CeO₂ Containing Thin Films as Bioactive Coatings for Orthopaedic Implants

Georgiana-Alexandra Prefac¹, Marina-Larisa Milea¹, Andreea-Mihaela Vadureanu¹, Sorin Muraru¹, Daniela-Ileana Dobrin¹, Gabriela-Olimpia Isopencu², Sorin-Ion Jinga¹,², Mina Raileanu³, Mihaela Bacalum³ and Cristina Busuioc²,*

¹ Faculty of Medical Engineering, University POLITEHNICA of Bucharest, RO-011061 Bucharest, Romania; georgiana.prefac@stud.fim.upb.ro (G.-A.P.); marina_larisa.milea@stud.fim.upb.ro (M.-L.M.); andreea.vadureanu@stud.fim.upb.ro (A.-M.V.); sorin.muraru@upb.ro (S.M.); daniela.dobrin2007@stud.fim.upb.ro (D.-I.D.); sorinionjinga@yahoo.com (S.-I.J.)
² Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, RO-011061 Bucharest, Romania; gabriela.isopencu@gmail.com
³ Horia Hulubei National Institute of Physics and Nuclear Engineering, RO-077125 Magurele, Romania; mina.raileanu@nipne.ro (M.R.); mihaela.bacalum@nipne.ro (M.B.)

* Correspondence: cristina.busuioc@upb.ro

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Abstract: Due to the fact of their ability to bond with human’s hard tissue, bioglasses have gained interest in the biomedical field with certain purposes regarding their usage in the replacement, healing or repair of bones. In the form of thin films, they trigger an increase in biocompatibility for the inert supports after implantation, based on surface engineering to ensure osteoinduction. For that, this research is focused on obtaining coatings based on cerium-enriched bioglass to generate bioactive and potential additional antimicrobial and antioxidant properties. The addressed oxide system was a novel and complex one, 46.10 SiO₂–2.60 P₂O₅–16.90 CaO–10.00 MgO–19.40 Na₂O–5.00 CeO₂ (mol%), while two different synthesis methods, laser ablation and spin coating, were tackled comparatively. In the case of the first technique, substrate temperature was selected as variable parameter (room temperature or 300 °C). After conducting a complex characterization, films’ deposition was validated, their bioactive behaviour was proven by the formation of calcium phosphate after immersion in simulated body fluid for four weeks, while the impact exerted on the tested human fibroblast BJ cells (ATCC, CRL-2522) confirmed the applicative potential.

Keywords: cerium dioxide; thin films; laser ablation; spin coating; bioactivity; Escherichia coli; fibroblast cells

1. Introduction

In the last decades, a variety of materials have been used for bone defects repair, such as natural and synthetic polymers, glasses and ceramics as well as their composites. Inorganic materials employed as scaffolds in bone tissue engineering mainly refer to the calcium phosphate family and bioactive glasses [7]. Despite the disadvantage of brittleness, bioactive glass is a valuable candidate for bone
tissue engineering. Its capability to degrade at a controllable rate and convert to a hydroxyapatite-like material that bonds firmly to hard and soft tissues, but also the potential to release ions during the degradation process, show the specificity and superiority of bioactive glass [8]. The results of several recent studies indicated that the delivered ions have a beneficial effect on osteogenesis, angiogenesis and chondrogenesis [9]. Moreover, due to the technological progresses, bioactive glass can be processed as a high specific surface area or large pore volume including material, being subsequently used as carrier for different types of active phases; the silanol-enriched surface can promote an efficient loading, as well as controllable release. Additionally, through degradation, it releases active elements (i.e., silicon and calcium) that could upregulate gene expression levels and induce osteoblast differentiation [10].

The glass matrix can accommodate various dopants while maintaining the vitreous character, together with the basic physical and chemical properties [11]. There are many metal ions, such as magnesium (Mg) [12], strontium (Sr) [13], manganese (Mn) [14], iron (Fe) [15], zinc (Zn) [16], silver (Ag) [17], cerium (Ce) [18] and other some rare earth metals that have been integrated successfully into bioactive glasses to enhance their mechanical and biological properties. Cerium was selected due to the fact of its antimicrobial and antioxidant properties as well as its positive impact on mineralization and cells differentiation [19]. The greatest antimicrobial activity was observed against Gram-negative and fungi such as Escherichia coli, Pseudomonas aeruginosa and Proteus [20–22].

