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Ion implantation induced nanotopography on titanium and bone cell adhesion

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ABSTRACT

Permanent endo-osseous implants require a fast, reliable and consistent osseointegration, i.e. intimate bonding between bone and implant, so biomechanical loads can be safely transferred. Among the parameters that affect this process, it is widely admitted that implant surface topography, surface energy and composition play an important role. Most surface treatments to improve osseointegration focus on micro-scale features, as few can effectively control the effects of the treatment at nanoscale. On the other hand, ion implantation allows controlling such nanofeatures.

This study has investigated the nanotopography of titanium, as induced by different ion implantation surface treatments, its similarity with human bone tissue structure and its effect on human bone cell adhesion, as a first step in the process of osseointegration. The effect of ion implantation treatment parameters such as energy (40–80 keV), fluence (1–2 e17 ion/cm^2) and ion species (Kr, Ar, Ne and Xe) on the nanotopography of medical grade titanium has been measured and assessed by AFM and contact angle. Then, in vitro tests have been performed to assess the effect of these nanotopographies on osteoblast adhesion.

The results have shown that the nanostructure of bone and the studied ion implanted surfaces, without surface chemistry modification, are in the same range and that such modifications, in certain conditions, do have a statistically significant effect on bone tissue forming cell adhesion.

1. Introduction

A wide variety of implants are used today in the clinical field, with titanium and its alloys the material of choice in bone engaging load bearing components. This is because titanium offers the best combination of bulk mechanical and surface properties, especially in relation to osseointegration [1]. On the other hand, shortcomings in terms of implant failure and patient treatment times, i.e. time required for osseointegration to happen and thus allow a safe loading of the implant, have led the development of implant surface modifications of surface chemistry, physical properties and topography to promote a faster and more complete osseointegration [2].

Macro and micro scale surface modifications are regularly applied on a commercial basis on Ti made implants to promote osseointegration, e.g. sandblasting, acid etching based solutions and its combinations, thermal spraying (either atmospheric plasma spraying of hydroxyapatite or titanium plasma spraying) and others. One accepted route in attempting to enhance bone differentiation and promote fast and direct bone growth on implants is based on studying the underlying reactions at nanoscale [3–14]. Among the different techniques under study, ion implantation based treatments have also been explored in recent years [15–17]. Some of the difficulties many of these techniques find when trying to explain the effect of nanoscale modifications on osseointegration lie in discerning between the separate effects of topographical, chemical and physical surface modifications [3,18], and in the study of the events occurring at the material and living tissue interface at the nanoscale [19].

The goal of the present study was to test the hypothesis that the nanotopography modifications of Kr, Ar, Ne and Xe ion implanted titanium surfaces under different processing condition have an

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effect on cell adhesion in vitro and those nanostructural features are in the same range of the inorganic phases of human bone tissue.

2. Materials and methods

2.1. Materials: titanium samples

Grade 4 titanium, in accordance with ASTM F67 and ISO 5832.2 (Acnis International, Villeurbanne, France), discs with a diameter of 8 mm and 3 mm thick were cut, ground and mirror polished. Samples were then sonicated in acetone and ethanol, before the ion implantation treatments. The chemical composition of the alloy was: 0.01%C, 0.28%O, 0.01%N, 0.001%H, 0.40%Fe and Ti balance.

2.2. Ion implantation

Ne^+, Ar^+, Kr^+ and Xe^+ ion implantation treatments were performed in a 1090 Danfysik high current ion implanter (Danfysik A/S, Jyllinge, Denmark) with gaseous ion sources. For each ion species three different treatment conditions were applied: 1×10^{17} ion/cm^2 fluence at 40 keV acceleration voltage, 1×10^{17} ion/cm^2 at 80 keV and 2×10^{17} ion/cm^2 at 40 keV. Samples were referenced as "nIONm", where n stands for fluence $\times 10^{17}$ ion/cm^2, ION for the ion species implanted, and m for the voltage used. The angle of ion incidence was always perpendicular to the sample surface. Non-ion implanted samples, subjected to the very same process conditions, other than ion beam exposure, were used as control.

2.3. Surface characterization

Atomic force microscopy AFM nanoscope IIIa (Veeco Bruker, MA, USA) was used. Rectangular cantilever type probe RESP (Bruker, MA, USA) with 290 kHz tuning frequency with a reflective backside cantilever Au-coating and a nominal tip radius of 12 nm was used in tapping-non-contact mode for surface imaging. The scan velocity was 0.5 Hz.

