blue for 3 hours. The dye was extracted with sodium dodecyl sulfate (SDS) and the optical absorption was read with enzyme linked immunosorbance assay (ELISA) at 630 nm. Experiments were performed in triplicate (n = 3) and corning 7059 glass slides pieces were used as a control.

For cytoskeletal organization of the osteoblasts cells cultured onto $ZnAl_2O_4$ films, the cells were seeded at a concentration of 1×10^3 cells and incubated for 24 hours in DMEM cultured medium. After 24 hours the samples were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT), permabilized with 0.2% Triton X-100 for 5 minutes, washed twice with PBS and incubated with α-actin antibody diluted 1:100 in 0.2% of bovine serum albumine (BSA)-PBS for 1 h at RT. The cells were then gentile washed twice with 0.2% BSA-PBS and twice with PBS. Then, cells were incubated with FITC secondary antibody diluted 1:1000 in PBS for 1 hour. The cells were gentile washed with PBS and visualized by means of indirect immunofluorescence (Axiophot, Carl Zeiss®, Germany). Slides lacking first antibody were used as negative controls.

For cell viability of osteoblasts cells cultured onto $ZnAl_2O_4$ films we used the MTT assay based on the ability of mitochondrial dehydrogenases to oxide 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 2×10^4 per well in triplicate and were incubated for 3, 5 and 7 days of culture. After each term, 10 µL of MTT was added and incubated for 3 hours. Then, the supernatant was removed and 500 µL of dimethyl sulfoxide (DMSO) was added to each well. After 60 minutes of slow shaking the absorbance was read at 570 nm.

For in vitro mineralization assays, osteoblasts were plated at high density (2×10^5) onto $ZnAl_2O_4$ films and cells were cultured for 5 days. The composition of the mineral-like tissue in the extracellular matrix formed by osteoblasts onto $ZnAl_2O_4$ films and the morphology of the mineral deposited by osteoblasts were analyzed by means of energy dispersive spectroscopy (EDS) and scanning electron microscopy (SEM). For these purposes, after 5 days of culture in DMEM "mineralizing media", the cultures were fixed with 70% ethanol, air-dried and surfaces of the cultures were covered with a thin gold film of about 100 nm thick to avoid electron disturbances that could affect microanalysis. Cultures were analyzed by means of a Leica-Cambridge 440 scanning electron microscope (Leica® Microsystems Inc., Bannockburn, IL USA), fitted with a Pentafet energy dispersive X-ray micro-analysis microprobe. All analyses were carried out at 20 kV for 300 seconds.

Statistics for experimental assay were performed with the Student's *t*-test, using Sigma Stat V 2.0 software (Jandel Scientific). Results of $p < 0.05$ values were considered significant to test $ZnAl_2O_4$ material films against the control.

3. Results and Discussion

Figure 1 shows the XRD pattern of a typical $ZnAl_2O_4$ film deposited on corning-glass 7059 at Ts = 550 °C. Considerable peaks

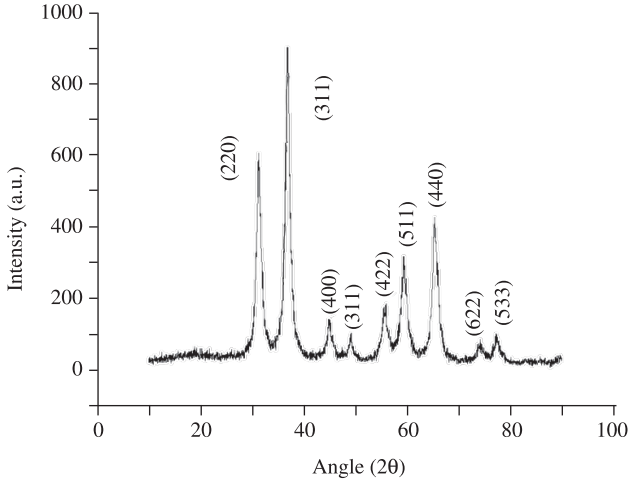


Figure 1. XRD pattern of $ZnAl_2O_4$ films which correspond to the cubic spinel phase. The peaks broadening are due to the nanostructured nature of the grains.

