BIOMIMETIC Ca-P COATINGS OBTAINED BY CHEMICAL/ ELECTROCHEMICAL METHODS FROM HANKS' SOLUTION ON A Ti SURFACE

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Abstract

The purpose of this study was to investigate the bioactivity of porous calcium phosphate coatings on titanium prepared using a two-step procedure (chemical etching or anodic oxidation of Ti followed by soaking in simulated body fluid or direct electrodeposition from Hanks' solution). In order to evaluate the potential use of the coatings for biomedical applications, the adsorption of serum albumin, the most abundant protein in the blood, and the attachment of living cells (osteoblasts, U2OS) were studied.

Keywords: *biomaterials, biomimetic, surface analysis, protein adsorption, U2OS cells*

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Introduction

The main requirements for metallic biomaterials, including titanium, are: (a) biocompatibility, (b) resistance to biological corrosion and (c) antisepticity. Those requirements may be met by developing perspective titanium biomaterials with surface layers of strictly-defined microstructure, chemical and phase composition. Recently, various surface modifications have been applied to form a bioactive layer on a Ti surface, which is known to accelerate osseointegration [1]. Chemical processes for modifying surfaces of Ti and its alloys are widely employed to increase the biocompatibility of those materials [2]. Methods, such as Ti etching in alkaline solutions (e.g. NaOH [2,3]), acidic solutions (H_2SO_4 [3,4]) or hydrogen peroxide (H_2O_2 [5]) at high temperatures, combined with subsequent prolonged soaking of samples in artificial physiological solutions (SBF- Simulated Body Fluid, Hanks' solution) of pH~7, allow to obtain porous oxide layers with built-in calcium and phosphorous ions [6-8]. Electrochemical processes are also commonly applied for modifying surfaces of Ti in order to increase its biocompatibility [9,10]. The chemical composition of the resulting coatings is close to that of hydroxyapatite, which is known to support bone osseointegration and ingrowth when used in orthopaedic or dental applications [1,7]. The anodic polarization at constant voltage of Ti and its alloys in acidic or neutral solutions containing fluorides is a typical electrochemical method for obtaining oxidized layers of uniform chemical composition, different thickness and refined nanoporosity [11-13]. The addition of a suitable fluoride concentration to an electrolyte ensures that a porous morphology is obtained, in the form of titanium dioxide nanotubes [11-13]. Such structures can provide very promising substrates which increase biological tolerance, since it is possible to precisely control the thickness of the layers (by the end voltage of the anodic polarization) and their surface morphology (porosity). Further chemical treatment aimed at introducing additional factors increasing biocompatibility, such as ions of calcium and phosphorus, can be carried out by immersing the oxide layers in artificial physiological solutions [14,15]. To evaluate a potential use of the Ca-P coatings, thus obtained, for biomedical implants protein adsorption and living cells attachment were examined. Serum albumin, the most abundant protein in blood, and U2OS cells were used in this study [8,16,17].

Materials and methods

a) Material substrate: Ti foil 0.25 mm-thick (99.5% purity, Alfa Aesar, USA).

b) Chemical pretreatment: the samples were soaked in a 3 M NaOH aqueous solution at 70°C for 24 h, or in an H_3PO_4 + H_2O_2 solution (with a volume ratio of 1:1) at room temperature for 24 h.

c) Electrochemical pretreatment: titanium oxide nanotube layers were fabricated by anodic oxidation of Ti in an optimized mixture of NH_4F (0.86 wt.%) + DI water (47.14 wt.%) + glycerol (52 wt.%) electrolyte at room temperature, applied voltage V_{max} = 20 V, 2 h. After anodization, the samples were annealed in air at 600°C for 2 h.

d) Deposition of calcium phosphate coatings: soaking in Hanks' solution (7 days, at 37°C) or electrodeposition from Hanks' electrolyte (E = - 1.5 V vs. OCP, for 2h 15 min). The Hanks' solution was prepared by dissolving reagent-grade (g/L): NaCl 8.00, KCl 0.40, $Na_2HPO_4 \cdot 2H_2O$ 0.06, KH_2PO_4 0.06, $MgSO_4 \cdot 7H_2O$ 0.20, $NaHCO_3$ 0.35, $CaCl_2$ 0.14 into distilled water and buffering at pH = 7.4.

