Materials Science and Engineering C 44 (2014) 254–261

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Zirconia coated titanium for implants and their interactions with osteoblast cells

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A R T I C L E I N F O

Article history:
Received 12 March 2014
Received in revised form 11 July 2014
Accepted 8 August 2014
Available online 17 August 2014

Keywords:
Zirconia
Anodic plasma-electrochemical oxidation
Implants
Osteoblasts
Bone sialoprotein
Osteocalcin

A B S T R A C T

The anodic plasma-electrochemical oxidation in aqueous electrolytes of $Zr(SO_4)_2$ was used to prepare new zirconia/titania-based surfaces **M1** (Ti, Zr and O: 7–10, 22–27 and 65–69 at.%) and **M2** (Ti, Zr and O: 11–13, 20–23 and 64–69 at.%). The chemical composition and the microstructure of these coatings were characterized by surface and solid state techniques such as scanning electron microscopy, electron probe microanalysis, Raman spectroscopy and X-ray diffraction. These mixed oxides of ZrO_2/TiO_2 surfaces consist up to 84% (m/m) of ZrO_2 and 16% (m/m) of TiO_2. Monoclinic zirconia was detected as the dominant microcrystalline phase. *In vitro* studies were conducted on primary human osteoblast cells. MTT and DAPI assays were used for assessment on cell proliferation. Immunohistochemical analyses of morphology, cell cluster formation and expression of bone sialoprotein (BSP) and osteocalcin (OC) were performed. Novel surfaces **M1** and **M2** induced proliferation and expression of OC and BSP similarly to Ticer, used in clinical practice. Furthermore, the presence of zirconia on titanium surface has a higher beneficial effect on the osteoblast morphological changes and cell cluster formation.
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1. Introduction

In modern dentistry the gold standard for replacing missing teeth is in many cases the introduction of dental implants in the jaw bone (endosseous dental implants) [1]. Those implants consist mainly of commercially pure titanium (Ti cp). The advantages of titanium implants are primarily in reasonably good biocompatibility and high mechanical stability [2]. Interactions of the bone matrix and osteoblasts with implants are influenced with the surface characteristics of biomaterials [3,4]. It was reported that the surface roughness of titanium implants affects the rate of osseointegration and biomechanical fixation [5,6]. Namely, surface topography, surface charges, composition, chemical states and wettability have a profound effect on implant–bone interaction. Various methods are used in preparation of dental implants (e.g. acid etching, plasma spraying, anodization, grit blasting, or combination techniques) [7–9]. Depending on the modification methods, obtained implants may possess diverse and distinctive surface properties affecting the host-to-implant response.

Despite high stability of the titanium dioxide on implant surfaces, it should be mentioned that some studies show a measurable increase of titanium ions in peri-implant tissue. Moreover, some authors confirmed significantly increased concentrations of titanium in the lung, liver, spleen and kidney after insertion of titanium implants [10]. In the process of anodic oxidation under spark discharge in different solutions the surface is enriched with other ions. This process offers fewer opportunities for the migration of the titanium ions in peri-implant tissue. Furthermore, by coating of materials with tantalum, niobium or zirconium migration of ionic species can be reduced by several orders of magnitude compared to conventional materials [11].

Beside improvement of titanium-based materials, there is an increased interest for a development of implants on the basis of zirconium oxide. Zirconia has mechanical properties suitable for dental use and comparable to those of stainless steel. Excellent biocompatible properties and the tooth-like color are further advantages of zirconia [12]. Nevertheless, the clinical use is limited due to poor osseointegration. To improve osseointegration the surface of such implant systems has to be modified which may lead to the formation of cracks and surface damage, followed with the total fractures in those implants. Zirconium-based implants are not causing inflammation response. Additionally, individual cells (giant cells) were unresponsive in the matrix, without formation of a fibrous capsule around implants [13,14]. Moreover, adhesion of bacteria is proved to be reasonably low [15,16]. Zirconia implants with titanium or titanium oxide coating of the intraosseous part are described [17]. The invention relates to implants having an implant body of zirconia, and a surface coating of titanium oxide. In this investigation, neither problems of mechanical stability of zirconia nor toxic potential of titanium oxide are solved.

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http://dx.doi.org/10.1016/j.msec.2014.08.032
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In the presented study novel titanium implant materials with zirconia coatings are prepared by anodic plasma-electrochemical oxidation. Namely, in this process both the mechanical stability of titanium and the biological properties of zirconia are conserved. The development of this implant surface is based on modern and reliable implants and quickly healing surfaces. *In vitro* studies were conducted on primary human osteoblast cells. MTT and DAPI assays were used for determination of cell proliferation. Immunohistochemical analysis of morphology, cluster formation and expression of bone sialoprotein (BSP) and osteocalcin (OC) was performed.

2. Materials and methods

2.1. Preparation of the coatings

For the investigations' specimens five different materials were used: two new **M1** and **M2** and three referent materials: the Ticer surface [18], chemical pure titanium Ti cp and a solid zirconia Cercon [19]. All samples are round blanks of 6 mm in diameter and 0.5 mm high. Ti cp and the Cercon were obtained as kind gifts from ZL Microdent, Germany, and were used without any treatment. Ticer was prepared as described in literature [18]. For the preparation of novel coatings **M1** and **M2** the anodic plasma-electrochemical coating process was employed.

