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CAN TITANIUM ANODIZATION LEAD TO THE FORMATION OF ANTIMICROBIAL SURFACES?

Abstract
In recent years, there has been observed a growing need for novel, multifunctional materials that would not only replace, but also heal the damaged tissues. In this paper, the titanium dioxide films manufactured by anodic oxidation method are investigated. The study of their structurization and antimicrobial properties of the coatings is presented. Samples anodized in water solutions of ethylene glycol exhibited various character -from structurized to porous ones. As the study revealed, all samples acted anti-adhesive in terms of bacterial (*Escherichia coli*) and fungal (*Candida albicans*) surface colonisation.

Keywords
Ti6Al4V, anodic oxidation, antimicrobial properties

Introduction

Biomaterials and medical devices market is growing every year, all the time introducing new technologies and materials leading to prevent post-implantation and post-treatment bacterial and fungal infections [1,2]. A great effort is put on designing not only materials that exhibit desired properties like haemocompatibility or enhancement of ossteointegration, but most importantly – having antimicrobial properties[3-5]. Bacterial and fungal infections are a very serious problem concerning implants – it affects every type of surfaces, no matter if they are made of metal, polymer or biological tissues[6]. In case of microbial colonization on biomaterials by pathogenic microorganisms, one of the best solutions seem to be the replacement of the whole implant to the new one. However, the risk of recurrent infection is very high [7]. According to literature, a relatively high percentage of reimplantation due to microbial infections takes place. Based on literature review and own experience, this work assesses the material properties that can influence the microbial biofilm formation – both bacterial and fungal.

Microbial biofilm is a microorganisms-based structure in which microbes are attached to each other and surrounded by extracellular matrix (made of polysaccharides, proteins and DNA), that is attached to biological or artificial surface [8, 9]. This structure has been recognized as the basic form in which microorganisms can live on the surfaces, while the endospores and planktonic forms only serve to move and inhabit new places [10,11]. Biofilm formation is a complex process, with its duration dependent on environmental conditions and type of microbes that create it. It can be divided into 4 consecutive processes[12], presented in the diagram on the Fig.1.

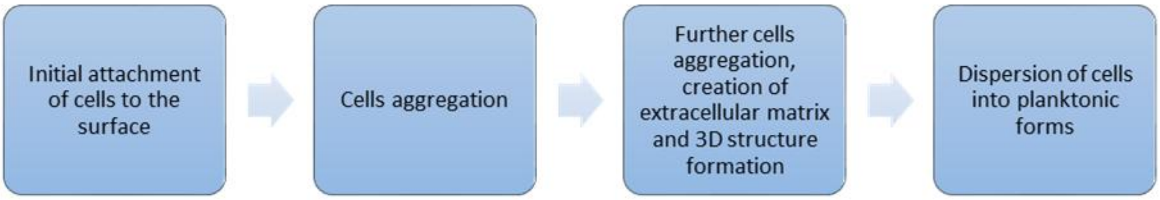


Fig. 1. Biofilm formation steps
Source: Author's

All these stages lead to the formation of a drug-resistant, complex structure that, when untreated, can cause the infection spreading around the whole human organism.

There are many factors that influence the formation of biofilms on biomaterials. Surface topography of the material, i.e. its roughness and presence of irregularities like scratches, cavities etc., can have a great influence on the adhesion of microorganisms[13]. One of the main suggestion when preparing the surface of biomaterial is that all the imperfections of the surface should be at least one order of magnitude smaller than the size of microorganisms that can inhabit the material. That is, the higher is the roughness of the surface, the higher will be the adhesion of microorganisms because of the fact that they will find 'a shelter' between the irregularities [14-16]. However, there exist theoretical models that do not agree completely with those statements. According to Seigismund et al., calculating the interaction energy between microorganisms and surface, it was seen that with the increasing Ra parameter, the distance between bacteria and surface is increased, thus reducing the interaction between microbe and material [17]. All published data suggest though, that the dependence between surface topography and microbial attachment is non-linear, and only when surface irregularities are on comparable level to adhered microbes, the contact between cells and material increases as well as the risk of increased microbial adhesion [18-20]. Chemical composition of the surface onto with microorganisms are about to attach plays a crucial role in determining microbial biofilm formation. Since decades, scientists are proposing new materials and coatings that can deal with inhibition of attachment of microorganisms. The most popular approach is ion implantation to the surfaces of materials. Those ions must exhibit desired properties like causing microbial apoptosis or reducing the amount of cells attachment. Very similar is deposition of coatings that would repel microorganisms or prevent them from colonization. Such materials involve e.g. diamond-like carbon coatings, titanium dioxide and coatings doped with antimicrobial agents like silver, copper, zinc, fluoride, silicon etc. [21-26].

