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Titanium dioxide nanotube films
Preparation, characterization and electrochemical biosensitivity towards alkaline phosphatase

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ABSTRACT

Titania nanotubes (TNTs) were prepared by anodization on different substrates (titanium, Ti6Al4V and Ti6Al7Nb alloys) in ethylene glycol and glycerol. The influence of the applied potential and processing time on the nanotube diameter and length is analyzed. The as-formed nanotube layers are amorphous but they become crystalline when subjected to subsequent thermal treatment in air at 550 °C; TNT layers grown on titanium and Ti6Al4V alloy substrates consist of anatase and rutile, while those grown on Ti6Al7Nb alloy consist only of anatase. The nanotube layers grown on Ti6Al7Nb alloy are less homogeneous, with supplementary islands of smaller diameter nanotubes, spread across the surface. Better adhesion and proliferation of osteoblasts was found for the nanotubes grown on all three substrates by comparison to an unprocessed titanium plate. The sensitivity towards bovine alkaline phosphatase was investigated mainly by electrochemical impedance spectroscopy in relation to the crystallinity, the diameter and the nature of the anodization electrolyte of the TNT/Ti samples. The measuring capacity of the annealed nanotubes of 50 nm diameter grown in glycerol was demonstrated and the corresponding calibration curve was built for the concentration range of 0.005–0.1 mg/mL.

1. Introduction

Although electrochemical growth of titanium oxide nanotubes (TNTs) on titanium and titanium alloys has a history of 14 years [1], the electrolytes, processing conditions and morphology of these nanostructured films are still an open discussion [2–7]. Also, several functionalization procedures of these films were proposed for various applications [8–12]. In the field of medicine, these functionalized surfaces can be used as biosensors for protein measurement in diagnostics of diseases.

Alkaline phosphatase (ALP) is an enzyme present mainly in the liver, osteoblasts and placenta, and its activity and expression are clinical references in the diagnosis of hepatic or bone diseases. The bone ALP represents a biochemical marker of bone formation and general osteoblast activity. This enzyme is also involved in bone mineralization process [13].

The paper defines controlled electrochemical growth conditions in order to obtain predetermined sizes of the nanotubes films, in organic electrolytes, both glycerol and ethylene glycol, for which growth equations are proposed, for exposure times of practical interest (short exposure). The ALP adhesion at the oxide layer interface is favoured by the specific nature, diameter and length of TNT [14].

TNT films grown on three of the most common titanium base materials, titanium and TiAl6V4 and Ti6Al7Nb alloys are interdisciplinary and comparatively investigated, as their mechanical strength, biocompatibility, non-toxicity, corrosion resistance and easy processing [15,16] are well known.

The purpose of this analysis is to select the best support and preparation conditions in order to maximize the nanotube film biosensitivity towards ALP. Therefore, specific morphology, crystallinity and biocompatibility of these films were pursued. Also, among the selection criteria of TNT films, the degree of electrochemical homogeneity and their wetting ability were also considered. All these features are important in the surface response to the presence of protein markers such as ALP enzyme, for direct electrochemical evaluation. Under polarization, specific oxidation and/or reduction processes can occur at the interface or the electrochemical double layer characteristics may be altered and these changes, related to the marker, can be measured by cyclic voltammetry or by electrochemical impedance spectroscopy.

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Table 1
Preparation and features of experimental samples.

