

On influence of anodic oxidation on thrombogenicity and bioactivity of the Ti-13Nb-13Zr alloy

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Purpose: This paper reports on the results of investigations on the blood response to the modified of surfaces titanium alloys. *Methods:* To enhance biocompatibility of the Ti-13Nb-13Zr alloy, anodisation was performed at 80 and 150 V. The oxidation process was carried out in a solution containing 4 mol dm⁻³ H₃PO₄ and 0.59 mol dm⁻³ Ca(H₂PO₄)₂. *Results:* The haemolytic activity of the titanium alloy surface was not altered much by the anodisation. The obtained values of the percentage of haemolysis were well below the levels required for the materials intended for blood contact. The clotting time of the blood was similar for the as-ground sample and the sample anodised at 80 V. For the sample anodised at 150 V the clotting time was shorter. The differences between these samples were observed in partial thromboplastin time after activation, prothrombin time and thrombin time, after 24 h. Extracts taken from the samples were not toxic towards the L-929 mouse fibroblast cells. *Conclusions:* The proposed treatment might be appropriate for the preparation of modified Ti-13Nb-13Zr surfaces intended for bone reconstruction or cardiovascular implants depending on process parameters.

Key words: vanadium-free titanium alloys, anodic oxidation, surface modification, blood compatibility

1. Introduction

Titanium and Ti-6Al-4V alloy have been widely used for bone surgery. Among all the metallic biomaterials, some of titanium alloys show the best biotolerance, good resistance to crevice and stress corrosion in chloride environment, paramagnetic properties and high fatigue strength. Their propensity for autospontaneous passivation in body fluids is one of the most important features of titanium and its alloys. The oxide layer improves the corrosion resistance of the substrate in human body [12], [6]. As corrosion products of the Ti-6Al-4V alloy are toxic [11], novel titanium alloys have been investigated as alternative materials for implants. Titanium alloys such as Ti-15Mo, Ti-13Nb-13Zr, Ti-18Nb-13Zr, Ti-35Nb-5Ta-7Zr, are composed exclusively of biocompatible elements, and exhibit much better mechanical properties than titanium and

Ti-6Al-4V alloy [13], [14]. To enhance the anticorrosion properties of the titanium alloys such as Ti-13Nb-13Zr, materials biologically compatible for human body, their surface should be modified [4].

Modification of the surface of a titanium implant by anodic oxidation is relatively easy and inexpensive. It guarantees good adhesion of the homogeneous oxide layer to the substrate. Depending on the voltage applied during the process and the composition of the anodising bath, the oxide formed on the surface may be enriched with biocompatible elements (i.e., calcium, phosphorus or particles of hydroxyapatite). The oxidation at a higher voltage than that of the dielectric breakdown of the oxide layer is called plasma electrolytic oxidation (PEO). The surface of the implant after application of this technique is modified and the formation of numerous surface micropores can be seen [19], [9]. The porous oxide layer is desired on the titanium alloy surface when the materials are meant to

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Received: April 22nd, 2016

Accepted for publication: July 4th, 2016

be used as bone-like implants. PEO coatings are duplex in structure, meaning there is an inner compact oxide layer with barrier properties and outer, much thicker porous layer. High surface area of the porous layer improves integration of the implant with bone tissue. In comparison, the compact oxide layer mainly increases the corrosion resistance of the titanium alloys. Oxide layers obtained at voltages below oxide breakdown can be used as coatings for cardiovascular implants.

The surface of titanium implants has an influence on immobilization of bioactive molecules from human body fluid, which may promote or support several interactions. The protein layer adsorbed on the implanted material affects the interaction between platelets and activation by interaction with platelet membrane receptors. Coagulation factors are then activated to form a fibrin network [7], [8]. The surface of the material should assure balance between bleeding and thrombosis. Little data on thrombogenicity of the anodised Ti-13Nb-13Zr alloy surfaces is available. Thus, in this paper, the results of in vitro investigations concerning influence of blood components on the physicochemical properties of the anodised titanium alloy surfaces are presented.

2. Experimental

2.1. Surface pretreatment and anodisation

Ti-13Nb-13Zr alloy (BIMO Metals, Wrocław, Poland) of the following specific composition was used throughout this investigation: 13.30 wt% Nb, 13.00 wt% Zr, 0.0009 wt% N, 0.05 wt% C, 0.005 wt% H, 0.08 wt% Fe, 0.1 wt% O and balance Ti. The samples were cut in the form of discs or rectangular coupons. The diameter of the discs was 9.5 mm and their height – 5 mm. The dimensions of the rectangular titanium alloy samples were: 4 mm × 9.5 mm × 3 mm. They were ground with #320 and #600 SiC abrasive paper and then cleaned ultrasonically for 5 min in a 2-propanol/deionised water mixture (sample TNZ). Afterwards, the samples were polished electrochemically according to the procedure described in [17].

