CORROSION RESISTANCE AND IN-VITRO OSTEOBLAST RESPONSE OF CALCIUM-PHOSPHATE COATED TITANIUM SUBSTRATES PREPARED BY LASER SINTERING

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Abstract

A rough surface texture was found on laser sintered titanium substrate, which was responsible for instability of the calcium phosphate layer from the point of view of obtaining good adhesivity to the substrate at given preparation conditions. After the mechanical treatment and following soaking of Ti substrate in 3 M NaOH solution, the surface texture was much smoother than in the original sample and a fiber-like titanate layer was created on the surface. This treatment improved the mutual bonding between titanium and the electrophoretically deposited calcium phosphate coating. The final coating contained two major phases – rutile and β–tricalcium phosphate. The deposition of calcium phosphate on Ti substrate significantly enhanced its corrosion resistance. Results of in-vitro testing confirmed a low cytotoxicity of both the NaOH treated and calcium phosphate coated Ti substrates. The calcium phosphate layer increased the in-vitro ALP activity of osteoblasts.

Keywords: calcium phosphate coating, electrophoretic deposition, titanium, laser sintering, in-vitro testing

INTRODUCTION

There is a high degree of biostability of titanium and its alloys, which are widely used as implant materials for failed hard tissue. It is necessary to improve properties such as their lifespan and bioactivity, which is low for pure metal implants. The bioactivity of implants and corrosion resistance can be enhanced by the deposition of biocompatible material e.g., calcium phosphates. For the deposition of calcium phosphate (as, e.g., hydroxyapatite) on the surface of titanium alloys, many methods have been used, including plasma spraying, the biomimetic method (immersion in physiological fluids) [1-3], the sol–gel methods [4,5] electrophoretic deposition or electrochemical (cathodic) deposition [6-10]. The chemically or electrochemically deposited hydroxyapatite layers can be decomposed to other types of calcium phosphates after additional annealing at higher temperatures. This step allows one to obtain good mechanical properties and optimal adhesion with the substrate surface. A cheaper and faster method of deposition of compact calcium phosphate layers are electrochemical methods (electrophoresis, etc.), where the conditions of electrolysis (e.g. electrolyte temperature, applied potential) are crucial from the point of view of the stoichiometry of the deposited calcium phosphate [7,11,12]. Very compact and strong layers (double layered coatings) on titanium substrates have been prepared by optimising the process or by the plasma spraying method [13], [14]; dip-

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coating of gel suspensions of hydroxyapatite or bioglass-hydroxyapatite [15], etc.. The advantage of these methods is the preparation of top layers containing a pure calcium phosphate phase (mainly pure hydroxyapatite), which differ in properties from the lower layers. The lower layers provide good adhesion with the metal surface due to interaction between different phases. Applying the AC-electrophoretic deposition of hydroxyapatite showed some advantage in denser and uniform HA coatings with a lowering of crack formation [16]. Selective laser sintering as rapid prototyping technique was effectively utilized for the preparation of Ti-SiO_2 biomedical scaffolds [17]. Similarly, the direct laser metal sintering fabrication was successfully applied for the preparation of Ti–6Al–4V samples and detailed analysis of microstructure verified the presence of columnar beta grains with alpha and beta laths within the grains. As the result of microporosity, the Young´s modulus was lowered to a value closer to bone [18].

It has been found that the high concentrations of calcium and phosphate ions after amorphous calcium phosphate dissolution can strongly affect the proliferation of osteoblast cells [19]. Osteoblasts were attached and adhered to the hydroxyapatite surfaces, proliferated and secreted extracellular matrix without any toxic effects [20]. The osteoblast detachment strength was surface roughness, fibronectin preadsorption and integrin subunit sensitive [21]. The osteoclastogenic response increased with surface roughness in human osteoclast precursors [22]. The initial cell attachment was not affected by the surface topography of the HA ceramics, but the protein synthesis, ALP (alkaline phosphatase) activity favoured surfaces with a smooth texture [23]. The crystallite size of HA may play an important role in governing the expression of osteoblast activities [24]. Good osteoblast and osteoclast proliferation and cell attachment were observed in pure biomimetically prepared apatite-like coatings [25].

