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Biocompatibility and bioactivity enhancement of Ce stabilized ZrO_2 doped HA coatings by controlled porosity change of Al_2O_3 substrates

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Abstract: Al_2O_3 substrates with controlled porosity were manufactured from nanosized powders obtained by plasma processing. It was observed that when increasing the sintering temperature the overall porosity was decreasing, but the pores got larger. In a second step, Ce stabilized ZrO_2 doped hydroxyapatite coatings were pulsed laser deposited onto the Al_2O_3 substrates. It was shown that the surface morphology, consisting of aggregates and particulates in micrometric range, was altered by the substrate porosity and interface properties, respectively. TEM studies evidenced that Ce stabilized ZrO_2 doped HA particulates ranged from 10 to 50 nm, strongly depending on the Al_2O_3 porosity. The coatings consisted of HA nanocrystals embedded in an amorphous matrix quite similar to the bone structure. These findings were congruent with the increased biocompatibility and bioactivity of these layers confirmed by enhanced growing and proliferation of human mesenchymal stem cells. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B:000–000, 2010.

Key Words: controlled surface porosity, biocompatibility and bioactivity, Al_2O_3 implants, Ce stabilized ZrO_2 doped HA coatings

INTRODUCTION

In tissue engineering, highly porous scaffold materials were found to supply the means for improved bone attachment and growth.[1]

Alumina (Al_2O_3) is now one of the most used biomaterials in orthopaedic and dental applications.[2,3] There is a growing interest in developing nanophase biomaterials that could be tailored to meet clinical requirements associated with either anatomical differences or patient age.[4] Microporous Al_2O_3 exhibits an increased surface area to volume ratio that could result in significant bioresorption and higher bioactivity. Many studies were carried out during recent years trying to combine the biocompatibility of calcium phosphates thin coatings with the strength of substrate ceramics such as Al_2O_3[5,6] or ZrO_2[7].

Because of its close resemblance to bone and capability to induce mineralization, hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, coatings were synthesized onto different biomedical substrates in view of improving the chemical bonding between the implant and the surrounding osseous tissues.[8] It was demonstrated that highly adherent HA thin structures are obtained on porous alumina, and the strength of the porous bioactive structure was substantially improved.[9] The addition of a metal oxide dopant has been proposed to reinforce the biomimetic layer[10] and improve its mechanical performances. Ce stabilized ZrO_2 doped HA (Ce-ZrO_2:HA) thin coatings on Al_2O_3 substrates were found to merge the biocompatibility and bioactivity of the shallow nanostructured layer with the high toughness and strength of the porous Al_2O_3 substrate.[11] Very recent studies showed the necessity to reproduce not only the composition in perfectly compatible and active layers but also the structure, morphology, and eventually the functionality of human bone.[12]

Thus, despite the fact that chemical properties of the coating surface are considered to be of significant importance under the exposure to body fluids, the surface roughness and porosity proved to play an essential role in osteointegration.[11–14] Moreover, recent studies proved the relevant significance of nanotopographical features on the control of human mesenchymal stem cell (hMSC) differentiation.[15] It is therefore of key importance to tailor the surface morphology, keeping the composition of the biomimetic coating unchanged.

Pulsed laser deposition (PLD) has proved to be a flexible method to process stoichiometric thin nanostructured layers as either simple or doped hydroxyapatite.[8,16,17]

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The technique confirmed the potential to produce a large diversity of coating morphologies ranging from smooth and dense to rough and porous.[18]

A thorough microstructural investigation of the Ce-ZrO_2:HA coatings synthesized by PLD onto porous Al_2O_3 is proposed herewith in view of correlating the porosities of the substrates and the modified morphologies of the bioactive layers with *in vitro* studies. To reach this goal, the PLD experimental parameters were kept unchanged while using Al_2O_3 substrates with different porosities.

MATERIALS AND METHODS

Substrate preparation

The alumina powder was produced by plasma chemical evaporation in a RF installation using 99.7% pure Al_2O_3 as raw material (dominant impurities: $SiO_2 \leq 0.02$ wt %, $Fe_2O_3 \leq 0.02$ wt %, MgO ≤ 0.02 wt %, Na and K ≤ 0.1 wt %). The specific surface area (SSA) of the synthesized powder was of 30 m^2/g. Plasma processed powder was next granulated from water suspension. The nanosized powders were axially pressed at 120 MPa in tablets of 12 mm diameter and 2–3 mm thickness, further used as deposition substrates. The sintering process was performed in air at 1400°C, 1500°C, and 1600°C respectively. The open porosity of obtained samples was of 32% to 36%, as measured with a high-pressure Hg porosimeter "Autopore IV", whereas the matrix density was of 3.93–3.95 g/cm^3, determined by the Archimedes method.