e) Surface characterization: SEM (Hitachi S-5500), XPS (Microlab 350, Thermo Electron), FTIR (Nicolet iN10-MX, Nicolet 6700, Thermo Electron Scientific).

f) Biological tests: bovine serum albumin (BSA) (Sigma, purity of 99.8%) was dissolved in phosphate buffered saline - PBS, pH=7.4 and used as a model protein. Human osteosarcoma U2OS cells were used to evaluate the biocompatibility of the Ca-P coatings under study. Dulbecco's modified eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% of a penicillin/streptomycin mixture was used as a cell culture medium. Cells were seeded on the sample surfaces at 1.0 × 10^4 cells/cm^2 and cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 120 h. Cells were transiently transfected with memb-mCherry 24 h before observation allowing the expression of fluorescent mCherry protein localized in the cell membranes. FuGENE HD (Roche Diagnostics, Switzerland) was used as a transfection reagent according to the manufacturer's recommendations. Cell nuclei were then stained 1 h before observation with Hoechst 33342 (Life Technologies, USA) according to the manufacturers' instructions. Cell morphology was examined using an optical microscope (Eclipse 80i, Nikon Instruments, Tempe, AZ).

g) All samples were sterilized by autoclaving at 121°C for 20 min prior to the cell culture experiments [18].

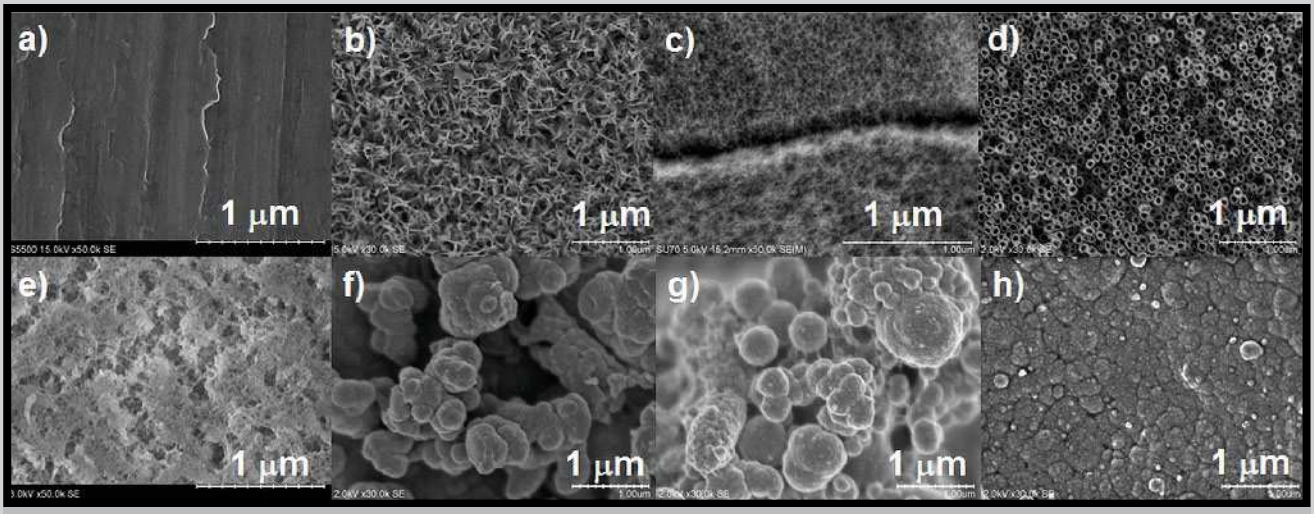

FIG. 1. SEM images of un-treated Ti (a); chemically treated in NaOH solution, 24 h at 70°C (b); H_3PO_4 + H_2O_2 solution: 24 h, room temperature (c); electrochemically treated in 0.86 wt.% NH_4F+glycerol+water DI electrolyte: 20 V, 2h (d); electrodeposited in Hanks' solution: - 1.5 V, 8000 s (e) and after subsequent immersion in Hanks' solution: 7 days, temp. 37°C, pH = 7.4 (f, g, h) – top view.