Glasses intended for medical purposes can be made by both traditional melt-quenching process and sol-gel route. The second method was pioneered by Li et al. [23] in the early 1990s and allows the production of both glass and ceramic materials at lower temperatures than those involved in the traditional manufacturing process. In the sol-gel method, colloids emerge within a suspension and assembly at room temperature, leading to the creation of a gel in the form of a wet inorganic network of covalently bonded entities, which is then thermally treated in order to produce a glassy phase [24]. The advantages in the biomedical field are remarkable, such as the possibility to functionalize the system with biomolecules during the formation of the vitreous matrix, without compromising their physicochemical properties by thermal degradation. Further advantages are linked to the compositional features of the approached systems, such as the possibility to simplify the glass formulation, avoiding the addition of high amounts of sodium oxide to lower the melting temperature and facilitate glass processing [25,26]. Differences in terms of physical properties between melt-derived and sol-gel bioactive glasses were deeply investigated by Sepulveda et al. [27]; their study, dedicated to the effect of nanoporosity and variations in chemical composition on degradation and in vivo behaviour, showed that the main difference between the two processing routes stays in the texture of the final product. Sol-gel glasses are indeed intrinsically nanoporous, while the melt-derived counterparts most of the time are in the form of highly dense monoliths [27].

In order to increase the bioactivity of inert substrates, like titanium or alumina, bioglass coatings can be deposited through several methods. Pulsed laser deposition (PLD) is a term used for a number of deposition techniques variants which refers to the general method of irradiating a target with a laser, commonly in vacuum, in order to evaporate its solid components onto a substrate [28]. The laser-induced expulsion produces a plasma plume for which the stoichiometry is alike to the target, the impurity content is low due to the external energy source, while the uniformity of the subsequent grown layers is excellent [29–31]. Another deposition technique is spin coating (SC), a process where a small amount of liquid precursor is placed at the centre of a substrate and rapidly rotated to spread the initial drop into a film and coat the targeted surface as a result of centrifugal force action; it has the advantage that is simple and generates fine and homogeneous coatings [32–34]. Since the deposition is carried out at room temperature, a heat treatment has to be applied to remove the solvent and promote the development of the desired bioactive coating; as well, this method includes the disadvantage of a dynamic dispense which becomes increasingly difficult to get complete substrate coverage when using either low spin speeds or very viscous solutions [35]. Compared with other deposition technologies, using PLD is usually easier to obtain the required film stoichiometry of multielement materials. PLD was frequently employed for the deposition of bioglasses on titanium.
samples, based on its main advantages: conceptual simplicity, stoichiometry preservation, versatility in terms of addressed materials and working conditions, cost-effectiveness and scalability [36–38].

Lately, there were many studies involving investigations on bioactive glass for biomedical applications. Concerning cerium, Deliormanli [39] found that doping 13–93 bioactive glass with cerium or gallium increases its antibacterial properties against Escherichia coli and Staphylococcus aureus, while maintaining good biocompatibility and no cytotoxicity. Additionally, Nicolini et al. [40] stated that 45S5 bioactive glass which contains cerium inhibits oxidative stress with the effectiveness depending on certain ratios of Ce$^{3+}$ and Ce$^{4+}$. Morais et al. [41] reported that doping a bioactive glass reinforced hydroxyapatite composite with cerium, enhancing it with an antibacterial effect against Gram-positive bacteria, specifically Staphylococcus aureus and Staphylococcus epidermidis. Goh et al. [42] concluded that a bioactive glass containing at least 5 mol% cerium in its composition effectively improves its antibacterial properties. Concerning the glass structure, Borges et al. [43] hypothesized that other rare earth elements, such as gadolinium or ytterbium, act similarly to cerium in terms of network connectivity, specifically given a decrease in the latter. Moghanian et al. [44] compared lithium and strontium doped 58S bioglass and found the former to be more effective for antibacterial properties. Silver embedding bioglass was extensively studied recently, and Krishnamacharyulu et al. [45] reported that silver ions increase the bioactivity. Moreover, Shahrbabak et al. [46] examined 58S bioactive glass containing zinc or silver and discovered that the latter promotes higher antibacterial effect and bioactivity. Baino et al. [47] summarized the effects of the ions from the doped bioactive glass types and reported that antibacterial activity is known to be enhanced by silver, manganese, zinc, copper, cerium and gallium. Moreover, cerium improves osteogenesis, which is shared by many other ions, such as lithium, gallium, iron or strontium.

