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Biocompatibility of Zinc Aluminate Nanostructured Material

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Abstract. We have used zinc aluminate nanostructured films deposited by spray pyrolysis to determine its biocompatibility assessed by cells attachment and cell differentiation. Cell attachment onto zinc aluminate showed an increase of 53, 81 and 86% at 180, 300 and 420 minutes ($p<0.05$) when compared to controls. Mineralization was analyzed at 5 and 14 days of culture by scanning electron microscopy, microanalysis and atomic force microscopy. Our results showed in experimental culture a higher density of mineral-like tissue with small needle-shaped crystal and granular nanoparticles with preferential orientation when compared to controls. The composition of the mineral-like tissue deposited in zinc aluminate nanostructured material had a Ca/P ratio of 1.6, whereas control culture had a Ca/P ratio of 1.50. Our finding revealed that $ZnAl_2O_4$ promoted higher expression of type I collagen, bone sialoprotein, osteocalcin and alkaline phosphatase, suggesting that zinc aluminate provides a microenvironment that favors mineral formation and cell differentiation. Our results point to the potential use of $ZnAl_2O_4$ for the osteoinductive process in biomedical implants.

Introduction

Nanoscale materials, also called nanomaterials, are commonly defined as those materials with very small components and/or structural features (such as particles, fibers, and/or grains) with at least one dimension in the range of 1–100 nm. Nanomaterials can be metals, ceramics, polymers, or composite materials which demonstrate novel properties compared to conventional materials due to their nanoscale features [1]. Recent progress in the synthesis and characterization of nanostructured materials (such as luminescence, electrical, mechanical and optical properties) has gained much attention for their wide variety of potential technological applications [2]. However, the potential for biological applications in particular for biomedical implant is scarce because the materials used for orthopedic and dental applications have almost exclusively been conventional metal alloys and ceramics with grain sizes greater than 100 nanometers [3-5]. The major goal for nanoscale materials application is to determine bone cell functions on nanophase ceramic or polymer composites with grain sizes less than 100 nm. Natural bone is now considered as a nanophase material, composed of approximately 30% of organic components (mainly type I collagen and non-collagen proteins -bone sialoprotein, osteocalcin and alkaline phosphatase-) and 65% of hydroxyapatite crystals as inorganic ceramic components.

To date, only few attempts to explore the bone cell responses on composites of poly (latide-co-glycolide) acid combined separately with nanophase ceramic of alumina, titania, and hydroxyapatite (30/70 ceramic/polymer weight ratio) have been conducted and partially understood [6]. Recently, in vitro and in vivo studies have demonstrated biological sensitivity to micro- and nanoporosity of ceramics and showed that the process of cell adhesion and differentiation of mineralizing cells depends on various parameters: the topography, the chemistry, or the composition of the material [7-11]. To acquire these characteristics to the material, novel nanofabrication techniques are opening up the possibilities for mimicking the inherently nano-world of the cell, i.e the nanotopography or nanochemistry [12]. Spray pyrolysis technique, with ultrasonic generation, is relatively simple and probably the least expensive non-vacuum nanofabrication technique suitable for deposition over large areas of nanophase material from solution-base chemical approaches and it 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 process has been successfully used in the synthesis of zinc aluminate ($ZnAl_2O_4$) films. Zinc aluminate is a well-known wide bandgap semiconductor with a spinel structure and also with unique catalytic, mechanical and surface properties in the range of nanometers [14, 15]. This material has been widely used as ceramic, electronic and catalytic material, in chemical and petrochemical industries [16, 17]. However, regarding biological applications, nanophase ceramic material properties of $ZnAl_2O_4$ films are very scarce.

The present study analyzed a nanostructured ceramic formulation of zinc aluminate, deposited onto corning glass 7059 by spray pyrolysis and sintered at low temperature to determine its biocompatibility and to examine its potential to promote mineralization and gene expression of bone-related molecules.

