Archives of Materials Science and Engineering

Volume 73
Issue 2
June 2015
Pages 53-61

International Scientific Journal published monthly by the World Academy of Materials and Manufacturing Engineering

Functionalized nanotubular oxide layer on Ti6Al4V as a regulator and biosensor of bone tissue remodeling

A. Kaczmarek-Pawelska*, E. Krasicka-Cydzik
Department of Mechanical Engineering, University of Zielona Gora, Licealna 9, 65-417 Zielona Gora, Poland
* Corresponding e-mail address: a.kaczmarek@ibem.uz.zgora.pl

ABSTRACT

Purpose: Our aim was to obtain functionalized nanotubular oxide layer (TNTs) on Ti6Al4V alloy and evaluate its efficiency as a platform for electrochemical biosensor of bone remodeling markers. It was also crucial to examinate does the amount of bonded bALP and BMP-2 and antibodies depends from nanotubes diameter and their electrochemical properties.

Design/methodology/approach: The antibody specific for bALP and BMP-2 were used to functionalize the TNTs on Ti6Al4V. The spectrophotometry and electrochemical measurements (CV and EIS) were used to examinate the functionalization efficiency and comfirmed sensing properties of the functionalized TNTs on Ti6Al4V alloy.

Findings: The obtained results confirmed that TNTs can strongly bind antibodies by physioabsorption and may be a proper platform for biosensing of the selected markers. The protein immobilization efficiency depends over the nanotube diameter and their electrical charge. Thermally modified TNTs with 50 nm diameter on Ti6Al4V strongly bind bALP antibodies and bALP and it can be detected amperometrically. BMP-2 quantitatively binds to the functionalized non annealed charged TNTs with 100 nm diameter, and it is possible to detect it using EIS.

Research limitations/implications: The biosensors presented in this work are simple and fast, but this construction is a prototype and need to be optimized to be used in bone remodelling diagnostics.

Practical implications: Development of the functionalized TNTs on the Ti6Al4V sensitive for physiological concentrations of the bone remodelling markers may be alternative for immunotests in diagnostic of bone diseases. Moreover the TNTs morphology generates nano roughness over the Ti6Al4V surface and functionalized by antibodies strongly bind bALP or BMP-2 and stimulate bone proliferation.

Originality/value: Unique value of this research is the statement the amount of bonded markers and antibodies depends from TNTs diameter and electrochemical properties, and that the prototype of novel biosensor electrode was developed.

Keywords: Materials; Biomaterials; Biosensors; Bone remodelling; Titania nanotubes

Reference to this paper should be given in the following way:

A. Kaczmarek-Pawelska, E. Krasicka-Cydzik, Functionalized nanotubular oxide layer on Ti6Al4V as a regulator and biosensor of bone tissue remodeling, Archives of Materials Science and Engineering 73/2 (2015) 53-61.

MATERIALS

1. Introduction

Absorption of proteins on inorganic biomaterials is a key topic in a wide range of research fields like biochemistry, biophysics, biocoatings and biosensing. Specific composition and morphology of the surface govern the final conformation and the activity of the absorbed protein [1]. Orthopaedic implants and novel diagnostic tests are the most crucial elements of the complex healing process, and they are improved by nanomaterials and nanocoatings. Injuries in the skeletal system are dangerous specially for patients with osteoporosis or younger peoples with bone cancer. Decreasing of bone mass in women begins above 30 years of age and significantly increases after menopause [2]. In the report of Polish Society of Orthopaedics and Traumatology from 2015 osteoporosis is called "silent epidemic". The number of bone fracture causes by this disease increased from 167.663 in 2009 to 2.710.000 in 2010 [3]. The diagnosis of bone diseases is complicate and it is expensive procedure. Bone density measurements are not effective in diagnostic of early stadium of skeletal diseases, so as detection of bone remodelling markers by immunotests like ELISA. Limited access to expensive laboratory diagnostics generates the need for a quick and cheap diagnostic tests. Quantification of markers specific for bone remodeling in eg. bone alkaline phosphatase (bALP) and bone morphogenetic protein-2 (BMP-2) may also support healing of other diseases like cancer and kidney dysfunctions [4-9]. bALP and BMP-2 are released from osteoblast during the ripening of bone matrix [10,11]. The other important issue in healing of bone injuries is increasing number of revisions after implantation, caused by infections, dislocations, and mechanical loosening. Sample statistics for revision after total knee arthroplasty (TKA) and total hip arthroplasty (THA) in the USA are presented on Fig. 1.