After electropolishing, the samples were oxidised anodically in a bath that contained phosphoric acid (4 M H₃PO₄) and calcium hypophosphite (0.59 M Ca(H₂PO₂)₂) [16], [18]. The anodisation was carried out at a voltage of 80 V (sample TNZ-80) and 150 V

(sample TNZ-150) at a current density of 0.2 A/dm⁻² for 5 min. After anodising, the samples were rinsed with distilled water and cleaned ultrasonically in 2-propanol/deionised water mixture. For the purpose of anodising, a DC power supply (PWR800H, Kikusui, Japan) was used. The anode was the Ti-13Nb-13Zr alloy specimen and the cathode was made from titanium.

2.2 Biological tests in vitro

The in vitro investigations consisted of examination of the blood and cytotoxic response of the modified titanium alloy samples. The titanium alloy samples were sterilized in an autoclave at 120 °C for 30 min and then the samples were moved into plate-parallel flow system [15], [1], [2].

2.2.1. Blood contact

The study was performed on human blood 0 Rh+ collected in a CPD solution anticoagulant (citrate-phosphate-dextrose). Consent of Bioethical Commission of Wrocław Medical University was granted for the tests (No. KB-400/2012). The study included the haemolytic activity determination, plasma activation of the coagulation system, and scanning electron microscope (SEM) observations of the test materials after the contact with blood.

Hemolysis tests

The experiments were performed on human whole blood and red blood cells. The study was conducted with the use of phosphate buffered saline (PBS) solution (Ca- and Mg-free).

Haemolytic activity with the use of whole blood

Citrated whole blood drops (0.2 cm³) were put onto the titanium alloy samples (sample surface area 0.71 cm²). Then, the samples were incubated in 6-well plates for 30 min at 37 ± 1 °C. Afterwards, 4 cm³ of PBS solution was added into the wells and they were incubated for another 60 min. Pure, PBS was used as a negative control (0% lysis) and injection water served as positive control (100% lysis). After incubation, the samples were centrifuged. The supernatant absorbance was measured at 545 nm and the percentage of haemolysis (H%) was calculated using the formula

$$H\% = \frac{A_{\text{sample}} - A_{\text{control}}}{A_{100} - A_{\text{control}}} \cdot 100\% \quad (1)$$

where A_{sample} – absorbance of the test sample, A_{control} – absorbance of the control sample, A_{100} – absorbance of the sample with 100% haemolysis. For materials intended for contact with blood, haemolysis should not exceed 1%.

Haemolytic activity with the use of erythrocytes

The test was performed with erythrocytes derived from whole human blood after centrifugation ($1018 \times G$ for 10 min) without plasma. The titanium alloy coupons were then incubated in PBS (5 cm^3 of PBS/ 15 cm^2 exposed sample surface) at $37 \pm 1 \text{ }^\circ\text{C}$ for 24 h. Negative and positive control samples were incubated in parallel to the measured samples. Next, the erythrocytes (0.02 cm^3) were contacted with the test samples and incubated at $37 \pm 1 \text{ }^\circ\text{C}$ for another 24 h. Subsequently, the samples were centrifuged ($366 \times g$ for 10 min) and their H% was determined analogously to those calculated for whole blood. This value cannot exceed the value of 3% for the materials intended for blood contact [21], [22].

Coagulation tests

Citrated plasma was obtained from human whole blood after centrifugation ($1467 G$ for 10 min) and separated from the morphotic blood elements. The ratio between the plasma volume to the exposed surface area of the sample was chosen experimentally.

Plasmatic coagulation test

Low-platelet plasma with the titanium alloy samples (0.8 cm^3 of plasma/ 0.71 cm^2 of exposed sample surface) and without the test material (control sample) were incubated for 30, 60, 120 min and 24 h at $37 \pm 1 \text{ }^\circ\text{C}$. Partial thromboplastin time after activation (APTT, s), prothrombin time (PT, s), thrombin time (TT, s) and fibrinogen (Fb, g dm^{-3}) concentration were determined for the plasma samples. The tests were performed using a Coag Chrom 3003 coagulometer (Bio-Ksel, Poland) at $37 \pm 1 \text{ }^\circ\text{C}$ and at a wavelength of 405 nm. The determination procedures were consistent with the manufacturer's instructions [21], [23].

Recalcification time test

A drop of whole blood (0.02 cm^3) was deposited on all the samples tested and the shape of the drop was then observed (Fig. 1). After incubation at $20 \text{ }^\circ\text{C}$ during 120 s, 0.025 cm^3 of 25 mM CaCl_2 was added, and then the clotting time was measured (Table 1). The measurement was stopped when the first fibrin thread was formed [22].

Table 1. Recalcification time (CT) on the material surfaces

Sample	CT, s
TNZ	364.00 ± 14.98
TNZ-80	366.50 ± 13.99 --
TNZ-150	316.00 ± 10.52 ## ++

Reference range: 520,00 s – 571,00 s.

$p < 0.01$ – differences in relation to the TNZ,

++ $p < 0.01$ – differences in relation to the TNZ-150/TNZ-80,

-- $p < 0.01$ – differences in relation to the TNZ-80/TNZ-150.