Materials and Methods

Deposition process of $ZnAl_2O_4$ nanostructured thin films

Films of zinc aluminate were deposited by ultrasonic spray pyrolysis technique described earlier [18]. Briefly, this technique consists of an ultrasonic generator used to produce a mist from the spray solution. This mist is carried to a host substrate placed on a tin bath trough a tubing setup using humid air as a carried gas (10 liters/minute). When the mist of the solution gets in touch with the hot substrate, the solvent in the solution is vaporized producing a solid coating onto the substrate. The nozzle in the system is localized approximately 1 cm above the substrate. The spraying solution consisted of 0.05M zinc acetate and aluminum chloride in de-ionized water as solvent. The solution flow rate was 3 ml/minute. The substrate temperature (Ts) during deposition was 550°C; the substrate used was Corning 7059 glass slides pieces. The deposition time was adjusted to 3 minutes to obtain films with the approximately the same thickness (~ 3.0 ± 0.03 μm).

Films of $ZnAl_2O_4$ nanostructured material characterization

The nanostructured material of interest in the present study was characterized for crystalline phase, chemistry and surface morphology according to standard techniques. The crystalline structured features of the deposited films were analyzed by X-ray diffractions (XRD) using a Siemens D-500 diffractometer with wavelength radiation of 1.5406 Å (Cu K_α). The chemical composition of the films was measured with a Cambridge-Leica electron microscope Stereoscan 440, equipped with a Beryllium window X-ray detector, using Energy Dispersion Spectroscopy (EDS). The standard used for EDS measurements was the Multi-element X-ray Reference Standard (Microspec), Serial 0034, part No. 8160-53. The surface morphology was analyzed by means of scanning electron

microscopy (SEM) cited above and by atomic force microscopy (AFM) using a Jeol JSPM-421 in AC mode with silicon cantilever CSC15/25 Ultrasharp.

Cell culture

In this study we have used human gingival fibroblast cells transfected with CEMP1 gene (HGF-CEMP1) as reported elsewhere [19]. The HGF-CEMP1 cells were cultured with DMEM media, supplemented with 10% fetal bovine serum (FBS), and antibiotic solution (streptomycin 100 μg/ml and penicillin 100U/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. HGF-CEMP1 at the 2nd passage was used for all the experimental procedures. The cells were grown in a medium with 10% FBS (cell adhesion) or in mineralizing media (10% FBS, 10 mM β-glycerophosphate and 50 μg/mL of freshly prepared ascorbic acid).

Cell adhesion

As first attempt to investigate how HGF-CEMP1 cells would interact with the $ZnAl_2O_4$ nanostructured material surface, adhesion assays were performed. For this purposed, HGF-CEMP1 were seeded ($1x10^4$ cells/cm^2) onto the $ZnAl_2O_4$ nanostructured material in DMEM supplemented with 10% FBS and were allowed to adhere in standard cell culture for 15, 30, 60, 180, 300 and 420 minutes. After the prescribed time period, substrates were rinsed three times using phosphate buffered saline (PBS) to remove non-adherent cells. The adherent cells were fixed with 3.5% paraformaldehyde. Evaluation of cell attachment was performed according to Hayman [20] with some modification. Briefly, fixed cells were incubated with 0.1% Toluidine Blue for 3 h. The dye was extracted with sodium dodecyl sulfate (SDS) and the optical absorption read with enzyme linked immunosorbance assay (ELISA) at 605 nm. Experiments were performed in triplicate (n=3). Corning 7059 glass slides pieces were used as a control.

Biomineralization assay

To investigate if $ZnAl_2O_4$ nanostructured material surface promotes HGF-CEMP1 mineralization, cells were plated at a density of $2x10^4$/cm^2 and left to adhere overnight. The next day media was changed to a mineralizing media. The media was changed every 2 or 3 days and cells were cultured for 5 and 14 days. At each term cells were fixed in 96% ethanol for 10 min, and mineral deposition was identifying by using a saturated solution of Alizarin red S pH 4.2 (Sigma Chemical Co., St. Louis, MO) added to the cell cultures and stained for 5 min. Residual staining was removed with PBS and the presence of nodules was documented by light microscopy. To determine the morphology and homogeneity of the mineral-like tissue deposited onto $ZnAl_2O_4$ nanostructured material we used an AFM, Jeol JSPM-421 in AC mode with silicon cantilever CSC15/25 Ultrasharp as reported elsewhere [21]. Evaluation of biomineralization and compositional examination of the mineral was performed by SEM. Cell cultures were fixed with 4% formaldehyde in 0.1 M phosphate buffer solution, and then dehydrated in graded of ethanol, sputter-coated with gold and analyzed in SEM equipped with X-ray microanalysis capability [22]. Corning 7059 glass slides pieces were used as a control for biomineralization assay.