TABLE 1. $Ca2p_{3/2}$, $P2p_{3/2}$ and O1s binding energies as determined from corrected XPS spectra after chemical/electrochemical treatment in Hanks' solution, and estimated Ca/P atomic ratio.

Ti surface modification	$Ca2p_{3/2}$ / eV	$P2p_{3/2}$ / eV	O1s / eV	Ca/P at.% ratio, average volume
NaOH pretreatment and soaking in Hanks' solution for 7 days	347.5 – 347.9 / Ca^{2+}	132.6 – 133.4 / PO_4^{3-}	531.1 – 531.6 / O_2^-	1.08
H_3PO_4 + H_2O_2 pretreatment and soaking in Hanks' solution for 7 days				1.09
after electrodeposition in Hanks' solution (- 1.5 V vs. OCP)				1.10
anodic oxidation pretreatment (20 V) of Ti and soaking in Hanks' solution for 7 days				1.37

Results and Discussions

SEM images of Ti before and after chemical/electrochemical pretreatment are shown in FIGs. 1a, b, c, d. The reference sample is smooth (pure Ti, FIG. 1a), with no particular morphological features. In contrast, immersion in 3 M NaOH at 70°C for 24 h results in the formation of a 'coral-like' topography (FIG. 1b). The surface layer exhibits a developed, rough morphology characterized by a network of sharp-edged pores. After pre-treatment in H_2O_2 + H_3PO_4 at room temperature, the morphology is quite different, and seems to be less developed (with shallower 'valleys') than that obtained after NaOH pre-treatment. Ti treated in H_2O_2 + H_3PO_4 exhibits sponge-like porosity, as FIG. 1c suggests. A distinct texture with round nano-sized pores is clearly seen. The nanopores are uniformly distributed across the surface. The optimized anodization conditions resulted in the formation of TiO_2 nanotubes (hollow cylinders) arranged perpendicularly to the substrate and separated from each other, as shown in FIG. 1d. SEM examinations revealed that the nanotubes were open at the top. It is generally accepted that rough and porous surfaces can stimulate nucleation and the growth of calcium phosphates. SEM images of a typical morphology of calcium phosphate coatings obtained on pretreated Ti by chemical/electrochemical methods are shown in FIGs. 1e, f, g, h. After immersion for 7 days in Hanks' solution, the Ca-P surface layer formed is composed of many spheroidal particles tightly packed together (FIGs. 1f, g, h). The Ca-P coating formed on TiO_2 nanotubes is denser, and seems to be better crystallized than on Ti chemically pretreated in alkali and acidic solutions.

As the SEM investigations show, an electrodeposited calcium phosphate coating exhibits a completely different morphology characterized by a network of longitudinal pores of different shapes (FIG. 1e). All the obtained morphologies are similar to that reported in the literature [5,6,9,10,15,19], and seem to be promising for biomedical applications.

XPS measurements were then performed to evaluate the chemical state of the calcium and phosphorous in the coatings deposited on chemically/electrochemically treated titanium.

TABLE 1 shows the binding energies of the O 1s, Ca $2p_{3/2}$, and P $2p_{3/2}$ signals, and the suggested chemical composition of the coatings. Position of the main peak of P $2p_{3/2}$ may change within a range of 132.6-133.4 eV for all coatings. The spectral data for Ca suggest as well the presence of calcium phosphate compounds (Ca 2p3/2: 347.5 - 347.9 eV). The main component of the O 1s peak at BE = 531.1 - 531.6 eV is attributed to PO_4^{3-} groups. X-ray photoelectron spectroscopy analysis revealed that the surface is enriched in calcium and phosphorous, where the Ca/P molar ratio is 1.08 (NaOH solution), 1.09 (H_2O_2 + H_3PO_4), 1.10 (electrodeposited layer) and 1.37 (TiO_2 nanotubes). This is less than the stoichiometric hydoxyapatite ratio of 1.67. However, our EDS results show that the atomic concentration ratio of Ca/P for the all samples may vary from place to place, oscillating around the average value indicated above. However, higher Ca/P ratios (up to 1.62, 1.64 and 1.75, respectively) were also measured locally.