In this paper we studied the corrosion resistance and in-vitro response of MC3T3 pre-osteoblast cell line of electrophoretically deposited calcium phosphate coating on laser sintered titanium substrates.

EXPERIMENTAL PROCEDURE

Nanohydroxyapatite (Hap) for electrophoresis was prepared by the precipitation of 0.5 M $Ca(NO_3)_2$ and 0.5 M $(NH_4)_2HPO_4$ aqueous solutions (molar ratio of Ca/P = 1.66). The $Ca(NO_3)_2.4H_2O$ solution was slowly dropped to an aqueous solution of $(NH_4)_2HPO_4$ in 1.5 hour. The pH at the end of the precipitation was kept at 11 by adding NH_3(aq) (1:1). The rotation of the stirrer was around 450 rpm and the reaction was done at 25°C. Ageing time was 72 h and the final hydroxyapatite powder was washed with distilled water, filtered and dried at 110°C for 2 h.

Samples were made of titanium alloy (Ti-6Al-4V) powder by the layer-by-layer method using the direct metal laser sintering (DMLS) technology (EOSINT M280, EOS GmbH, Germany). The production layer thickness was about 30 μm with laser sintering precision of 50 μm. The other parameters of laser sintering process were the following: sintering speed for the support material equals 400.0 $mm \cdot s^{-1}$, the laser power was 80.0 W, 1250.0 $mm \cdot s^{-1}$ contouring speed of the part, the 150.0 W laser power with the beam offset of 0.015 mm, and the sintering speed of the core at 1250.0 $mm \cdot s^{-1}$, the 170.0 W laser power and the beam offset 0.015 mm.

The surfaces of titanium alloy (Ti2Al3V) sheets of 10 x 20 x 1 mm were ground on 1000 SiC grit paper. The sample surfaces were treated with 3M NaOH solution at 70°C for 1 hour (Ti-NaOH). Following this the sheets were rinsed with acetone in an ultrasonic bath for 5 minutes. Titanium substrate was used as cathode and Pt basket as anode to complete the electrolytic cell. Electrophoresis of calcium phosphate (hydroxyapatite) was carried out in

ethanol - hydroxyapatite suspensions (2 g HAP in 100 mL of ethanol (analytical grade, Merck)) at the current densities of 10 mA/cm^2 for 3 minutes [9]. Suspensions were ultrasonically dispersed for 5 min before electrophoresis. Samples were dried at 70°C for 2 h and annealed at 950°C in air for 20 min. The heating rate was 10°C/min.

The corrosion resistance of samples was evaluated by the Tafel equation from potentiodynamic measurements. Potentiodynamic curves were obtained by the three-electrode scheme, where the sample was used as working, Pt basket as counter and standard calomel electrode (SCE, saturated KCl electrolyte) as reference electrodes. Measurements were carried out in 0.9% NaCl solution at a scan rate of 1 mV/sec using the polarographic analyser PA3 (Czechoslovakia). The phase composition of the calcium phosphate layers was analyzed by X-ray diffraction (Philips X´ PertPro, using Cu Kα radiation). The surface texture and morphology of particles in deposits were observed by field emission scanning electron microscopy (JEOL FE SEM JSM-7000F). The roughness of Ti substrates was determined by a confocal microscope (PluNeox 3D).