One of the biomaterials that is frequently used in manufacturing of the implants and prosthesis is titanium. Its excellent mechanical properties combined with biocompatibility make this material very promising in the development of new solutions in biomaterials and surface engineering. One of the very interesting features of the titanium is that it has the possibility to form in an environment where oxygen is present, a very thin, oxide passive layer – titanium dioxide [27-30]. However, this layer is formed spontaneously and it can easily be removed from the material surface. Many coatings deposition technologies were tested, so that the more durable titanium dioxide coating could be formed. One of them – anodic oxidation, is a fast, non-expensive coatings manufacturing methods that allows to obtain the TiO_2 of the desired properties simply by changing the process parameters or electrolyte composition [31-35].

Anodic oxidation (also called anodization) is a method of electrolytic passivation of metals in the electrolytes, in presence of electric current. During the process, the material being coated serves as an anode, and the electrolytes are most often the water-based solutions of acids (very frequently these are sulfuric, phosphoric or hydrofluoric acid). While the voltage is applied to the system, the dissolved oxygen reacts with the metal immersed in the electrolyte, which forms a thin oxide layer on the anode surface [36-40].

This work is devoted to the analysis of titanium anodized in viscous electrolytes in terms of the layers structurization and their microbiological properties.

Materials and methods

Samples of titanium alloy Ti6Al4V (Bibus Metals Sp. z o.o.) were used as a substrate material. All samples were disks of ϕ = 16 mm. Prior to anodization processes all disks were washed with deionized water and acetone to remove all the contamination. Anodization of titanium was performed in a bath containing different concentrations of ethylene glycol (Chempur) in aqueous solutions. Each electrolyte had an addition of 2% vol. of hydrofluoric acid (Chempur). All electrochemical processes were conducted in constant voltage of 20V with 20 minutes deposition time. Voltage was controlled by the digital multimeter and devoted software. Prepared coatings list is presented in table 1.

Table 1. List of deposited TiO2 coatings

Sample	Water concentration [% vol.]	Ethylene glycol concentration [% vol.]
G1	50 %	50 %
G2	40 %	60 %
G3	30 %	70 %
G4	20 %	80 %
G5	10 %	90 %

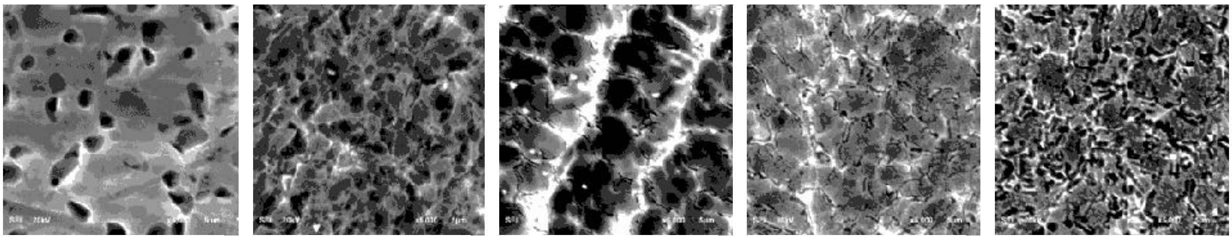
Source: Author's

The topography of the prepared coatings was investigated with the use of Scanning Electron Microscopy (SEM) [41,42]. The observations were performed in high vacuum, under the accelerating voltage of 20 kV and magnification 5000x. What is more, the roughness of surfaces was evaluated by mechanical profilometer. For all samples, the values of Ra parameter were calculated.