TABLE 2. Results of local EDS analysis (Ca/P atomic ratio) of calcium phosphate coatings deposited on pure Ti or pretreated Ti from Hanks' solution by chemical/electrochemical methods.

EDS results	Ca/P at.% local ratio	Ca/P at.% ratio, average volume
Ti/Ca-P -1.5 V vs. OCP	1.17 – 1.64	1.38
Ti(NaOH) + Ca-P chemical treatment in NaOH solution + immersion in Hanks' medium	1.14 – 1.62	1.29
Ti(H_3PO_4+H_2O_2) + Ca-P chemical treatment in H_3PO_4+H_2O_2 solution + immersion in Hanks' medium	1.15 – 1.75	1.33
Ti(TiO_2 NT) + Ca-P electrochemical treatment in NH_4F+glycerol+water electrolyte + immersion in Hanks' solution	1.13 – 1.75	1.34

TABLE 2 presents the average value of the Ca/P molar ratio – 1.38 for electrodeposited Ca-P coating on Ti, 1.29 for Ti(NaOH), 1.33 for Ti(H_2O_2 + H_3PO_4) and 1.34 for Ti(TiO_2 NT). It is worth to mention that, the XPS measurements provide surface information from the few uppermost nanometers of the samples. This may suggest that a nucleation of CaP phases with lower Ca/P ratio is limited to the outermost surface only. This observation does not concern calcium phosphate layers obtained on TiO_2 NT. The differences in the thickness and crystallinity of the titanium oxide layers fabricated by chemical etching and anodic polarization may play a role here [20].

The differences in the molar Ca/P ratio may result from different formation stages within the bulk comparing to those in the outermost layer of the coating. Local supersaturation and pH fluctuation during precipitation of the hydroxyapatite generally cause the formation of metastable transient phases, where Ca/P ratio may change from 1.2 to 2.2, which then transform into HAp in the process of hydrolysis [21,22]. The estimated molar Ca/P ratio by EDS measurements suggest formation of octacalcium phosphate (OCP, Ca/P = 1.33), and probably various intermediate Ca–P phases [23]. The OCP compound is thought to be a precursor for the crystallization of bone-like apatite/hydroxyapatite [21].

To evaluate the potential use of our materials for biomedical applications, we examined protein adsorption on the surfaces studied. Serum albumin (SA) was used in this study, as it is the most abundant protein in blood. FTIR spectra of SA adsorbed on titanium before and after chemical/electrochemical modification are presented in FIG. 2.

Plasma protein was found to interact with the surfaces studied. The bands at ~1650 cm^{-1} and ~1540 cm^{-1} are assigned to amides I and II, respectively [24]. However, changes in the shape and frequency of the amide II band for the electrodeposited Ca-P layer are noticeable. This may be a result of some changes in the protein tertiary structure (3D) due to the interactions with the investigated surfaces.

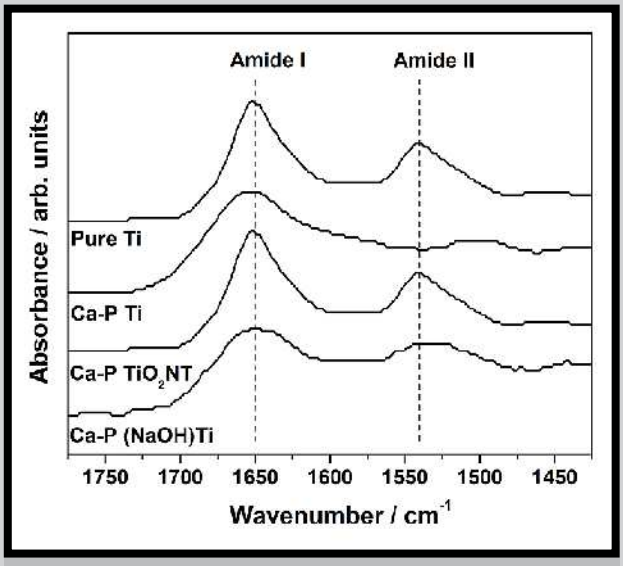

FIG. 2. FTIR spectra of amide I and amide II bands of SA adsorbed on pure Ti and on chemically/electrochemically-treated Ti.

