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Physico-chemical and biological evaluation of doxycycline loaded into hybrid oxide-polymer layer on Ti–Mo alloy

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ABSTRACT

Oxide-polymer coatings were formed on the surface of the vanadium-free Ti–15Mo titanium alloy. The Ti alloy surface was modified by the plasma electrolytic oxidation process, and then, the polymer layer of a poly (D, L-lactide-*co*-glycolide) with doxycycline was formed. The polymer evenly covered the porous oxide layer and filled some of the pores. However, the microstructure of the polymer surface was completely different from that of the PEO layer. The surface morphology, roughness and microstructure of the polymer layer were examined by scanning electron microscopy (SEM) and a confocal microscope. The results confirmed the effectiveness of polymer and doxycycline deposition in their stable chemical forms. The drug analysis was performed by high-performance liquid chromatography. The ^{1}H NMR technique was used to monitor the course of hydrolytic degradation of PLGA. It was shown that the PLGA layer is hydrolysed within a few weeks, and the polyglycolidyl part of the copolymer is hydrolysed to glycolic acid as first and much faster than the polylactide one to lactic acid. This paper presents influence of different microstructures on the biological properties of modified titanium alloys. Cytocompatibility and bacterial adhesion tests were evaluated using osteoblast-like MG-63 cells and using the reference *S. aureus* and *S. epidermidis* strains. The results showed that the optimum concentration of doxycycline was found to inhibit the growth of the bacteria and that the layer is still cytocompatible.

1. Introduction

Implantology is a field of science that has a huge impact on the quality of human life. It enables patients to recover efficiently and supports the treatment of many serious diseases. Requirements for materials from which implants are made include very good bioactivity, lack of toxicity, excellent mechanical properties and resistance to factors in the surrounding implant environment. For the production of implants dedicated to hard tissue, titanium and its alloys have been widely used for many years. Currently, modern titanium alloys that do not contain vanadium and aluminum, which are cytotoxic and hazardous to human health, are desirable. In the passive layer, depending on the elemental composition, there are nontoxic oxides, e.g., Nb_2O_5, ZrO_2, Ta_2O_5, and MoO_3, that dissolve to a small extent in the environment of body fluids [1]. The very low solubility and diffusion of elements in the form of ions or particles is due to the presence of a passive layer on the surface of the material that delimits the tissue environment from the substrate metal. For example, in a water environment where the pH value is close to that of human body fluids. Additionally, the electric charge of particles dissolved in the tissue environment has an effect on the biocompatibility of the substance, for example, by affecting the properties of the proteins. Because the molecules of the abovementioned oxides are electrically neutral, they do not cause undesirable reactions in contact with human tissues [1]. With

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regard to biomaterials, the properties of the surface layer are a significant problem because this layer is in direct contact with the tissue after implantation into the human body. Titanium has a high affinity for oxygen; thus, a stable oxide layer is quickly formed on its surface. However, the coating is not very biologically active, though it undergoes various reactions, as a result of which its thickness, structure or composition may change. Improvement in the bioactivity of the titanium surface as well as modern titanium alloys can be achieved by modifying the surface layer by various physical, chemical or electrochemical methods. Plasma electrolytic oxidation (PEO) is a well-known method. It is used for the preparation of ceramic-oxide coatings on the surfaces of light metals. The obtained layers are porous, are hard and adhere well to the substrate. In addition, they are characterized by high corrosion resistance, good biocompatibility and increased abrasion resistance. The process runs under a high voltage. The thickness of the barrier layer increases, but it is not uniform over the entire surface; thus, the resistance and current vary. The gas evolves, the oxide layer on the surface of the alloy is penetrated, and when the breakdown voltage is reached, glow discharge appears. The porous structure of the coatings can be intentionally enriched with chemical compounds or solid particles from the solution. This is possible because glow discharge causes a local increase in temperature and pressure. When sparks disappear, places near discharges cool quickly, and the chemical compounds including ceramic particles might be are built in. In this way, the porous oxide layer may be enriched in substances (present in the electrolyte solution) that stimulate the crystallization of hydroxyapatite, which supports the growth of cells on the surface of the implant that can be introduced into the system [2,3].

Hybrid oxide-polymer layers can be a modern solution because they combine the advantages of different surface modification techniques. These coatings may be obtained using the dip-coating technique. It is an easy and cheap method that involves immersing the object in a sol and then pulling it out at a constant speed. It causes sol drainage and immediate gelling. By changing the dipping speed, drying time and viscosity of the sol, the properties of the coating, such as its thickness and morphology, can be changed. The dip-coating technique is useful for materials with a complicated shape. One of the popular synthetic polymers is poly (lactide-*co*-glycolide) (PLGA). Production of this copolymer has allowed overcoming the disadvantages of the pure starting of the starting polymers and has facilitated the control of their properties, e.g., increasing the solubility [4–7]. These coatings may be enriched with biologically active substances, e.g., medicines, which can effectively reduce the risk of bacterial infection after surgery. Research into the controlled drug release system is underway. One of the worthy antibiotics is doxycycline, which is the most stable substance in the group of tetracyclines. It is a long-acting bacteriostatic antibiotic used against gram-positive and gram-negative bacteria, such as *Mycoplasma, Brucella, Moraxella Catarrhalis, Streptococci, Staphylococci, Propionibacterium anaerobes and Bacteroides fragilis*. The drug is used in the treatment of infections, e.g., in soft tissue, in the upper and lower respiratory tract, in the gastrointestinal tract, in the genitourinary system, for ophthalmic and other infections, and in the prevention of malaria. However, the use of doxycycline over a long period of time results in the resistance of certain strains to drug development, which is manifested by the limitation of the degree of penetration of the antibiotic inside the bacterial cells, as well as the action of “pushing” it back out [8]. There are systems that, apart from using high-quality raw materials, facilitate therapeutic treatment through the use of a controlled drug release system, which reduces the risk of rejection of the implant by the human body [9].

Doxycycline is effectively absorbed from the gastrointestinal tract in less than 2 h and is easily assimilated. The dosage depends on the patient's age, weight and grade of infection, where initially take a daily dose of 100–200 mg. The drug is half-metabolized in the liver, and its half-time is 18–22 h [10]. The stability of doxycycline depends on the pH and temperature. There are different forms of the drug. The proportion of these forms changes entirely depending on the pH of the environment. Depending on the environment, the configuration changes around the carbon atom as the stereogenic center. The lower the pH value is, the better the stability of the drug. Studies have shown that for a pH below 6, there is a slow process of transition to a less active form, while an increase in pH increases the degree of deactivation of doxycycline [11].