Gene expression

To investigate if $ZnAl_2O_4$ nanostructured material promoted HGF-CEMP1 cell differentiation and expression of bone-related molecules, HGF-CEMP1 cells were plated at a density of $5x10^4$/cm^2 and cultured in the conditions described above. Gene expression was assessed after 14 days of culture. Total RNA was isolated using RNAeasy Mini Kit (Quiagen, Valencia CA, USA) as previously

described [23]. One μg of total RNA was used to perform one-step RT-PCR (Invitrogen Carlsbad, CA, USA) according to the manufacturer's protocol during 35 cycles in a thermal cycler (MJ Research, Watertown, MA, USA). Previously published primer sequences and reaction conditions were used for amplification of the following molecules: alkaline phosphatase (ALP), osteocalcin (OCN), collagen 1 (COL I) and bone sialoprotein (BSP), the GAPDH was used as internal control under the same conditions [23]. Corning 7059 glass slides pieces substrates were used as a control.

Statistical analysis

Statistics for adhesion assay was 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 nanomaterial against the control.

Results and Discussion

Characterization of $ZnAl_2O_4$

In Fig. 1, it is shown the XRD pattern of the $ZnAl_2O_4$ sample, where a considerable peaks broadening due to the nanometric dimension of the grains can be observed. The diffraction peaks show that cubic $ZnAl_2O_4$ spinel-gahnite is the only crystalline phase present. The calculated lattice parameter: a= 0.8086 nm, is in good agreement with the reported value: a= 0.8084 nm, for cubic spinel $ZnAl_2O_4$ (ICCD Card File N°. 5-669) [24].

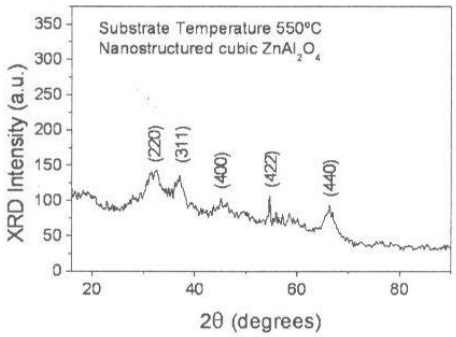


Figure 1. XRD pattern of $ZnAl_2O_4$ which correspond with the cubic spinel phase. The peaks broadening are indicative of nanostructured grain size.

Using the Debye-Scherrer formula for the line broadening fitting curve XRD program, the particle size was evaluated. The average particle diameter was around 20-30 nm, calculated by considering that the grains were spheres.

Fig. 2 (A) shows the SEM surface morphology of the coating. It is possible to observe grapefruit-like grains with nanometric dimension between 25 to 80 nm. This result indicate that XRD pattern peaks broadening is due to the smallest nanometric crystallites and that probably the semispheric agglomerates are constituted by several small crystallites in the 20-30 nm dimension. In Fig. 2 (B) is shown an AFM close view surface image of one of the agglomerates that shows crystallites of nearly uniform size of about 30-50 nm, with porous of nanometric dimension (around 50-100 nm), testing that the $ZnAl_2O_4$ layer is nanostructured in nature. The experimental chemical composition was determined by EDS technique. The experimental results indicate a composition of : 56.49 at.% of oxygen; 13.57 at.% of zinc; 27.83 at.% of aluminum and 2.11 at.% of chlorine, which is compared with the theoretic composition: 56 at.% of oxygen; 14 at.% of zinc and 28 at.% of aluminum. The result of this comparison indicates a stoichiometric compound of $ZnAl_2O_4$ doped with chlorine [25-27].

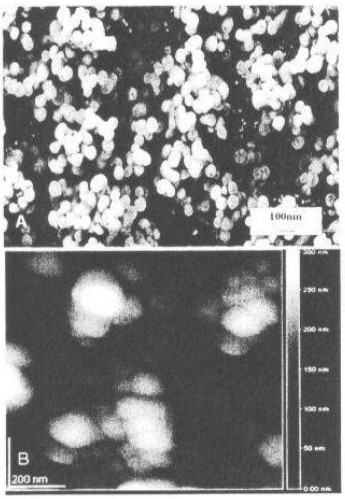


Figure 2. Scanning electron microscopy and Atomic force microscopy images of $ZnAl_2O_4$ nanostructured material. (A) Grapefruit-like nanostructured grains morphology determined by SEM and (B) agglomerate morphology of semi-spherical nanocrystallites of nearly uniform size with nanoporous regions determined by AFM.