MC3T3 mouse osteoblasts (ECACC, Salisbury, UK) were cultured in culture flasks (SPL Life Sciences, Korea) with MEM (Minimum essential medium) with Earles balanced salts, 2 mM L-glutamine (SAFC Biosciences, Hampshire, UK), 10% fetal bovine serum (Sigma-Aldrich) and ATB-Antimycotic (Penicillin, Streptomycin, Amphotericin) solution (Sigma-Aldrich) at 37ºC in 5% CO_2 atmosphere with 95% humidity in an incubator (Memmert). The medium was changed every 2 days. After the cells reached about 80% confluence, they were harvested by trypsinization with 0.25% Trypsin-EDTA (Sigma-Aldrich) solution followed by the addition of fresh medium to create cell suspension. Ti substrates were sterilized in an autoclave at 121°C and they were placed in a 48-well suspension plate, seeded with $2.0x10^4$ cells in 500 µL of complete medium and cultured at 37°C in 5% CO_2 and 95% humidity in an incubator. The cell proliferation was examined using MTS test (Cell titer 96 aqueous one solution cell proliferation assay, Promega, Madison, USA) and scaffolds were evaluated for 48 hours and 6 days after cell seeding. After 4 hours of incubation, the intensity of colouring, which characterizes the formazan concentration in a culture medium, was evaluated using a UVVIS spectrophotometer (Shimadzu 1800) at a wavelength of 490 nm. The measured absorbances of medium from wells with cell seeded substrates were compared with absorbances of medium from wells in the tissue culture polystyrene plate (control, treated 48-well tissue culture plate, Santa Cruz Biotechnology, Santa Cruz, USA). The pure complete culture medium was used as a blank. Acridine orange (AO), (Sigma-Aldrich) used to stain the MC3T3 cells proliferated on scaffolds; the samples were rinsed with phosphate buffered saline solution (PBS); cells were fixed in 96% ethanol for 20 minutes and stained with 0.01% AO solution for 2 minutes in the dark. The cells on substrates were observed using a fluorescence microscope (Leica DM IL LED, blue filter) to investigate the cell distribution on the scaffold. The ALP activity of osteoblasts was determined using the phosphatase substrate (Sigma-Aldrich, 5 mg tablet in 5 mL diethanolamine buffer, pH = 9.8). Attached osteoblasts were lysed by adding 200 µL phosphate buffer saline with 0.1% (v/v) of Triton X-100, 20 mM Tris and 1 mM $MgCl_2$ to each well, followed by freezing at -20°C for 1 h. The 100 µL phosphatase substrate was added to 100 µL of cell lysate and the mixture was incubated at 37°C for 1 h. The alkaline phosphatase reaction was stopped by the addition of 50 µL of 3 M NaOH. ALP activity was measured by the amount of released p-nitrophenol after catalysis of the p-nitrophenol phosphate. The concentration of p-nitrophenol was determined from a calibration curve using UVVIS spectrophotometry at 405 nm. The statistical evaluation of results (n = 3) was performed using ANOVA analysis at level $\alpha = 0.05$.

RESULTS AND DISCUSSION

The surface textures of native, NaOH treated and calcium phosphate coated Ti (Ti-NaOH-CaP) sheets are shown in Fig.1. The native Ti sample surface contains (Fig.1a) a large number of spherical, approximately 20 μm sized Ti particles, which are strongly attached to compact titanium. These particles represent original powder Ti particles, which were only partially melted during laser sintering. The measured roughness R_a of the surface of this sample was about 18 μm. Note that such a surface was not appropriate for hydroxyapatite deposition because of a weak mutual bounding between rough Ti substrate and calcium phosphate particles. The NaOH treatment had a large effect on the surface texture of ground Ti sheets. A continuous and homogeneously microporous layer of fiber sodium titanate was formed during the soaking of Ti in 3M NaOH solution and R_a was ‹ 1 μm. C. Kim et al. [26] found the formation of a similar fine fibrous layer composed of sodium titanate after the immersion of Ti sheets into 5 M NaOH solution. In the case of KOH solutions, nanotubes were observed on Ti surfaces. Besides this, the created titanates significantly improved the hydrophilicity of sample surfaces and alkaline phosphatase activity of osteoblasts cultured in-vitro. Q.L. Feng et al. [27] observed increase in the deposition rate of hydroxyapatite from supersaturated calcification solution on Ti surfaces soaked in NaOH solutions. The annealing of sodium titanate layers at higher temperatures in air improved adhesivity between these ones and underlying Ti surfaces [28].