The aim of this work was to study the formation of hybrid oxide-polymer coatings containing a biologically active substance on the surface of a titanium alloy: Ti–15Mo. The scope of this work includes the modification of the Ti–15Mo titanium alloy surface by plasma electrolytic oxidation, followed by the application of a degradable polymer with doxycycline on the porous oxide layer. The morphology of the modified surface and its qualitative composition were investigated in detail using scanning electron microscopy and confocal microscopy. The process of hydrolytic degradation of the hybrid layer, the release of the drug from the degrading surface and the biological properties, such as the cytocompatibility and antibacterial activity, were also examined using the most representative cells (osteoblast-like cells MG-63) and the reference bacterial strains, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*.

2. Experimental

2.1. Surface pretreatment and coating formation

The titanium alloy Ti–15Mo (TM), with a composition of 14.73–14.98 wt% Mo, 0.15 wt% O, 0.06 wt% Fe, 0.08 wt% C, 0.016 wt % N, 0.01% H, and Ti balance (BIMO Metals, Poland), was used for the surface modifications. The samples were cylindrical in size: diameter of 10 mm and height of 5 mm. The surface was ground by using abrasive papers with a gradation up to 1000 and then etched in a mixture of 4 M H_2SO_4 with 1 M HF. The sample was cleaned with distilled water in an ultrasonic cleaner.

The Ti–15Mo alloy was anodized in a solution of 0.1 M $Ca(H_2PO_2)_2$ (Alfa Aesar, Germany) with an initial current density of 100 mA/cm^2 and a limited voltage of 300 V for 5 min (DC power supply (Kikusui, Japan)) according to the procedure presented in Ref. [4].

The porous oxide layer was coated by a poly (D,L-lactide-*co*-glycolide) (PLGA) (50/50), Mn = 19 000 (Sigma Aldrich, Poland), using a dip-coating technique. The samples were placed in a chloroform solution (density of 1.48 g/cm^3) with 5% PLGA (v/w). In the case of applying drug-containing layers, a solution consisting of PLGA and the addition of 10 wt% doxycycline was used (Across, Poland). The dipping speed of the samples was determined experimentally by measuring the weight gain due to the application of the polymer layer and visual assessment of the surface. The homogeneous coatings were obtained when the parameters of the dip coatings were as follows: speed of immersion and drawing of 1 cm/s, immersion time of 60 s and drying time of 10 min.

2.2. Coating characterization

2.2.1. Surface morphology and roughness

The surface morphology of the modified titanium alloys was tested using scanning electron microscopy (SEM) (Phenom Pro, backscattered electron imaging, accelerating voltage of 15 keV). The hybrid oxide layer with the drug was also analyzed using confocal microscopy to determine the thickness of the oxide layer with doxycycline. Imaging was performed using an Olympus FV 1000 confocal microscope with 40 × /NA 0.95 objectives. Z-stack images were generated using a 488 nm laser for the FITC dye. 3D data sets were analyzed as volume-rendered data sets using Imaris (custom software developed by Bitplane Scientific Software, Zurich, Switzerland). The surface roughness parameters for the investigated investigatedsamples were determined using the 3D reconstruction software associated with the SEM. The arithmetic

mean deviation of the roughness profile Ra and roughness height by 10 points in 5 selected places on the surface of the sample Rz were observed.

2.2.2. Degradation of the hybrid oxide-polymer layer

Degradation of the polymer was examined by ^{1}H NMR spectroscopy. Polymer samples were placed in Falcon tubes and immersed in 5 ml of artificial body fluid solution (0.7 g/dm^3 NaCl, 0.5 g/dm^3 KCl, 0.26 g/dm^3 Na_2HPO_4, 1.3 g/dm^3 $NaHCO_3$, 0.33 g/dm^3 KSCN, and 0.13 g/dm^3 urea – Across. Poland). Then, they were placed in an incubator at 37 °C and shaken at 60 rpm. The samples were removed at weekly intervals over a period of 28 days. The solution was lyophilized and analyzed by ^{1}H NMR at 25 °C using a $CDCl_3$ solvent. ^{1}H NMR spectra were recorded with a UNITY-INOVA spectrometer (Varian 300 MHz). The internal standard used was tetramethylsilane (TMS).

2.2.2.1. Drug release. The stability of the doxycycline and drug release from the coatings was analyzed by high-performance liquid chromatography. The samples with coatings were immersed in 5 mL of phosphate buffered saline (PBS w/o Ca, Mg, VWR) in Falcon tubes and shaken at 60 rpm in an incubator at 37 °C. After 30 min, 100 μl were collected from the 5 samples and filtered using a nylon filter of 0.2 μm. Then, to the Falcon tubes with the samples, 100 μl of fresh PBS solution was added. The procedure was repeated for the three independent series of samples.

2.2.2.2. High-performance liquid chromatography. HPLC analyses were performed using a Merck-Hitachi chromatograph equipped with an L-7100 pump and L-4500A diode array detector (DAD). Chromatographic separations were carried out on a TSKgel ODS-100 V column, 150–4.6 mm, with a particle size of 5 (TOSOH BIOSCIENCE) at 22 °C. Isocratic elution with 0.05% trifluoroacetic acid in water and acetonitrile (70:30, v/v) was applied. The detection of doxycycline was performed with the use of a DAD detector, where the wavelength was set to $\lambda = 270$ nm. A calibration curve for the determined doxycycline in an artificial saliva matrix was prepared. The calibration curve was prepared for solutions in the range of 10–550 μg/mL. The calibration curve parameters were 13293 for the slope (a) and 58491 for the intercept (b). The number of experimental points taken for regression was $n = 15$. Every solution was injected thrice. The volume of the solution was 20 μL for every single injection. The limit of detection (LOD) and limit of quantification (LOQ) as a signal-to-noise ratio was determined. The LOD for doxycycline was 3 ng/mL, while the LOQ was 10 ng/mL.

Studies on the doxycycline stability were conducted at four different concentrations selected to represent the range of concentrations in real samples. Doxycycline model solutions were prepared in PBS with concentrations of 10 μg/mL, 50 μg/mL, 100 μg/mL and 300 μg/mL. Three parallel samples of a given concentration were prepared, and each sample was injected twice. Analyses were performed immediately after solution preparation (time zero) and then after 6.5 h, 13 h and 21 h of storage at room temperature.