PLD experiments

The thin film coating process was performed in a stainless steel chamber using a UV KrF* COMPEX Pro 205 excimer laser source ($\lambda = 248$ nm, $\tau \sim 25$ ns). The chamber was first pumped down to a residual pressure of 10^{-4} Pa.

The Ce-ZrO_2:HA nanostructures were synthesized in 50 Pa H_2O vapors on Al_2O_3 substrates of different porosities. The substrates placed at 4 cm separation distance were heated at 400°C during the experiments. The heating and cooling rate of the substrate was kept at 6°C/min to avoid films deterioration by cracking or peeling. The laser was working at a frequency repetition rate of 10 Hz, while the beam was focused by an AR coated MgF_2 lens to get an incident fluence of 5.5 J/cm^2 for ablation. During the multipulse laser ablation, the target was rotated and translated along two orthogonal axes to avoid its piercing and to ensure a uniform deposition. Five thousand subsequent pulses were applied for the deposition of one structure. According to previous calibration, this corresponds to a layer of 350–400 nm thickness. A posttreatment at 380°C for 6 hours in a water vapor enriched atmosphere completed the sample preparation procedure.

Series of 12 identical samples were prepared for the biocompatibility and bioactivity studies.

Morphology investigations

The scanning electron microscopy (SEM) images were acquired with a Hitachi S-4800 apparatus. Transmission electron microscopy (TEM) studies were performed using an electron microscope TEM Philips CM 120 ST operating at 120 kV, having a point-to-point resolution of 0.24 nm, and equipped with facilities for selected area electron diffraction (SAED) analysis.

Biocompatibility assays

Fluorescence microscopy. For sterilization, samples were placed in Petri dishes and autoclaved in water vapor at 121.1°C for 30 minutes in a Falcon 30 Autoclave (LTE Scientific). Adult human mesenchymal stem cells (MSC) were isolated by density gradient centrifugation from bone marrow, as previously mentioned[11] and cultured *in vitro* for several passages. Five thousands cells per 1 cm^2 sample were seeded for biocompatibility tests in 24-well plates (Nunc). The cells were cultured on the Ce-ZrO_2:HA film surface for 48 hours. Then, they were labeled *in vivo* using ER-Tracker Blue-White DPX (Molecular Probes), a blue fluorescent dye which specifically localizes in the endoplasmic reticulum. After 30 minutes, the samples were rinsed three times with fresh media and then analyzed using a Nikon Eclipse E600W fluorescent microscope. Pictures were taken with a Nikon Digital Light DS-SM camera. Images were captured with the LuciaNet Software and processed using Adobe Photoshop 7.0 software. The controls were cells grown on standard tissue culture materials (borosilicate cover glass).

MSC adhesion assay. MSCs adhesion to thin coatings of Ce-ZrO_2:HA onto Al_2O_3 substrates was compared with the cells' adhesion to standard microscopy cover slips (CS). Two samples of each type were coated with heat-inactivated fetal bovine serum overnight- and two samples were left uncoated. Next day, the samples were washed with phosphate-buffered saline (PBS) and MSCs were added at a concentration of 3×10^4 cells/disc in serum free DMEM. They were allowed to adhere for 90 minutes at 37°C. Unattached cells were removed by three washes with PBS, whereas the attached ones were lysed by repeated freeze-thaw cycles after carefully removing the liquid from the wells. The plates were stored at −80°C for 1 hour and then thawed at room temperature (RT). The samples were covered by 500 μL dH_2O per well and the plates were incubated at 37°C for 1 hour. They were stored again at −80°C for 1 hour and then thawed at RT. The DNA content of the attached cells was assayed by addition of SYBR Green reagent 10,000× (Molecular Probes) prepared at a concentration of 5× in 50 m*M* Tris HCl (pH 7.4), 150 m*M* NaCl, and 5 m*M* EDTA (TNE buffer). After 30 min incubation at 37°C, the fluorescence was quantified at 530 nm (with excitation at 495 nm; Mithras microplate reader; Berthold). The cell number was determined using a standard curve of fluorescence. The experiments were performed two times consecutively on duplicate samples using cells isolated from two different donors.

RESULTS

The Al_2O_3 tablets porosity modification was controlled by changing the sintering temperature applied after pressing.

