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Biocompatible and bioactive nanostructured glass coatings synthesized by pulsed laser deposition: *In vitro* biological tests

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ABSTRACT

We report on the synthesis by pulsed laser deposition with a KrF* excimer laser source (λ = 248 nm, τ = 25 ns) of bioglass thin films of 6P57 and 6P61 types. Physiology, viability, and proliferation of human osteoblast cells were determined by quantitative *in vitro* tests performed by flow cytometry on primary osteoblasts cultured on pulsed laser deposited bioglasses. Both types of glass films proved to be appropriate mediums for cell survival and proliferation. In a parallel investigation, cell morphology and adhesion to the surface was studied by fluorescence microscopy and scanning electron microscopy. Strong bonds between the materials and cells were found in both cases, as osteoblast pseudopodes penetrated deep into the material. According to our observations, the 6P57 glass films were superior with respect to viability and proliferation performances.

1. Introduction

Titanium and its alloys are currently the most employed metallic materials in implantology. Nevertheless, thin fibrotic encapsulation is often formed around the implant, isolating it from the organism. This reaction is commonly named foreign body response.

To increase implants and prostheses biocompatibility, metallic surfaces were coated with bioactive ceramic materials, with hydroxyapatite being used the most [$Ca_{10}(PO_4)_6(OH)_2$ – HA], due to its resemblance to the mineral constituent of the bones [1,2,3].

The new expected generation of implants will be biomimetic, thus touching the final level of biocompatibility, i.e. the perfect integration of a foreign structure into the human body. New materials such as bioglasses were intensively studied due to their behavior in prolonged contact with the extracellular fluid [4,5]. Through bioglasses degradation inside the human body and due to ions exchange between glass and extracellular fluid, a new carbonated bioapatite layer is naturally synthesized, replacing the initial coating material [6,7,8]. The forming apatite is similar in structure to biological apatites, therefore the body effectively recognizes the newly formed material as inorganic bone-like structure with higher biocompatibility than the initial bioglass coating [9].

We recently reported on PLD synthesis of bioglass thin films and their physical–chemical characterization by different techniques [10,11,12]. We resumed our studies by investigating the functionality of the obtained structures, i.e., the behavior of human osteoblast cells cultivated on bioglass thin films surfaces, throughout viability, proliferation and adhesion tests.

It is believed that the 60% SiO_2 content in glasses composition in the system SiO_2–Na_2O–K_2O–CaO–MgO–P_2O_5 represents a threshold value for biocompatibility and stability inside the human body. In general, SiO_2-rich glasses with silica contents higher than 60 wt% have a better mechanical stability and adhesion to the metallic substrate, but are not soluble in body fluids [5,6,13].

The current paper focuses on thin films synthesis by PLD of bioglasses in the system SiO_2–Na_2O–K_2O–CaO–MgO–P_2O_5 with two different compositions. One composition contains a slightly lower SiO_2 content than the threshold value (57% SiO_2) and the other a slightly higher than 60% SiO_2 content (61% SiO_2).

2. Materials and methods

2.1. Glass preparation

We used as targets, glasses in the system SiO_2–Na_2O–K_2O–CaO–MgO–P_2O_5 containing 61 wt% (further denoted 6P61) or 57 wt% (further denoted 6P57) silica. Their composition is 56.5% SiO_2, 11%

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Na_2O, 3% K_2O, 15% CaO, 8.5% MgO, 6% P_2O_5 in case of 6P57 and 61.1% SiO_2, 10.3% Na_2O, 2.8% K_2O, 12.6% CaO, 7.2% MgO, 6% P_2O_5 in case of 6P61 [14].

2.2. PLD experiments

PLD targets were prepared by pressing at 3 MPa and sintering for 6 h at 650 °C of the base powder materials.

The experiments were conducted inside a stainless steel deposition chamber. A UV KrF* excimer laser source (λ = 248 nm, τ = 25 ns) was used for the multipulse ablation of bioglass targets. The incident laser fluence was set at about 5.7 J/cm^2. The targets were cleaned by a preliminary ablation with 1000 pulses. A shutter was interposed between target and substrate, stopping the ablated flux containing impurities.

The laser beam hit the target at an angle of 45°. For the deposition of one bioglass layer, we applied 5000 subsequent laser pulses. Targets were continuously rotated and translated in order to avoid drilling and cracking of their surface. All thin films were deposited onto chemically-etched high-purity (97%) Ti disks of 15 mm in diameter and 1.5 mm in thickness. Prior to deposition, the Ti substrates were carefully cleaned with alcohol in an ultrasonic bath and rinsed in deionized water.

The Ti substrates were placed parallel to the targets, at 4 cm in front of them. All of the depositions were carried out on substrates heated to a constant temperature of 400 °C. The bioglass thin films were deposited in a dynamic flux of 13 Pa O_2. After the depositions, the samples were allowed to slowly cool down in the same atmosphere.

