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Osteoblast behavior on various ultra short pulsed laser deposited surface coatings

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ABSTRACT

Ultra short pulsed laser deposition technique was utilized to create amorphous diamond, alumina and carbon nitride, and two different titania coatings on silicon wafers, thus producing five different surface deposited films with variable physico-chemical properties. The surface characterizations, including the roughness, the contact angle and the zeta potential measurements were performed before we tested the growth properties of human osteoblast-like Saos-2 cells on these surfaces (three separate experiments). The average roughness and hydrophobicity were the highest on titania-deposited surfaces, while carbon nitride was the most hydrophilic one. Osteoblasts on all surfaces showed a flattened, spread-out morphology, although on amorphous diamond the cell shape appeared more elongated than on the other surfaces. On rough titania, the area covered by the osteoblasts was smaller than on the other ones. Cell proliferation assay did not show any statistically significant differences.

1. Introduction

The borderline between osteoblasts and the prostheses creates an interface, which is critical for the osteointegration and longevity of the implanted prostheses [1]. The coatings of the prostheses can be modified in order to find the best possible implant materials with good cellular adhesion and biocompatibility. The implant surface properties, including the surface chemistry and topography, and electric charge, influence the biocompatibility and long-term fixation of the implant, and are important factors to be considered in the search for better biomaterials for implants.

Titanium has excellent surface properties as a biomedical metal implant. It is also highly resistant to corrosion and has good biocompatibility. Thus, it has been widely used in various types of implants and heart valves [2]. However, even the best present-day prostheses (metallic or ceramic) are prone to fail due to aseptic loosening, and none of the cemented prostheses provided excellent (>90% at 15 years) survival in the patients 55 years or older in age [3]. The host response to titanium or other wear debris can cause this unfavorable event [4]. There is also a need of implant prostheses for the younger, more active patients [5,6], which sets even higher requirements for the quality of the prostheses. Thus, the development of medical implants using alternative materials has become necessary for the progress of orthopedic treatments, including new ceramics and coatings, and modified materials [7,8].

Several methods are nowadays available for manufacturing thin film coatings on top of various materials. These include physical vapor deposition techniques, which are basically evaporation and sputtering in nature [9]. They enable the manufacture of thin films of metals, alloys, ceramic and polymer on a wide range of materials. For the processing they generally utilize plasma, which can contain both charged and neutral atoms and particles. Ionized plasma is more favorable since the motion of neutral atoms is more difficult to control than ions [10]. Chemical vapor deposition techniques utilize a gaseous phase, which reacts chemically to deposit a selected surface [11]. It has been particularly useful for inorganic thin films [12]. However, the required high temperatures and high powers can damage polymer structures leading to undesirable coating characteristics, thus, modifications of the method have been developed [13]. By definition, laser ablation is removal of material from the surface of sample/target material. The advantage of laser treatments is that a precise control of width and depth is possible during processing, and they enable the processing of specific areas of the sample [14]. Ultra short pulsed laser deposition (USPLD) is a technique, which utilizes pulsed high power laser beam vaporization of target coating material in vacuum chamber to coat a selected substance with a thin film of the deposited material [15]. This technique has the advantage in giving possibility to produce materials with complex stoichiometry and narrow distribution of nanoparticle size [16]. In this study, Saos-2

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osteoblast behavior on variable coatings produced by USPLD technique was investigated.

2. Materials and methods

2.1. Materials and cell culturing

Alpha-modified Eagle's medium (α-MEM), L-glutamine, fungizone and penicillin/streptomycin were obtained from Euroclone (Pero, Italy). Fetal bovine serum was from Hyclone UK (Thermo Scientific, Cramlington, UK). TrypLE was purchased from Invitrogen (Taalstrup, Denmark). All the other chemicals were from Sigma-Aldrich (Steinheim, Germany).

Human osteosarcoma-derived osteoblast-like cells (Saos-2) at cell density of 250,000 cells/10 cm plate were cultured as monolayers until they reached 90–95% confluency in cell culture medium, which contained α-MEM supplemented with FBS, penicillin/streptomycin and L-glutamine at 37 °C in a humidity incubator with 5% CO_2 and 20% O_2. The cells were washed once with PBS, and the cells were detached with TrypLE at 37 °C for 5 min. Then 50,000 Saos-2 osteoblasts were resuspended in 1 ml of cell culture medium in 1.5-ml Eppendorf tube, and seeded on top of the different coated surfaces. The morphology of osteoblasts grown on regular polystyrene cell culture plastic (seeding density 50,000 cells/35 mm plate) was used as a reference for morphology inspection. The experiments were repeated three times, always using a new patch of Saos-2 osteoblasts.