Surface morphology investigations

The titanium alloy samples after the blood coagulation investigations were washed with PBS. The cells were fixed with 2.5% glutaric aldehyde in a 0.1 mol dm^{-3} cacodylic buffer for 2 h, then rinsed with ethyl alcohol with increasing concentration (from 30% to 100%). The samples were sputtered with gold and their morphology was examined using scanning electron microscope (SEM, Hitachi 3400-S, Japan; accelerating voltage = 25 kV).

2.2.2. Cytotoxicity tests

Mouse fibroblast cells L-929 (NCTC clone 929: CCL 1, American Type Culture Collection ATCC®), were cultured in a culture medium composed of Eagle's minimum essential medium (EMEM) with L-glutamine (ATCC®) and supplemented with 10% foetal bovine serum (FBS, Lonza®). The tests using extracts prepared from the culture medium with serum were performed.

Extract preparation

For the cytotoxicity tests the following extracts were prepared: negative control – high density polyethylene (HDPE, U.S. Pharmacopeia – Rockville, MD, USA), the titanium alloy samples with surface area of $6 \text{ cm}^2/\text{cm}^3$ of culture medium and positive control – sodium lauryl sulfate (SLS, Sigma-Aldrich®) in a medium with the following concentrations: 0.15, 0.1, 0.05 mg/cm^3 . In order to conduct the in vitro cytotoxicity assessment, the samples of the titanium alloy extracts were prepared with the following concentrations: 100, 50, 25, 12.50% vol. In addition, a blank sample was prepared, i.e., medium with serum without the sample. The samples were incubated for another 24 h at $37 \pm 1 \text{ }^\circ\text{C}$.

MTT test

L-929 cells were seeded in 96-well flat-bottomed cell culture plates (1×10^4 cells in 100 μL of medium per well) at $37 \pm 1 \text{ }^\circ\text{C}$ for 24 h to allow for the attachment and then the medium was replaced with 100 μL

of the test extract or the control group extract. Cytotoxicity was assessed after another 24 h of incubation. The changes in cell morphology were evaluated with the use of an inverted optical microscope (CKX 41, Olympus®). The viability of the cells was assessed with the use of MTT assay (1 mg/1 cm³ MEM without phenol red, without glutamine and without NaHCO₃). To perform the MTT assay 100 µL of MTT reagent was added to each well and then the plates were incubated at 37 ± 1 °C in 5% CO₂ for 3 h. Next, the MTT solution was discarded and 100 µL of isopropanol (analytical grade, Stanlab®) was added to each well. After 30 min, the absorbance values were measured at 570 nm using Epoch Microplate Spectrophotometer (BioTek®).

Cell viability was calculated according to the formula

$$\text{Viab.}\% = \frac{A}{B} \cdot 100\% \quad (2)$$

where *A* – mean value of the measured optical density extracts of the test sample, *B* – mean value of the measured optical density of the blanks.

Evaluation of the cytotoxicity

The cytocompatibility of the modified titanium alloy samples was determined using cell viability assay with the use of 5 g dm⁻³ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Germany). The evaluation of cytotoxicity was performed after 24 h incubation of the cells in the extracts from the test materials. The morphological changes occurring upon the contact with the materials

were observed under a contrast reverse-phase microscope CKX 41 (Olympus). The degree of toxicity of the materials was evaluated on the basis of changes in cell morphology and cell viability. According to the 0–4 degree scale (Table 2), changes in the cell cultures of the degree higher than 2 and a decrease in cell viability greater than 30% are considered to be caused by the cytotoxic effect.

Statistical analysis

The results were statistically analysed using Statistica ver. 9.00. Arithmetic mean (*X*), standard deviation (±SD) and level of significance (*p*) were determined. Statistical analysis was performed using Student's *t*-test. Significant differences were assumed at **p* < 0.05.

3. Results

The appearance of the test samples is presented in Fig. 1. The untreated titanium alloy sample and the one anodised at 80 V exhibited a slight degree of lustre which was not observed for the sample treated at 150 V. This phenomenon is typical of anodic oxide layers obtained at different voltages. When the anodisation is performed below the dielectric oxide breakdown voltage the anodic oxide is growing uniformly with the increasing voltage and the surface obtained resembles the original metallic surface [28] and when the dielectric oxide breakdown voltage is breached the surface of the oxide layer is markedly changed due to concurrent metal dissolution, gas generation and plasma formation [19], [9]. This exerts an impact on the surface appearance and its characteristics. The colour observed after the anodisation at 80 V is due to visible light reflection and interference off the oxide and metal surfaces, with a few hundred nm of space between them (typical thickness of the oxide layers obtained at low voltages).

The results of haemolytic activity are summarised in Fig. 2. The percentage of haemolysis, regardless of the type of material and whether whole blood (1%) (Fig. 2a) or erythrocytes (3%) (Fig. 2b) were used for determination did not exceed the standard the values. The values of the haemolysis index and the percentage of haemolysis determined for the materials investigated were well below the upper limits in the case of application in contact with blood, which implies lack of haemolytic activity of Ti-13Nb-13Zr alloy.