In this study, to increase the bioactivity of inert substrates, like titanium or alumina, bioactive coatings were deposited. The films were obtained through two different techniques: pulsed laser deposition and spin coating. The target necessary for the laser ablation experiments was obtained through the sol-gel method. In the spin coating treatment of substrates, a solution was prepared using the same initial precursors. The morphology, elemental composition, structure and phase composition of the coated surfaces were investigated. The improvement of bioactivity was evaluated by immersion in simulated body fluid for 28 days, while the biological impact was assessed in relation to human fibroblast BJ cells. The novelty of the paper is sustained in the first place by the complexity of approached oxide system, defined starting from the well-known 45S5 bioglass but including magnesium, an element that generates superior mechanical properties and improved biological response, as well as cerium, an element that trigger antibacterial activity; secondly, the comparative character in terms of layers growing routes completes the value of the work by proposing alternatives for achieving tunable features for the final material.

2. Materials and Methods

2.1. Materials

Tetraethyl orthosilicate (Si(OCH$_3$)$_4$, TEOS, 98%, Aldrich, St. Louis, MO, USA), triethyl phosphate ((C$_2$H$_5$O)$_3$PO, TEP, 99%, Merck, St. Louis, MO, USA), calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$·4H$_2$O, 99%, Merck, St. Louis, MO, USA), magnesium nitrate hexahydrate (Mg(NO$_3$)$_2$·6H$_2$O, 99%, Merck, St. Louis, MO, USA), sodium nitrite (NaNO$_2$, 99%, Riedel-de Haën, Charlotte, NC, USA) and ammonium cerium (IV) nitrate ([NH$_4$]$_2$Ce(NO$_3$)$_6$, 98%, Sigma–Aldrich, St. Louis, MO, USA), were employed as sources of cations. The oxide composition of targeted material in terms of molar concentration can be found in Table 1.
Table 1. Oxide composition and employed reagents.

| Oxide | SiO₂ | P₂O₅ | CaO | MgO | Na₂O | CeO₂ |
|-------|------|------|-----|-----|------|------|
| Oxide concentration (mol%) | 46.10 | 2.60 | 16.90 | 10.00 | 19.40 | 5.00 |
| Reagent | Si(OC₂H₅)₄ (C₂H₅O)₃PO | Ca(NO₃)₂·4H₂O | Mg(NO₃)₂·6H₂O | NaNO₂ | (NH₄)₂Ce(NO₃)₆ |

2.2. Synthesis of the Precursor Powder

The nitrite/nitrate-type powders were weighed and dissolved in distilled water. TEOS was diluted with ethanol, and nitric acid (HNO₃) was added to reach pH = 2. TEP was hydrolysed and added over the salts solution, then TEOS solution was finally poured. All solutions were subjected to strong magnetic stirring or ultrasounds in order to achieve a clear final solution, with totally hydrolysed or solubilized compounds. To gelling, the solution was kept 24 h in the oven at 40 °C, after which the gel was dried at 80 °C for another 24 h. In the next step, the obtained light-yellow mass was calcined at 650 °C for 2 h. Thus, by applying a simple sol-gel protocol, an oxide powder with complex composition, high purity and homogeneity as well as small particle size was prepared.

2.3. Fabrication of the Final Target

The previous powder was granulated by adding 2% polyvinyl alcohol aqueous solution as binding agent and then pressed at 150 MPa with the help of a hydraulic press, taking the shape of a pellet 25 mm in diameter. This was sintered in two stages at 900 °C for a total of 10 h, in order to get a target suitable for coatings deposition through physical vapor deposition.

2.4. Deposition of the Thin Films

The deposition trials were carried out by two different techniques. The first one was of physical nature, pulsed laser deposition (PLD), where the material is taken from the target surface in the form of plasma with a nanosecond pulsed laser, transported through the deposition chamber and grown on the surface of the employed substrate. The second one fell into the category of chemical methods, spin coating (SC), and started from the precursor solution described in Section 2.2; a drop of this was placed on the surface of the desired substrate, rotated with high speed in order to ensure a balanced coverage, after which a preliminary drying process was applied by hot plate laying at 200 °C for 5 min. It should be mentioned that the solution must be processed quickly from the moment of realization, so that the gelling process does not take place, hindering through the high viscosity the spread of liquid due to the action of centrifugal force. Silicon was selected as substrate for PLD experiments, imposed by a configurational necessity, while titanium and alumina were chosen for SC trials, as representatives of bioinert implants category. All other parameters and corresponding values involved in the layers’ deposition are detailed in Table 2.