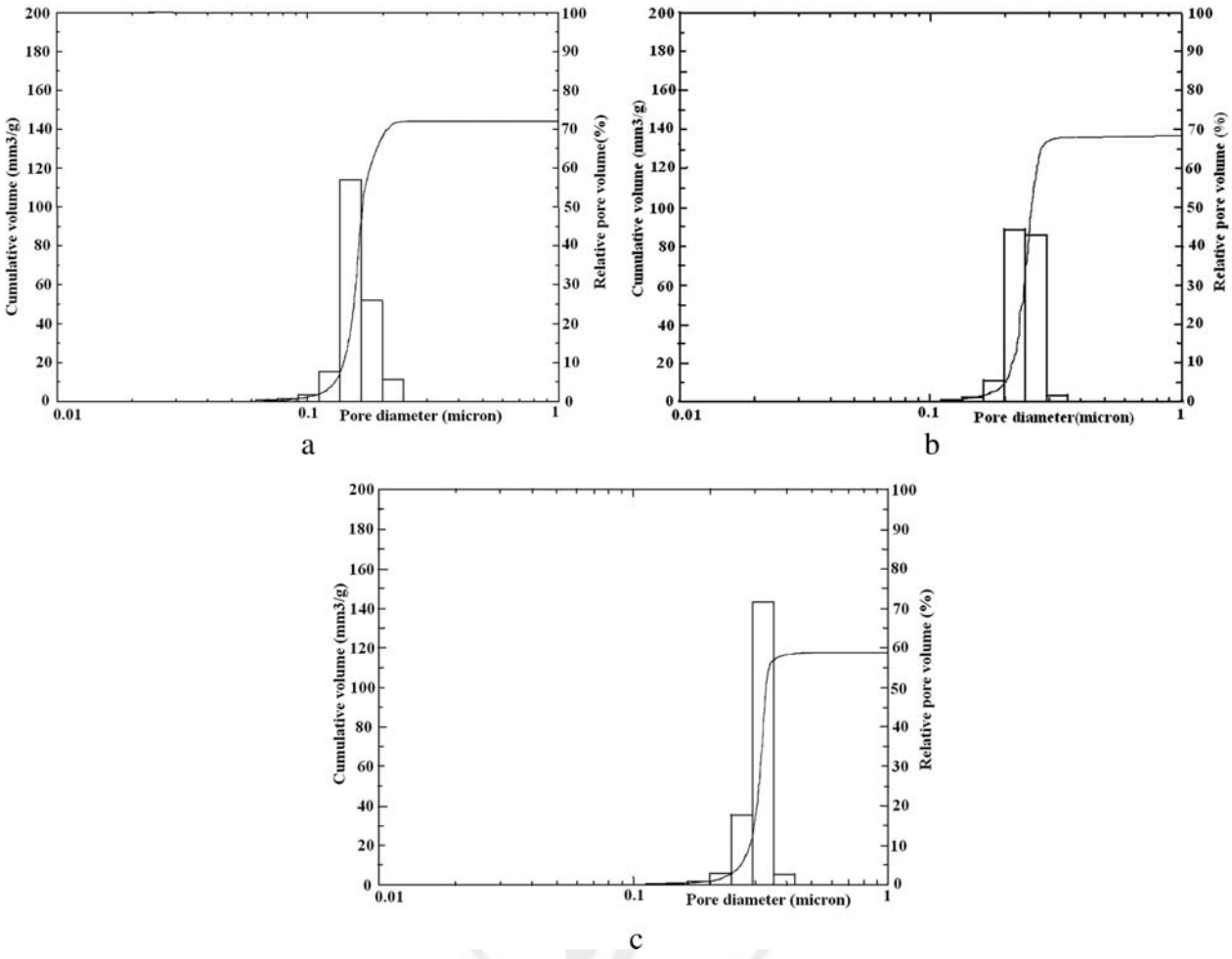

FIGURE 1. Cumulative and relative volumes vs. pores diameter in case of Al_2O_3 substrates sintered at (A) 1400°C, (B) 1500°C, and (C) 1600°C.