Table 2. Toxicity degrees on the basis of morphological evaluation for the test of direct contact of materials extracts with cells [14]

Grade	Reactivity	Condition of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no cell growth; cells cover the entire hole plate; many cells in division.
1	Slight	Not more than 20% of the cells are round loosely without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells present; only slight growth inhibition observable.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis, not more than 50% growth inhibition observable.
3	Moderate	Not more than 70% of the cell layers contain rounded; cells or are lysed, cell layers not completely destroyed, but more than 50% growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

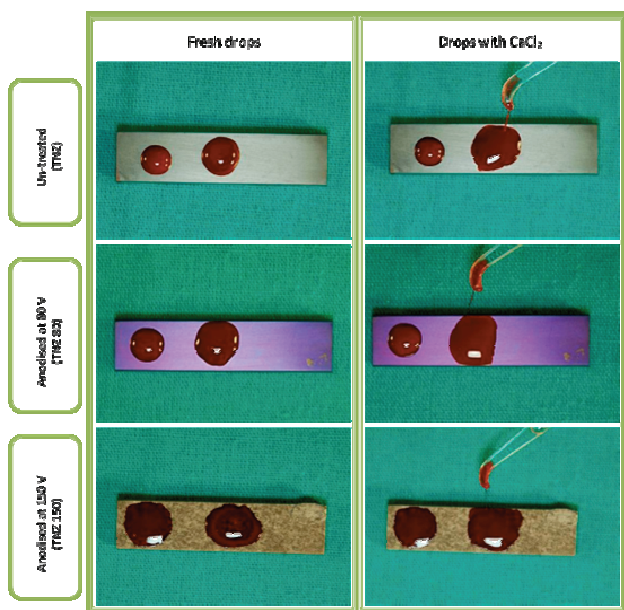


Fig. 1. Citrated whole blood drops (0.02 cm^3) on the surface of the titanium alloy samples (left) and the same drops with addition of $25\text{ mmol/dm}^{-3}\text{ CaCl}_2$ at the moment of appearance of the first fibrin clot threads

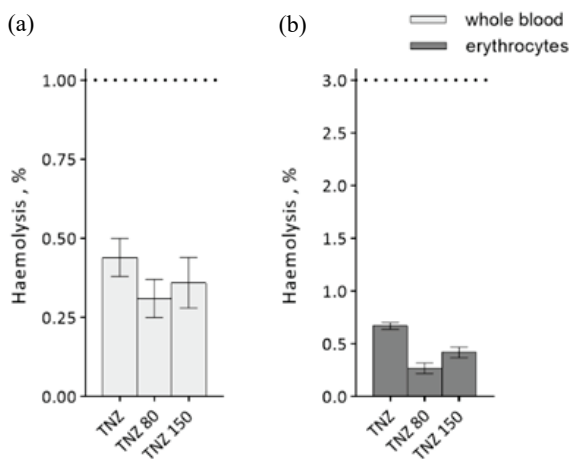


Fig. 2. Haemolytic activity results determined for the titanium alloy samples. The percentage of haemolysis (H%), determined on the basis of contact with whole blood (a) and erythrocytes (b), was calculated with the use of diluted blood. Dotted lines indicate the acceptable activity levels in accordance with [16]–[18]

The results of the plasmatic coagulation system activation tests including activated partial thromboplastin time (APPT), prothrombin time, thrombin time and fibrinogen concentration are presented in Figs. 3 and 4. The APTT (Fig. 3) is one of the basic tests to characterise blood coagulation. The greatest prolongation of the time was determined for sample TNZ-150 ($p < 0.01$, $p < 0.001$), and significant difference increased along

with contact time of plasma. The APTT values for the TNZ-150 were significantly higher compared to the TNZ and TNZ-80 samples, and exceeded the reference range. APTT determined for all of the titanium alloy samples was similar to the time determined for the control samples, when the samples were incubated for 0.5 h. After 1 h, the APTT was much higher for sample TNZ-150 compared to the others. After 2 and 24 h APTT for all of the samples increased. The results indicated that the sample TNZ-150 exhibited much higher APTT compared to the other samples. The APTT for the TNZ and TNZ-80 samples was similar to that of the control starting from 0.5 hour cubatron and ending at 2 hours. The values of activated partial thromboplastin time in the treated groups were significantly prolonged compared to the control group, after 24 h of measurement. The values of prothrombin time (Fig. 4a) were similar for the TNZ and TNZ-80 samples after each time point, and similar to the values determined for the control sample. For the sample TNZ-150 the prolongation of the time was observed after 2 h ($p < 0.001$), and higher compared to the control sample. The thrombin time after 0.5 and 1 h (Fig. 4b) was similar for all of the samples. After 2 and 24 h a significant prolongation of the time was observed ($p < 0.001$). A significant difference was observed only for sample TNZ-150. This relationship was also observed in the thrombin ratio determined. No significant differences were observed in the fibrinogen concentration (Fig. 4c) determined for all of the samples.