Table 2. Details of the coatings’ deposition.

| Sample Code | Deposition Method | Substrate Type | Processing Temperature (°C) | Other Parameters |
|-------------|------------------|----------------|-----------------------------|------------------|
| PLD-RT      | Pulsed laser deposition | Silicon plate | Room temperature | • 385 nm wavelength |
|             |                  |                |                             | • 73–74 mJ/pulse energy |
| PLD-300     |                  |                | 300                         | • 15,000 pulses |
| SC-Ti       | Spin coating     | Titanium plate | 650                         | • 2000 rpm/s acceleration |
| SC-Al₂O₃    |                  | Alumina plate  |                             | • 8000 rpm rotation speed |

2.5. Characterisation Techniques

The morphology and elemental composition of the samples were investigated using scanning electron microscopy (SEM) and energy-dispersive x-ray spectroscopy (EDX) from a FEI Quanta Inspect...
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F microscope equipped with an EDX probe (FEI Company, Hillsboro, OR, USA); before imaging, some of them were coated with gold employing a magnetron sputtering system. The structural analysis was done by Fourier transform infra-red spectroscopy (FTIR) with a Thermo Scientific Nicolet iS50 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), within the wavenumber range 2000–400 cm$^{-1}$. The crystalline features of the samples were studied by means of X-ray diffraction (XRD) using a Shimadzu XRD 6000 diffractometer (powder and target, Shimadzu Corporation, Kyoto, Japan) or a PANalytical Empyrean diffractometer (thin films, Malvern Panalytical, Royston, UK), within the 2θ range 10–80°.

2.6. Bioactivity Assessment

The in vitro bioactivity was estimated by immersing the four coated plates in simulated body fluid (SBF) for 28 days, at 37 °C; SBF solution preparation was previously reported by Kokubo [48]. The modifications occurred at the surface of the samples were evidenced by SEM, EDX and FTIR.

2.7. Antibacterial Activity Evaluation

To determine antibacterial activity, Escherichia coli (K12-MG1655) was employed as microbial strain, Nutrient Agar Medium as cultivation environment and adapted Kirby–Bauer Agar Disc Diffusion as protocol. Petri dishes with culture medium were inoculated with 100 μL cellular suspension with a concentration of 0.5 in McFarland standards. The samples were placed on the surface of inoculated medium and incubated at 37 °C, the results being recorded after 24 h of incubation.

2.8. Biocompatibility Evaluation

Cell viability was assessed through the MTT assay, as previously described [30]. Human fibroblast BJ cells (ATCC, CRL-2522) were plated on the investigated surfaces in 24 well plates, at a density of 20,000 cells/well. They were grown for 48 h, after which MTT was added to each well (final concentration of 1 mg/mL). The cells were further incubated for 4 h, then the medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formed crystals. The absorbance of the solution was recorded at $\lambda = 570$ nm using the plate reader Mithras LB 940 (Berthold, Bad Wildbad, Germany). For the Control condition, the cells were plated on glass slides. Cell viability was calculated based on the following equation: cell viability = (corrected absorbance of treated cells/corrected absorbance of control cells) × 100.

The morphological changes induced in the cells found around the samples after 48 h of incubation were observed under a CKX53 bright-field microscope (Olympus, Tokyo, Japan) employing a 10× objective and photographed with a WAT-902H camera (Watec, Saint-Lambert-la-Potherie, France).

The morphological modifications of the cells grown on the tested surfaces were also investigated by fluorescence microscopy. The actin filaments were stained using Phalloidin-FITC (Sigma–Aldrich, St. Louis, MO, USA), while the nucleus with Hoechst 33342 (Invitrogen, Karlsruhe, Germany), as briefly described. The cells were grown on the surfaces placed in 24 well plates, in similar conditions as described above. After 48 h, they were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde, washed again with PBS and permeabilized with 0.1% Triton X-100 in PBS. The cells were washed again with PBS and the two fluorescent dyes were added together on the cells and left in the dark, at room temperature for 1.5 h. Finally, they were washed with PBS and fixed with FluorSave™ (Merck, Darmstadt, Germany). The fluorescence images were taken with a confocal spinning disk microscope (Andor DSD2 Confocal Unit) mounted on an Olympus BX-51 epifluorescence microscope, employing a 40× objective. The images were recorded using the DAPI/Hoechst filter cube (excitation filter 390/40 nm, dichroic mirror 405 nm and emission filter 452/45 nm) for nucleus and the GFP/FITC filter cube (excitation filter 466/40 nm, dichroic mirror 488 nm and emission filter 525/54 nm) for actin filaments. The two images were further processed (pseudo-coloured and overlaid) using the ImageJ software (version 1.53a).
No approval by an ethics committee was necessary for the presented work, since the chosen procedures are performed on commercially available immortalized cell lines.