broadening due to the nanometric dimension of the grains can be observed. The diffraction peaks show a crystalline material which was identified as pure phase $ZnAl_2O_4$ with cubic structure. The calculated lattice parameter a = 0.8086 nm, is in good agreement with the reported value a = 0.8084 nm, for cubic spinel-gahnite $ZnAl_2O_4$ (ICCD Card File Nº. 5-669)[31]. Using the Debye–Scherrer formula for the broadening fitting curve XRD program, the particle size was evaluated. The average particle diameter was around 20-30 nm, considering that the grains are spheres. Taping mode AFM measurement on the same film is shown in Figure 2. The $ZnAl_2O_4$ nanoparticles appear semi-spherical in shape and they are uniformly distributed in the whole deposition region. In Figure 2(a) is shown an AFM close view of the image which depict crystallites of nearly uniform size of about 30-85 nm, with porous of nanometric dimension of around 50-100 nm. This result indicate that XRD pattern peaks broadening is due to the smallest nanometric crystallites and that probably the semispherical particles are constituted by several small crystallites in the 20-30 nm dimension. Furthermore, the boundaries between nanoparticles are well defined as observed in the 3D image in Figure 2(c). The experimental chemical composition of the same film was determined by EDS. Results indicate a chemical composition of: 56.5 at.% of oxygen; 13.6 at.% of zinc; 27.8 at.% of aluminum and 2.1 at.% of chlorine, which is compared with the theoretical composition: 56 at.% of oxygen; 14 at.% of zinc and 28 at.% of aluminum (Figure 7a). The result of this comparison indicates a stoichiometric compound of $ZnAl_2O_4$ doped with chlorine[18-20].

In order to evaluated the biological response of the osteoblasts cells, in vitro methods were used to provide information about biocompatibility of $ZnAl_2O_4$ films.

In this study we evaluated two different stages; in the first one, we studied the adhesion properties of the material surface morphology and the second stage let to detect any toxic effects of the $ZnAl_2O_4$ films surface. Figure 3 shows the evaluation of the cellular adhesion of osteoblasts cells after an incubation period of 12 and 24 hours as the first step to asses the compatibility of the material surface morphology. The results are presented as the cellular percentage of attached cells in relation to control cultures (Corning 7059 glass). The adhesion of osteoblasts cells can be seen to be favored exceeding 100% of attachment and had a statistical difference between $ZnAl_2O_4$ surface films and control corning 7059 surface glass at $p < 0.05$. Although,

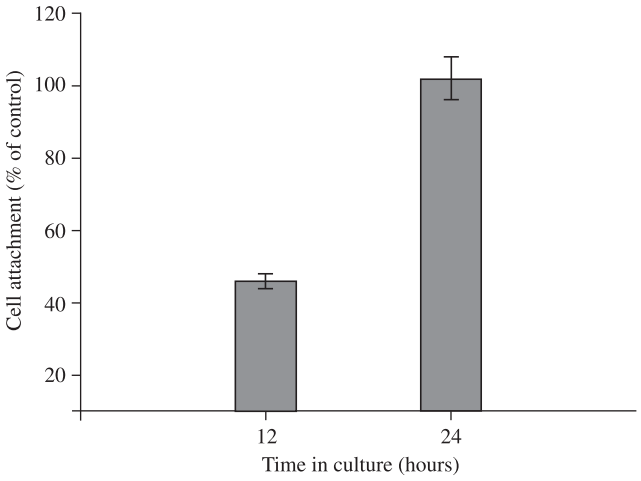


Figure 3. Adhesion of osteoblasts cells cultured onto $ZnAl_2O_4$ film after an incubation period of 12 and 24 hours expressed as the percentage of attached cells in comparison with control culture.