The antimicrobial character of the coatings was analysed with 2 strains – bacteria *Escherichia coli* (gram negative) and fungi *Candida albicans* (yeasts). *E.coli* was grown in Luria-Bertani medium, while *C.albicans* in YPG. All samples were incubated in culture medium for 24 hours at temperature 37C. When the samples were taken out from the growth medium, the specimens were rinsed with deionized water so the not-adhered microorganisms were removed from the surface. The observations were conducted under the fluorescent microscope Olympus GX 71, and the visualization of both live and dead cells was possible due to the use of two fluorescent dyes – propidium iodide and bis-benzamide. After 5 minutes incubation in dark, samples were observed. For the purpose of comparison of the samples, stainless steel (Medgal sp. z o.o.) substrate was used as a control. The number of adhered cells being the % of the cells attached to the control samples was calculated.

Results and discussion

Figure 2 presents the images from the scanning electron microscope, revealing the topography of the obtained coatings. Water to ethylene glycol ratio marked below.



10:90 20:80 30:70 40:60 50:50

Fig. 2. Topographical SEM images of the deposited coatings in 5000x magnification
Source: Author's

In the electrochemical processes involving viscous electrolytes, the general trend is that the reduction of amount of water in the electrolyte may cause help the formation of longer tubular structures [43-45]. That is due to the fact that that in organic electrolytes the donation of oxygen to the formed layer is more difficult. What is more, the anodic oxidation processes performed in viscous electrolytes may result in the formation of much different shapes than are observed for aqueous oxidizing baths[46-48]. However, in this study, as the scanning electron microscope examination revealed, the samples prepared in ethylene glycol electrolytes (see fig.2) did not exhibit tubular structures.

For the sample being prepared in the electrolyte containing 70% vol. of ethylene glycol and 30% vol. of water, a nearly tubular but not very deep structures were observed. Surprisingly, for the electrolyte with the highest concentration of ethylene glycol (90% vol. of ethylene glycol and 10% vol. of water), a porous character rather than rough and tubular is observed.

Also, the roughness measurements showed that porous surfaces had an average roughness Ra being almost 3 times higher than for structurized samples, as it is presented in the Table 2.

Table 2. Average roughness (Ra) measurements.

Water to ethylene glycol ratio				
50:50	**40:60**	**30:70**	**20:80**	**10:90**
0.28 ± 0.06 µm	0.26 ± 0.07 µm	0.18 ± 0.06 µm	0.06 ± 0.01 µm	0.06 ± 0.01 µm

Source: Author's

Antimicrobial performance of the coatings was assessed in terms of bacterial and fungal adherence to the formed layers. Figure 3 and 4 present the microbial adhesion being expressed by the total area occupied by microorganisms being the % of the control sample. In this investigation, the number of cells adhered to the control sample is expressed as 100%.

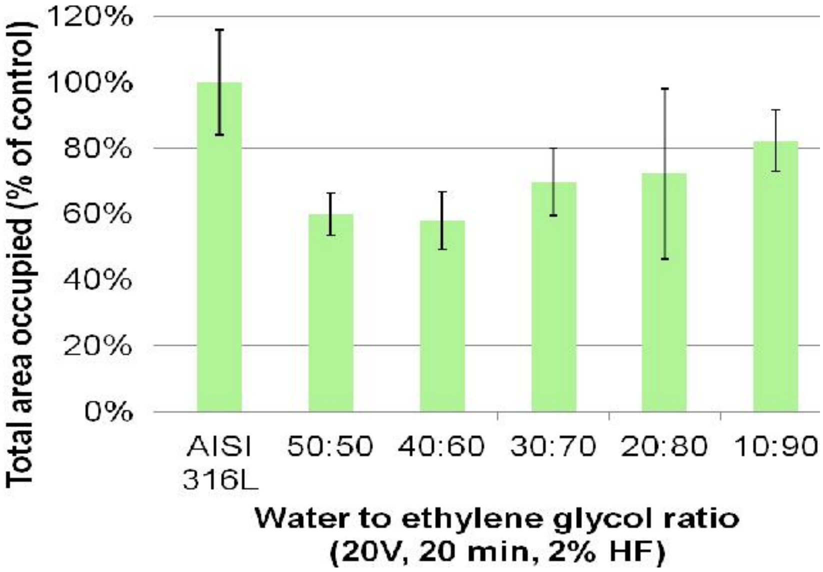


Fig. 3. Bacterial adhesion to anodized surfaces expressed by percentage of control
Source: Author's

