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Biological effect of hydrothermally synthesized silica nanoparticles within crystalline hydroxyapatite coatings for titanium implants

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ABSTRACT

Development of functional coatings for artificial bone implants that strengthen the osseointegration and accelerate bone healing processes is urgently needed in the biomedical field. In this study we present biological effect of novel composite coatings with different concentration of silica nanoparticles within crystalline hydroxyapatite matrix (HAp-SiO_2) synthesized on titanium under hydrothermal conditions. Samples were analyzed for their elemental composition, structure, bioactivity and *in vitro* cytotoxicity. The results indicate the formation and homogeneous distribution of silica nanoparticles on the surface of hexagonal hydroxyapatite (HAp) crystals. The coatings show improved bioactivity in comparison with pure HAp after 4 days of immersion in simulated body fluid (SBF). The responses of human osteoblast-like cells (MG-63) cultured onto the synthesized materials provide evidence that HAp-SiO_2 composites exhibit good biocompatibility. We propose that this is because HAp-SiO_2 composites favor biomineralization process with cell proliferation remaining unaffected, regardless of the amount of silica. Furthermore, SEM and fluorescence measurements demonstrate that HAp-SiO_2 had positive effect on cell morphology, favoring cell adhesion.

1. Introduction

Titanium and some of its alloys are materials of choice for orthopedic and dental metallic implants. This is due to their beneficial mechanical properties, such as relatively low modulus, excellent strength to weight ratio, toughness, ductility, and formability. Additionally, they exhibit superior biocompatibility and corrosion resistance [1]. Despite these beneficial characteristics, metallic implants suffer from some disadvantages, discussed extensively by Alvarez and Nakajima [2]. The material surface lacks from biological recognition and without a strong chemical bonding with the living tissue implant is poorly stabilized and the micro motions occurring at the implant-bone interface can lead to its loosening. The wear of the material, as well as biocorrosion, may lead to shedding of the metallic ions or particles which in turn can cause inflammation and immune response in the adjacent tissue [3]. These limitations can be addressed with the dedicated surface coatings that protect the implant material while at the same time increase surface biocompatibility [4].

Hydroxyapatite (HAp) is the most commonly used coating material, as well as the main constituent of bone-tissue scaffolds [5–7]. In spite of bioactivity and good biocompatibility, in some *in vivo* tests bonding of HAp to the surrounding bone is much weaker than for bioactive glass or tricalcium phosphate [8]. In order to enhance the bioactivity of synthetic HAp and create chemical composition more similar to the mineral component of natural bone, dopant additions or ionic substitutions are often used [9–11]. Silicon plays the essential role in metabolic processes involving normal bone and cartilage formation and preventing their degradation. Carlisle in 1970 [12] identified abnormal bone formation due to deficiency of Si. This was later confirmed by Schwarz [13] who recognized Si as crosslinking agent in the connective tissues along with its importance to vascular health. Silicon promotes collagen type I synthesis and osteoblast differentiation. At wounded sites, the concentration of silicon increases several dozens of times facilitating bone repair [14]. According to other reports, bone mineralization also requires some amount of soluble silicon [15]. Furthermore, research assessing patient diet showed a correlation between increased nutrition with silicon and higher bone mineral density (BMD) in the cortical bone of hip [16]. Silica (SiO_2) is the most widespread source of silicon and considered to be non-toxic and highly biocompatible. Hench et al. [17] showed the integral role that silica plays

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in the bioactivity and osteogenic potential of bioactive glass (biomaterials containing 46–60% SiO_2). In addition to the high surface area, silica nanoparticles in their hydrated form exhibit high concentration of silanol groups (Si-OH) exposed on their surface, acting as crystal nuclei for apatites, and therefore strongly influencing the bioactivity. Furthermore, studies on the biological effect of silica nanoparticles reveal their osteoblastogenic activity and ability to suppress bone-resorbing osteoclast differentiation [18]. In the form of biodegradable osteoinductive composite with silk, silica was found to upregulate the osteoblast differentiation marker (bone sialoprotein) and increase the formation of collagen/calcium phosphate extracellular matrix [19]. Moreover, this work also showed that particles of amorphous silica undergo biodegradation and can release biologically active silicon, facilitating bone repair, in contrast to silica-based bulk materials.

In this study, we present novel hydrothermally synthesized HAp coatings enriched with different concentration of silica nanoparticles. We are first to report on the synthesis of silica on the surface of pure HAp crystallized directly on titanium. Our results reveal strong enhancement of nucleation rate of bone-like apatite for HAp-SiO_2 coatings in comparison with pure HAp, and an increase in bioactivity with an increase of silica content. Only one study so far reported on the formation of silica nanoparticles on the surface of calcium phosphate particles during hydrothermal process [20]. Our research therefore provides valuable information on the mechanism of the coatings synthesis.