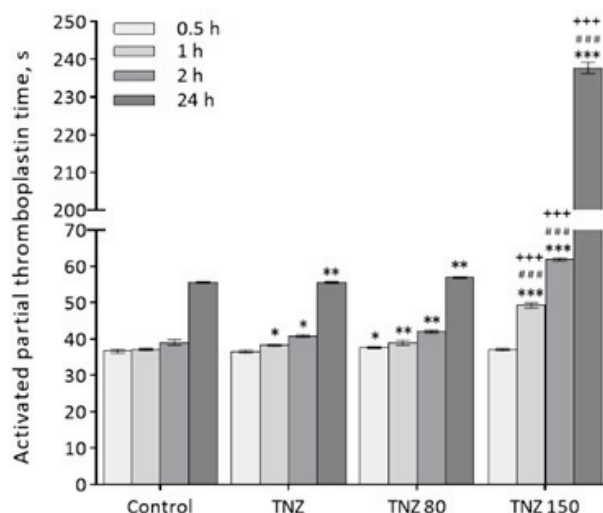


Fig. 3. Activated partial thromboplastin time in the citrated blood plasma after contact with titanium alloy samples and control plasma. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – differences in relation to the control, ### $p < 0.001$ – differences in relation to TNZ, +++ $p < 0.001$ – differences in relation to TNZ-80

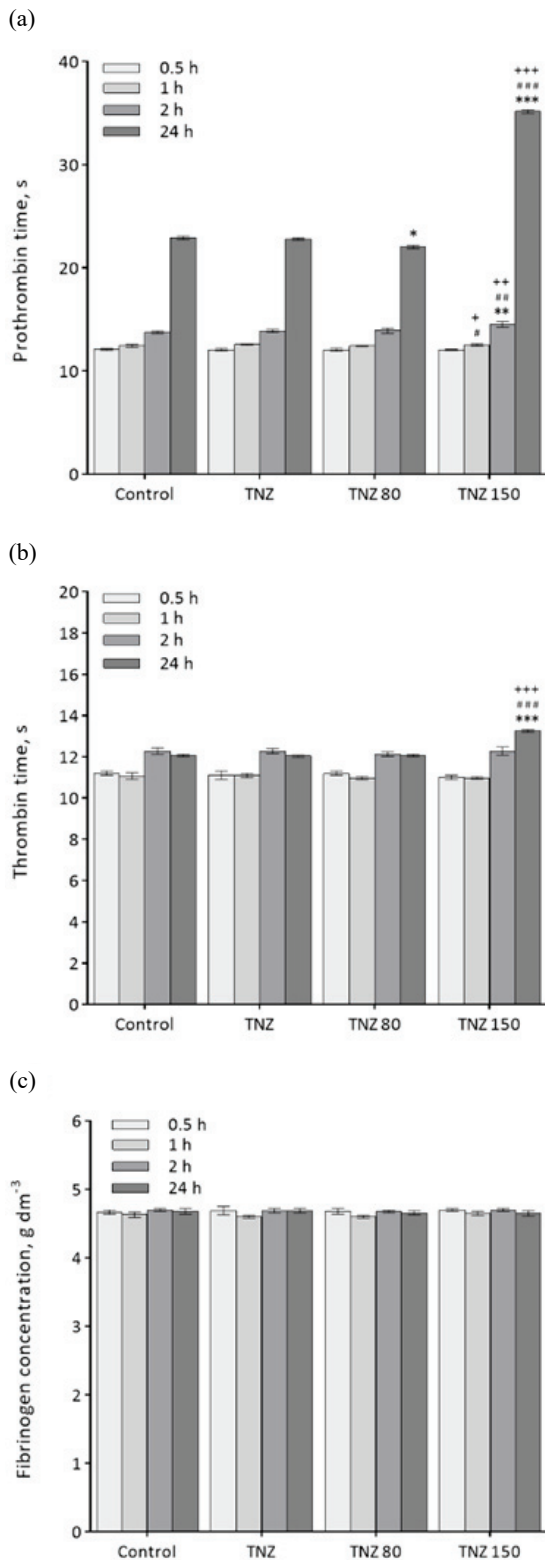


Fig. 4. Prothrombin time (a), thrombin time (b) and fibrinogen concentration (c) in the citrated plasma after contact with titanium alloy samples and control plasma. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – differences in level of significance in relation to the control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ – differences in level of significance in relation to TNZ, + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ – differences in level of significance in relation to TNZ-80

The results of the recalcification time (CT) for all the samples are collected in Table 1. For the TNZ sample the clotting time was $364.00 \text{ s} \pm 14.98$. For the anodised samples the clotting time was determined to be $366.50 \text{ s} \pm 13.99$ and $316.00 \text{ s} \pm 10.52$ for the TNZ-80 and TNZ-150 samples, respectively. For the TNZ-150 sample, a significant shortening of the recalcification time was observed compared to the TNZ ($p < 0.01$) and TNZ-80 ($p < 0.01$) samples. On the other hand, for the TNZ-80 a significant prolongation of the CT was observed compared to the TNZ-150 ($p < 0.01$). The clotting time strongly depends on the morphology and chemical composition of the surface. Addition of CaCl_2 into the blood induces the transformation of inactive prothrombin into active thrombin. Afterwards, the fibrinogen changes into fibrin, a primary protein in the blood clot. Anodic oxidation at 80 V did not alter the Ti alloy enough to have a strong influence on the clot formation. However, anodisation at 150 V decreased clotting time significantly, which is due to surface alterations caused by PEO (larger specific surface area as well as the presence of Ca and P).