The pore diameter was inferred from the adsorption isotherm curves of Hg vapors recorded by the "Autopore IV" porosimeter. As alumina is stable and does not react with water, the pore diameter does not change in aqueous medium. Figure 1 presents the graphs where cumulative and relative volumes are represented as a function of pore diameter for Al_2O_3 substrates sintered at 1400°C, 1500°C, and 1600°C, respectively. It was noticed, in accordance with Ref. 19, a significant increase of the pore diameter with sintering temperature, as visible from the shift of the position of maximum pores diameter from 0.15 μm for Al_2O_3 sintered at 1400°C [Figure 1(A)] to 0.22 μm for Al_2O_3 sintered at 1500°C [Figure 1(B)] and 0.32 μm for Al_2O_3 sintered at 1600°C [Figure 1(C)], respectively. Nevertheless, the total cumulative volume of pores decreased with 20%, from 144.79 to 141.11 and 121.71 mm^3/g. Finally, the total porosity determined by Archimedes method diminished from 33.6% to 32.74% and 30.7 %.

PLD experiments were conducted using identical deposition parameters in view of comparing Ce stabilized ZrO_2 doped HA thin layers on Al_2O_3 tablets with different porosities. By optical microscopy, the films appeared to cover all the deposition area.

The porosity of Al_2O_3 substrate [Figure 2(A,B)] and the morphology of the deposited Ce-ZrO_2:HA coating [Figure 3(A,B)] were visualized by SEM investigations. One can see from Figure 2 that the pores are well interconnected, but the Al_2O_3 structure was rather compact and homogenous. The investigated Ce-ZrO_2:HA thin coatings were uniform and adherent to the substrate. From SEM studies (Figure 3) it was remarked that the morphology of the deposited films was significantly modified by the substrate porosity. An agglomeration tendency of tiny particles on small areas forming a rather uniform coating was observed by SEM in the case of Ce-ZrO_2:HA obtained on Al_2O_3 substrates sintered at 1400°C. Conversely, larger and disordered particles forming a layer with open porosity were visualized in the case of the synthesized nanostructures on Al_2O_3 substrates sintered at 1600°C.

To get a better insight into the process of the coating formation, TEM investigations of all nanostructures were conducted. Figure 4 presents the Ce-ZrO_2:HA nanocrystals formed after PLD on Al_2O_3 substrates sintered at 1400°C [Figure 4(A)] and Al_2O_3 sintered at 1600°C [Figure 4(B)], respectively. Typical quite regular nanocrystals were visualized.[20]

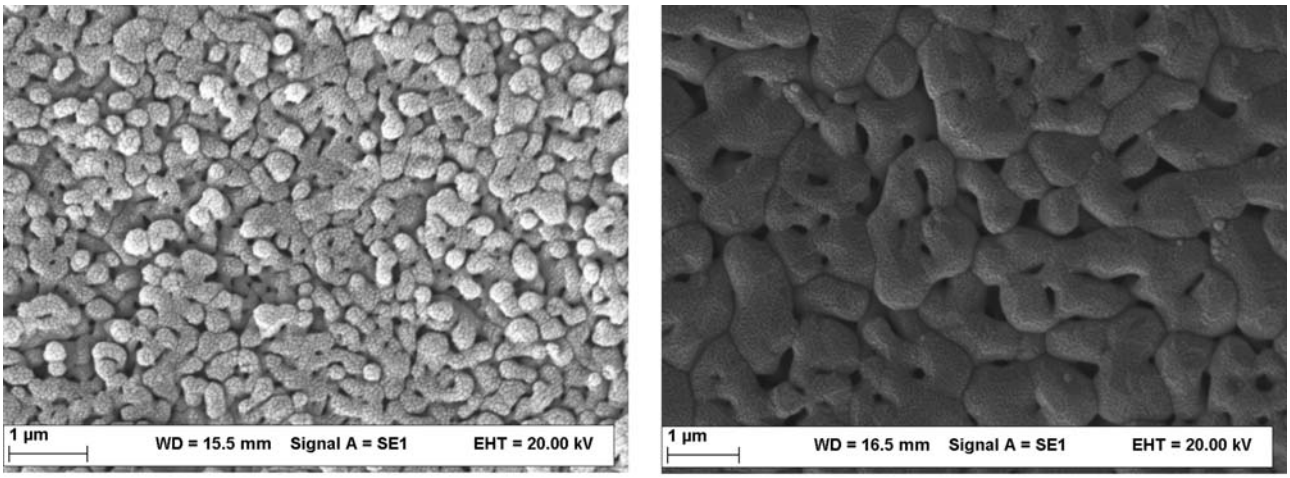

FIGURE 2. Characteristic SEM micrographs at different magnifications of Al_2O_3 sintered at (A) 1400°C and (B) 1600°C.