Dominant problems in the tissue-implant interaction of the tissue are related with differences in their chemical compositions and reactions on the border between organic and inorganic matter (tissue- body fluid- implant). During the presence of a wide range of metallic implant materials and their protective layers, it is not clearly established which chemical reactions and biological processes occur during tissue overgrowth implant surface. In molecular level the response bone cells to implant materials depends on the topography, physicochemistry, mechanics, and electronics of the implant surface and the influence for cells behavior, such as adhesion, proliferation, shape, migration, survival, and differentiation [15]. Research on biochemical bonds and osteointegration with metallic implants is a very difficult scientific challenge and still an open task. Titanium alloys are still commonly used in surgery and their oxidation is still the most proper method for isolation the metallic surface from the environment [16,17]. Depending on the forming conditions the oxide layers have a varied morphology (compact, porous, nanotubular), thickness, roughness, chemical composition and wettability [18-20]. Titanium surface modifications with respect to the topography and chemistry induce differential inflammatory response [21,22]. Oxide nanotubes (TNTs) formed on titanium and its alloys by anodization are able to support in e.g. antibiotic therapy, smooth muscle cell proliferation, or growth factors delivery [23,24]. The creation of nano-roughness on implants by formation of TNTs *in vitro* induce multiplication of osteoblast, endothelial cells and smooth muscle cells [26,27]. The wettability and electrical charges of titanium oxide nanotubes layer plays a crucial role in the biomedical applications and adhesion of cells. TNTs with diameters from 50 up to 100 nm present excellent biocompatibility, although there are still controversial results regarding the optimum nanotubes diameters for biomedical applications [28].

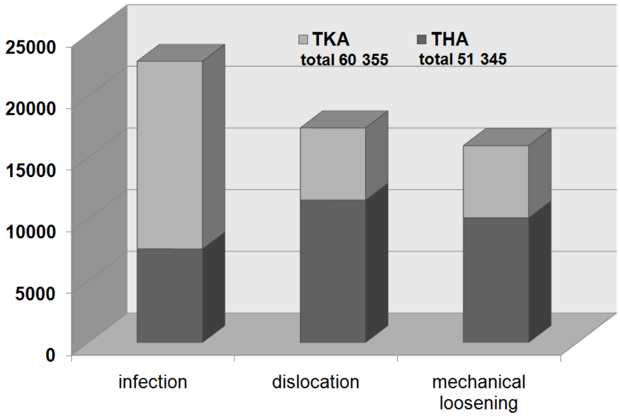

Fig. 1. US National Inpatient Sample (NIS) statistics- revision of total hip (THA) and knee (TKA) arthroplasty [12-14]

Oxide nanotubes specially from TiO_2 and vanadium oxides accept of bioactivity presence excellent sensing properties [29,30]. There is a possibility to use biological activity and sensing properties of oxide nanotubes made from titanium and vanadium to create a double functional surface- biosensor for bone remodeling markers and bone tissue stimulator. Titanium and vanadium reach nanotubular layer on the popular implant alloy - Ti6Al4V, after functionalization by antibodies specific for bone remodeling markers: bALP and BMP-2, should be the proper platform for detection of bone remodeling markers and support bone in-growth into the surface of surgical implants. Binding of proteins to the surface in practice is carried by using three mechanisms: chemical linkers; physiosorption (different surface and protein charge), and linker-free immobilization [25]. In our research to bind proteins into the TNTs layer on Ti6Al4V alloy we use the second mechanism, due to the presence of charges in nanotubular oxide layer.

In present work our aim was to produced functionalized nanotubular oxide layers with different diameters on Ti6Al4V alloy and evaluate its efficiency as a electrochemical biosensor. It is assumed that the amount of bonded bALP and BMP-2 and antibodies depends from nanotubes diameter and their electrochemical properties.

2. Materials and methods

Scope of the work included three stages: preparation of oxide nanotubes on the Ti6Al4V alloy; functionalization by antibodies specific for bALP and BMP-2; evaluation of the antigen binding efficiency and sensing properties by specific antigen-protein reaction (Fig. 2).

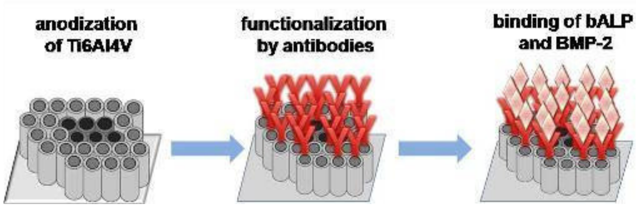

Fig. 2. Schematic illustration of research stages

2.1. Preparation of nanotubular oxide layer on the Ti6Al4V alloy

It was decided to investigate nanotubes with diameters 50 and 100 nm, due to the contradictory reports in the literature describing the relation between the diameter and the amount of bound protein and the rate of osteoblasts growth.