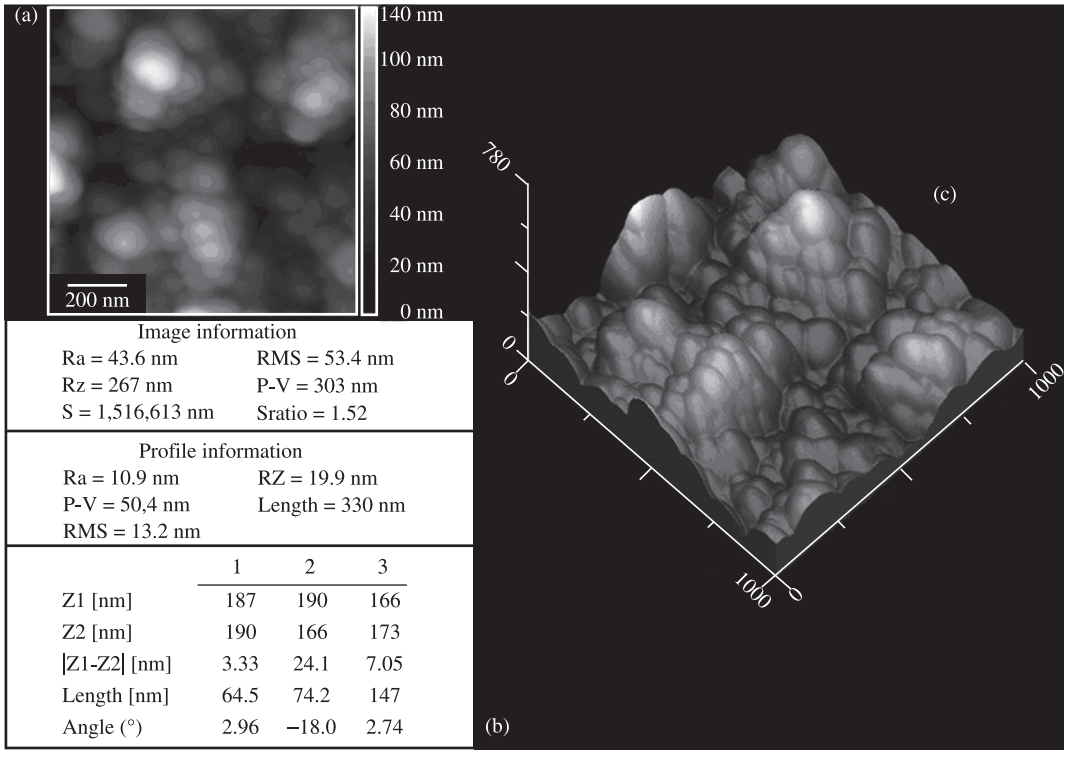


Figure 2. Atomic force microscopy images of the semi-spherical morphology of the surface topography of zinc aluminate ceramic film (a). Analysis measurement of the semi-spherical morphology of zinc aluminate ceramic films (b). A three-dimensional image features of the surface topography of zinc aluminate ceramic film (c).

it is important to remark that increased cellular attachment obtained in the $ZnAl_2O_4$ films is a good indicator that the surface is not toxic to the cells. We perform the cell viability test assessed by the MTT assay to confirm it. The results of the MTT assay are presented as the optical absorbance at 570 nm as shown in Figure 4. We found high levels of MTT conversion, comparing it to the control on day 3 and continue until day 7. This increment is directly proportional to the increase of metabolic active cells on the surface of $ZnAl_2O_4$ films and inversely proportional to the toxicity effect of the surface topography of the material. Not statistical significances in MTT activity were determined between osteoblasts culture onto $ZnAl_2O_4$ films and osteoblasts culture in corning 7059 glass substrate control at 3 days of culture. However, statistical difference were found between the viability of osteoblasts culture onto $ZnAl_2O_4$ films and osteoblasts culture in corning 7059 glass substrate at 5 and 7 days at $p < 0.05$. This increase in adhesion and MTT activity of osteoblasts cells could be favored for the semi-spherical nanophase of the zinc aluminate material. The semi-spherical nanophase as showed by the images of the topography by AFM has a grain size of around 30-100 nm and it is clear that the cell functionality and biocompatibility of the osteoblasts cells to $ZnAl_2O_4$ films will be influenced by the semi-spheroid mor-

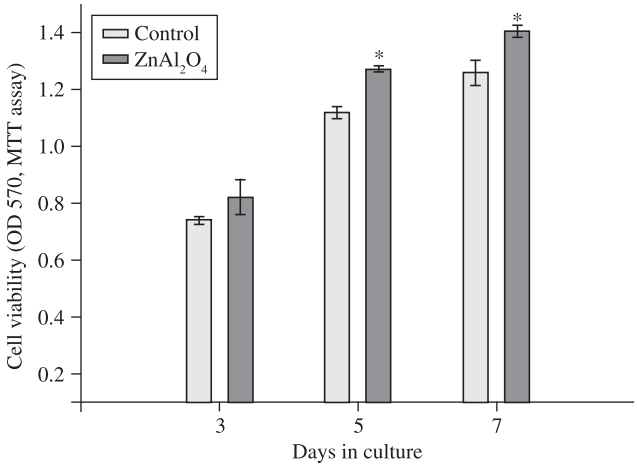


Figure 4. Cell viability of osteoblasts cells (MTT test) expressed as the absorbance at 570 nm for control corning glass (□) and zinc aluminate ceramic film (▯) cultures. Asterisks indicate statistical significance ($p < 0.05$).