Biocompatibility response

In order to evaluate the biocompatibility of the $ZnAl_2O_4$ nanostructured material we used an *in vitro* cell culture system to investigated cell attachment and cell differentiation. As shown in Fig. 3, the adhesion of HGF-CEMP1 cells with the $ZnAl_2O_4$ nanostructured material surface was measured at 15, 30, 60, 180, 300 and 420 minutes after plating. The number of HGF-CEMP1 cells attaching to $ZnAl_2O_4$ nanostructured material surface showed an increase of 53, 81 and 86% at 180, 300 and 420 min ($p<0.05$) when compared to controls. No statistically significant differences in HGF-CEMP1 adhesion were observed between nanostructured material and corning glass at 15, 30 and 60 minutes of time. However, this increased in adhesion of the HGF-CEMP1 cells is favored for the spherical nanophase of the formulation of the zinc aluminate material. The spherical nanophase as showed by the images of the topography by AFM and SEM has a grain size of around 20-30 nm. It is clear that the cell functionality and biocompatibility of the HGF-CEMP1 cells to $ZnAl_2O_4$ nanostructured material is influenced by the nanometer surface features. Similar results have been reported for enhance adhesion to nanostructured materials and reported that critical grain size plays a crucial role in mediating adhesion to nanophase ceramics and also reported that enhanced adhesion is independent of the specific chemistry of the material surface and dependent only on the optimal surface topography of the material [2,28,29].

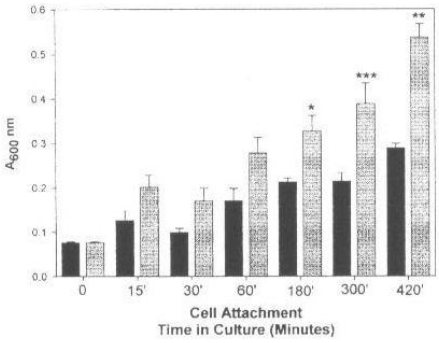


Figure 3. Adhesion of HGF-CEMP1 cells on the $ZnAl_2O_4$ nanostructured material (▒) and Corning 7059 glass slides (■) after an incubation period of 15, 30, 60, 180, 300 and 420 minutes. Asterisks indicate statistical significance ($p<0.05$).

Cell differentiation of the HGF-CEMP1 cells cultured onto $ZnAl_2O_4$ nanostructured material were evaluated by testing its potential in promoting mineralization and gene expression of bone-related molecules. Scanning electron microscopy images examination showed a sequence of morphological disposition of the mineral-like tissue deposited by HGF-CEMP1 cells. The features observed in experimental cultures at 5 days showed a tiny small globular morphology attached to nanostructured surface (Fig. 4B). At 14 days showed large and higher amount of mineral deposition with a nanospherical porous agglomerates or bone-like nodules distributed on the whole surface of the nanostructured material (Fig. 4D). Control at 5 and 14 days, revealed a smaller globular or spherules mineral formed by agglomerate structures (Figs. 4A and 4C for 5 and 14 days of culture respectively). The density and the mineral-like tissue deposit increased with time for both control and experimental cultures. AFM images showed a sequence of the three-dimensional morphological disposition of the mineral-like tissue deposited by HGF-CEMP1 cells. Our results revealed a tiny needle-shaped crystal formation and granular nanoparticles with preferential orientation deposited by experimental cultures at 5 and 14 days of culture Fig. 5 (B and D for 5 and 14 days of culture respectively). Nevertheless, control cultures showed a granular and grain agglomeration that favored the formation of crystalline plaquetes deposited by control cultures at 5 and 14 days of culture (Fig. 5 A and C for 5 and 14 days of culture respectively). In both cases, X-ray microanalysis of the mineral-like tissue deposited by HGF-CEMP1 cells showed the presence of calcium and phosphorus. The Ca/P composition ratio of the mineral-like tissue was 1.64 for experimental cultures and 1.50 for control cultures, which correspond well with the biological hydroxyapatite value.