3. Results and Discussion

3.1. SEM Analysis

The SEM technique was firstly used to investigate the fracture surface of the target fabricated for PLD experiments and then the surface features of the films deposited by the aforementioned methods, both before and after SBF immersion; the corresponding images are displayed in Figures 1–3. In the case of target (Figure 1), the morphology was specific to a sintered material, but the densification degree was quite poor, which is not a surprise if we take into account the fact that the final thermal treatment was performed only at 900 °C. However, the micrometric pores were separated by massive walls or blocks that should supply a satisfactory amount of material during the laser ablation trials, without affecting the quality of the achieved layers. Only at higher magnification, can some small shapes that resembled grains be distinguished, these being embedded in a continuous matrix. It has to be added that the evolution of target was monitored from a dimensional point of view and interesting aspects were evidenced: after the first sintering step at 900 °C for 2 h, the disc volume increased by 132.6%, probably as a consequence of crystallization in a structure with higher unit cell, while the second sintering step at 900 °C for 8 h led to a contraction of solely 1.6%, showing that the previous process was completed and no further improvements in terms of porosity reduction were possible at such a low temperature.

![Figure 1. SEM images of the target: (a) 1000× and (a') 10,000×.](image)

The subsequent images (Figure 2) shows clear distinctions among three of the obtained coatings, especially between the PLD films and SC one. The former had rather flat and uniform surfaces, with nanometric grains, showing approximately a 20 nm average size and a very narrow size distribution, while the latter exhibited a rough appearance, copying substrate irregularities but were otherwise continuous and homogeneous in respect to the grain size, approximately 35 nm in diameter, and they were difficult to discern among neighbouring entities. PLD layers were covered with a family of perfect spherical droplets of different diameters which give the surface a certain roughness; their number seems to be increased for the 300 °C processed sample, in addition to them having a larger size. Similarly, when comparing the grain dimensions, a slight increase was observed for the higher substrate temperature. Overall, this means that despite the similarities between the two PLD films, a less rough surface was obtainable when the operation took place at room temperature. This can be explained by the fact that the energy delivered by the substrate surface modifies, to a certain extent, through the process of thermodynamics in the sense that it accelerates the nucleation and growth, compromising quality. In contrast, SC generates a wavy surface, formed out of directional ridges separated by abrupt troughs or even extended and deep craters. Additionally, the creases become
seemingly flat plateaus at higher magnification, but the general aspect stays uneven, which should represent a stimulating factor for the adhesion and proliferation of tested cells.

Figure 2. SEM images of the deposited thin films: (a and a’) PLD-RT, (b and b’) PLD-300 and (c and c’) SC-Ti.

The layered samples were immersed in SBF for 28 days in order to evaluate their capability of rapid mineralization. Comparing the images from Figure 2 with the ones from Figure 3, a completely different surface morphology can be noticed. In the case of PLD-produced coatings (Figure 3a,b), some quasi-spherical structures composed of randomly and loosely arranged nanosheets can be identified which highlights a pronounced interaction between the deposited film and SBF, with hydroxyapatite formation during the immersion period as demonstrated by EDX and FTIR analyses (Figures 4 and 5). The individual globular entities can exceed 1 µm in diameter, but the constituent laminas are light and fine, with a thickness of few nm and intertwined as a crumpled ball. The characteristics of the primary surface are slightly shielded by the newly grown shell, that looks more diffuse for the sample deposited at room temperature; a possible explanation can be correlated with the surface roughness, as 300 °C processed coating ensures a larger surface area through the presence of more droplets. Unlike these, the SC-derived sample (Figure 3c) showed much more rumpled and better delimitated quasi-spherical formations but integrated in a thinner layer, since the morphological properties induced the formation hydroxyapatite (proven by EDX and FTIR analyses) by following the directional and deepness particularities of support. Each distinct globe expands up to 300 nm by connecting a smaller number of lamellar structures compared to the previous case and in a more fortuitous way.
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Figure 3. SEM images of the deposited thin films after SBF immersion for 28 days: (a and a’) PLD-RT, (b and b’) PLD-300 and (c and c’) SC-Ti.

3.2. EDX Analysis

The EDX analysis was performed on the sintered target, respectively, on both types of coatings, before and after SBF immersion for 28 days to confirm the elemental composition transfer from bulk/solution to films and, subsequently, its modification as a consequence of hydroxyapatite emergence. All desired elements added through the precursors in synthesis were found within target, including cerium as Figure 4a evidences. The situation is similar for PLD layers (Figure 4b), with the only exception that the intensity of silicon line gets very big because of the substrate contribution that cannot be blocked by the thin coating from the top.