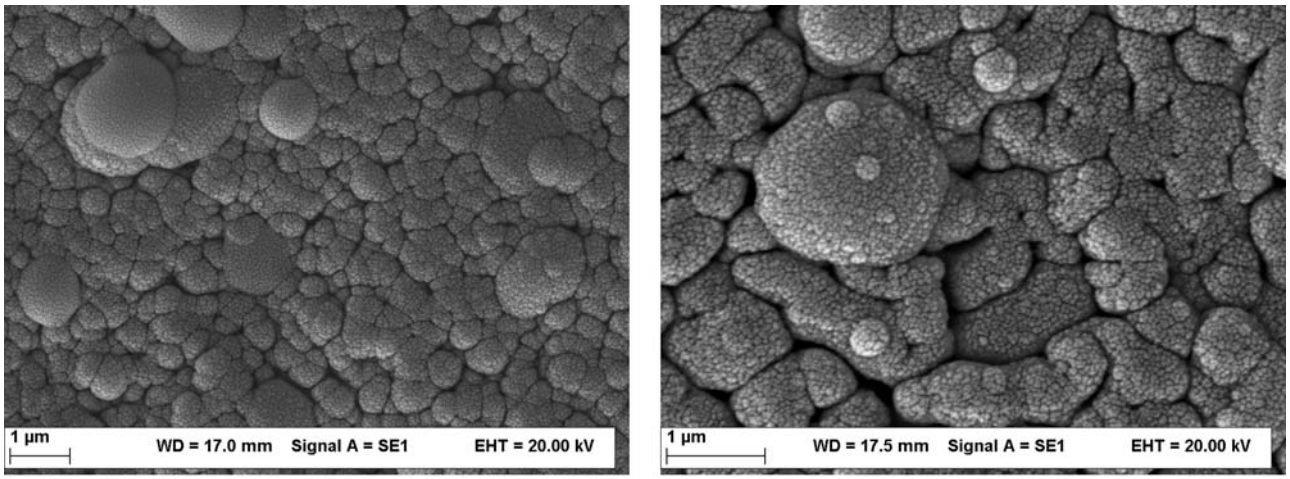

FIGURE 3. SEM micrographs at different magnifications of Ce-ZrO_2:HA thin films on Al_2O_3 sintered at (A) 1400°C and (B) 1600°C.

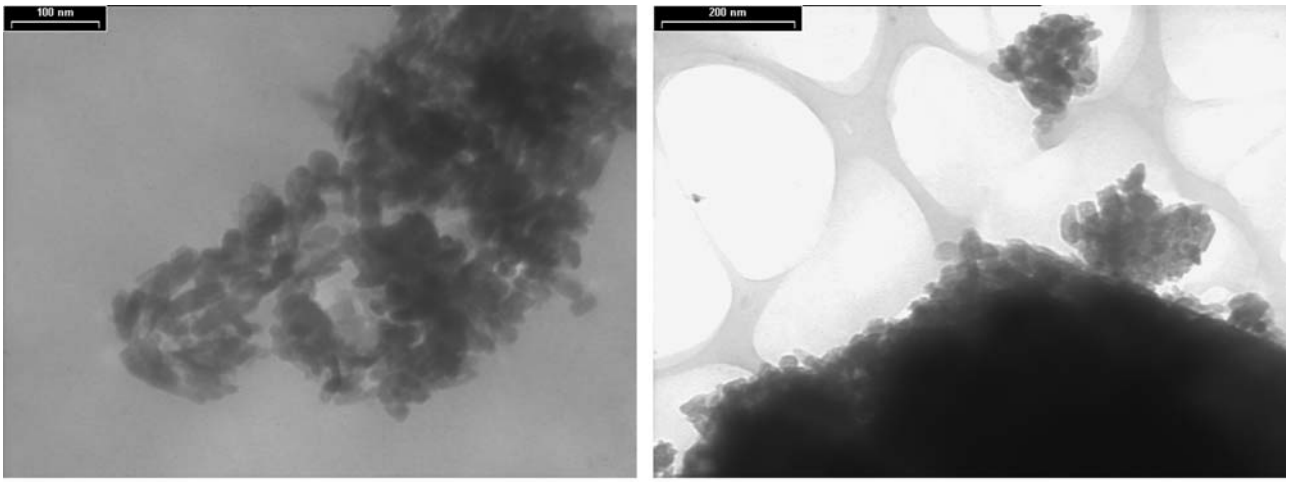

FIGURE 4. Characteristic TEM images of Ce-ZrO_2:HA films deposited on Al_2O_3 substrates sintered at (A) 1400°C and (B) 1600°C.