2. Experimental procedures

2.1. Preparation of silica-enriched hydroxyapatite (HAp-SiO_2) coatings

Titanium substrates were prepared according to the protocol reported by us previously [5]. Three hydroxyapatite coatings with different SiO_2 content were produced by the hydrothermal method with the final molar concentration of TEOS of: 3 mM, 5 mM and 14 mM. Samples were labelled as HApSi3, HApSi5, HApSi14, respectively. Reagent grade calcium nitrate [$Ca(NO_3)_2{\cdot}4H_2O$] (0.1 M) (POCH, Poland), ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] (0.06 M) (POCH, Poland) and tetraethylorthosilicate (TEOS) (Sigma-Aldrich, Poland) were used as Ca, P and silica source, respectively. Additionally, ethylenediaminetetraacetic acid [$Na_2EDTA{\cdot}2H_2O$] (0.1 M) (POCH, Poland) was used as a chelating agent. The concentration of calcium nitrate and ammonium hydrogen phosphate was adjusted to keep the molar ratio of Ca/P = 1.67 (weight ratio Ca/P = 2.15) for all solutions. First, Ca $(NO_3)_2{\cdot}4H_2O$ was mixed with $Na_2EDTA{\cdot}2H_2O$ in 50 ml of distilled water. In another beaker, TEOS was added to 50 ml of $(NH_4)_2HPO_4$ solution. After the reagents were completely mixed, both solutions were combined together and subsequently stirred at room temperature for 10 min. The pH was adjusted to pH = 10 with ammonia. The final stock solution was ultrasonically treated for 15 min and then transferred into 200 ml glass vessel and sealed in an autoclave (Carl Roth 2098.1). The hydrothermal process was carried out in 200 °C for 7 h. Afterwards, samples were rinsed with distilled water and left to dry in air. Stoichiometric hydroxyapatite coating (HAp) was synthesized as a reference sample using the same chemical reagents, excluding TEOS [5], and was labelled as HAp.

2.2. Surface characterization

The Raman spectrometer used in the study was a high resolution confocal micro-spectrometer (Almega XR of Thermo Electron Corp). The chosen excitation light wavelength was 532 nm, objective magnification 100×, and a pinhole aperture of 25 μm. The Raman scattered light was energy-dispersed by diffraction grating and registered with the CCD camera. The spectrometer's spectral resolution was 2 cm^{-1}. The laser spot size was approx. 1 μm and the confocal depth of the microscope was approx. 2 μm, thus the sample volume probed in each spectrum collection run was approx. 2 μm^3. Data was recorded in the spectral range from approx. 100 cm^{-1} up to 4000 cm^{-1}. The X-ray diffraction (XRD) measurements were performed with PANalytical X'Pert Pro diffractometer operating at 30 mA and 40 kV. The radiation wavelength (Cu Kα) was 1.54 Å. X-ray diffraction patterns were taken over the 2Θ range of 20°–80° with a 0.05° step size. The angular resolution of the instrument was calibrated using LaB6 line profile standard (SRM660a-NIST certificate). The sample morphology was examined using secondary electron (SE) detector of a Tescan Vega 3 SEM instrument equipped with a tungsten cathode. Chemical composition of the samples was measured using two instruments, the Energy Dispersive X-ray Spectrometer (EDS) Quantax with XFlash detector (Brucker) and the Proton Induced X-Ray Emission microprobe (μPIXE). The μPIXE analysis was performed using 2 MeV protons from the electrostatic Van de Graaff accelerator, at the Institute of Nuclear Physics, Polish Academy of Sciences, Cracow (IFJ PAN). Rectangular areas (0.25 × 0.25 mm^2) were scanned with the beam of approx. 20 μm in diameter. The semiconductor Si(Li) detector, with the resolution of 160 eV for the energy of 5.9 keV and the active area of 80 mm^2, was placed 25 mm away from the irradiated sample and was operating without any attenuation filter. Obtained data were quantified using the software package GUPIX [21].

2.3. Bioactivity test in simulated body fluid (SBF)

To assess the bone-like apatite formation ability, samples were immersed in SBF at 37 °C for 7 days. The solution was replenished after 2 days of incubation. The SBF was prepared by dissolving reagent grade (POCH, Poland) NaCl, KCl, $NaHCO_3$, $CaCl_2$, $KH_2PO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$ and Na_2SO_4 in distilled water and buffered to pH = 7.42 with trishydroxymethyl aminomethane (TRIS) and 1 M HCl at 37 °C [22]. After incubation samples were left to dry.

2.4. In vitro biological study

2.4.1. Cell culture

Human osteoblast-like MG-63 cells (ATCC, USA) were cultured in EMEM (Eagle's Minimum Essential Medium) growth medium supplemented with 10% inactivated FBS (Fetal Bovine Serum) (ATCC, USA) in humidified atmosphere of 5% CO_2 at 37 °C. Cells were cultured until passage 4 before being used for the study. Cell suspension was obtained by addition of 5% trypsin with EDTA (HyClone, USA). After flushing and centrifugation, cell suspension was concentrated in complete culture medium to 2×10^4 cells/ml. Next, cells were seeded on sterile biomaterials samples placed in 24-well tissue culture plates (ThermoSci, Nunc, Denmark). Prior to the experiment the samples were sterilized by soaking in 70% ethanol, followed by 30 min exposure to UV light on each side.

2.4.2. Cytotoxicity and cell proliferation assays

Cytotoxicity of biomaterials was determined with the bioluminescent ToxiLight Bioassay (Lonza, USA) in accordance with the manufacturer's procedure. Cell proliferation was assessed with ToxiLight 100% Lysis reagent set together with ToxiLight Bioassay kit (Lonza, USA). After 7 days of culture, media from human osteoblast-like cells were collected for the cytotoxicity assay. ToxiLight Bioassay kit was used to measure the levels of AK (adenylate kinase) released into the culture medium from damaged cells. The cells grown on the surface of studied samples were lysed with ToxiLight lysis reagent. Obtained lysates were used for the estimation of total AK concentration with the aid of ToxiLight Bioassay kit. The amount of AK released from all cells in the culture corresponds to the levels of cell proliferation. Cells cultured on commercial tissue culture polystyrene (TCPS) were used as a control sample. Tests were conducted in quadruplicates. All measurements were carried out with microplate reader PolarStar Omega (BMG Labtech, Germany).