2.2.2.3. Cytocompatibility. Cell metabolic activity was evaluated using the Alamar Blue reagent. The exact procedure was described in our previous work [12]. Briefly, the samples were sterilized in ethanol and then placed in 24-well plates. In each of them, 8000 osteoblast-like cells were embedded in a 1 cm^3 solution of the EMEM 66 medium. The indicator was added to the cell culture, and the entire system was maintained at 37 °C for 4 h in a humidified atmosphere containing CO_2. The number of active cells was determined from three samples. Tissue culture polystyrene (TCPS) was used as a reference in cytocompatibility test. A calibration curve and an average and a standard deviation were used. Statistical analysis of the results obtained was also carried out using Student's t-test. The viability, distribution and connection to the ground of the deposited cells were analyzed using live/dead staining and fluorescence microscopy.

2.2.2.4. Antibacterial properties. The antibacterial properties of the material were evaluated using the solution with the doxycycline released from the coatings after 1 h, 2 h, 4 h, 6 h, and 12 h of immersion in artificial saliva at 37 °C, with shaking performed at 60 rpm. The bacterial strains used throughout this experiment were *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 700296). The bacterial strains were precultured in the TSA (Triptic Soy Broth) culture medium at 37 °C for 18 h (Incubator ES-20/60, Biosan). Then, the bacteria were sent into the agar diffusion plate (Diag-Med, Poland), and 100 μl of previously filtered solution with doxycycline was added. The samples were cultured at 37 °C for 18 h (Incubator ES-20/60, Biosan).

The solution collected after various times of doxycycline release was also used to determine the minimal inhibitory concentration. Two milliliters of the filtered solution with the drug was added to 2 mL of the TSB with 10^6 CFU/mL of the bacteria (*S. aureus* (ATCC 25923) or *S. epidermidis* (ATCC 700296)), and the optical density (OD) was measured using a densitometer (Densilameter, Erba Lachema). The samples were cultured at 37 °C for 18 h, and the OD was again measured. The results are presented as an average value obtained according to the McFarland scale. The experiment was carried out for each bacterial strain using 3 independent samples.

3. Results and discussion

The results of the test carried out for:

- the Ti–15Mo titanium alloy (TM sample),
- the titanium alloy after the plasma electrolytic oxidation process (TM-PEO sample).
- the titanium alloy with the PLGA layer (TM-PEO-PLGA sample).
- the titanium alloy with the doxycycline-consisting polymer layer (TM-PEO-PLGA-DOX) are presented below.

Surface morphology and roughness.

Fig. 1 shows the surface morphology of the Ti–15Mo alloy after the plasma electrolytic oxidation process (Fig. 1a and b) and after coating the anodized surface with a PLGA polymer layer (Fig. 1c and d).

Many pores were created under the anodization process as a result of conditions prevailing during the anodizing process. When the oxide barrier layer broke down, spark discharges caused the formation of pores in the layers. The surface roughness for the TM-PEO sample was as follows: $Rz = 1.5 \pm 0.3$ μm and $Ra = 0.5 \pm 0.09$ μm. In the case of the TM-PEO-PLGA sample, the characteristic porous oxide layer was still visible after the formation of an additional polymer layer. The polymer layer reproduced the structure of the porous oxide layer, when the layer was observed under SEM microscope. The microstructure of the coatings was still porous after PLGA deposition. As a result of formation of PLGA layer on previously anodized titanium alloy surface, the value of the roughness parameters increased, and a greater difference was noted for $Ra = 1.2 \pm 0.2$ μm than for $Rz = 2.3 \pm 0.5$ μm.

Fig. 2 shows the 2D and 3D SEM images of the TM-PEO-PLGA-DOX sample surface. The pores were still visible in the coatings, and the particles of doxycycline were not detected by SEM. The modification of the surface layer with the drug resulted in an increase in the mean value of the Rz parameter and a decrease in the value of the Ra parameter in relation to the layer containing only the polymer ($Rz = 4.9 \pm 0.9$ μm, $Ra = 1.2 \pm 0.1$ μm). The coatings with the drug, which may find application in medicine, were analyzed in detail using a confocal microscope. The coatings were immersed in a solution with SYTO9 and then rinsed with water. Fig. 2c and d presents the microstructure of the polymer layer with the drug deposited on the previously anodized Ti alloy surfaces (TM-PEO-PLGA-DOX sample). It is clearly visible that the microstructure of the polymer layer is completely different from those

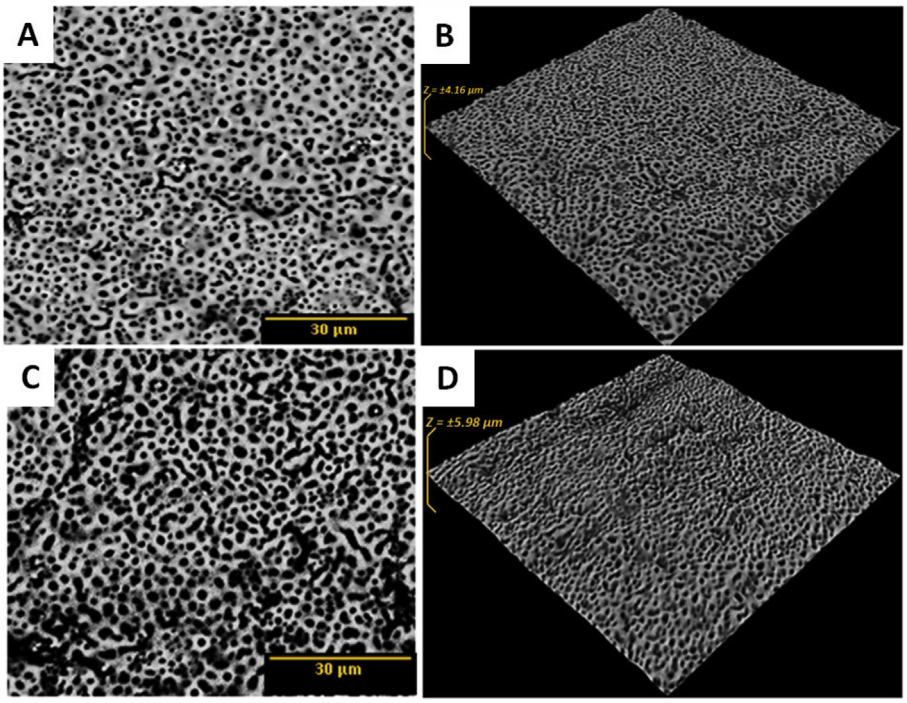

Fig. 1. SEM images of the anodized surface (A, B: TM-PEO sample) and the sample with the oxide and polymer layers (C, D: TM-PEO-PLGA).

obtained on the oxide layer. The polymer was irregular but formed a coherent layer. The thickness of the polymer layer was measured at representative locations in the optical image, and it is indicated that the thickness of the polymer layer was between 22.1 and 39.1 µm.