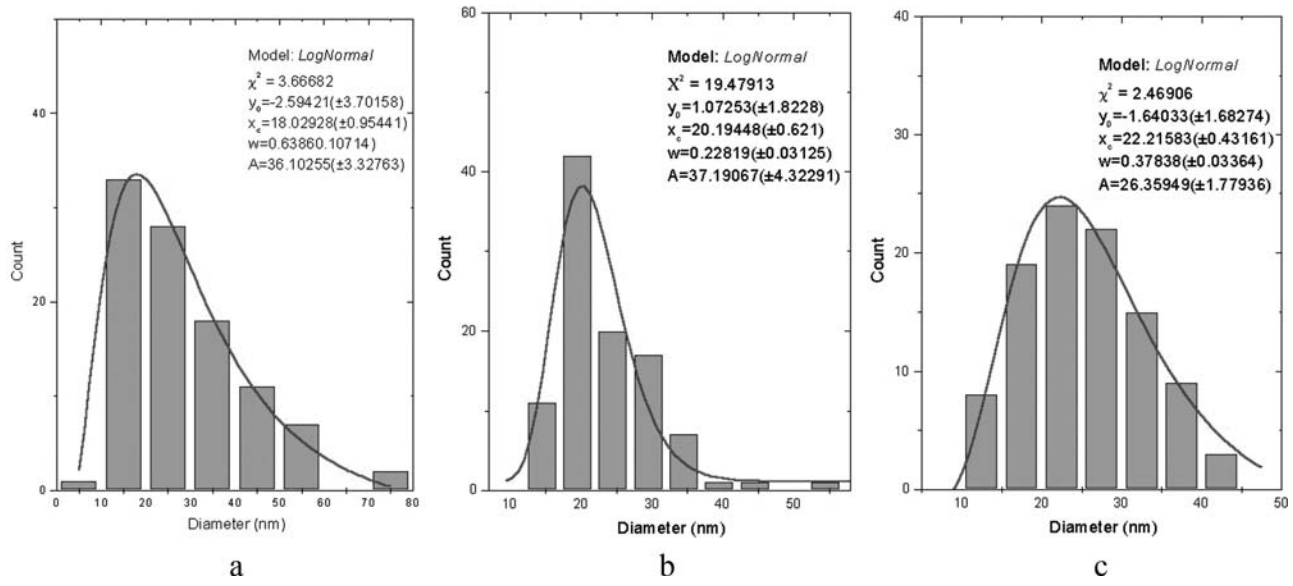

FIGURE 5. TEM histograms of Ce-ZrO_2:HA nanocrystals deposited on Al_2O_3 substrates sintered at (A) 1400°C, (B) 1500°C and (C) 1600°C. [Color AQ6 figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

F5 TEM histograms (Figure 5) were drawn on randomly selected areas in view of inferring the particles size distribution. A slight increase of nanocrystals size with increasing Al_2O_3 substrate porosity was observed. Thus, a predominant distribution of particles with dimensions in the range 10–20 nm after deposition on Al_2O_3 sintered at 1400°C, and of particles of 20–30 nm size after deposition on Al_2O_3 sintered at 1600°C substrates could be noticed.

F6 Figure 6 shows a HRTEM image of the PLD film grown onto Al_2O_3 sintered at 1400°C substrates, wherefrom HA nanocrystals in an amorphous matrix could be seen. The respective FFT analysis presented the interference fringes characteristic to HA plans (100) and (200), respectively.

These studies were followed by fluorescence microscopy investigations of the interaction between the Ce-ZrO_2:HA coatings deposited on different porous Al_2O_3 substrates with human mesenchymal stem cells. At 48 hours after seeding, the bone marrow-derived MSCs adhered well to the surface of all tested samples and the standard. A uniform coverage of all films was observed. However, it could be F7 noticed from Figure 7 a higher spreading of single cells on PLD coatings with complex and compact morphology [Figure 7(B)], rather than on layers exhibiting random, irregular discontinuities and big droplets [Figure 7(C,D)]. The last two samples showed cell colonies growing in discrete areas of the films.