Samples of the Ti6Al4V alloy Gr5 (5×10×0.5 mm) were cut off from the alloy sheet (William Gregor Co., UK), sonicated in acetone, deionized (DI) water and dried in nitrogen stream. Anodizing and electrochemical tests were performed with the AutoLab PGSTAT 302N potentiostat/galvanostat, equipped with voltage multiplier (to 30 V), Frequency Response Analyzer 2 and Nova 1.8 software. Based on our previous experiments [32-35] nanotubular oxide layer on Ti6Al4V alloy was produced by anodization in organic electrolytes. Anodization was carried out in ethylene glycol (99%, water solution, Sigma Aldrich) to produced nanotubes with 50 nm diameter, and glycerol (99%, water solution, Sigma Aldrich) to obtain 100 nm nanotubes. Both electrolytes contain 0.6% wt. of NH_4F (Sigma Aldrich). Samples were polarized from OCP to 20 V and respectively 30 V with scan rate 500 mV/s and hold at 20 V for 1200 s and at 30 V for 2 h. To convert amorphous titanium and vanadium oxides to more conductive anatase and rutile and change the electrical charge of the layer samples were annealed at 600°C (heating rate 30°C/min) in nitrogen atmosphere for 2 h. After anodization and thermal treatment samples of Ti6Al4V with TNTs layers were characterized using SEM, EDS analysis (JEOL FE-SEM 7600F), wettability measurements (using Klimtest goniometer), and in electrochemical measurements (OCP, CV, EIS). Anodization on Ti6Al4V alloy samples was carried in two-electrode system with sample as a working electrode and platinium foil as a counter electrode. All electrochemical examinations were carried out in 5 ml of 0.01 M PBS (pH 7.4) solution room temperature in the three electrodes system, with the sample as a working electrode, platinum foil as a counter electrode, and the Ag/AgCl reference electrode (+0.220 V, 25°C). The EIS spectra were registered in the frequency ranging from 10^4 to 0.1 Hz with the AC amplitude 10 mV at 0 DC bias voltage and the CV curves recorded in potential range from -0.5 to 1.5 V (vs Ag/AgCl) for 30 cycles with the scan rate of 50 mV/s with 0.5 ml of p-NPP in 5 ml of PBS solution.

2.2. Functionalization of nanotubular oxide layer

All functionalization experiments was carried for each antibody on separate samples. All proteins solutions and washing solution were taken from bALP ELISA test (BlueGene) and BMP-2 ELISA test (abcam®).Nanotubular oxide layers on Ti6Al4V with 50 and 100 nm of diameter were functionalized by direct dropping of 20 µl bALP and BMP-2 antibodies onto sample area (5×5 mm). Samples were incubated at 37°C for 1 h and washed 5 times (each wash 180 µl). Average efficiency of bALP antibody immobilization was evaluated by the spectrophotometric measurements of antibody concentrations in 900 µl solution after washing using Spectroflex spectrophotometer at a wavelength 450 nm.

2.3. Evaluation of bALP and BMP-2 binding efficiency and sensing properties

In this step functionalized nanotubular oxide layers were contacted with bone alkaline phosphatase (bALP) and bone morphogenetic protein (BMP-2). To estimate the quantity of bonded proteins to functionalized oxide layer 60 µl of solutions containing bALP and respectively BMP-2 were directly dropped onto the antibody functionalized the sample area. To obtain utility results proteins concentration was close to physiological, for bALP 1.0; 2.5; 5.0; 10.0 ng/ml, for BMP-2 0.5; 1; 2; 3 ng/ml [36,37]. Incubation and washing steps were the same as in previous stage. Average efficiency of bALP and BMP-2 immobilization was evaluated by the spectrophotometric measurements of antibody concentrations in 900 µl after each washing using Spectroflex spectrophotometer at a wavelength 450 nm.

Sensing properties of functionalized nanotubular oxide layer was examined in electrochemical measurements. To evaluate the sensing abilities functionalized TNTs on Ti6Al4V alloy as a platform for detection of bALP a model reaction of conversion of p-nitrophenylophosphate (p-NPP) catalysed by bALP was chosen (Fig. 3). This reaction was also used in testing other sensor materials, for instance gold nanowires [38], in detection of bALP released from osteoblasts with accuracy close to ELISA test [39]. Intensity of oxidation and reduction reactions is proportional to amount of bALP bonded into the functionalized nanotubular oxide layer and can be measured by cyclic voltametry.

The functionalized biosensor Ti64NTs electrodes were examined by cyclic voltammetry in range -0.5 V to 1.5 V with the rate scan 50 mV/s in PBS solution (pH 7.4) with the presence of p-NPP.

Bone morphogenetic protein does not presence any characteristic redox reactions and the amount of BMP-2 bonded into functionalized nanotube oxide layer on Ti6Al4V alloy was measured using electrochemical impedance spectroscopy (EIS) [40].

3. Results and discussion

3.1. Anodization of Ti6Al4V

During anodization of the Ti6Al4V alloy nanotubular oxide layers with diameter of 50 nm ± 10 nm and 100 nm ± 10 nm were formed (Fig. 4a,b). After thermal modification in nitrogen at 600°C for 2h nanotubes on both phases of the Ti6Al4V alloy titanium and vanadium oxides are presented (Fig. 4c,d).

Table 1 shows the results of wetting angle measurement and corrosion potential values for obtained nanotubular oxide layers with 50 and 100 nm diameter before and after thermal treatment. For all obtained TNTs contact angle values are below 90° and they are hydrophilic. After annealing hydrophility of nanotubular oxide layers on Ti6Al4V alloy rise, and the open circuit potential for examinated oxide layer also increased to positive values.