Figures 5 and 6 present the SEM images of the samples before and after exposure to blood. The oxide layer formed on sample TNZ-80 was smooth without any cracks. On sample TNZ-150 a porous oxide was formed due to application of higher voltage and the occurrence of spark discharges during plasma electrolytic oxidation. Morphology of the oxides strongly depends on the conditions of surface anodizing. Previously, chemical composition of the layers was examined using an energy-dispersive X-ray spectrometer and X-ray photoelectron spectroscopy [16], [18]. Oxide layer formed on sample TNZ-80 was mainly composed of alloy oxides such as: TiO_2 , Nb_2O_5 , ZrO_2 , and small amount of TiO , Ti_2O_3 . The coatings formed on sample TNZ-150 were enriched in calcium and phosphorus compounds incorporated from the solution. Round cells with pseudopodia adhered onto the TNZ sample surface were observed. In some places, the cells were agglomerated. The cells were more uniformly distributed on sample TNZ-80 surface. Furthermore, the pseudopodia from the cells were clearly visible. The porous surface of sample TNZ-150 was the most fitted for the erythrocytes. Correct shapes and forms of acanthocytes were also clearly visible. The cells were well distributed on the top of the coating and within its pores.

The results of the cytotoxicity evaluation are presented in Tables 3 and 4. No significant differences in the cytotoxicity of the extracts were observed. The number of L929 cells was not below of 95%. Additionally, there were no significant differences between the number of cells in 100% extracts between the titanium

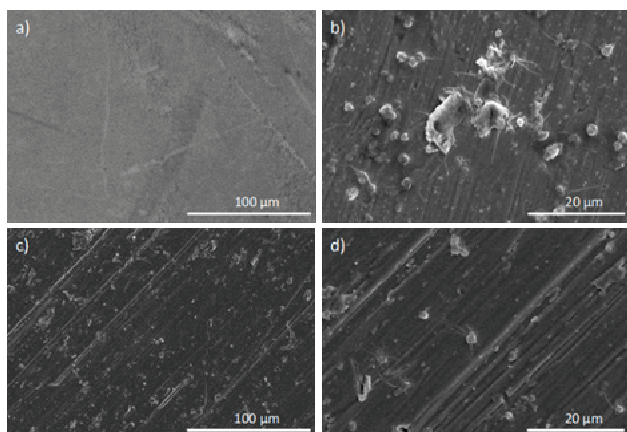


Fig. 5. SEM images of the surfaces of Ti-13Nb-13Zr alloy samples before (a – TNZ) and after (c – TNZ-80) anodisation at 80 V. (b) and (d) depict the respective surfaces after exposure to blood for 15 minutes

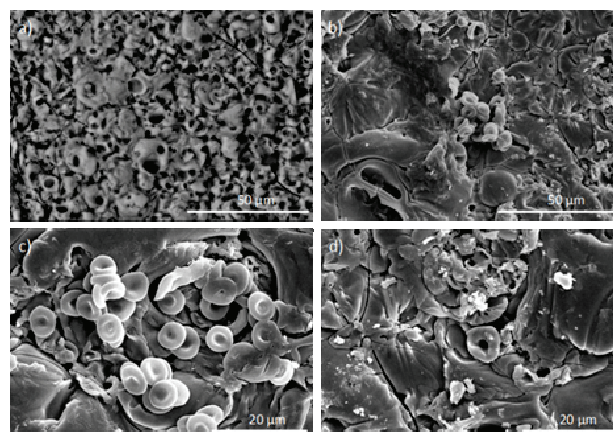


Fig. 6. SEM images of the surface of the Ti-13Nb-13Zr alloy sample anodised at 150 V (TNZ 150) before (a) and after (b, c, d) exposure to blood for 15 minutes. Erythrocytes with correct shapes and forms of acanthocyte are visible in the images. Single platelets are observed to adhere to the medium or form aggregates

Table 3. Cells viability (MTT test) on the basis of morphological evaluation for test of direct contact of the control materials

Test	Extract	Cells viability, %	Description	Grade
Negative	100%	94.20	Discrete intracytoplasmatic granules, no cell lysis. The cells cover the entire hole plate. Many cells in division (Fig. 7a).	0
Positive	SLS 0.05 mg/ml	61.15	Over 40% of the cells rounded, shrunk, loosely attached separating from the ground without densities cytoplasm, single cells disrupted. Empty spaces between cells (Fig. 7b).	2
	SLS 0.1 mg/ml	33.8	Completely destroyed cell culture. Extensive cell lysis (Fig. 7c).	4
	SLS 0.15 mg/ml	32.28	Completely destroyed cell culture. Extensive cell lysis.	4