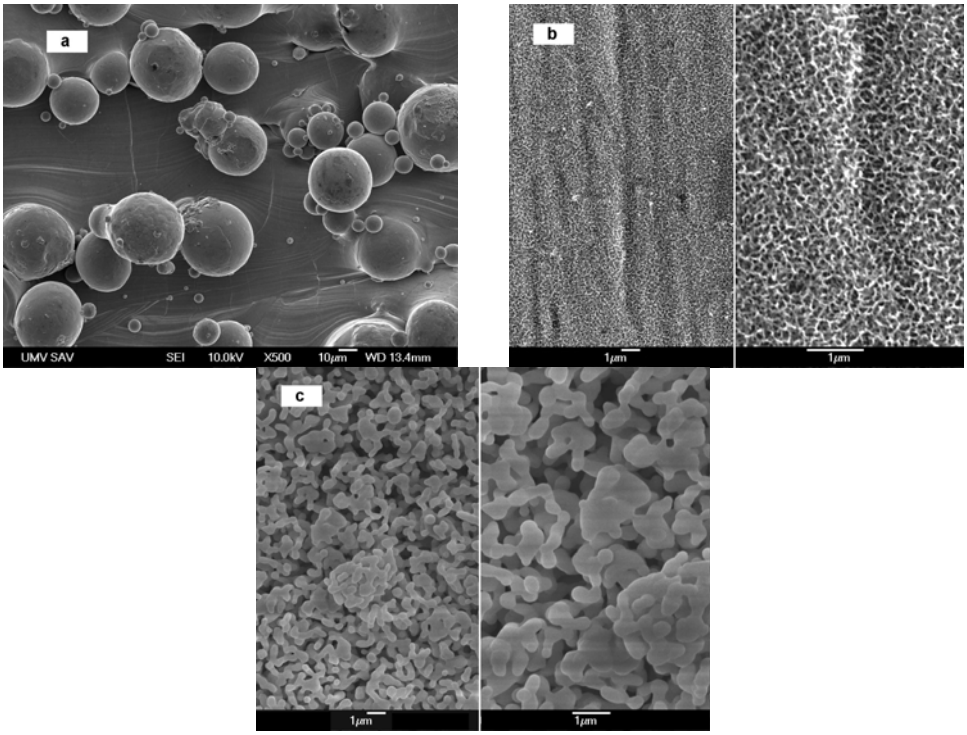

Fig.1. Microstructures of sample surfaces: a) native Ti; b) NaOH-Ti; c) CaP-NaOH-Ti after annealing.

In Figures 1 b,c, the microstructures of calcium phosphate coating on NaOH treated Ti samples after annealing at 950°C for 20 min in air are shown. The surface is uniformly coated with a calcium phosphate layer via the electrophoretic deposition of

nanohydroxyapatite, a higher number of microcracks in size of approx. 10 μm can be visible in the surface texture due to different thermal expansion coefficients. The calcium phosphate particles which resulted in the layer were sized ‹1 μm and in detailed micrograph, partial sintering and coarsening of individual calcium phosphate particles was observed. It is clear from the figures that a high fraction of micropores is present in the coating.

Measured potentiodynamic curves of individual differently treated sample surfaces are shown in Fig.2. The location of the corrosion potential (E_{corr}) of native Ti and Ti-NaOH-CaP samples determined from the Tafel equation were at -0.192 and -0.167 V respectively. A small potential shift of Ti-NaOH-CaP corrosion potential to the anodic region verified a lower ability of calcium phosphate coated Ti surface to oxidation. Similarly, the corrosion currents (i_{corr}) of Ti- NaOH-CaP sample (i_{corr} =12 nA/cm^2) was approximately five times lower than i_{corr} of native Ti sample (56 nA/cm^2). Thus the corrosion resistance of calcium phosphate coated samples was much higher than the uncoated.

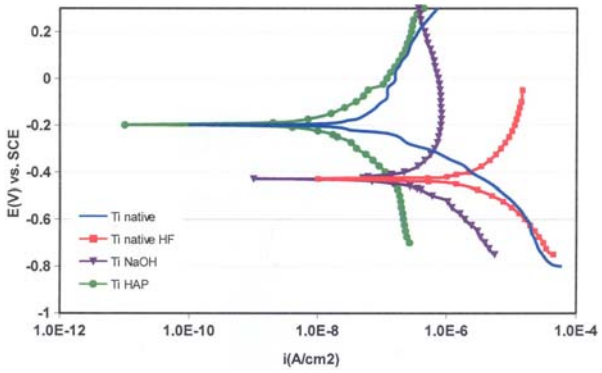

Fig.2. Potentiodynamic curves of Ti substrates after different treatment.