Table 1.
Wettability measurements results for nanotubes oxide layer on Ti6Al4V before and after thermal modification

Nanotube diameter	Non annealed		Annealed	
	50 nm	100 nm	50 nm	100 nm
Wetting angle	43°	64°	24°	32°
OCP	-250 mV	-92 mV	~10 mV	~1 mV

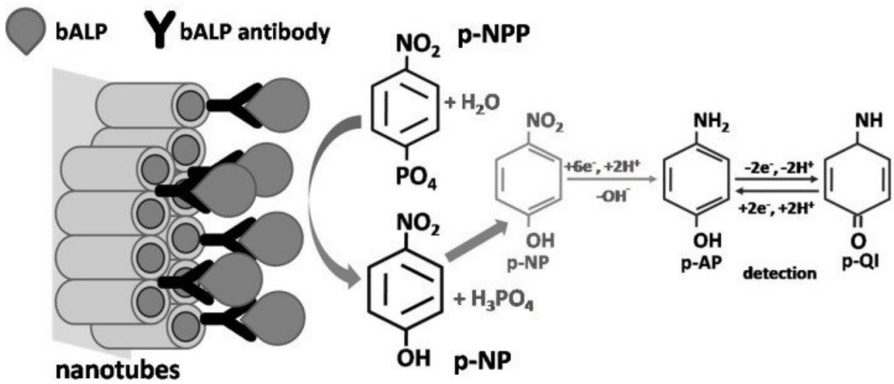

Fig. 3. Scheme of construction bALP biosensor based on functionalized nanotubular oxide layer on Ti6Al4V alloy (p-NPP-p- nitrophenylophosphate, p-NP-p-nitrophenol, p-AP-p-aminophenol, p-QI-p-quinoneimine)

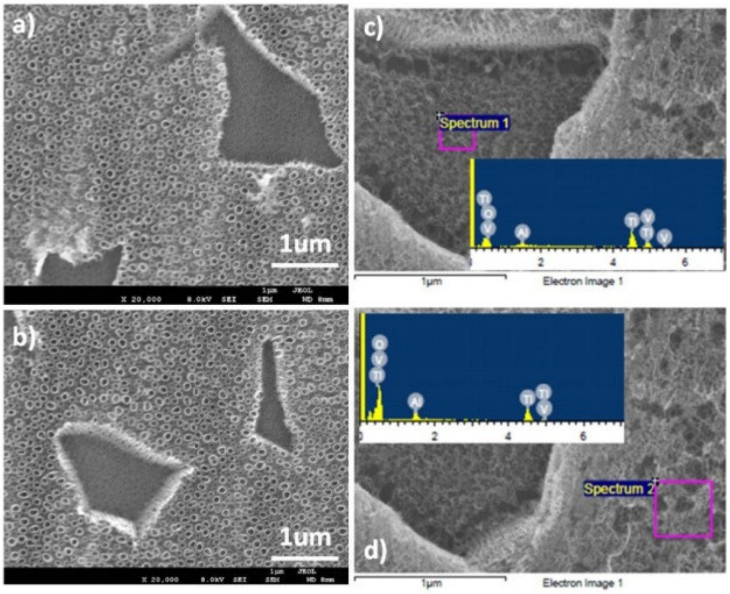

Fig. 4. Nanotubes formed by anodization in 99% ethylene glycol solution with 0.6% NH_4F at 20 V for 20 min with scan rate 500 mV/s with 50 nm diameter (a) and in 99% glycerol solution 0.6% NH_4F at 30 V for 2 h with 100 nm with diameter. EDS analysis of nanotubes after thermal modification at 600°C in nitrogen for 2 h (c,d)

3.2. Functionalization of nanotubes oxide layer on Ti6Al4V

Figure 5 shows average immobilization efficiency of bone alkaline phosphatase (bALP) antibody and different bALP antigen concentrations. During the immobilization procedure, after five time washing bALP antibodies are strongly bonded onto annealed nanotubular oxide layer on Ti6Al4V with 50 nm diameter, the immobilization efficiency is 80%. Immobilization of bALP protein in physiological concentrations: 1.0; 2.5; 5.0; 10.0 ng/ml is the highest also for 50 nm nanotubes thermally modified with positive value of OCP. The immobilization efficiency increased linearly with increasing the bALP concentration. In case of annealed 100 nm diameter oxide nanotubes layer the bALP immobilization efficiency is close to 60%, for the other examined nanotubular oxide layers immobilization efficiency is lower. This behaviour suggest that positively charged nanotubular oxide layer is able to strongly bind by physioabsorption small proteins. Thermally modified nanotubular oxide layer on Ti6Al4V with 50 nm diameter functionalized with bALP antibodies are proper layers for further electrochemical examination as a platform for bALP biosensor.

Immobilization efficiency for bone morphogenetic protein-2 (BMP-2) is presented on Figure 6. BMP-2 antibodies strongly bind with negatively charged non annealed nanotubes with 50 and 100 nm diameter and the immobilization efficiency is around 60%, for thermally modified and positively charged nanotubes efficiency drops below 50%. Similar occurrence is presented for BMP-2 antigen immobilization, which quantitatively bind into non annealed TNTs layer on Ti6Al4V alloy with 100 nm diameter, and its linearly depends over protein concentration. For 50 nm diameter nanotubes without thermal modification the efficiency of immobilization is above 70%, but does not collaborate with BMP-2 concentration.