Table 4. Cells viability (MTT test) on the basis of morphological evaluation and grade for test of direct contact of the titanium alloy samples

Titanium sample	Extract	Cell viability, %	Description	Grade
TNZ	100%	96.9	Discrete intracytoplasmatic granules, no cell lysis.	0
	50%	97.9	Discrete intracytoplasmatic granules, no cell lysis.	0
	25%	100.3	Discrete intracytoplasmatic granules, no cell lysis, density of culture comparable to density of negative control culture.	0
	12.5%	97.4	Discrete intracytoplasmatic granules, no cell lysis, density of culture comparable to density of negative control culture.	0
TNZ-80	100%	98.5	Discrete intracytoplasmatic granules, no cell lysis (Fig. 2c).	0
	50%	100.7	Discrete intracytoplasmatic granules, no cell lysis.	0
	25%	100.2	Discrete intracytoplasmatic granules, no cell lysis.	0
	12.5%	100.2	Discrete intracytoplasmatic granules, no cell lysis, density of culture comparable to density of negative control culture.	0
TNZ-150	100%	99.4	Discrete intracytoplasmatic granules, no cell lysis (Fig. 2b).	0
	50%	100.4	Discrete intracytoplasmatic granules, no cell lysis, density of culture comparable to density of negative control culture.	0
	25%	100.5	Discrete intracytoplasmatic granules, no cell lysis, density of culture comparable to density of negative control culture.	0
	12.5%	100.3	Discrete intracytoplasmatic granules, no cell lysis, density of culture comparable to density of negative control culture	0

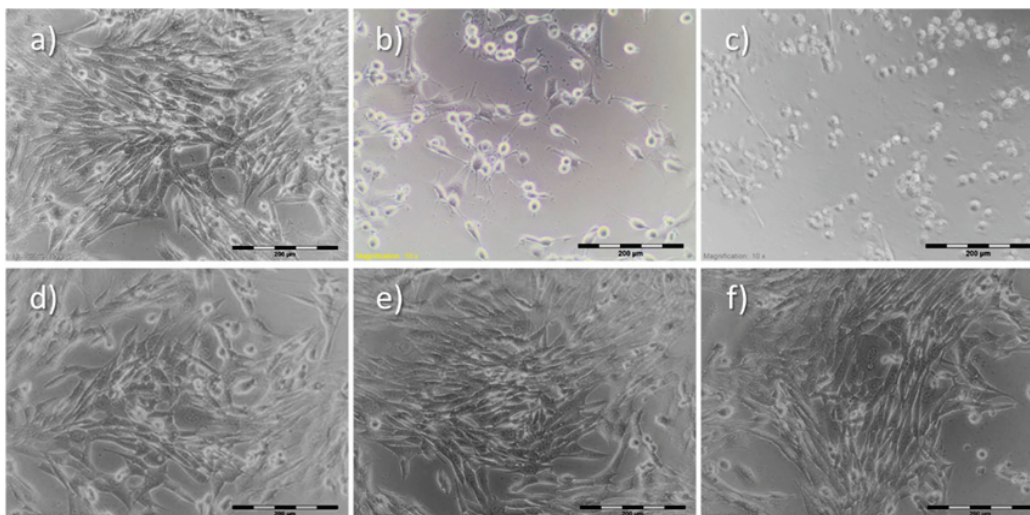


Fig. 7. Morphology of the cells adhered on the plates in contact with control medium with addition of (a) HDPE extract (non-diluted), (b) 0.05 mg ml^{-1} and (c) 0.1 mg ml^{-1} SLS solution. The images d–f show the adhered cells in the control medium with non-diluted (100%) extracts from the (d) TNZ, (e) TNZ-80 and (f) TNZ-150 samples

alloy samples. The number of cells for the TNZ, TNZ-80 and TNZ-150 was 96.9%, 98.5% and 99.4%, respectively. In a reduced concentration of the diluted extracts an increase in cell survival rate was observed.

Representative images of the morphology of the cells adhered onto the plates with control medium and the medium with the extracts from the titanium alloy samples are presented in Fig. 7. The morphology of the cells indicated that all of the titanium alloys were non-toxic towards the cell line investigated. No toxic substances were extracted from the samples into the medium and no significant difference was observed between the samples and the control group. There were no significant changes in cells viability and morphology between test samples anodised at 80 and 150 V.

4. Discussion

Biomaterials for temporary and permanent contact with the organism should be characterised in terms of their physical and chemical properties as well as bioinertness. The knowledge of the interaction between a novel material and blood is in constant need of improving. Several parameters of the materials intended for implantation influence the response of the organism. Protein adsorption, haemolytic reaction and blood coagulation play an important role in biocompatibility of the implanted materials during first seconds after implantation, therefore, a deep understanding of these processes should be attained before in vivo and clinical studies.