From the comparison of E_{corr} and i_{corr} native Ti and Ti samples cleaned in HF solution (Ti-HF) it clearly resulted that i_{corr} of Ti-HF sample was about two orders higher (2.3 μA/cm^2) than i_{corr} of native Ti, whereas the corrosion potential of Ti-HF sample had the lowest value (E_{corr} = -0.430 V) among all measured E_{corr} of samples. A significant rise in i_{corr} of Ti-HF sample confirms the existence of a compact oxide surface layer in the untreated native Ti sample, which is the result of intensive oxidation during the sample preparation by laser sintering. The decrease in i_{corr} to 0.29 μA/cm^2 and increase in E_{corr} to -0.384 V related to the Ti-HF sample were found after soaking of the native Ti sample in 3 M NaOH solution. On other hand, the i_{corr} of Ti-HF sample was higher than i_{corr} of native Ti sample, from which resulted lowering corrosion resistance of the sample due to dissolution and transformation of the compact oxidic shell on the native Ti sample surface. It is clear that a very porous fiber-like surface titanate layer is more permeable and causes susceptibility to oxidation of the underlying clean Ti surface. From the above facts it comes as a result that the Ti-NaOH-CaP sample with calcium phosphate coating had the best corrosion resistance properties. Nie et al. [29] found improving corrosion properties by deposition of the bottom TiO_2 layer and followed by coating with hydroxyapatite. H.W. Kim et al. [30] verified that formation of the first dense TiO_2 layer under hydroxyapatite coating significantly enhances both the bonding strength of coating to Ti surface and its corrosion resistance.

The results of XRD analysis are shown in Fig.3. From the comparison of XRD patterns of the original nanocrystalline Hap powder (JCPDS 24-0033) used for

electrophoresis with the pattern of annealaed calcium phosphate coating at 950°C clearly results in the transformation of hydroxyapatite to well-recrystallized β-tricalcium phosphate (whitlockite, JCPDS 09-0169) and rutile (JCPDS 78-1510). Besides this, a small amount of $Ca_2Ti_5O_{12}$ (JCPDS 33-0315) phase created by the interaction between hydroxyapatite with TiO_2 was present in the final layer. Note that no peaks corresponding to the sodium titanate phase were found in patterns contrary to results shown in ref. [31] after annealing of the Ti-NaOH substrates.

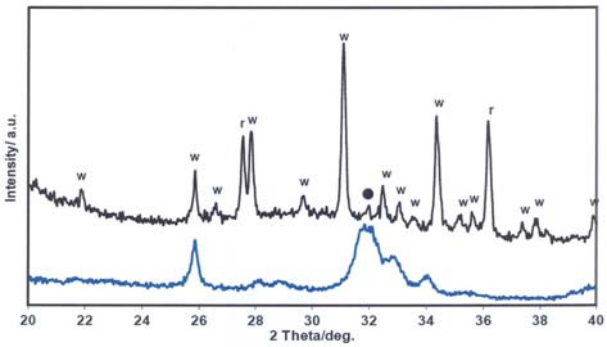

Fig.3. XRD patterns of origin nanocrystalline Hap (bottom curve) and CaP-NaOH-Ti (upper curve). (w:whitlockite, r:rutil,● $Ca_2Ti_5O_{12}$).