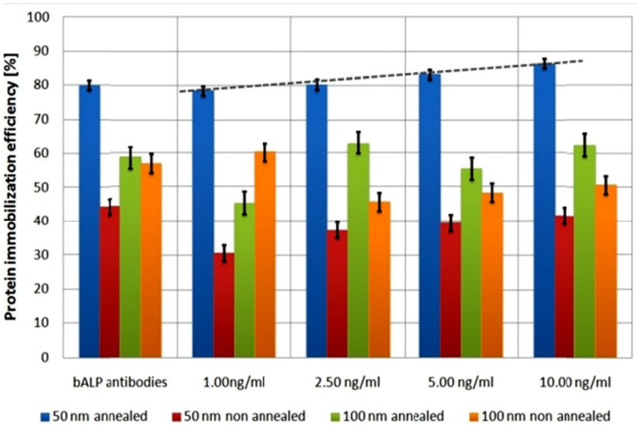

Fig. 5. Average immobilization efficiency for bALP antibody and bALP antigen of different concentrations on the nanotubular oxide layers with 50 and 100 nm of diameter with and without thermal modification

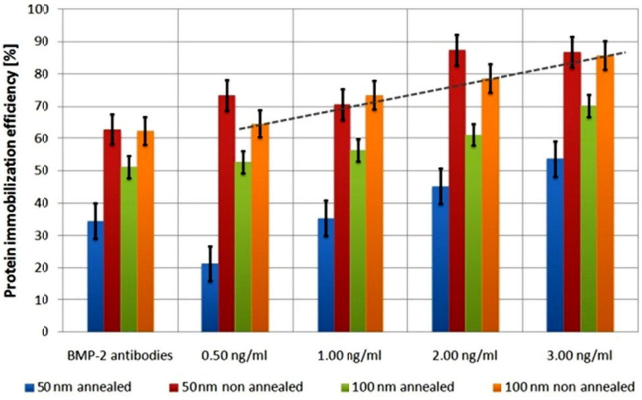

Fig. 6. Average immobilization efficiency for BMP-2 antibody and BMP-2 antigen of different concentrations on the nanotubular oxide layers with 50 and 100 nm of diameter with and without thermal modification

a)

b)

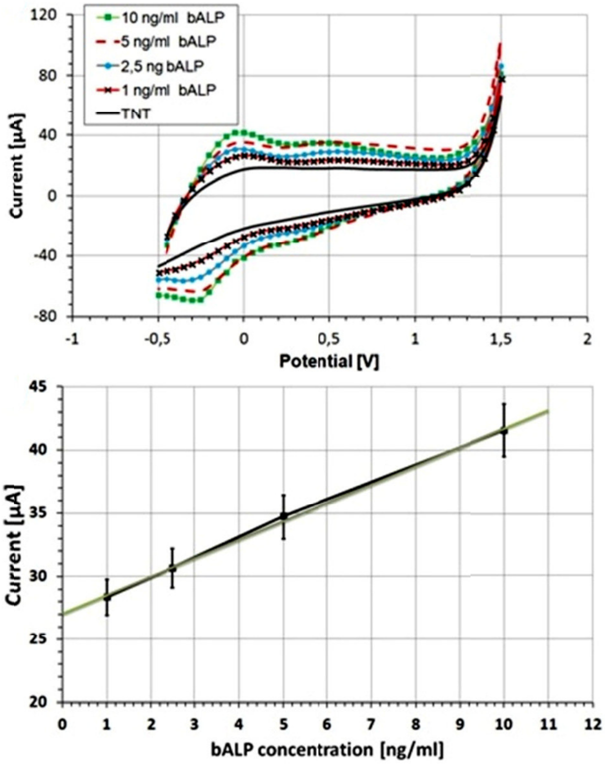

Fig. 7. Voltammograms for the annealed nanotubular oxide layer with 50 nm diameter, the layer functionalized by bALP antibody and the layers incubated in solution with bALP antigen of 1.0, 2.5, 5.0, 10.0 ng/ml concentrations, recorded in 5 ml of PBS solution (pH 7.4) with addition of 0.5 ml of p-NPP, (b) calibration curve for amperometric detection of bALP on the TNTs layer on Ti6Al4V

a)

b)

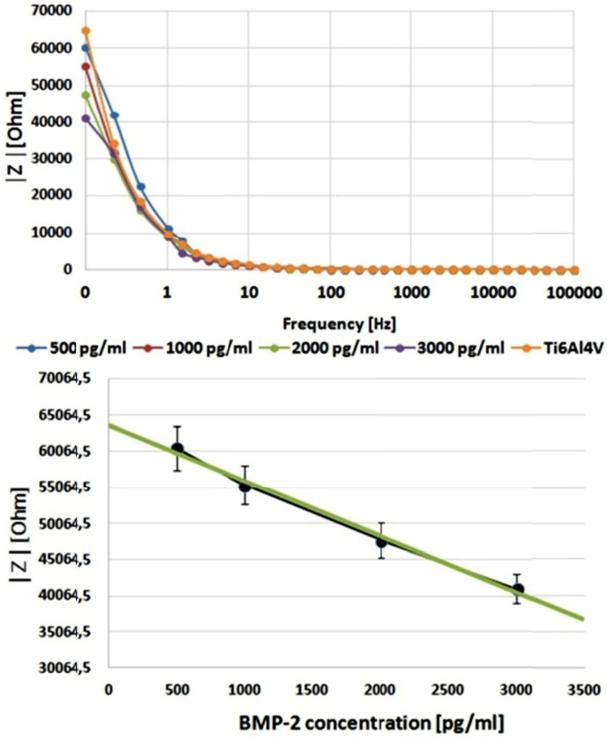

Fig. 8. Niquist plot for functionalized nanotubular oxide layer with 100 nm diameter on Ti6Al4V alloy with immobilized BMP-2 in different concentrations (a), calibration curve of impidimetric BMP-2 biosensor (b)

Non annealed nanotubular oxide layer with 100 nm diameter was chosen for examinations as a platform for biosensor of BMP-2.