The SEM images confirmed the formation of a porous oxide layer on the surface of the titanium alloy as a result of plasma electrolytic oxidation. The final results of the surface treatment strongly depends on the electrochemical composition of the PEO process, and the bath composition and substrate, as well [13,14]. The characteristic porous structure of the coatings usually is composed of the chemical compounds incorporated from the anodizing bath. In our case, the PEO process was carried out in solution with calcium and phosphorus, and these ions were built in the coatings. The chemical analysis of the PEO coatings anodized in $Ca(H_2PO_2)_2$ solutions were presented in our paper [15]. If calcium or phosphate are built in, apatite may crystallize, which is beneficial for bone tissue growth and the formation of a good connection between the implant and a given bone. Formation of the apatite on the polymer surface is favorable for osseointegration process [16].

3.1. Degradation of hybrid layers

The progress of hydrolytic degradation of the hybrid coatings containing doxycycline was examined by the ^{1}H NMR technique. Fig. 3 shows the spectra of the PLGA before degradation and of the PLGA

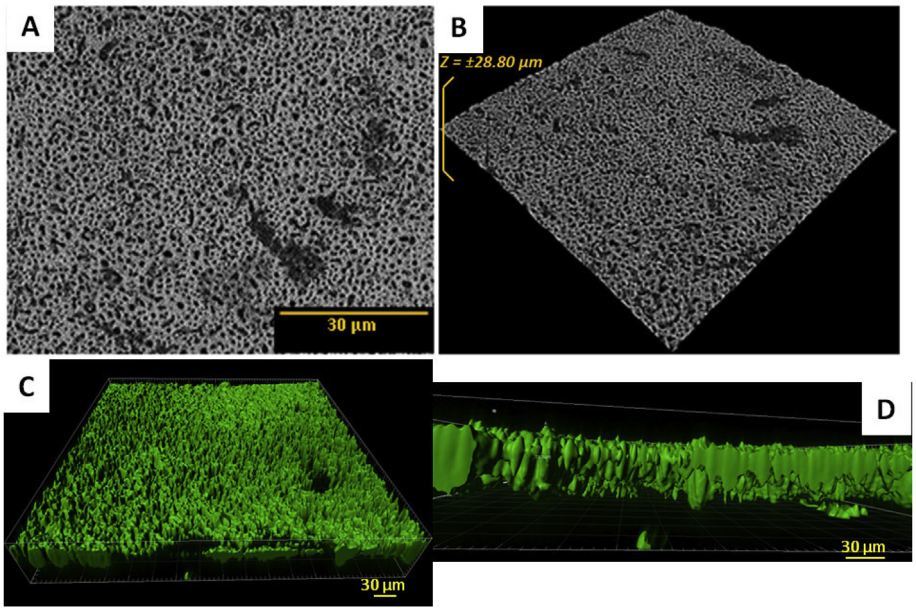

Fig. 2. SEM images of the oxide-polymer layer with doxycycline (A, B: TM-PEO-PLGA-DOX sample), and images of the polymer layer loaded with doxycycline (C) and the polymer layer thickness (D) determined by confocal microscopy.