2.3. Cell cultivation

We used primary human osteoblasts obtained by differentiation from mesenchymal stem cells (hMSCs) to test cell adhesion on bioglass surfaces. Adult hMSC were isolated by density gradient centrifugation from whole bone marrow of a 65-year-old patient receiving a bone implant. Cells were plated in T150 flasks at a density of 5×10^4 cells/cm^2 and grown in minimum essential medium MEM Earle, supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 mg/ml streptomycin. The MSC phenotype was validated at the second passage by flow cytometry. Cells were found negative for the hematopoietic markers CD14, CD34, CD45 and positive for the MSC markers CD13, CD29, and CD90. MSCs were differentiated *in vitro* to osteoblasts using osteogenic media containing 82 μg/ml ascorbic acid magnesium phosphate salt, 100 nM dexametasone and 10 mM β-glycerophosphate.

2.3.1. Cell adhesion

Differentiated osteoblasts were cultured on bioglasses for 3 days then washed with PBS, fixed with 4% paraformaldehyde (20 min, 4C), and permeabilized with 0.2% Triton X-100 for 3 min. Actin filaments were labeled with Alexa Fluor 594 phalloidin and vinculin with anti-vinculin antibody (mouse monoclonal from Sigma) followed by Alexa Flour 488 goat anti-mouse antibody. Cells were mounted with Vectashield and visualized on Nikon Eclipse E600 microscope. Images were recorded using a Nikon DS-U1 camera controlled by NIS Elements software.

2.3.2. Cell proliferation

Osteoblasts were fluorescently labeled with CFDA (carboxifluorescein diacetate) of CellTrace CFSE Cell Proliferation Kit (Molecular Probes). CFDA penetrates cell membrane, is cleaved by intracellular esterases and binds proteins as CFSE (carboxifluorescein succinimil ester). After cell division, daughter cells receive half of the fluorescence of precursor cells; each generation could be visualized as a peak in FACS histograms. After labeling, 3000 cells were seeded per 24-well plates. Six days after seeding cells were harvested using Trypsin/EDTA solution. Samples were centrifuged at 1500 × rpm for 5 min at 4C, washed twice and resuspended in 200 μL FACS buffer. Cell suspension was analyzed with FACSCalibur flow cytometer on the FL-1 channel. Proliferated cells percentage was determined using Cell Quest Pro Software.

2.3.3. Cell viability

Primary osteoblasts (5000 cells per 24-well plates) were cultivated in osteogenic media for 72 h, detached with Trypsin/EDTA, washed with FACS buffer and labeled with propidium iodide (PI). Samples were analyzed with FACSCalibur on the FL-2 channel. The percentage of viable cells was determined using the Cell Quest Pro software.

2.3.4. Scanning electron microscopy (SEM)

Cells were grown for 3 days on cover glass (control experiment) and PLD bioglasses, fixed with PFA 4%, washed with PBS and gradual dehydrated in 70%, 90%, and 100% ethanol solutions. The samples were air-dried and analyzed with SEM-FEG Quanta Inspec F microscope.

3. Results and discussions

Differentiated osteoblasts were cultivated on bioglasses for 3 days, detached and labeled with PI for FACS analysis. Since only dead and permeabilized cells were able to internalize PI, cell viability was determined measuring the percentage of unlabeled cells from the pool of analyzed cells. Usually cultured cells will have 90–95% viability depending on cell type, days from splitting, number of passages, etc.

The PLD samples showed good viability. Primary osteoblasts grown on 6P57 bioglass displayed a better viability as compared with the 6P61 structures (88.7% and 82.3%, respectively). Control experiment showed 96.3% viability (Fig. 1).

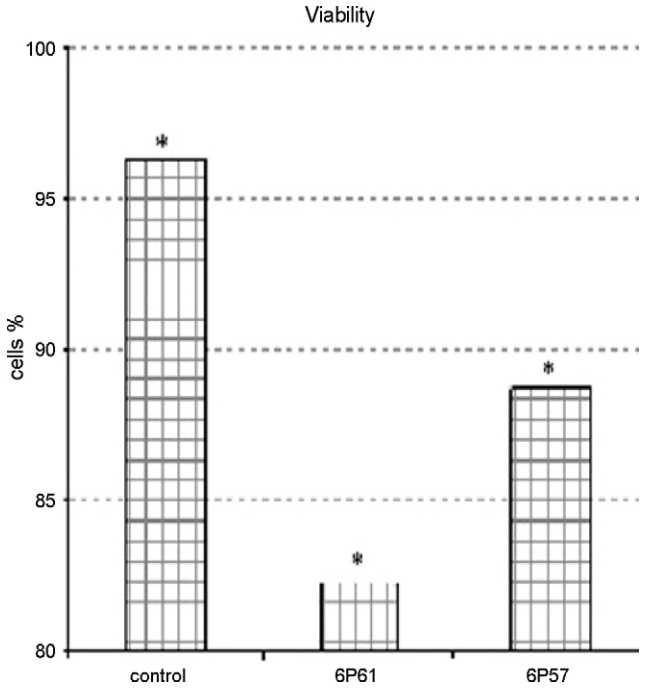

Fig. 1. 72 h viability of osteoblasts (passage no. 9) cultivated on 6P61 and 6P57 bioglasses. 2265 events were recorded.