Figure 7a shows voltammograms recorded during examination of the annealed nanotubular oxide layer on the Ti6Al4V with 50 nm diameter functionalized by bALP antibody and bALP antigen in concentrations 1.0, 2.5, 5.0, 10.0 ng/ml. For bALP functionalized electrodes anodic peaks with maximum current values were registered at potential -0.04 V and cathodic peaks at potential -0.24 V. These potential values are characteristic for p-nitri-phenyl-phosphate decomposition and oxidation-reduction of its products p-aminophenol and p-quinoneimine (Fig. 3). Values of anodic peak current increase proportionally to bALP antigen concentrations. The dependency between the current values and bALP antigen concentrations can be illustrated as the calibration curve for amperometric biosensor of bALP (Fig. 7b).

During the fact that bone morphogenetic protein does not presence any characteristic redox reactions and the amount of BMP-2 bonded into functionalized nanotube oxide layer on Ti6Al4V alloy was measured using electrochemical impedance spectroscopy (EIS) [40]. Results of EIS examinations of nanotubular oxide layer on Ti6Al4V alloy with 100 nm diameter functionalized and bonded with BMP-2 are presented on Niquist plot on Figure 8a. For the lowest examinated frequency 0.1 Hz impedance modulus value decrease over for different BMP-2 concentrations. On the base of obtained results it was possible to plot a calibration curve (Fig. 8b).

The antibody nanotubular oxide layer on Ti6Al4V alloy with 100 nm diameter and non annealed is a proper biosensor for impidimetric detection of BMP-2 in physiological concentration range.

4. Conclusions

Nanotubular oxide layers on Ti6Al4V with 50 and 100 nm of diameter were obtained during anodization in organic electrolytes. To increase their hydrophility and the charge of the layer TNTs were thermally modified in 600°C nitrogen. Functionaliztion by direct dropping of bALP and BMP-2 antibody shows that immobilization efficiency is different for 50 and 100 nm TNTs diameter. Thermally modified and positively charged nanotubular oxide layer with 50 nm diameter on Ti6Al4V strongly bind bALP antibodies and bALP antigens in physiological range. The functionalized nanotubular oxide layer can be a biosensor platform, cause amount of bALP bonded to this surface can be detected amperometrically. In case of BMP-2, this protein quantitatively bind to the functionalized non annealed - negatively charged TNTs with 100 nm diameter, and it is possible to detect the amount of BMP-2 using electrochemical impedance spectroscopy. During the research it is proved that TNTs functionalized by antibodies may be a platform for fast, simple and precise biosensor for detection bone remodeling markers and diagnostics of bone diseases with accuracy comparable to ELISA test.

The results obtained during this research suggest that functionalized nanotubular oxide layer on Ti6Al4V alloy with strongly bonded bALP or BMP-2 will stimulate osteoblasts proliferation during the fact that they are released into environment physiologically and are able to activate cell signaling patchways. In osteoblasts proliferation it is important to signalize to other cells that something positive is happening on implant surface, and it can be archived by presence of bALP and BMP-2 on the surface. Moreover the nanotubular oxide layer morphology generates nano roughness over the Ti6Al4V surface which also stimulate bone remodeling [41-43].

Acknowledgements

This work was financially supported in the project ERA-NET-/MNT/TNTBIOSENS/1/2011 by the NCBR (Polish National Centre for Research and Development).

Additional information

Selected issues related to this paper are planned to be presented at the 22nd Winter International Scientific Conference on Achievements in Mechanical and Materials Engineering Winter-AMME’2015 in the framework of the Bidisciplinary Occasional Scientific Session BOSS'2015 celebrating the 10th anniversary of the foundation of the Association of Computational Materials Science and Surface Engineering and the World Academy of Materials and Manufacturing Engineering and of the foundation of the Worldwide Journal of Achievements in Materials and Manufacturing Engineering.

References

[1] T. Utesch, G. Dominelli, M.A. Mroginski, Molecular Dynamics Simulations of the Adsorption of Bone Morphogenic Protein-2 on Surface with Medical Relevance, Langmuir 27 (2011) 13144-13153.

[2] T.V. Nguyen, P.N. Sambrook, J.A. Eisman, Bone loss, physical activity, and weight change in eldery women: the Dubbo Osteoporosis Epidemiology Study, Journal of Bone and Mineral Research 13 (1998) 1458-1467.

[3] Polish Society of Orthopedics and Traumatology, Report: Osteoporosis-silent epidemic in Poland, 10-02-2015 (in Polish).

[4] P. Urena, M. Fruby, A. Ferreira, K.S. Ang, M.C. Vernejoul, Plasna Total Versus Bone Alkaline Phosphatase as Marker of Bone Turnover in Hemodialysis Patients, Journal of American Society of Nephrology 7 (1996) 506-512.