Sample	Substrate	Conditions	Features
1	Ti	Ethylene glycol[a], 40 V, 300 min[b]	Cavities, Ø = 85 nm
2	Ti	Ethylene glycol[a], 10 V, 120 min[b]	Ø = 25 nm; l = 1400 nm
3	Ti	Ethylene glycol[a], 15 V, 30 min[b]	Ø = 50 nm; l = 850 nm
4	Ti	Ethylene glycol[a], 15 V, 60 min[b]	Ø = 50 nm; l = 1100 nm
5	Ti	Ethylene glycol[a], 15 V, 120 min[b]	Ø = 50 nm; l = 1990 nm
6	Ti	Ethylene glycol[a], 20 V, 30 min[b]	Ø = 55 ± 5 nm; l = 1000 nm
7	Ti	Ethylene glycol[a], 20 V, 60 min[b]	Ø = 65 ± 5 nm; l = 1300 nm
8	Ti	Ethylene glycol[a], 20 V, 120 min[b]	Ø = 65 ± 5 nm; l = 2200 nm
9	Ti	Ethylene glycol[a], 30 V, 30 min[b]	Ø = 90 nm; l = 1600 nm
10	Ti	Ethylene glycol[a], 30 V, 60 min[b]	Ø = 95 ± 5 nm; l = 1900 nm
11	Ti	Ethylene glycol[a], 30 V, 120 min[b]	Ø = 90 ± 10 nm; l = 3000 nm
12	Ti	Glycerol[c], 2.5 V, 120 min[b]	Ø = 11.5 nm; l = 700 nm
13	Ti	Glycerol[c], 5 V, 120 min[b]	Ø = 17.5 nm; l = 1500 nm
14	Ti	Glycerol[c], 10 V, 120 min[b]	Ø = 45 nm; l = 425 nm
15	Ti	Glycerol[c], 15 V,120 min[b]	Ø = 65 nm; l = 550 nm
16	Ti	Glycerol[c], 20 V,120 min[b]	Ø = 80 nm; l = 560 nm
17	Ti	Glycerol[c], 30 V, 120 min[b]	Ø = 110 nm; l = 650 nm
18	Ti	Ethylene glycol[a], 22 V, 10 min; annealed[d]	Ø = 50 ± 10 nm, l = 500 ± 50 nm
19	Ti6Al4V	Ethylene glycol[a], 22 V, 15 min; annealed[d]	Ø = 50 ± 5 nm, l = 500 ± 50 nm
20	Ti6Al7Nb	Ethylene glycol[a], 15 V, 30 min, annealed[d]	Ø = 52 ± 4 nm, l = 800 ± 100 nm
21	Ti6Al7Nb	Ethylene glycol[a], 15 V, 30 min[b]	Ø = 47 ± 5 nm, l = 750 ± 50 nm
22	Ti	Glycerol[c], 10 V, 120 min, annealed[d]	Ø = 50 ± 10 nm; l = 500 ± 25 nm
23	Ti6Al4V	Glycerol[c], 10 V, 120 min, annealed[d]	Ø = 50 nm; l = 500 nm
24	Ti6Al7Nb	Glycerol[c], 10 V, 120 min, annealed[d]	Ø = 45 ± 5 nm; l = 475 ± 25 nm
25	Ti	Polished titanium plate	–
26	Ti	Ethylene glycol[a], 10 V, 120 min, annealed[d]	Ø = 20 nm; l = 1400 nm
27	Ti	Ethylene glycol[a], 30 V, 60 min, annealed[d]	Ø = 100 nm; l = 1900 nm
28	Ti	Glycerol[c], 5 V, 120 min, annealed[d]	Ø = 20 nm; l = 1500 nm
29	Ti	Glycerol[c], 30 V, 120 min, annealed[d]	Ø = 100 nm; l = 650 nm

[a] 98.45% ethylene glycol, 0.55% NH_4F, 1% H_2O.
[b] As-formed.
[c] 90% glycerol, 9.3% H_2O, 0.7% NH_4F.
[d] 550°C, 1 h.

2. Experimental

The samples, Table 1, were prepared on 10 × 10 mm titanium plates (Al = 0.30; Cd = 0.003; Cr = 0.010; Cu = 0.020; Fe = 0.040; Mg = 0.05; Mn = 0.005; Mo = 0.005; Ni = 0.009; Pb = 0.40; Sb = 0.020; Si = 0.05; Zn = 0.005), Ti6Al4V alloy plates (N = 0.0051; C = 0.030; Al = 5.53; V = 3.90; Fe = 0.13; Si = 0.05–0.1; Ni = 0.01–0.05; Cr = 0.005–0.01; Co < 0.005; Cu ≅ 0.001; Pb < 0.005), and Ti6Al7Nb alloy plates (C < 0.08; N < 0.05; Fe < 0.25; H < 0.009; O < 0.20; Ta < 0.5; Al 5.5–6.5; Nb 6.5–7.5).