The surface roughness was analyzed with Nanoscope 1.20 software. All images were sequentially plane-fitted to correct for the sample slope and flattened to remove low frequency noise. Roughness analysis was applied to each image, statistical values were calculated according to the peaks, valleys and plateau of a surface with different numerical parameters. Roughness was measured in 1 μm × 1 μm boxes. Images were sectioned to learn about their surface profiles and to determine the peak to peak distance.

The topography of human bone tissue and ion implanted dental implant bone interface were also characterized by AFM, with the same Nanoscope IIIa Atomic Force Microscope working in contact mode. Samples were obtained of the posterior zone of the mandible from a 52-year-old male patient after a period of three months since implant placement in the framework of the 277/06/EC clinical trial (approved by the Spanish Agency of Medicines and Medical Devices and the corresponding Committees of Ethic and Clinical Research, and conducted according to the ethical principles for medical research in humans as adopted by the Helsinki Declaration, the regulations of directive 93/42/CEE relative to medical devices and the circular number 07/2004 regarding clinical research with medical devices of the Spanish Agency of Medicines and Medical Devices). Before AFM analyses, samples had been dried at 37 °C, dehydrated on different consecutive ethanol solutions and embedded in low temperature curing resin, before being cut and polished, following a method reported elsewhere [20].

2.4. Wettability tests

The hydrophilicity/hydrophobicity of the treated and control samples were assessed by contact angle measurements. The contact angle was measured by the static sessile drop method, using a Digidrop Contact Angle Meter (GBX Instruments, Bourg de Peage, France). A water drop was deposited at the surface of the sample, which was observed using a video camera and determined using image analysis software (Microsoft AFX-Digidrop Windows, USA). Five measurements were carried out on each sample, and the average values were calculated.

2.5. Cell culture and adhesion

hFOB 1.19 cells were obtained from the American type culture collection and were cultured in accordance with ATCC recommendations, namely at 34 °C in a 1:1 mixture of DMEM (high glucose) and Hams F12 supplemented with 0.3 mg/mL G418, 10% foetal bovine serum (FBS), 100 U/mL penicillin and 10 mg/mL streptomycin. This immortalized cell line displays osteoblast-specific phenotypic markers and mineralizes extracellular matrix (ECM).

For all experiments, similar passages were used from passage 5 to passage 10. The cells were cultured at 34 °C in a humified atmosphere of 5% CO_2. After reaching confluence of about 80%, the cells were trypsinized and seeded. For the assay, 1.5×10^4 cells were seeded onto the 8 mm diameter Ti samples, and incubated for 4 h and 24 h ($n = 3$). Besides, cells were also plated on untreated Ti-samples ($n = 5$) as a control. In all experiments, Ti samples were first washed with soap and water and sterilized with EtOH, in the same clean room (Class 10,000) environment where the cell culture test were carried out.

Cell adhesion was assessed using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay, an established method for the spectrophotometric measurement of cell proliferation as a function of mitochondrial activity in living cells [21]. After each endpoint, Ti samples were transferred to a new well plate and 10 μL of WST-1 solution were added to each well containing 100 μL of tissue culture media. After 4 and 24 h incubation at 34 °C, the optical density of the solution in each well was measured at a wavelength of 450 nm using a microplate reader (Biotek, Powerwave XS, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Results of the wettability, cell culture and adhesion tests were subjected to statistical analysis, which established the statistical significance of the differences in number of adhered cells and the contact angle between the ion implanted samples and the untreated control samples, by means of the Student's t test (significance for $p < 0.05$; strong significance for $p < 0.01$).

3. Results

AFM analysis of the ion implanted samples showed marked differences in nanoroughness depending on the ion species implanted and the ion implantation process conditions (Fig. 1). The induced nanoroughness ranged from Ra 0.5 nm for the Ne ion implanted samples (1Ne80), close to that of the polished untreated control samples with Ra 0.3 nm, to Ra 7.3 nm for the Xe ion implanted samples (2Xe40) (Table 1). The peak to peak distance for the ion implanted samples evaluated ranged from around 67 to 217 nm.

The contact angle measurements showed that most of the treated surfaces became more hydrophobic, as compared to the control sample, although the change was small, i.e. it was only statistically significant for the samples 2Ne40 and 2Xe40 (Table 1; $p < 0.05$). The contact angle varied most for titanium samples ion

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