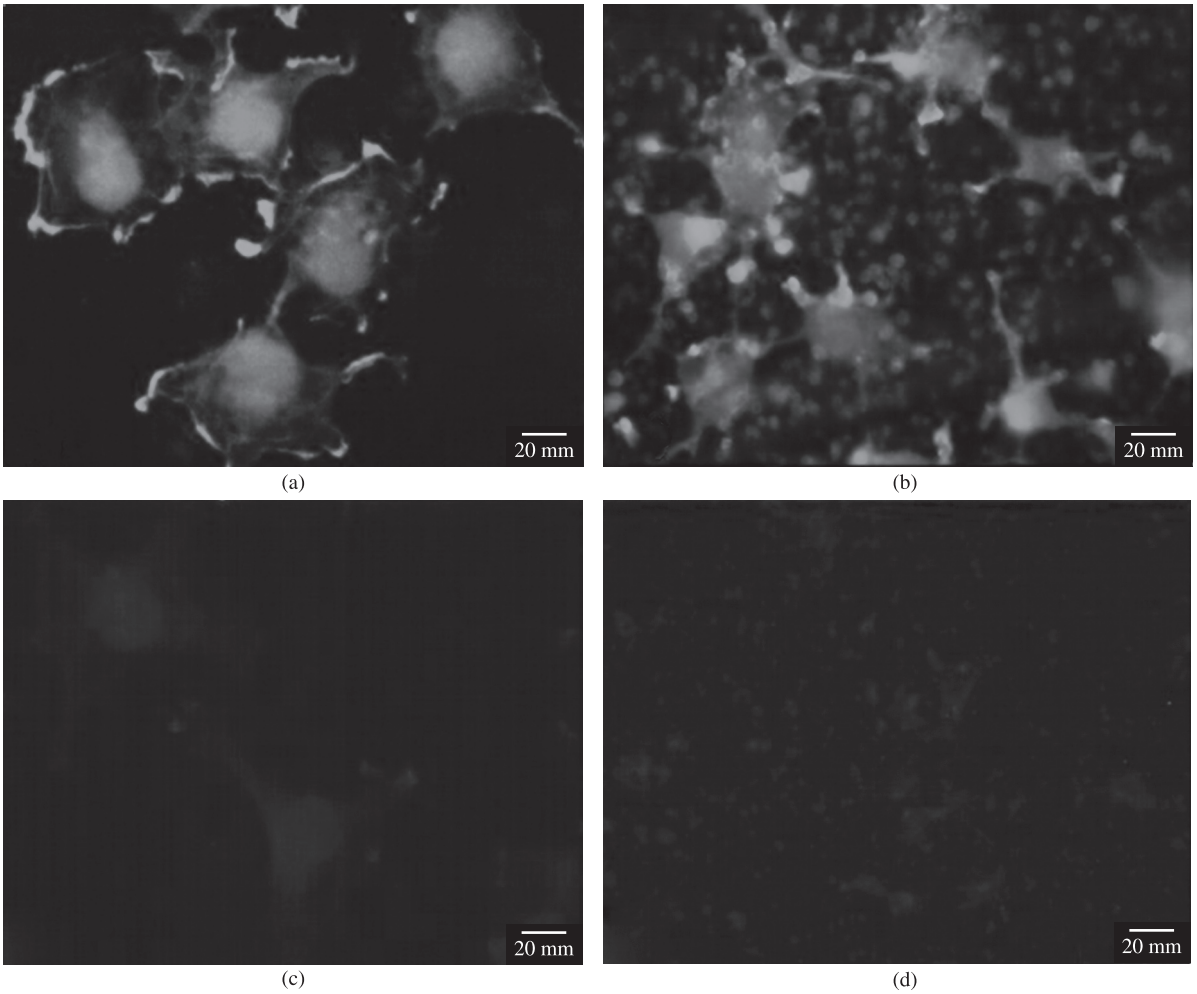
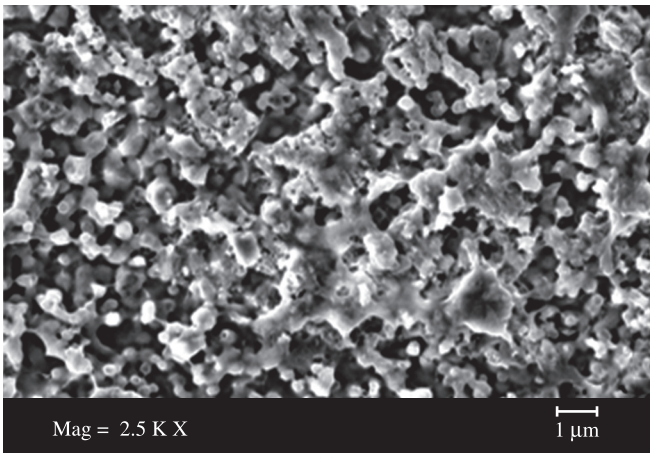