[5] F. Sarac, F. Saygth, Causes of high bone alkaline phosphatase, Biotechnology & Biotechnological Equipment 21 (2007) 194-197.

[6] S. Sardiwal, P. Magnusson, D.J.A. Goldsmith, E.J. Lamb, Bone Alkaline Phosphatase in CKD Mineral Bone Disorder, American Journal of Kidney Diseases 62 (2013) 810-822.

[7] E. River, F. Chopin, G. Coiffeier, T.F. Brentano, B. Bouvard, P. Gamero, B. Cortet, Bone turnover

markers for osteoporotic status assessment? A systematic review of their diagnosis value in osteoporosis, Joint Bone Spine 79 (2012) 20-25.

[8] Y.L. Hu, M.S. Huang, Ch.J. Yang, J.Y. Hung, L.Y. Wu, P.L. Kuo, Lung Tumor- associated Osteoblast-derived Bone Morphogenetic Protein-2 Increased Epithelial-to-Mesenchymal Transition of Cancer Runx2/Snail Signaling Pathways, The Journal of Biological Chemistry 286 (2011) 37335-37346.

[9] L.D. Hover, C.D. Young, N.E. Bhola, A.J. Wilson, D. Khabele, C.C. Hong, H.L. Moses, P. Owens, Small molecule inhibitor of the bone morphogenetic protein pathway DMH1 reduces ovarian cancer cell growth, Cancer Letters 368 (2015) 79-87.

[10] F.G. Draenert, A.L. Nonnenmacher, P.W. Kammerer, J. Goldschmitt, W. Wagner, BMP-2 and bFGF release and in vitro effect on human osteoblast after absorption to the bone grafts and biomaterials, Clinical Oral Implants Research 24 (2013) 750-757.

[11] E. Mornet, E. Stura, A.S. Lia-Baldini, T.S. Stigbrand, A. Merez, M.H. Le Dus, Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization, The Journal of Biological Chemistry 276 (2001) 31171-31178.

[12] D.J. Jaekel, K.L. Ong, E.C. Lau, H.N. Watson, S.M. Kurtz, Epidemiology of Total Hip and Knee Arthroplasty Infection, in: B.D. Springer, J. Parvizi (ed.) Peroprosthetic joint infection of the hip and knee, Springer, 2013.

[13] K.J. Bozic, S.M. Kurtz, E. Lau, K. Ong, T.P. Vail, D.J. Berry, The epidemiology of revision total hip arthroplasty in the United States, The Journal of Bone & Joint Surgery. American vol. 91 (2009) 128-133.

[14] K.J. Bozic, S.M. Kurtz, E. Lau, K. Ong, V. Chin, T.P. Vail, H.E. Rubash, D.J. Berry, The epidemiology of total knee arthroplasty in the United States, Clinical Orthopaedics and Related Research 468 (2010) 45-51.

[15] S. Minagar, J. Wang, C.C. Berndt, E.P. Ivanowa, C. Wen, Cell response of anodized nanotubes on titanium and titanium alloys, Journal of Biomedical Materials Research A 101A (2013) 2726-2739.

[16] Y.T. Sul, C. Johansson, E. Byon, T. Albrektsson, The bone response of oxidized bioactive and non-bioactive titanium implants, Biomaterials 26 (2005) 6720-6730.

[17] K. Kim, B.A. Lee, X.H. Piao, H.J. Chung, Y.J. Kim, Surface characteristics and bioactivity of an anodized titanium surface, Journal of Periodontal Implant Science 43 (2013) 198-205.

[18] J.M. Macak, H. Tsuhiya, L. Taveira, A. Ghicov, P. Schmuki, Self-organized nanotubular oxide layers on Ti6Al7Nb and Ti6Al4V formed by anodizing in NH_4F solutions, Journal of Biomedical Materials Research Part A 75A (2005) 928-933.

[19] R.A. Gittens, L. Scheideler, F. Rupp, S.L. Hyzy, J. Geis-Gerstorfer, Z. Schwartz, B. Bayan, A review on the wettability of dental implant surfaces II: Biological and clinical aspect, Acta Biomaterialia 10 (2014) 2907-2918.

[20] E. Krasicka-Cydzik, K. Bialas-Heltowski, I. Glazowska, T. Klekiel, A. Kaczmarek, Effect of fluorides on bioactivity of titania nanotubes in SBF solution- EIS studies, Archives of Materials Science and Engineering 51 (2011) 33-39.

[21] M.A. Alfarsi, S.M. Hamlet, S. Ivanowski, Titanium surface hydrophilicity modulates the human macrophage inflammatory cytokine response, Journal of Biomedical Materials Research A 102A (2014) 60-67.

[22] F. Schwarz, I. Mihatovic, V. Golubovic, S. Eick, T. Igihaut, J. Becker, Experim ental peri-implant mucosits at different implant surfaces, Journal of Clinical Peridontology 41 (2014) 513-520.

[23] P. Chennell, E. Feschet- Chassot, T. Devers, K.O Awitor, S. Descamps, V. Sautou, In vitro evaluation of TiO_2 nanotubes as cefuroxime carriers on orthopaedic implants for the prevention of periprosthetic joint infections, International Journal of Pharmaceutics 455 (2013) 298-305.