Haemocompatibility tests were performed on Ti-19Zr-10Nb-1Fe alloy by Xue et al. and the results of the $H\%$ were compared with the haemolysis percentage determined for other popular titanium alloys [26]. The results obtained for Ti-13Nb-13Zr alloy presented in this paper are comparable with those mentioned above. Furthermore, the proposed surface modification treatment had only a little beneficial effect on the titanium alloy surface. This effect was not surprising, since the alloy surface before anodisation was covered with a passive oxide layer. The treatment increases the thickness of the oxide but does not influence its chemistry and topography to a large extent when performed at low voltages. Only at higher voltages, which allow for plasma formation, extra elements from the electrolyte solution can be incorporated into the oxide layer, and therefore, alter the surface chemistry of the material. Additionally, surface morphology of the oxide layer was modified with micropores. The effect of microstructure on haemolytic activity was demonstrated by others [20], [10]. Nevertheless, no rise of haemolytic activity was noted after anodisation at 150 V, which suggests that incorporation of Ca and P into the oxide and morphological changes in the surface had no negative effect on haemolytic properties of the Ti alloy. The effect of PEO in P-containing electrolytes on haemocompatibility of Ti alloys was also investigated by others [25]–[27]. Xu et al. [25] found that the porous ceramic-like coating produced on the NiTi shape memory alloy during the process reduced $H\%$ and the number of platelets adsorbed on the material surface compared with non-treated Ti alloy. Similar behaviour was reported by

Yu and Yu [27] on Ti-3Zr-2Sn-3Mo-15Nb after PEO. The authors attributed the loss of haemolytic activity of the materials after the treatment to the surface energy changes, and hence, to changes in their wettability.

The chemical composition, surface roughness and wettability have an impact on the interaction of blood plasma components at the solid–liquid interface of the biomaterial surface. The surface properties undergo changes allowing adsorption and aggregation of blood plasma components depending on the time of exposure. Titanium alloys have been extensively investigated and several studies have reported on in vitro investigations. Surface morphology and surface roughness influence the blood clotting time and the plasmatic coagulation. The surface roughness of TNZ-150 sample ($R_a = 8.58 \mu\text{m}$) was much higher compared to the TNZ-80 ($R_a = 0.26 \mu\text{m}$), which was determined in our previous paper [18]. The results indicate that the TNZ-150 samples exhibited shorter clotting time, higher APTT and prothrombin time than the other samples. It was hypothesised that thrombogenicity of titanium surface influences the osseointegration process [5]. Thrombin generation on the modified surface could induce cell proliferation, bone formation and inhibit the apoptosis of osteoblast cells [3]. It seems that TNZ-150 sample is a favourable material for dental implants. Thor et al. [24] investigated the importance of whole blood for thrombin generation and the subsequent platelet activation in contact with various Ti-13Nb-13Zr alloy surfaces. It was shown that the most coagulation system activation promoting effect (due to the generation of thrombin-antithrombin complex TAT) was observed on the surface with the highest roughness (TiO_2 grid blasted and hydrofluoric acid treated, $S_a = 1.50 \pm 0.08 \mu\text{m}$). The blood coagulation was also connected with the chemical composition of the coatings. In our case, the coating formed on the TNZ-150 sample was composed of Ca and P ions [16], [18], and probably could increase thrombogenic properties of the sample surface.

In the present paper influences of anodizing voltage on biologic properties of formed oxide layers on promising TNZ alloy surface were presented. On the porous oxide layer the activated partial thromboplastin time was prolonged compared to result obtained for thin, uniform coating. However, the remaining haemolytic parameters were similar. Many papers reported that surface morphology and chemical composition strongly affect the interaction between a sample and L-929 mouse fibroblast. In this paper, we present methods for the Ti-13Nb-13Zr surface modification where both compact and porous oxide layers are high cyto-compat-

ible. Without any trouble the surface of titanium alloys can be very easily prepared as a material for cardiovascular or bone-like implant applications.

5. Conclusions

The hemocompatibility investigations have shown that the Ti-13Nb-13Zr (TNZ) alloy and anodised titanium alloy surface do not induce haemolytic reaction. No significant difference was observed in blood coagulation between the non-treated and the anodised at lower of the voltages (80 V) Ti alloy, whereas the anodisation performed at 150 V resulted in a formation of a surface which exhibited faster blood coagulation as well as shorter thrombin and prothrombin time after 24 h of incubation. At the same time, the fibrin concentration was similar for all of the samples investigated. No toxic reaction was observed during cytotoxic investigations using the L-929 mouse fibroblast cells. In the light of the results obtained it can be concluded that the proposed technique might be appropriate for the preparation of modified Ti-13Nb-13Zr surfaces intended for bone reconstruction (sample TNZ-150) or cardiovascular implants (sample TNZ-80).

Acknowledgements

The work was supported by the project No. ST-668 of the Wrocław Medical University.

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