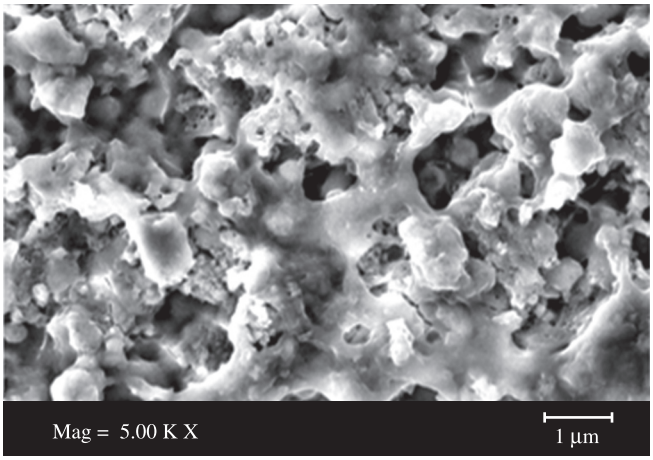
Figure 5. Optical images of cytoskeletal immunofluorescence of the osteoblasts cells cultured onto control corning glass (a, c) and onto $ZnAl_2O_4$ films (b, d). On control cultures the osteoblasts showing a circular and lenticular morphology of the cytoskeleton (a) and on experimental cultures osteoblasts cells showing a flattened and spreaded cell morphology indicated that cell cytoskeleton is oriented along the grooves of the nanostructure ceramic films (b). Negative controls of the immunofluorescence assay for both control (c) and experimental culture (d).

phology of the surface features. Similar results have been reported for enhance adhesion and biological response to nanostructure materials and had indicated that grain size plays a crucial role in mediating on the cell adhesion to nanophase ceramics and also had been reported that spheroids material surface should be more beneficial to proliferation of osteoblastic cells[32-36]. Finally, Figure 5 shows the morphology of osteoblasts cells cultured onto $ZnAl_2O_4$ films. The images show a well attached cell to the semi-spherical nanostructure substratum covering long extensions of area with very spreading, elongated and lamellae morphology. This morphology demonstrated a good state of the membrane that extend from the cell and try to attach to more nanospherical substratum with a preferential orientation of the elongated and flattening with the extensions appearance of the cytoskeleton (Figure 5b). Our results showed that the topography of semi-spheroid particle of $ZnAl_2O_4$ has a significant influence on cell morphology. These results are supported by previous works where it has been reported that the distance between particles plays a crucial role in the response of cells to nanomaterials[37] and the preferential orientation is a well known phenomena called contact guidance that refers to the tendency of cells to be guided in the direction of the substrate shape[38,39].