Since EDX spectroscopy is a precious tool for unveiling sensitive compositional variations, it was also employed for the mineralized samples and the corresponding results revealed the majority presence of calcium, phosphorous, and oxygen on the investigated surfaces (Figure 4c,d) with different Ca/P ratios (Table 3). Considering the values of the Ca/P ratios both before and after SBF immersion, it is obvious at first sight that the elemental composition completely changed after 28 days in contact with the testing solution with a significant increase in phosphorus concentration. For the SC coating, the Ca/P ratio was 1.55 which represents the closest value to that of stoichiometric hydroxyapatite (1.67). In addition, PLD films processed at room temperature or 300 °C display significant differences in this regard, with 0.73 and 0.63, respectively. These findings indicate that the calcium phosphate layer formed on the surface was calcium-deficient carbonated apatite [49]. An overlap of spectra before and after SBF immersion would emphasize an increase in phosphorus line intensity reported to the level of calcium, as well as a flattening of magnesium, sodium and cerium maxima due to the compositional evolution and barrier effect exerted by apatite.
The spectra of both PLD films were quite similar compared to the target, the only substantial difference being observable at the level of the degree of definition and separation among singular bands because of the reduced amount of material that produces signals. Moreover, a new broad maximum centered at approximately 1245 cm\(^{-1}\) seemed to occur after converting the bulk into a two-dimensional structure; this may be related to the modifications emerged in the coupling way of SiO\(^4\)\(^-\) groups or surface functionalization with OH\(^-\) entities and generation of a thin shell of silica gel [53–55]. But the spectrum of SC deposited layer was more focused on the substrate features, showing a large band associated to the vibrations of the structural entities found in titanium dioxide layer [56], naturally formed at the surface of titanium plate by oxidation.

### Table 3. Ca/P ratios extracted from the EDX spectra before and after SBF immersion for 28 days.

| Sample     | Before SBF Immersion | After SBF Immersion |
|------------|-----------------------|---------------------|
|            | Ca (wt.%) | P (wt.%) | Ca/P | Ca (wt.%) | P (wt.%) | Ca/P |
| PLD-RT     | 15.64    | 3.07    | 5.09  | 30.67    | 42.30    | 0.73  |
| PLD-300    | 15.04    | 2.71    | 5.55  | 27.63    | 43.79    | 0.63  |
| SC-Ti      | 15.44    | 3.79    | 4.07  | 6.25     | 4.04     | 1.55  |

### 3.3. FTIR Analysis

FTIR analysis is a powerful tool that enables us to determine exactly the bonding and grouping tendency within the coatings prepared through the two approached techniques. The absorption bands show the way in which the synthesis parameters trigger changes at the lowest dimensional level, these becoming visible due to the profiling of new vibrational characteristics. Figure 5a presents the spectrum of target and highlights specific chemical entities attributed to most of the integrated cations in coordination with oxygen. Si–O contributions individualize between 600 and 1200 cm\(^{-1}\), while those of \(\text{Ca–O}, \text{Mg–O}\) and \(\text{Ce–O}\) at wavenumber values below 600 cm\(^{-1}\) as frequently reported for glassy matrices or silicate-type compounds [50–52].

The spectra of both PLD films were quite similar compared to the target, the only substantial difference being observable at the level of the degree of definition and separation among singular bands because of the reduced amount of material that produces signals. Moreover, a new broad maximum centered at approximately 1245 cm\(^{-1}\) seemed to occur after converting the bulk into a two-dimensional structure; this may be related to the modifications emerged in the coupling way of SiO\(^4\)\(^-\) groups or surface functionalization with OH\(^-\) entities and generation of a thin shell of silica gel [53–55]. But the spectrum of SC deposited layer was more focused on the substrate features, showing a large band associated to the vibrations of the structural entities found in titanium dioxide layer [56], naturally formed at the surface of titanium plate by oxidation.
After 28 days of SBF immersion, all samples induce new absorption bands in the corresponding spectra, which were assigned to the vibrations of PO$_4^{3-}$ groups from the newly formed apatite layer [57]; their clear detection is proof of its quantitative consistency.

![FTIR spectra](image)

**Figure 5.** FTIR spectra of: (a) target, (b) SC-Ti thin film, (c) PLD-RT thin film and (d) PLD-300 thin film, both before and after SBF immersion for 28 days.