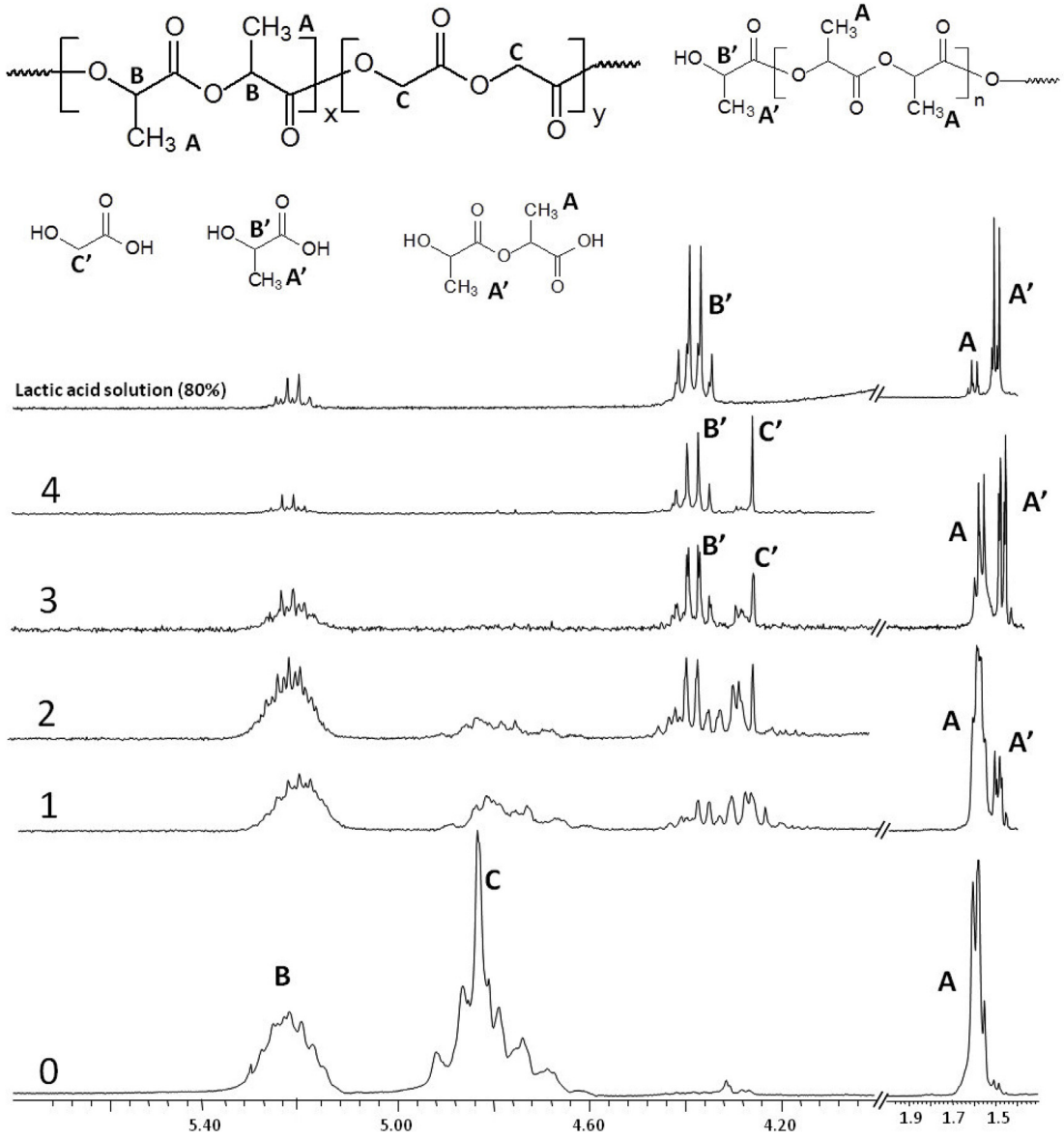

Fig. 3. ^{1}H NMR spectra of PLGA before degradation and of PLGA layer of the TM-PEO-PLGA-DOX samples obtained during the 4-week degradation process in the artificial body fluid solution.

Table 1
Ratio of the amount of lactide to glycolide units during 5 weeks of the TM-PEO-PLGA-DOX layer degradation.

Degradation time [weeks]	Ratio of LA: GA units in the polymer[a] [mol/mol]	Ratio of LA units in the polymer to LAc[b] [mol/mol]	Content of the LAc [%]
0	1 : 1	1 : 0	0
1	1 : 0.38 (2.6 : 1)	1 : 0.28 (3.5 : 1)	22.0
2	1 : 0.2 (4.8 : 1)	1 : 0.38 (2.6 : 1)	27.6
3	1 : 0	1 : 0.77 (1.3 : 1)	43.5
4	1 : 0	0.52 : 1 (1 : 1.9)	65.8
5	1 : 0	0.46 : 1 (1 : 2.2)	68.5

[a] Calculated on the basis of the intensity ratio of signals A and B.
[b] Calculated on the basis of the intensity ratio of signals C and C′.

layer from TM-PEO-PLGA-DOX samples after 1–4 weeks period of degradation carried out in the artificial body fluid solution at 37 °C.

The obtained spectra were compared with the spectrum of lactic acid (LAc) solution in water (80%). The observed signals in spectra were assigned to particular groups of protons: [A] (O)COCH–CH3 (m, 3H; 1.50–1.70 ppm), [B] CHOC(O) (m, ^{1}H; at 5.10–5.35 ppm) and [C] $CH_2OC(O)$ (m, 2H; 4.60–5.00 ppm). For each signal, the integral was calculated, and the ratio of their values A:B:C was approximately 3:1:2, which is consistent with the number of protons giving individual signals. When calculating the intensity ratio of signals A:C or B:C (after dividing by the number of protons), the lactidyl (LA) to glycolidyl (GA) units ratio was determined to be 1:1 in the initial polymer. During the degradation process, the intensity of signals from the above groups decreases, and after three weeks of samples immersion in the artificial body fluid, the peak from methylene protons [C] practically disappears. It means that polyglycolide part of copolymer is completely hydrolysed after such period of time. The polylactidyl part undergoes hydrolysis significantly slower. Basically, the products of PLGA degradation were identified as a short-chain oligomers and low-molecular-weight hydroxyacids such as lactic (LAc) and glycolic acid. The chemical structure of polymer degradation products is presented in Fig. 3, with the corresponding signals at 4.35–4.50 ppm (q, ^{1}H, $CH_3(OH)C\mathbf{H}C(O)OH$) [B′], 4.25–4.35 ppm (s, 2H, $HO(O)CC\mathbf{H}_2OH$) [C′], and 1.45–1.55 ppm (d, $C\mathbf{H}_3(OH)CHC(O)OH$) [A′]. Table 1 presents changes in the ratio of the number of lactidyl to glycolidyl units and the percent amount of LAc

formed during 5 weeks of degradation. At the beginning of the experiment, the ratio of LA:GA units is 1:1. It changes in a relatively short time, and after the first week of being in the artificial body solution, it is 1:0.38, while after two weeks, it drops to 1:0.20. At the same time, the lactic acid content increases. After 7 days of immersion, it is 22%, and after 14 days, it is slightly higher – 26%. After 3, 4, and 5 weeks of conducting the experiment, signals indicating the presence of glycolide units in the polymer structure are undetectable; thus, the ratio of LA:GA units is 1:0. The largest increase in LAc content is observed between the second and fourth weeks. Finally, after 5 weeks in the artificial body solution, its content is 68.5%.

It can be concluded that as the degradation process progresses, the percentage of lactic acid increases slowly because hydrolysis of PLGA chains proceeds slower in polylactidyl part than that of polyglycolidyl one. These changes indicate the PLGA degradation process in the body fluid environment, which is in line with expectations and confirms the possibility of using the tested polymer as a matrix for the controlled drug release systems.

The hydrolytic degradation of PLGA occurs in a short time with the increase in lactic content. E. Vey et al. reported that the degradation rate constant for polylactide is smaller than for polyglycolide and may differ depending on the lactic unit content [17]. Poly (lactide-*co*-glycolide) is used in many drug delivery systems, combined with various drugs and in various carriers [18]. The PLGA is widely used for nanoparticles formation and loaded by various drugs [19]. Drug release play a key role in the biomaterial characterization, however the stability of the drugs plays a key role in the beginning of the drug release determination.

3.1.1. Drug release and its stability

The concentration of doxycycline in artificial body fluid was determined by high-performance liquid chromatography. This technique allows confirming that the drug was not degraded during the elution process from the modified surface. However, doxycycline is unstable in artificial saliva, and the time of its stability depends significantly on its concentration in solution. Fig. 4A presents the results of the drug concentrations in the artificial saliva after the various times of exposure in the vials.