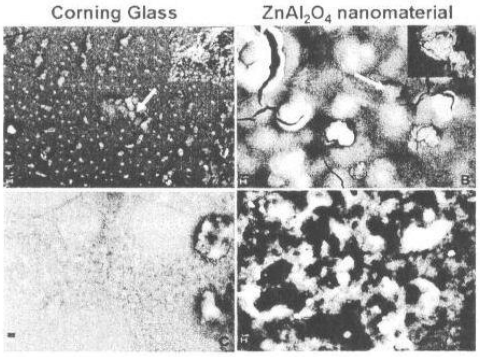
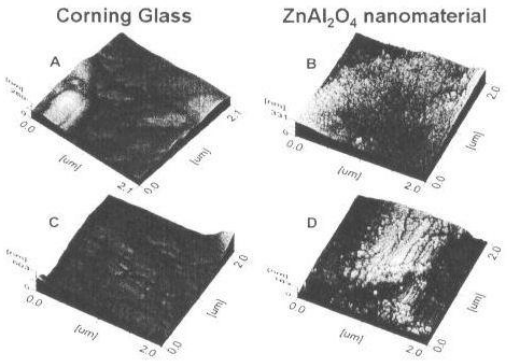


Figure 4. Scanning electron microscopy photographs of the mineral-like tissue deposited by HGF-CEMP1 onto $ZnAl_2O_4$ nanostructured material after 5 (B) and 14 (D) days revealed higher amount of mineralized-like tissue nodules. The inset magnification of the nanospherical globular-like structures of the mineral-like tissue onto $ZnAl_2O_4$ nanostructured material is showed by the arrow. Control cultures at 5 days (A) and 14 days (C) show the lower mineral density with globular and agglomerated morphology. This morphology is showed in the inset magnification marked by the arrow. Bar size = 1 μm.

Figure 5. Three-dimensional features observed by AFM showed the grain agglomeration that favored the formation of crystalline plaquetes in control cultures at 5 (A) and 14 (C) days of culture. Experimental cultures revealed at 5 (B) and 14 (D) days of culture, a preferential spatial arrangement of hydroxyapatite crystallites with homogeneous and needle-shaped crystals deposited onto $ZnAl_2O_4$ nanostructured material.



In order to analyze the genes that have been associated with cell differentiation of mineralized tissues deposition we employed RT-PCR. The results of electrophoresis of RT-PCR products obtained from HGF-CEMP1 cells cultured onto corning glass and $ZnAl_2O_4$ nanostructured material are shown in Fig. 6. The gene expression encoding for COL I, BSP, OCN and ALP showed a significantly higher levels of expression at 14 days in experimental culture when compared to control cultures. These molecules are considered to play a very important role in the process of mineralization and in this study, the high levels of gene expression of these matrix proteins are consistent with the elevated mineral-like tissue deposited in $ZnAl_2O_4$ nanostructured material as seen in Figs. 4 and 5. These differences in gene expression and the mineral-like tissue deposition could be associated to the nanostructured surface roughness of the material when compared to the smoother glass surface. Multiple reports have examined gene expression of bone cells during differentiation or in response to the surface material and reported high expression of alkaline phosphatase, osteocalcin, bone sialoprotein, collagen 1, osteopontin, and also reported that osteoblasts function were substrate-sensitive, either with respect to roughness or to the bulk composition of the material [30-35].

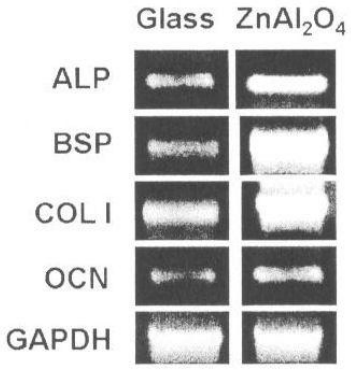


Figure 6. Representative gene expression using conventional RT-PCR from HGF-CEMP1 cells cultured onto $ZnAl_2O_4$ nanostructured material and Corning 7059 glass at 14 days of culture for ALP, BSP, COL I and OC products. GAPDH was used as house keeping gene control.

In summary, our results indicated that $ZnAl_2O_4$ nanostructured material provides a microenvironment with an increased bioactivity, cell differentiation and holds a future potential use for the osteoinductive process in biomedical implants. However, further studies may be focused on the effects of the nanotopography of $ZnAl_2O_4$ nanostructured material on proliferation, viability and changes in cytoskeleton proteins of mineral-like cells.

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