Cell differentiation of the osteoblasts cultured onto $ZnAl_2O_4$ films were evaluated by its potential in promoting mineralization. Scanning electron microscopy images showed a sequence of morphological disposition of the mineral-like tissue deposited by osteoblasts cells at 5 days of culture. The micrograph showed that osteoblasts form a tiny small spherical structures or agglomerates of mineral deposit. These mineral deposits were seen interspersed among osteoblasts cell layer as tiny small globular morphology, well organized as a tiny spherical-like nodules attached to nanostructure surface of $ZnAl_2O_4$ films (Figure 6). X-ray microanalysis of the mineral-like tissue deposited by osteoblasts cells showed the presence of calcium (56.1 at.% of Ca) and phosphorus (34.2 at.% of P). The Ca/P composition ratio of the mineral-like tissue was 1.63 which corresponds well with the biological hydroxyapatite value (Figure 7b). These



(a)



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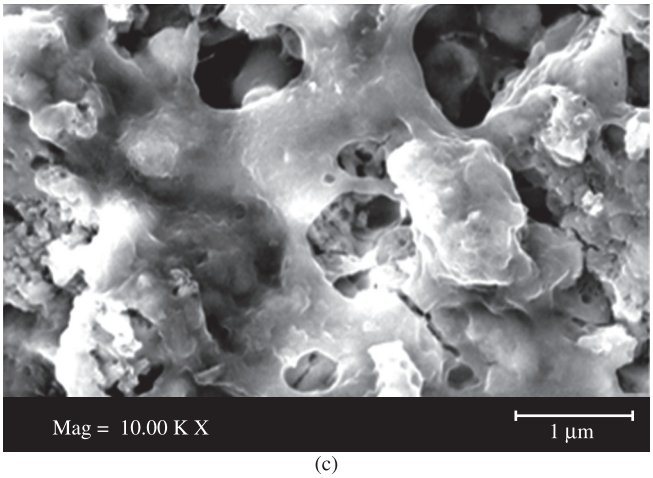
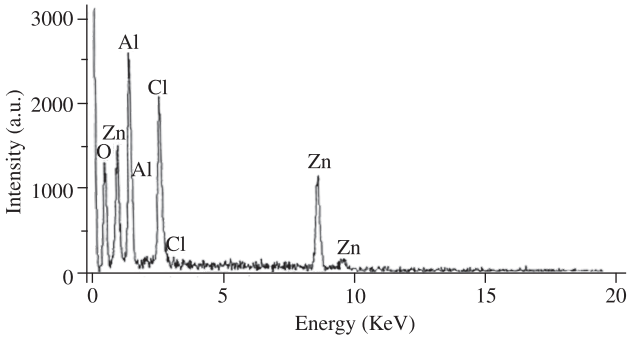
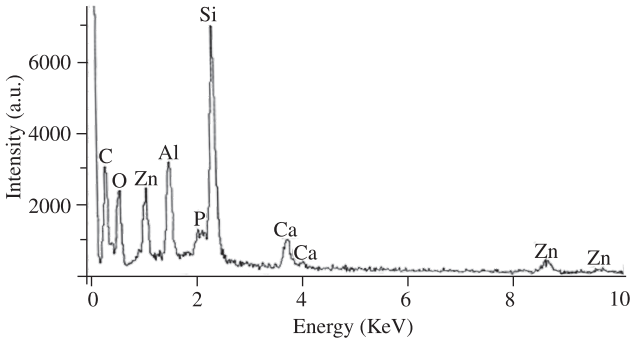


(c)

Figure 6. Scanning electron micrographs at different magnifications of the mineral-like tissue deposited by the osteoblasts cells cultured onto $ZnAl_2O_4$ films after 5 days of culture. It is revealed a higher amount of nanospherical globular-like structures of the mineral-like tissue.



(a)



(b)

Figure 7. (a) EDS spectrum that shows the compositional energy peaks of zinc, aluminum and oxygen of the $ZnAl_2O_4$ films and (b) the compositional spectrum energy peaks of calcium and phosphorus elements of the mineral-like tissue deposited by osteoblasts cells cultured onto $ZnAl_2O_4$ films after 5 days of culture.

results show that mineralization was not affected by the $ZnAl_2O_4$ composition of the films and the mineral deposition enhance was in concordance with previous studies of zinc-containing biomaterials that had shown an osteoconductive material for bone-related tissue engineering applications[40-43].

4. Conclusions

In this study we investigated the biological response of osteoblasts onto zinc aluminate ceramic films and our results did not show cytotoxicity effects to osteoblasts cells, because they exhibited good cell functionality as cellular adhesion, cellular viability and mineralization in the period investigated. Based in our results we concluded that $ZnAl_2O_4$ films provides a microenvironment with an increased bioactivity, cell differentiation and holds a future potential use in biomedical implants. However, further studies may be focused on the effects of the roughness topography of $ZnAl_2O_4$ nanostructured material in the cytoskeleton response of osteoblasts cells.

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