Before the coating process titanium samples were chemically polished for 10 s in a mixture of 85% phosphorus, 38% hydrofluoric and 53% nitric acid ($H_3PO_4/HF/HNO_3$) in a ratio of 49/24/27% (V/V/V), and ultrasonically cleaned in water followed by 2-propanol rinsing. The coatings were prepared in an electrochemical cell by the anodic spark deposition technique using a DC Delta Elektronika BV power supply SM 300-5 assisted by a Hameg function generator HM 8131-2 for the pulse generation [20]. As electrochemical cell a 250 ml double-wall glass beaker was used, equipped with the titanium samples as the anode and a platinum wire as the cathode. The samples **M1** and **M2** were obtained by a coating of Ti cp in aqueous solutions of 0.124 M and 0.062 M $Zr(SO_4)_2$ electrolyte, respectively. The commercially used anodic Ticer was generated by a coating of Ti cp in aqueous solutions of 0.12 M $Ca(H_2PO_4)_2$ electrolyte yielding formation of titanium oxide along with hydroxyapatite on the surface. At first an anodic prespark film with 50 mA/cm^2 was potentiodynamically formed until sparking occurred. The anodic spark current density was up to 0.9 A/cm^2. The electrolytes were continuously magnetically stirred and the electrolyte temperature was thermostatically controlled and maintained at 30 °C. Afterwards, the specimens were rinsed in distilled water and 2-propanol, dried and stored in air.

2.2. Surface characterization

For characterization, specimens were examined by a scanning electron microscope JEOL JSM 840 A (SEM) in planar view. To determine the concentration of the chemical elements electron probe microanalysis (EPMA) was carried out on a Cameca SX100. Raman spectroscopy was performed on a DILOR XY with an optical multi-channel analyzer. The spectra were excited by the radiation of the 514.5 nm line of an argon laser with 30 mW power. X-ray diffraction patterns (step size 2 = 0.01°) were collected at room temperature on a Bruker D8-advanced diffractometer working with CuKα radiation and equipped with a one-dimensional silicon strip detector (LynxEye™).

2.3. Cell seeding and culture conditions

All procedures used in this study were approved by the Ethics Committee of the University of Leipzig (No. 086-2008) and performed according to the rules of the Declaration of Helsinki from 1975 (revised in 1983). Human mandibular bone samples without any clinical or radiographic pathological evidence were obtained from one male donor, who was undergoing lower wisdom tooth surgery at the Department of Oral, Maxillary, Facial and Reconstructive Plastic Surgery at the University Hospital of Leipzig. The bone sample was placed in a sterile tube containing 0.05 M sterile phosphate buffered saline (PBS) at pH 7.4, and penicillin/streptomycin at 100 IU/ml each (PromoCell, Heidelberg, Germany). Subsequently, all samples were processed under sterile conditions. The bone samples were cut into 0.1 × 0.1 cm pieces. After rinsing several times in PBS, the material was incubated with 0.25% (m/m) collagenase type IV (166 U/mg; Biochrom, Berlin, Germany) for 30 min at 37 °C. Afterwards, suspension was discharged and the rest incubated for 2 h in the presence of 0.25% (m/m) collagenase type IV at 37 °C. Then, the cells were washed, centrifuged (300 *g* for 10 min) and cultured in an Osteoblast growth medium (PromoCell) and supplemented with 10% fetal bovine serum (PromoCell) in an atmosphere of 5% CO_2 at 37 °C. The medium was changed twice a week, and cells were grown to confluence in culture flasks (Greiner Bio-One, Frickenhausen, Germany). Thereafter, the cells were subcultured from initially isolated primary cells and seeded at a density of 4000 cells/ml (2000 cells/well) in chamber slides [21].

2.4. Experimental design

96-Well plates were used for MTT assay and eight chamber slides for all other experiments. Five sterile implant materials (round discs: diameter 6 mm, thickness 0.5 mm) were tested. Thus, novel materials **M1** and **M2** and three referent clinically-employed materials (Ti cp, Ticer and Cercon) were examined. Each experiment was performed in triplicate. For each MTT or immunocytochemistry assay one 96 well plate or eight chamber slide, respectively, with the osteoblast cells was removed from the incubator on day 3, 5, 7 or 10. Additionally, for immunocytochemistry assay the cells were fixed in paraformaldehyde (4% in PBS) for 15 min and rinsed in PBS.

2.5. Proliferation assays

2.5.1. MTT assay

Osteoblast cells were seeded at a density of 5000 cells/ml (2000 cells/well) in 96 well plates already containing investigated materials. Cells were incubated at 37 °C in 5% CO_2 for 3, 5, 7 and 10 days. MTT assay was performed as previously described [22]. Before measurement, liquid contents from the wells were transferred in new 96 well plate, and then absorbance was measured at 570 nm using a 96 well plate reader (Tecan Spectra, Crailsheim, Germany).

2.5.2. DAPI assay

Cells attached to the materials from the eight chamber slide were rinsed several times with PBS. Then, the cell nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Serva, Heidelberg, Germany) in order to quantify cell proliferation as the number of cells on the investigated surfaces [21].

2.6. Morphology of the cells

The cells were stained with acridine orange (AO, 15 μl, 3 μg/ml) and the cell morphology was determined as described previously [23]. In short, cell morphology was determined by measuring the footprint area of the cell on the surface after attachment and using a shape factor, $\phi = (4\pi A) / p^2$ (A = footprint area; p = the perimeter of the cell).

2.7. Cell cluster formation

Cells grown of different materials in the eight chamber slide were rinsed several times with PBS and then the cell nuclei were stained with DAPI (Serva). Sections were screened at 100× magnification.

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