### 3.4. XRD Analysis

Figure 6 illustrates the mineralogical evaluation performed from the stage of powder to the stage of mineralized surfaces. Thus, the XRD pattern of precursor powder (Figure 6a) was registered, because it emphasizes the situation of SC film, since both materials were thermally treated at 650 °C for 2 h in order to convert the dried gel into a mineral mass; this shows one crystalline phase with low crystallinity due to the fact that the full-widths at half-maximum were wide. Corroborating the former information with the low intensity of peaks, it can be stated that the powder and the SC-derived layer come in the form of a vitreous matrix containing nanocrystalline domains of cerium dioxide (CeO$_2$) with cubic symmetry.

Going to the target (Figure 6b), the associated X-ray diffractogram included signals for two crystalline phases: CeO$_2$ (ceria, cubic crystal system) and CaMgSi$_2$O$_6$ (diopside, monoclinic crystal system). Because only a part of the constituent cations was integrated in crystalline compounds, the existence of an embedding glassy phase was evident. In the presented case, there were sharp and tall diffraction interferences which were characteristic of a large crystallite size. As it was expected, the temperature increased from 650 °C (powder) to 900 °C (target) and promoted the occurrence of more crystalline phases, together with the increase of their ordering degree.

In the XRD patterns of PLD-deposited coatings (Figure 6c), a small contribution of CeO$_2$ was visible, accompanied by other three maxima at low-angle values, as protrusions from the base halo. These peaks were impossible to attribute despite the efforts made in this regard; however, we concluded that the crystalline domains were of small dimension and could be linked to the existence of several types of SiO$_2$ entities, as the FTIR investigation (Figure 5) indicated a certain modification in terms of Si–O bonding and grouping tendency from target to films.
CeO$_2$ is a very useful compound in tissue engineering. Ponnurangam et al. [58] found that nanoceria, which represents cerium dioxide nanoparticles, can improve both the biochemical and mechanical properties of in vitro cartilage tissue when embedded within engineered cartilage constructs. The antibacterial activity of CeO$_2$ has been extensively studied. Kannan and Sundrarajan [59] concluded that nanoceria has antibacterial properties after investigating its effects on both Gram-negative (Escherichia coli) and Gram-positive (Staphylococcus aureus) bacteria. Similarly, Reshma and Ashwini [60] reported an important antibacterial effect of nanoceria when evaluating its activity against both Gram-positive (Corynebacterium diphtheria and Sarcina lutea) and Gram-negative (Escherichia coli and Proteus vulgaris) bacteria. The role of nanoceria in bioactive glass foam scaffolds for bone regeneration was studied by Karakoti et al. [61], who discovered that this compound can increase collagen production by human mesenchymal stem cells. On the other hand, diopside scaffolds were found to be adequate for bone tissue engineering by Ghomi et al. [62], with highly porous structures and a compressive strength comparable to spongy bone.

A preliminary test was conducted in terms of antibacterial activity assessment on an Escherichia coli microbial strain, the results being displayed in Table 4, together with suggestive digital images captured after 24 h of incubation. High antibacterial activity was observed in the case of PLD-RT coating, followed by the other two investigated films with moderate influence and, finally, a poor response from the target side. Taking into account that the active material consists of a layer of maximum 200 nm in thickness and the investigated areas are reduced in size, the outcome achieved for PLD-RT was excellent, being a potential candidate for bone implants in infected environments. Additionally, the behaviour of PLD films against the target was understandably based on the differences in crystalline features (available in Figure 6). Thus, the target was highly crystalline, the constituent cations being

![Figure 6. XRD patterns of: (a) powder, (b) target and (c) PLD-RT and PLD-300 thin films.](image-url)
strongly fastened inside the ordered network, a fact that hinders potential ionic release; contrariwise, PLD layers were weakly crystallized with cerium cations embedded in a glassy matrix that was more prone to degradation. It was obvious that the increase of substrate temperature to 300 °C favours ions ordering and strengthens the chemical bonds, with detrimental effects on antibacterial activity. The situation is quite similar with SC grown sample, which displays a partially crystalline character after calcination at 650 °C.

**Table 4.** Details of antibacterial activity evaluation.

| Sample       | Area of Sample (mm²) | Inhibition Zone (mm²) | Antibacterial Activity Evaluation |
|--------------|----------------------|-----------------------|----------------------------------|
| PLD-RT       | 12.00                |                       | High                             |
| **72.82**    |                      |                       |                                  |
| PLD-300      | 11.25                |                      | Moderate                         |
| **43.73**    |                      |                       |                                  |
| SC-Al₂O₃     | 11.50                |                      | Moderate                         |
| **41.12**    |                      |                       |                                  |
| Target       | 9.00                 |                      | Poor                             |
| **13.11**    |                      |                       |                                  |