[24] S. Zhong, R. Luo, X. Wang, L. Tang, J. Wu, J. Wang, R. Huang, H. Sun, N. Huang, Effects of polydopamine functionalized titanium dioxide nanotubes on endothelial cell and smooth muscle cell, Colloids and Surface B: Biointerfaces 116 (2014) 553-560.

[25] Y.T. Sul, D.H. Kwon, B.S. Kang, S.J. Oh, C. Johansson, Experimental evidence for interfacial biochemical bonding in osteointegrated titanium implants, Clinical Oral Implant Research 24 (2011) 8-19.

[26] K.S. Brammer, S. Oh, C.J. Cobb, L.M. Bjursten, H. van der Heyde, S. Jin, Improved bone-forming functionality on diameter- controlled TiO_2 nanotubular surface, Acta Biomaterialia 5 (2009) 3215-3223.

[27] J. Lu, H. Li, D. Cui, Y. Zhang, S. Liu, Enhanced enzymatic reactivity for electrochemically drives drug metabolism by confining cytohrome P450 Enzyme in TiO_2 nanotube Arrays, Analytical Chemistry 86 (2014) 8003-8009.

[28] Y. Wang, C. Wen, P. Hodgson, Y. Li, Biocompatibility of TiO_2 nanotubes with different topographies, Journal of Biomedical Materials Research A 102 A (2014) 743-751.

[29] C.A. Grimes, G.K. Mor, TiO_2 Nanotubes: Synthesis, Properties and Applications, Springer, USA, 2009.

[30] A.V. Grigorieva, A.B. Tarasov, E.A. Goodilin, S.M. Badalyan, M.N. Rumyantseva, A.M. Gaskov, A. Birkner, Y.D. Tretyakov, Sensor properties of vanadium oxide nanotubes, Mendel Communications 18 (2008) 6-7.
[31] S. Bauer, K. von den Mark, J.C. Park, P. Schmuki, Engineering biocompatible implant surfaces Part I: Materials and surfaces, Progress in Materials Science 58 (2013) 261-326.
[32] A. Kaczmarek, T. Klekiel, E. Krasicka-Cydzik, Fluoride concentration effect on the anodic growth of self-aligned oxide nanotube array on Ti6Al7Nb Alloy, Surface and Interface Analysis 42 (2010) 510-514.
[33] E. Krasicka-Cydzik, A. Kaczmarek, I. Glazowska, K. Bialas-Heltowski, Scan rate and fluoride concentration effect on the anodic growth of self-aligned titanium dioxide nanotubes in phosphates, ECS Transactions 50 (2013) 263-270.
[34] E. Krasicka-Cydzik, K. Arkusz, A. Kaczmarek, A mathematic model for selection of formation parameters of TiO_2 nanotubes by anodizing, Engineering of Biomaterials 114 (2012) 34-40.
[35] A. Kaczmarek-Pawelska, E. Krasicka-Cydzik, Morphological and Chemical Relationships in nanotubes formed by anodizing of Ti6Al4V alloy, Advances in Materials Science 14 (2014) 12-20.
[36] P. Urena, M. Fruby, A. Ferreira, K.S. Ang, M.C. Vernejoul, Plasma Total Versus Bone Alkaline Phosphatase as Markers of Bone Turnover in Hemodialysis Patients, Journal of American Society of Nephrology 7 (1996) 506-512.
[37] J.B. Albilia, H.C. Tenenbaum, C.M.L. Clokie, D.R. Walt, G.I. Baker, D.J. Psutka, D. Backstein, S.A.F. Peel, Serum levels of BMP-2,4,7 and AHSG in patients with degenerative joint disease requiring total arthroplasty of the hip and temporomandibular joints, Journal of Orthopaedic Research 31 (2013) 44-52.
[38] C. Halling Kinder, S. Narisawa, J.L. Millán, P. Magnusson, Glycosylation differences contribute to distinct catalytic properties among bone alkaline phosphatase isoforms, Bone 45 (2009) 987-993.
[39] A. Baoteng, A. Brajter-Toth, Nanomolar detection of p-nitrophenol via in situ generation of p-aminophenol at nanostructured microelectrodes, Analyst 137 (2012) 4531-4538.
[40] S.H. Chan, Y.H. Lin, Ch.Y. Chu, Ch.M. Cheng, G.T. Chen, Y.Ch. Lu, S.Y.S. Thomas, Device and method for measuring prothrombin time and hematocrit by analyzing change in reactance in a sample. Patent no. US 20110303556.
[41] L. Mierelles, A. Arvidsson, T. Albrektsson, A. Wennerberg, Increased bone formation to unstable nano rough titanium implants, Clinical Oral Implants Research 18 (2007) 326-332.
[42] Y.Li, Q. Fu, Y. Qi, M. Shen, Q. Niu, K. Hu, L. Kong, Effect of a hierarchical hydrid micro/nano-rough strontium-loaded surface on osteointegration In osteoporosis, RSC Advances 65 (2015) 52296-52306.
[43] Z. Lewandowska, P. Piszczek, A. Radtke, T. Jedrzejewski, W. Kozak, B. Sadowska, Evaluation of the impast of titania nanotube covers morphology and crystal chase on their biological properties, Journal of Material Science: Materials in Medicine 26 (2015) 163-175.