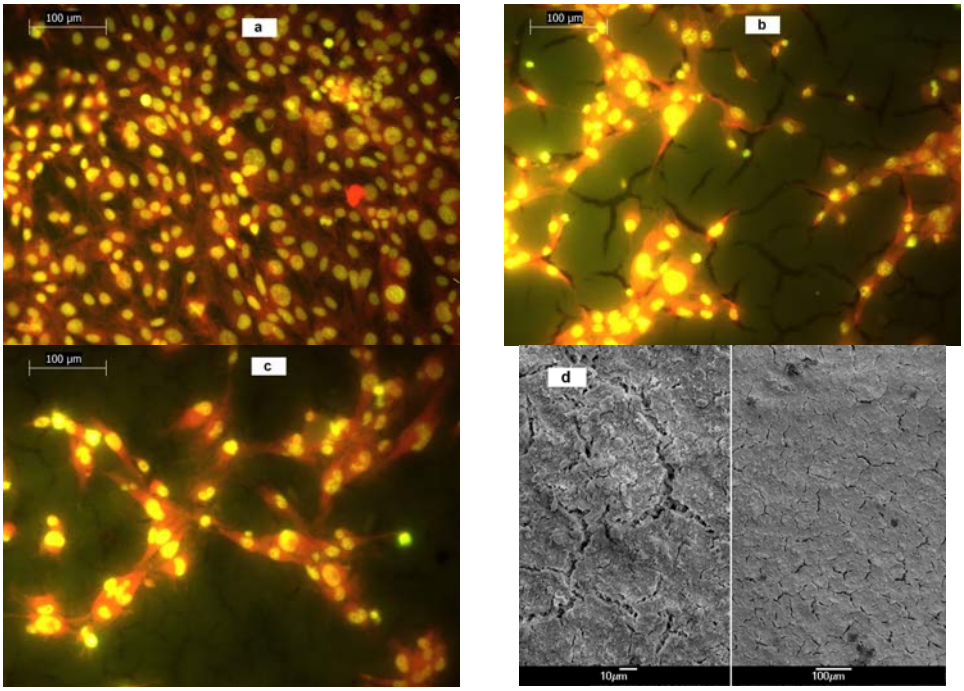

Fig.4. Cell density on surface of native Ti (a), CaP-NaOH-Ti (b) after 3 days proliferation and CaP-NaOH-Ti (c) after 6 days proliferation observed by fluorescence microscopy (acridine orange); microstructure of CaP-NaOH-Ti surface after 6 days of culture.

In-vitro testing verified the low-toxic properties of laser sintered Ti substrates (Fig.4a). In Figure 4b, a high density of osteoblasts was visible on the surface of the NaOH treated Ti substrate after 48 hours of culture. Osteoblast cells were well spread out and adhered to the substrate surface. A lower number of cells were observed on surfaces of the CaP-NaOH-Ti substrates (Fig.4c) after 48 hours of cultivation. Cells are arranged into bundles, which are uniformly distributed on sample surfaces. In the figure, some tendency toward cracks filling and preferential adherence inside or at the edges of microcracks in the calcium phosphate coating are visible. Note that insignificant growth in the osteoblast population was observed after 6 days of culture (Fig.4d), but the width of microcracks was reduced as can be seen in Fig.4d when compared with Fig.1c.

The reduction in microcrack dimensions caused their gradual filling during the production of extracellular matrix by osteoblasts. The 54 and 35% proliferation of osteoblasts in relation to the control sample (wells with seeded cells without sample) were found on NaOH-Ti and CaP-NaOH-Ti samples after 48 hours of cultivation at 37°C in complete medium. The ALP activity after 48 hours of culture was approx. 3-fold in the CaP-NaOH-Ti sample (7.9 nmol/min/μg of protein) than in NaOH-Ti (1.1 nmol/min/μg of protein) or control samples, which confirms the high concentration of this enzyme in osteoblasts proliferated on calcium phosphate surfaces and enhancing of the bioactivity of Ti samples after the calcium phosphate deposition. Note that differences in proliferation or growth of the cell number were affected by surface texture, porosity and surface roughness. The CaP layer had a much higher roughness and porosity than NaOH-Ti substrate and this fact was probably the reason for the decrease in growth of the cell population on CaP-Ti surfaces with cultivation time. It has been found that the protein synthesis and ALP (alkaline phosphatase) activity favoured surfaces with a smooth texture [23]. Apart from this, the crystallite size of HA may play an important role in governing the expression of osteoblast activities [24] and coarser hydroxyapatite or α- and β- tricalcium phosphate support the cell viability of osteoblast cells [32], contrary to nanosized calcium phosphates.

CONCLUSION

The laser sintered titanium substrates had a large surface roughness (R_a = 18 μm) corresponding with the average diameter of spherical Ti particles joined to the compact surface in the sintering process. On the surface of untreated substrates was verified a compact protected oxide layer, which improved the corrosion behaviour of these samples. The CaP-NaOH –Ti substrates showed about 5 times and more than 100-fold lower corrosion current than the native Ti and cleaned Ti-HF surfaces, respectively. Thus, the corrosion resistance of laser sintered titanium substrates was significantly increased by the calcium phosphate deposition. The original nanocrystalline hydroxyapatite was transformed to two major phases – the rutile and β–tricalcium phosphate after annealing at 950°C. Both the NaOH treated and CaP coated Ti substrates had a low cytotoxicity and the ALP osteoblast activity was enhanced with calcium phosphate layer deposition.

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