2.2. Preparation of the coatings

The deposition of different coatings on (100) silicon wafers 4 in in diameter was carried out using USPLD technique [17]. Before deposition, the wafer surfaces were cleaned with Ar^+ ion sputtering (SAM-7KV, Minsk, Belarus) operated at 4.5 kV and 30 mA for 5 min with a scanning. For deposition, a mode-locked fiber laser (Corelase Ltd, Tampere, Finland) and Coldab™ deposition technology (Picodeon Ltd Oy, Helsinki, Finland) were utilized in order to attain optimal laser parameters for USPLD [18]. The maximum average power was 20 W at 4 MHz, which results in a 5 μJ pulse energy. The pulse length was 20 ps. High-purity graphite (>99.9%, Carbone Lorraine, Paris, France) was used as the target for amorphous diamond deposition, while for titania (TiO_2) films high-purity titanium (99.9%, Koch-Light Laboratories Ltd, Colnbrook, England) in reduced oxygen vacuum of 10^{-4}–10^{-2} mbar was used. For alumina (Al_2O_3) film deposition, high-purity ceramic target was exploited. In the case of carbon nitride (C_3N_4) deposition, a high-purity sintered C_3N_4 target (Carbodeon Ltd, Helsinki, Finland) was utilized. The deposition parameters were adjusted to obtain a stable plasma plume, typically 15% above the threshold fluence for ablation. Deposition time was adjusted to obtain approximately 150 nm thick deposition layer. After deposition, the samples were cut into the size of 10 mm × 10 mm. The samples were then ultrasound-cleaned for a few minutes in 2% 7X® PF detergent (MP Biomedicals Ltd, Solon, OH, USA), and rinsed many times with deionized water.

2.3. Atomic force microscopic characterization of the surfaces

Surface roughness measurements were done with a PSIA XE-100 (Park System Corp., Suwon, South Korea) atomic force microscope (AFM) to scan the surfaces at nanometer scale [19]. Average surface roughness profiles were recorded randomly at three different locations on six samples per group, using the scan size of 2 × 2 μm^2. The scan rate was below 0.25 Hz, and the Z voltage gain varied between 1 and 3. The data from the images were analyzed with the instrument's own software.

2.4. Contact angle measurements

The contact angles were determined using the sessile drop (15 μl) method with a custom made apparatus, which consists of an optical microscope SZ-PT Olympus equipped with digital camera Olympus Camedia C-3030ZOOM (Olympus Corp., Tokyo, Japan). In brief, the contact angles for water were measured within 5 s after placing the water drop on the surface at 22 °C and 45% relative humidity. The drop image was stored in a digital camera, and image analysis software (www.gimp.org) was used to calculate the left and right margin contact angles of five sessile drops of each sample to obtain the average of the contact angle [19].

2.5. Zeta potential measurements

An electrokinetic analyzer (SurPASS, Anton Paar GmbH, Austria) was used to determine the zeta-potential of different coatings [19]. The streaming potential/current is generated when an electrolyte circulates through the measuring cell and is forced to flow through a small gap (about 100 μm) formed between the surfaces of two identical sample surfaces. Two Ag/AgCl electrodes were placed at both sides of the sample, and used to measure the potential difference between the ends of channel. The measurements were performed using 1 mM KCl as an electrolyte solution at a pH of 7.4 ± 0.2.

2.6. Scanning electron microscopic analysis of films and the osteoblasts

Scanning electron microscope imaging and energy-dispersive X-ray spectroscopy (EDS) analysis of the coated surfaces were carried out using a Hitachi S-4800 FE-SEM (Hitachi Science. System Ltd., Japan) equipped with an EDS detector at an accelerating voltage of 5–10 kV.

Forty-eight hours after cell cultivation on the different surfaces in 24-well plate, the cells attached to the different surfaces were fixed with 2.5% w/v glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature after washing with PBS. The samples were then dehydrated with series of gradually increasing concentration of ethanol (50% for 5 min, 70% for 5 min, 80% for 5 min, 90% for 5 min, 94% for 5 min, and 99% twice for 5 min). Finally, the specimens were treated with hexamethyldisilazane twice for 5 min. The specimens were then air-dried, covered with gold by sputtering (Sputter Coater E5100, Polaron Equipment Ltd, England) [20], and imaged by a scanning electron microscope XL30 ESEM TMP (FEI Company Philips Ab, Eindhoven, the Netherlands) at an accelerating voltage of 20 kV. The surface area covered by the osteoblasts was analyzed from the digitized scanning electron microscopic images with software Image J (http://rsbweb.nih.gov/ij/).

2.7. Cell proliferation assay

The cell proliferation after cultivation on various surfaces was assayed with MTT colorimetric assay. Briefly, after 48 h cultivation, the cells attached to the surfaces were carefully transferred to a new 24-well plate, and 2 ml of fresh medium containing 0.5 mg/ml of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added after one wash with PBS, and the medium was discarded after 3-hour-incubation at 37 °C. The samples were then carefully washed with PBS, then the formed MTT formazan salts were dissolved in 1 ml of dimethylsulphoxide/ethanol (1:1, v/v), and the absorbance was measured at 595 nm with a 96-well plate reader.

2.8. Statistical analysis

One-way ANOVA (SPSS, 16.0) variance analysis followed by Tukey Post-Hoc Test was used to examine the statistical significance of the