It was determined that the concentration of doxycycline significantly influenced its stability in the artificial saliva solution. When the concentration of doxycycline was higher than 75 μg/mL, more than 90% of the doxycycline was stable up to 21 h. The stability of doxycycline drastically decreased when its concentration in the solution was 10 μl. The primary content of doxycycline was below 90% after 6.5 h, then decreased below 80% after 13 h and then significantly decreased below 70% after 21 h. The marked bars in each column represent the coefficient of variation value. Concentration of doxycycline in the matrix determined after 21 h differs statistically from previous concentration values. The results indicate that the determined amount of doxycycline in the solution will be lower than the initial concentration due to its low stability in the solution. Thus, the doxycycline released from the polymer coatings was determined up to 10 h to obtain reliable results. This time is also the most important to prevent bacterial adhesion and biofilm formation.

Fig. 4B presents the concentration of the doxycycline released into the artificial saliva after up to 10 h of incubation at 37 °C. The first hours of drug release are the most important in bacterial adhesion and biofilm formation. The total concentration of doxycycline loaded into the polymer layer was 385.35 μg/cm^2. After 30 min of material immersion, more than 23% of the loaded drugs were released from the coating (91.67 μg/mL). Then, the concentration of doxycycline released into the artificial saliva slightly increased. The difference between the 30 min and 10 h concentrations of drug released from the material was 32.84 μg/mL. After 10 h of coating immersion, 32.3% of doxycycline was released.

Doxycycline presents 18 different forms depending on the pH of the solution. Due to the similarity of the physicochemical properties of the compounds, it is not always possible to determine each individual form. In this study, we used HPLC-DAD system with C18 stationary phase, the pH of the mobile phase was 2. The pH of the matrix solution to which the drug was released from the surface was also important. The applied measurement conditions allowed to observe three signals coming from doxycycline. The chromatographic system used in the study made it possible to separate the three of compound form and to validate the chromatographic procedure. Hence, the reported changes in drug concentration during release from the hybrid layer to the artificial saliva solution are the sum amounts for the forms present at the given pH values. Even if the percentage of individual antibiotic varieties is known in a given pH environment, the concentration of each of these forms in the artificial saliva solution cannot be unequivocally determined based on the absorbance value. The largest variety of forms occurs in the pH range from 6 to 8, and their content is often very similar. The accurate determination of the concentration of a particular substance would be inexact; thus, we presented forms, the occurrence of which at a given pH is most likely [11,20].

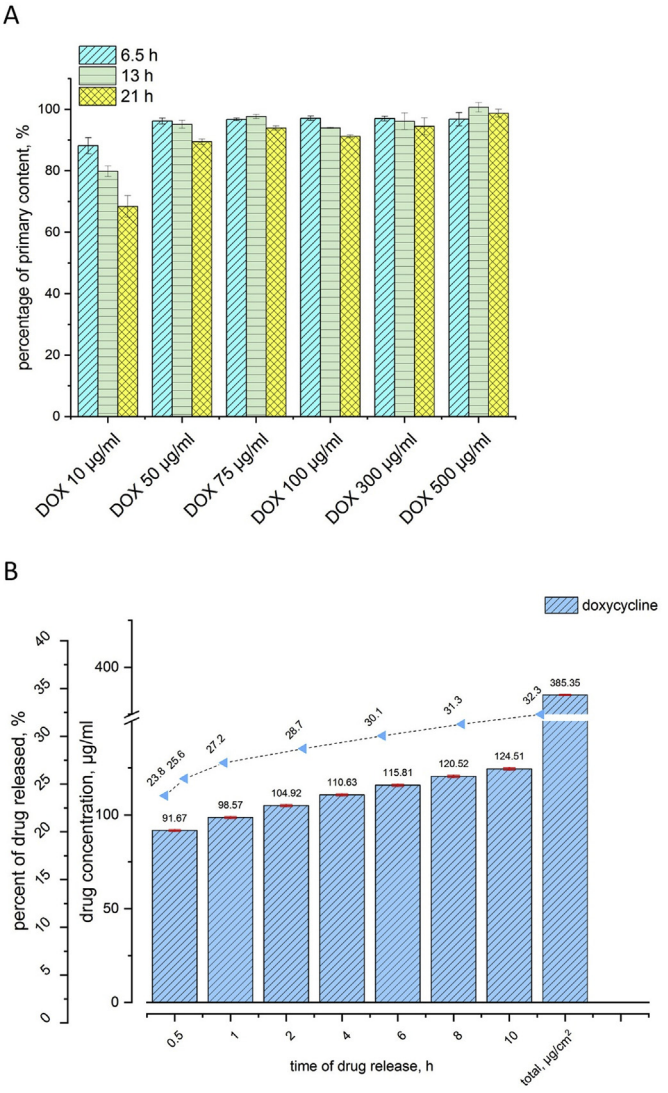

Fig. 4. Concentration of doxycycline after various exposure times in artificial saliva (A) and concentration of the doxycycline released from the oxide-polymer layer into artificial saliva, and the total drug concentration loaded into the coating (B). The changes are correlated with the concentration of doxycycline and strongly influence the determination of the amount of doxycycline released from the coating.

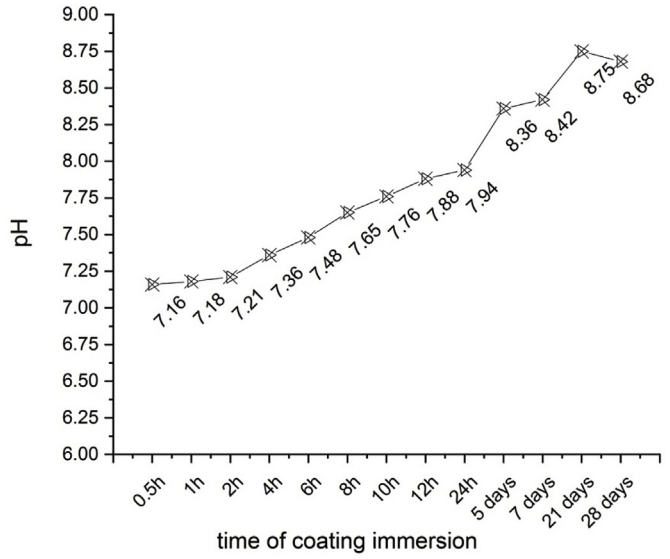

Fig. 5. Changes in the pH of artificial saliva during immersion of the oxide-polymer layer with doxycycline (TM-PLGA-PEO-DOX).