Glycerol and ethylene glycol-based electrolytes (90% glycerol, 9.3% H_2O, 0.7% NH_4F and 98.45% ethylene glycol, 1% H_2O, 0.55% NH_4F, respectively, Sigma-Aldrich pro analysis reagents) were used, due to their high viscosity which influences the diffusion of ionic species, the kinetics of nanotubes formation and their morphology. Prior to anodization, metallic plates were initially polished employing emery paper in successive grits of 320, 400 and 600 and finally with diamond paste, degreased by sonication in acetone followed by rinsing with deionised water, and drying in hot air stream.

Anodization was performed in a standard two-electrode bath with circular platinum mesh cathode and MLW DC power source, of 150 V and 10 A. The anodization temperature was fixed to lab temperature, 25 °C. Afterwards, the resulting TiO_2 nanotube films were rinsed with distilled water. Some samples were subsequently heat-treated in a VULCAN 3-350 furnace, in air, at 550 °C for 1 h [17,18].

The nanotube films were investigated by scanning electron microscopy (SEM, QUANTA INSPECT F) equipped with energy dispersive X-ray spectroscopy analyzer (EDAX), high resolution transmission electron microscopy (HRTEM, TECNAI F30 G^2) with a line resolution of 1 Å (selected area electron diffraction (SAED) images were also generated) and X-ray diffraction (XRD, PANalytical X' PERT MPD), in order to define film morphology and crystallinity, as well as the diameter and length of nanotubes. The length was typically measured in a section of the film of nanotubes [19].

The contact angle was measured using a PG-3 goniometer (Klimatest), using double distilled water. Due to the high porosity of the tested samples, the evaluation of the contact angles was carried out in the dynamic sessile drop mode.

Biocompatibility tests were carried out using G292 osteoblastic cells (ATCC CRL-1423) cultured in McCoy's 5a medium (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, in a humidified atmosphere (5% CO_2) at 37 °C. The culture medium was changed every 2 days until cells reached confluence and then were trypsinized with 0.25% trypsin–0.03% EDTA (Sigma-Aldrich). The cells were seeded onto titanium samples or cultured directly on tissue culture polystyrene (TCPS) in a six-well plate at a density of 2×10^4 cells/well, for 24 h. The samples were sterilized at 180 °C for 30 min prior to biological experiments. In order to detect alkaline phosphatase, the cells were fixed in 4% paraformaldehyde for 20 min at 4 °C and permeabilized with 0.5% Triton X-100 in phosphate buffered saline (PBS, Sigma-Aldrich) for 10 min. Cells were then blocked with 2% BSA in PBS for 30 min. Primary antibody against human alkaline phosphatase (Santa Cruz Biotechnology) was added on cells for 2 h at room temperature. After three washes with PBS, cells were incubated with TRITC-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) for 30 min. The nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) and the cells were observed using an Olympus IX71 fluorescent microscope and fluorescence intensity was quantified using ImageJ 1.43 software. The data were expressed as average ± SD (three independent experiments) and analyzed for statistical significance using Student's test. A value of $P < 0.05$ was considered significant.

Electrochemical measurements were performed using a VoltaLab PGZ 301 Radiometer potentiostat connected to a classical three-electrode cell. TNT samples were used as working electrodes (electrode surface at 0.2826 cm^2), a platinum plate was used as a counter electrode and a saturated calomel electrode (SCE) was used as reference electrode. Time evolution of the open circuit potential (OCP) was monitored for 30 min in PBS and several simulated body fluids (SBF): Carter–Brugirard artificial saliva (prepared according to AFNOR/NF (French Association of Normalization) 591-141), synthetic human plasma and synthetic human blood, both supplied by the "Babes-Bolyai" University of Cluj Napoca [20–22]. Cyclic voltammetry measurements were performed in the range of −500–1500 mV vs SCE, with 25 mV/s, without ohmic drop compensation. Impedance spectra were acquired in the frequency range of 100 kHz to 50 mHz. The applied amplitude of the AC potential was 25 mV. The DC potential was 0 mV vs SCE. The analyses and interpretations were based on the Nyquist curves and on a specific parameter denoted L, which represents half of the rough loop diameter measured on the real part axis of impedance, Fig. 18a.

Solutions of bovine intestinal alkaline phosphatase (ALP, Sigma-Aldrich, pro analysis reagent) were prepared with double distilled water just before use, in the range of 0.005–0.1 mg/mL.