The electrodeposited Ca-P coating seems to have a different effect on adsorbed SA conformation than does the Ca-P layer obtained by chemical methods, but the implication of this finding is not clear at present. The chemistry and morphology of the substrates may play a role here.

FIG. 3 shows the cell morphology on an un-treated Ti surface and on Ti after chemical/electrochemical pretreatment (with a calcium phosphate coating). Fluorescence microscopy observations revealed that the cells are well-extended, and exhibit an elongated morphology, similar to those on pure Ti (reference sample). Nuclei are clearly defined (dark gray color), although cell membranes form a dendritic like-structure (light gray color).

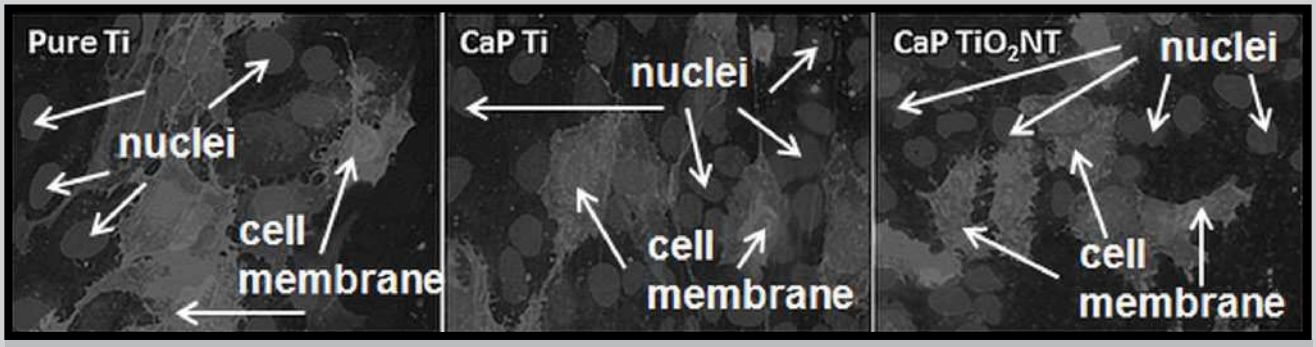

FIG. 3. Fluorescence microscopy images of U2OS cells cultivated for 120 h on pure Ti (a), an electrodeposited Ca-P coating on Ti (b), and a Ca-P layer covered with TiO_2 nanotubes (c). Cells express fluorescent mCherry protein localized in the cell membranes (light gray) and cell nuclei (dark gray) are stained with Hoechst 33342.

After 120h of incubation the cells on presented coatings exhibited cytoplasmic links, as shown in FIG. 3. Ca-P coatings induce cell membrane extension, as shown in FIG. 3, meaning that the cells adhered well to the Ca-P coated surfaces [17,25]. Preliminary results of the response of human osteosarcoma U2OS cells to the surfaces investigated are in qualitative agreement with the protein adsorption experiments.

Conclusions

The present investigations show that biomimetic calcium phosphate coatings of porous apatite-like structure can be grown following a specific morphology by chemical/electrochemical methods from Hanks' solution on a titanium surface. FTIR investigations showed that serum albumin (SA) adsorbed readily on calcium phosphate coatings. Such a Ca-P layer enhances osteoblast-like cell attachment as well. Thus, one may anticipate that chemically/electrochemically Ca–P coatings on Ti may promote early bone apposition and implant fixation by enhancing the adhesion between new bone and the surface of an implant.

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