The biological results achieved on human fibroblast BJ cells are depicted in Figure 7 for cell viability assay and Figure 8 for morphological evaluation of cells grown in the presence of the coated samples. As presented in Figure 7, in the case of SC-deposited films, it was found that SC-Ti presents a good biocompatibility for cells with a cell viability around 90% and no significant difference
compared to Control conditions. However, SC-Al₂O₃ did not prove to sustain cell proliferation and considerably affected cell metabolism, reducing cell viability down to 60%. Based on these findings, it can be concluded that alumina substrate hindered the deposition of high-quality coatings, favourable for cell growth, probably through the surface texture, that impeded a homogenous spreading of precursor solution, mainly by affecting the flowing and friction processes. For the other two layers, PLD-RT and PLD-300, cell viability was around 90%. These results indicate that coating the substrate through PLD can produce a bioactive surface which favours cell proliferation and demonstrate that the associate coatings do not influence negatively the cells development and, therefore, they are highly biocompatible.

![Figure 7](image_url)

**Figure 7.** Cell viability in the case of BJ cells grown on the coated samples compared to Control cells grown on glass slides. Statistical analysis was performed using One-way analysis of variance with Dunnett’s multiple comparison test. * p < 0.05, *** p < 0.001.

To further determine how the samples are affecting cell development, the morphology of cells found around them was analysed by transmission light microscopy, the recorded images being available in Figure 8a. As it can be seen, the general aspect of cells is not affected, but only their number, especially in the case of alumina substrate. The results are in accordance with MTT assay, proving that the surfaces of PLD-RT, PLD-300 and SC-Ti are not inhibiting the biological path of evolution.

The fluorescence images (Figure 8b) are in the same trend as the other tests conducted. Cell morphology, both the actin filaments and nucleus are clearly visible and not affected by the culture environment, with the best result for PLD-RT coating. Although for SC derived films the number of cells is decreased as compared to Control condition, their morphology is not altered. Moreover, in the case of PLD layers, cells spread filopodia and establish contacts with neighbouring cells, creating an extended network.

Our results showed a good biocompatibility for three of the studied samples (PLD-RT, PLD-300 and SC-Ti). The results are supported by various studies, which reported cerium-doped glasses biocompatibility on various cell types: osteoblasts, fibroblasts or epidermal keratinocytes [18,63–67]. Their biocompatibility and properties can be tweaked by changing the ratio of cerium ions: Ce³⁺ has a negative impact on cell proliferation, while Ce⁴⁺ has an opposite effect [68–70]. Aside from a good biocompatibility to various cell lines, cerium-doped bioactive glasses have shown to be suited for various applications in biomedical sciences such as bone regeneration, wound healing, anti-inflammatory, antimicrobial and even anticancer activity [64,71].

As a preliminary test, our findings are encouraging and can justify further studies in this direction. In order to develop better medical devices, with good biocompatibility and well characterized properties, one must respect the guidance of specialized organisms in the field, as well as ISO 10993
standards. Considering that in some cases the devices have to be applied at system level, aside from the cytotoxic studies, the samples hemocompatibility has to be investigated, as well as the genotoxicity (chromosomal aberration and micronucleus test). Following in vitro testing and validation, in vivo studies can be also performed. In perspective, we plan to further investigate the cyto- and genotoxicity against fibroblast and osteoblast cells. The investigations can be doubled by studies which can monitor if the glasses can promote osteogenesis or wound healing.

![Figure 8](image-url)

**Figure 8.** (a) Optical microscopy images and (b) fluorescence microscopy images of BJ cells grown on the coated samples compared to Control cells grown on glass slide. Scale bar is 10 μm for all images.

### 4. Conclusions

With the aim of increasing the bioactivity of inert substrates and subsequently speeding the healing process of those patients who need orthopaedic implants, bioactive glassy coatings containing CeO$_2$ nanocrystals were deposited by two different methods, pulsed laser deposition and spin coating. The emergence of new formations of hydroxyapatite with specific morphology at the surface of all layers after 28 days of immersion in simulated body fluid was highlighted through several characterization techniques. Furthermore, the results of cell proliferation assay were consistent with the images provided by optical microscopy and fluorescence microscopy, proving that the surfaces are not affecting cells morphology; the best response was recorded in the case of those films grown by pulsed laser deposition.

In conclusion, the materials obtained through the specified approaches have excellent properties and appropriate biological behaviour to be used in biomedical applications. Future aspects can be
managed, like using another surface deposition method in order to investigate how it influences the mineralization process and elucidate the interaction with cells in order to choose the appropriate one for the targeted application.

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