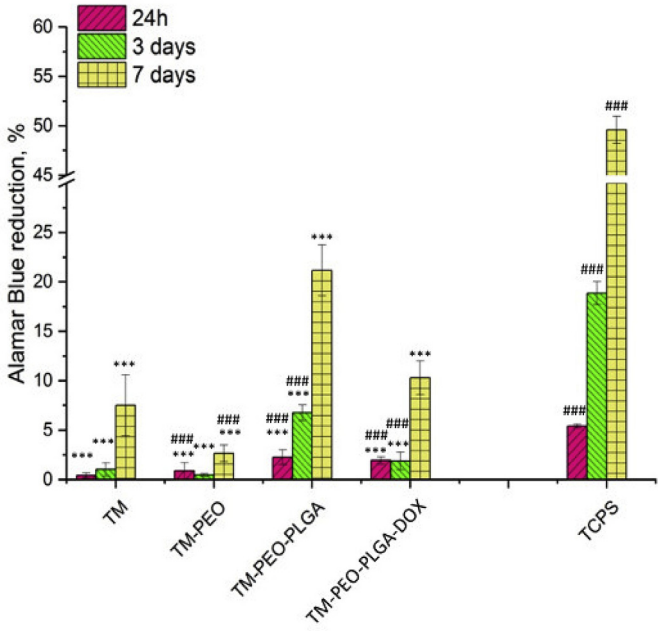

Fig. 6. The number of viable MG-63 cells on the modified Ti–15Mo titanium alloy surfaces, expressed as the Alamar Blue reduction rate. Statistical significance from the TCPS (*) and unmodified Ti–15Mo (#) are is marked; *, #p < 0.05, **, ##p < 0.01 and ***, ###p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

During the experiment, the pH value of the taken solutions was also measured. The results are presented in Fig. 5.

The form in which doxycycline occurs depends on the pH of the environment. Hence, knowing the pH of the solution into which the drug is released, we can estimate the percentage of each variety of the antibiotic in the sample. The differences in the pH were clearly observed. The initial pH of the coated Ti alloy with doxycycline in artificial saliva was 7.4. After 30 min, the pH decreased to 7.16. Then, the pH slightly increased, and after 10 h, it was 7.65. The pH significantly changed 24 h after the materials were coated (up to 7.88) and after 5 days (8.36), 21 days (8.75) and 28 days (8.68).

3.1.2. MG-63 osteoblast-like cell cytocompatibility

Fig. 6 shows the results of MG-63 osteoblast-like cell viability studies after 1, 3 and 7 days.

The number of viable cells was determined using the Alamar Blue dye, and the results are expressed as a reduction rate. The higher the degree of Alamar Blue reduction, the greater the number of live cells marked on the surface of a given titanium alloy sample. After 24 h of the experiment, the majority of viable cells were determined on the surface of the TM-PEO-PLGA-DOX sample (percent of Alamar Blue reduction was 2.85%), slightly less in the case of TM-PEO-PLGA (2.28%) and the least on the unmodified alloy Ti–15Mo (0.41%).

After 3 days of incubation, the reduction rate was the highest for the TM-PEO-PLGA sample – 6.75%. This is a significant increase compared to the first day (2.28%). In the case of drug-containing layers, the number of live cells decreased: for the sample with 5% by weight of doxycycline, it was only slightly lower – 1.89%; for the sample with a higher antibiotic content, the difference was greater – 1.72%. Thus, the degree of Alamar Blue reduction on modified surfaces differing in drug content became similar; the cell culture was influenced in a similar manner. After 7 days, the percent of reduction increased for all of the samples, but the highest increase was again noted for the TM-PEO-PLGA – 21.17%. In the case of samples containing doxycycline (TM-PEO-PLGA-DOX) the proliferation was 10.30%. The tendency of a slightly worst cell proliferation on the surface of the layer containing the smaller amount of drug was observed. After 3 and 7 days of culture, the lowest Alamar Blue reduction rate was observed for the sample after only the PEO process – 0.47% and 2.66%, respectively. The results indicate that the post-treatment technique increase the cytocompatibility of the investigated Ti alloy.

Fig. 7 shows the morphology of osteoblast-like cells after 1, 3 and 7 days of incubation.

To characterize the morphology of the surface of living cells and the number of dead cells, live/dead staining was used. Optical microscopy with a fluorescent attachment allows the observation of living cells, which, under these conditions, are green, while dead cells are red. After 24 h of incubation, most cells were adhered to the surface of the TM-PEO-PLGA sample, while the least number of were adhered to the surface of the pure Ti–15Mo alloy. For each sample, the cells were flattened, which indicates their good adhesion to the substrate. Their shape was also extended; only for the TM-PEO-PLGA-DOX surfaces were they more spherical. The arrangement on the surface of the TM was homogenous, whereas on the TM-PEO-PLGA and TM-PEO-PLGA-DOX samples, agglomerates of living cells were visible. After 24 h, only single dead cells were observed on the examined surfaces. After 3 days of the experiment, the number of living cells increased for each of the samples, with the highest dye reduction rate recorded for the TM-PEO-PLGA sample (6.75%), which confirms the largest number of preloaded cells visible in the picture (Fig. 7). The smallest coverage level was observed for the TM-PEO surface, which, in relation to the state after 24 h, increased by only 0.06% points. Based on the images (Fig. 7), the cells were found to be well flattened, to be regularly spaced and to not form agglomerates, especially in the TM-PEO-PLGA and TM-PEO-PLGA-DOX layers. After 7 days, the Alamar Blue reduction rate increased for each of the samples tested; thus, the number of osteoblast-like adhered cells again increased. After 3 days of incubation, the largest amount was on the surface of TM-PEO-PLGA and subsequently on TM-PEO-PLGA-DOX. They were well flattened and arranged in a homogenous manner. A significantly smaller number of cells were adhered to the TM-PEO layer. After both 3 days and 7 days of the experiment, dead cells were very rarely observed, being visible as red cells.