For a proper assessment of cell adhesion, a quantitative DNA content assay was performed using the SYBR Green dye. The MSCs attachment on material surfaces without serum and on films precoated with serum 90 minutes after F8 seeding was examined. As visible from Figure 8, cells adhesion was increased on films covering 1600°C sintered Al_2O_3 in comparison with 1400°C sintered substrates. Nonetheless, the adhesion to standard material (CS) was found to be the highest, whether in the presence of serum or in serum-free conditions. After 48 hours the number of cells growing on 1600°C sintered alumina continued to increase, explaining the colonies formation observed by fluorescence microscopy [Figure 7(D)]. On the other hand, MSCs on 1400°C sintered substrate divided less frequently, not reaching the cell number obtained after attachment on serum coated films at 90 minutes.

DISCUSSION

Numerous studies revealed the importance of the morphological and topographical features of thin films obtained by different techniques onto substrates of biomedical significance in promoting the cells growth and proliferation and rapid implant osseointegration. A recent research

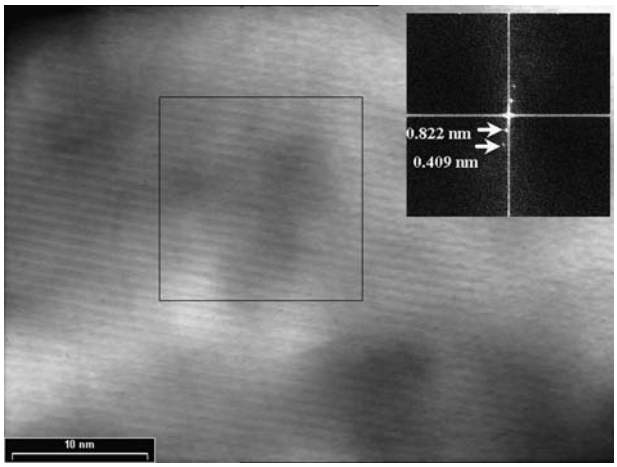

FIGURE 6. HRTEM and the corresponding FFT analysis of Ce-ZrO_2:HA nanocrystals on Al_2O_3 sintered at 1400°C.

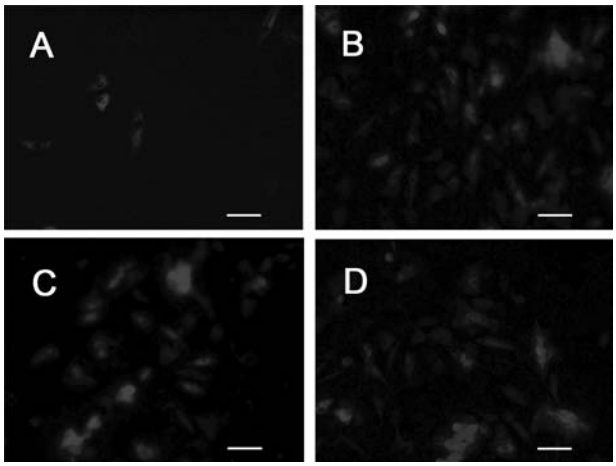

FIGURE 7. MSCs 48 hours after seeding on PLD Ce-ZrO_2:HA thin films. Cells were labeled with ER-Tracker: (A) on standard material, (B) Al_2O_3 sintered at 1400°C, (C) Al_2O_3 sintered at 1500°C, and (D) Al_2O_3 sintered at 1600°C (10×) (bar = 100 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

demonstrated the nanotopography significance on hMSC differentiation.[15] Nevertheless, this approach met with significant difficulties related to the possibility to replicate on a regular basis the substrates and synthesize reproducible thin coatings.[4,21] It is therefore of key importance to tailor the surface morphology at the nanometric scale preserving the composition of the biomimetic coating unchanged. This study could stand for an attempt to generate a biologically appropriate morphology of bioactive hydroxyapatite films via the controlled porosity of the Al_2O_3 substrates with the aim to implement this technology for manufacturing suitable coatings for new biomimetic implants.

Different surface morphologies of the coatings function of the substrate porosities were thus obtained (Figures 2 and 3). For low porosity substrates [Figure 2(B)], the slow evaporation of the dispersion medium allowed for the rearrangement of particulates in form of a dense packed structure [Figure 3(B)]. Consequently, large nanocrystals [Figure 5(C)] were formed on the top of this packed structure. For the porous substrate [Figure 2(A)], the dispersion medium quickly vanished because of capillary forces[22] and compact agglomerations of smaller nanocrystals [Figure 5(A)] appeared.