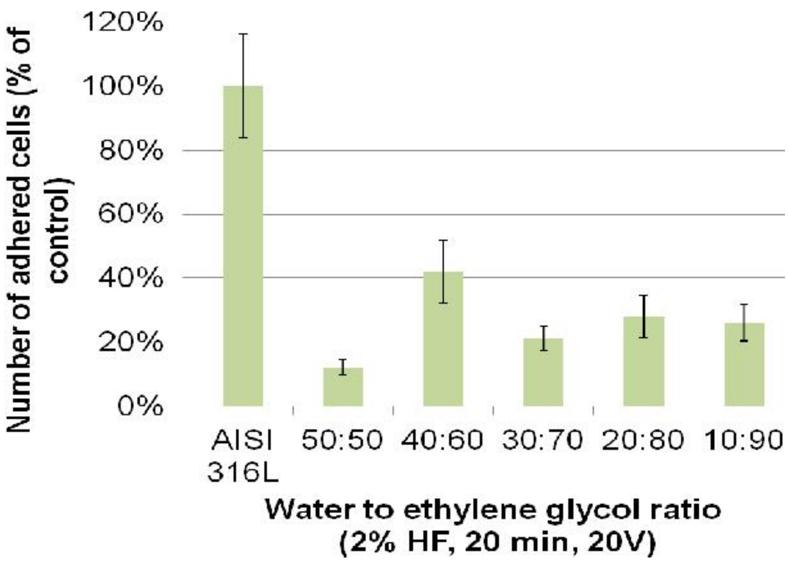


Fig. 4. Fungal adhesion to anodized surfaces expressed by percentage of control
Source: Author's

Numerous publications show that depending on the type of model organism selected for the experimental procedure, the obtained biological answer may differ. Presented researches were conducted on microorganisms belonging to different model groups: gram negative bacteria (*E coli*) and yeasts (*C albicans*). Those models are divergent for example on the basis of their physiology - like structure and composition of cell walls and in that way presence of different proteins, like integrins, responsible for adhesion to abiotic surfaces. Gram negative bacteria are characterised by three-component cell wall: two outer lipopolysaccharidic membranes and thin layer of peptidoglycan in the periplasmic space between them. Such structure results in for example low permeability for lipophilic molecules. In case of yeasts, the cell wall consists mostly of various glucans, with chitin dominating in inert part and mannoproteins in outer one [49]. Considering the above the type of the model organism might affect how the bacteria and yeasts were interacting with surfaces of various roughness.

When studying the bacterial colonisation on the manufactured surfaces, when the ethylene glycol content in the electrolyte was increasing, the almost linear growth of the total area occupied by bacteria in comparison to control sample was observed. However, considering fungal adhesion, for *Candida albicans* no linear dependence between the electrolyte composition and fungal surface colonisation was observed. The number of fungal cells occupying the surface was similar in all cases, with one exception. For the sample that was deposited in the electrolyte containing water to ethylene glycol ratio being 40:60, the number of *Candida* cells attached was much higher in comparison to others.

Conclusions

The study performed proved that the anodization process of titanium alloys can be possible when viscous electrolytes are used. However, as the most general trend should be that the reduction of water content in electrolyte help the formation of more tubular and longer titanium dioxide structures, no such dependence was observed . Each of the samples however, exhibited different character and structure. The anti-adhesive character of the obtained TiO_2 surfaces was maintained in terms of bacterial and fungal biofilm formation (the biological model employed in this manuscript were gram negative bacteria and yeasts) - all samples exhibited the antimicrobial character in comparison to the control sample.

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