As shown in the previous section, a high porosity of Al_2O_3 substrates within the range 32% to 36%, proved appropriate for efficient cells growth and proliferation. Moreover, the material strength, which is essential for load bearing applications, appeared to not change significantly with the sintering temperature and pores size. Thus, Al_2O_3 samples with a porosity of about 42% (obtained from powder with specific surface area of 30 m^2/g) exhibited a bending strength in the range of 75–95 MPa. For a porosity of ~50% (powder with specific surface area of 50 m^2/g) the bending strength was in the range of 35–40 MPa. One can emphasize that in both cases, the bending strength was independent of eithersintering temperature or pores size. These findings are in good agreement with the data in Ref. 23.

Bone marrow MSCs were used to evaluate the biocompatibility conferred by the PLD coating with bone forming cells. Indeed, significant differences have been reported between osteosarcoma and primary cells.[24] Accordingly, the use of primary cells is mandatory for the correct characterization of any bone implant-type material. As known, MSCs are precursors for obtaining various cell types. Their classical differentiation pathways yield osteoblasts, condrocytes, and adipocytes. Also, their plasticity makes them ideal for various therapeutic applications including bone regeneration and repair.[25]

HA nanocrystals with dimensions within the range of tens of nanometers depending on the substrate porosity were identified in all synthesized thin films. One can stress that the obtained HA nanocrystals of 10–20 nm embedded into an amorphous matrix are similar to those specific to human bone structure[26] and exhibited an ideal dissolution and cell compatibility properties.[27,28]

The fluorescence microscopy examination has shown that hMSCs adhered to Ce-ZrO_2:HA coated Al_2O_3 sintered at temperatures ranging between 1400°C and 1600°C. While the substrate sintered at 1400°C presented uniform cell covering, the alumina sintered at higher temperatures progressively induced larger stem cells colonies, which could be indicative for enhanced cells division. To further analyze the role of substrate sintering temperature in cell attachment, the exact number of cells covering the Ce-ZrO_2:HA films was quantified using a DNA content assay 90 minutes after seeding.[29] Around 6000 cells were attached to the film deposited on 1400°C sintered Al_2O_3, while 10,000 were present on the coating on the 1600°C sintered substrate. As expected, the number increased when samples were precoated with serum. The data support the fact that the coatings synthesized on alumina sintered at higher temperatures increases cell attachment. Moreover, when cells were left to grow for 48 hours, their number increased with 165% (from 10,400 to 27,400) in case of 1600°C sintered substrate versus 27% only (from 8000 to 10,000) for 1400°C sintered Al_2O_3. The control material induced a 175% (from 14,000 to 38,500) increase in MSCs number.

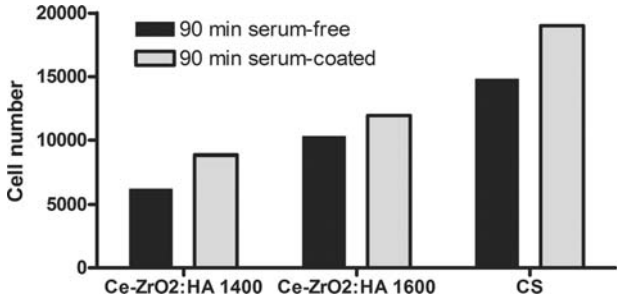

FIGURE 8. Quantitative analysis of MSCs adhesion to PLD Ce-ZrO_2:HA thin films. Cells were cultured for the specified intervals in the presence or absence of serum and then quantified by DNA content assay. Values represent mean of duplicate samples. One representative experiment out of two performed was represented.

These differences could be because of the pore size effect rather than to porosity.[30] Indeed, when increasing the substrate sintering temperature from 1400°C to 1600°C, the porosity is reduced with 10% only, while the pore size increased two times from 0.15 to 0.32 μm. As seen from SEM images (Figure 3), the Ce-ZrO_2:HA layers onto Al_2O_3 substrates present an open porosity more evident when they were growing onto 1600°C than on 1400°C Al_2O_3. These data support a strong dependency of cell attachment and growth on the topography of the bioactive layers grown by PLD onto porous Al_2O_3 substrates, tightly connected to their sintering temperature.

CONCLUSIONS

Al_2O_3 substrates with controlled porosities were manufactured for biomedical applications. The different porosities were reflected in specific morphologies of the pulsed laser deposited Ce-ZrO_2:HA biocompatible and bioactive layers which influenced the MSCs response. Ce-ZrO_2:HA coatings presented an open porosity and similar microstructure with human bone confirmed by the good biological performances. By monitoring the Al_2O_3 substrate porosity one can grow on its surface thin bioactive HA coatings which could generate different behavior of cell attachment and distribution. The surface structure and the open porosity were playing a key role in cell attachment and next proliferation and differentiation.

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