T. Wang et al. investigated the biological properties of PLGA. The polymer positively influences the growth of bone cells and forms a permanent connection between tissue and titanium implants. Investigations with the use of mouse MC3T3-E1 cells proved no toxic effects of PLGA and its biodegradation products and hence good

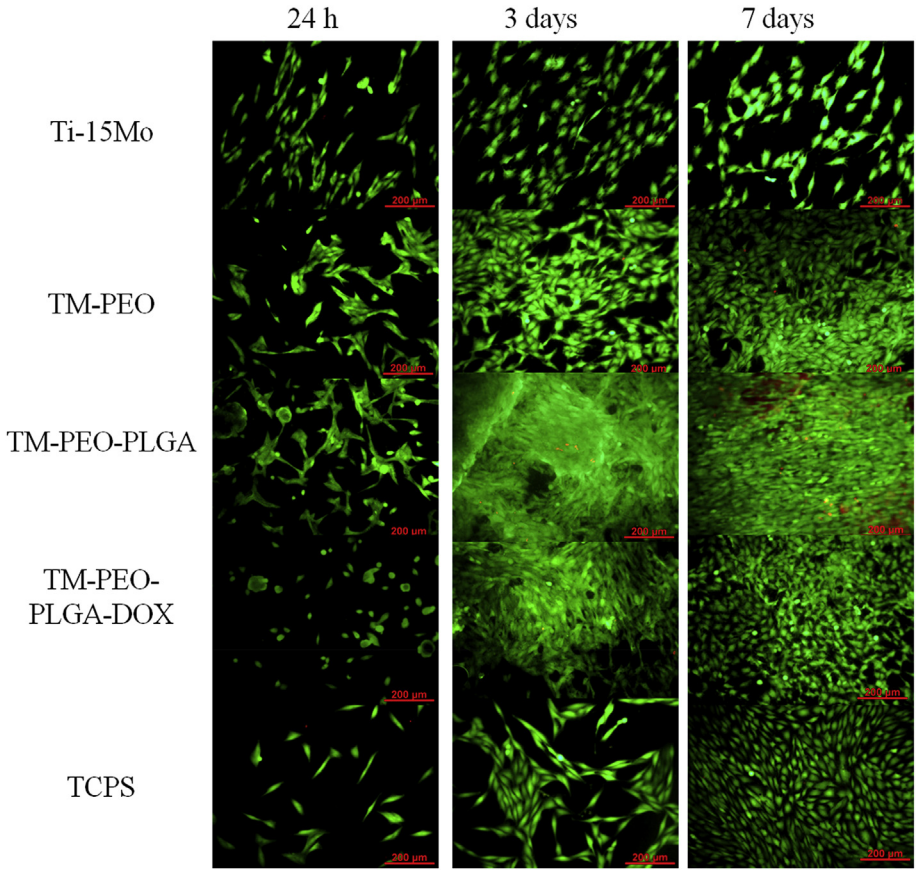

Fig. 7. Fluorescence microscopy images of the titanium alloy surfaces and reference samples after 1, 3 and 7 days of culture. Live/dead staining, where green indicates the live cells and red indicates the dead cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

biocompatibility of the polymer [21]. L. J. Smith et al. also agreed that coating the anodized titanium surface with PLGA enhances the proliferation and adhesion of osteoblast cells to the substrate [22]. The same effect was also obtained in the case of titanium grade 4 coated with zirconium oxide/polyethylene glycol with various percentages of PEG content [23].

The utility of PLGA in implantology was also analyzed by L. Zhao et al. The polymer was applied to bioceramics composed of calcium silicate. The bioactivity of the obtained materials was evaluated by immersion in simulated body fluid and assessment of the amount of crystallized apatite on the modified surface. Applying PLGA does not impair the ability to deposit apatite molecules. The thinner the polymer layer is, the higher the concentration of apatite. In addition, PLGA coatings have a good impact on the pH value of the environment surrounding the implant by binding the alkaline ions derived from the decomposition of calcium silicate by acidic products of polymer degradation. Reducing the pH value is also beneficial for cell growth, proliferation, spreading and morphology on the modified surface [24]. Wei Ji et al. proposed another solution: modification of the PLGA-based nanofibrous scaffolds with nanoapatite particles [25]. The addition of nanoparticles results in slowing down the degradation process. This is marked by the higher molecular weight and depends on the number of nanoapatite particles acting as a weak alkaline salt, giving the buffering effect. Investigations showed fewer inflammatory cells and foreign body giant cells in the case of samples with nanoapatitic particles. The authors claim that this is due to a lower degree of polymer biodegradation. In addition, acidic degradation products are homogenously distributed on the implant surface. As in our case, the polymer products are acidic. However, the pH measurements near the coatings indicate alkalization. Probably, it is an effect of changing in the doxycycline, and possibility of phosphates-based compounds formation on the surface of the sample. Especially, that the Ti–15Mo surface was anodized in calcium hypophosphate solution and calcium and phosphorous compounds exist in the coating [15]. It is possible that the small calcium-phosphate nanoparticles were formed on the surface, or the changes occurred in the solution, thus the pH was changed. The explanation of the changing the doxycycline is also connected with the results of the microbiology test. Where, the doxycycline released after 4 h was less active against bacteria, than the solution with doxycycline released up to 4 h.

3.1.3. Bacteria

To evaluate the effectiveness of surface modification of the Ti–15Mo titanium alloy, the antibacterial activity of hybrid coatings against the *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 700296) bacteria was examined. Initially, the antibacterial properties of the modified surfaces containing 5% (by weight of the polymer) doxycycline were tested. However, this concentration was not enough to significantly inhibit bacterial growth. Therefore, studies with more antibiotics (10% vs. PLGA weight) were performed. This amount was sufficient to inhibit the growth of both bacteria (*S. aureus* and *S. epidermidis*). Fig. 8 presents the inhibition zones of bacterial strains after culture with artificial saliva, with drug released from the coatings after 1, 2, 4, 6, and 12 h of sample immersion, and the control (artificial body fluid).

In the case of *S. epidermidis*, the lowest inhibition of bacterial growth was observed after 2 h (21 mm), and the greatest inhibition was observed after 4 h (32 mm). The effect of the drug clearly increased between these measurement points. Then, the drug weakened again, and the inhibition zone decreased. Significantly greater sensitivity to doxycycline was shown by *S. aureus*. The diameter of the growth inhibition zone exceeded 30 mm even for the solution obtained after 1 h of sample immersion – 36 mm, which was the best result. The same effect was noted for the sample after 12 h of incubation. Inhibition zone sizes remained at a similar level throughout the experiment, which suggests better stability of the drug on *S. epidermidis* than that on *S. aureus*.

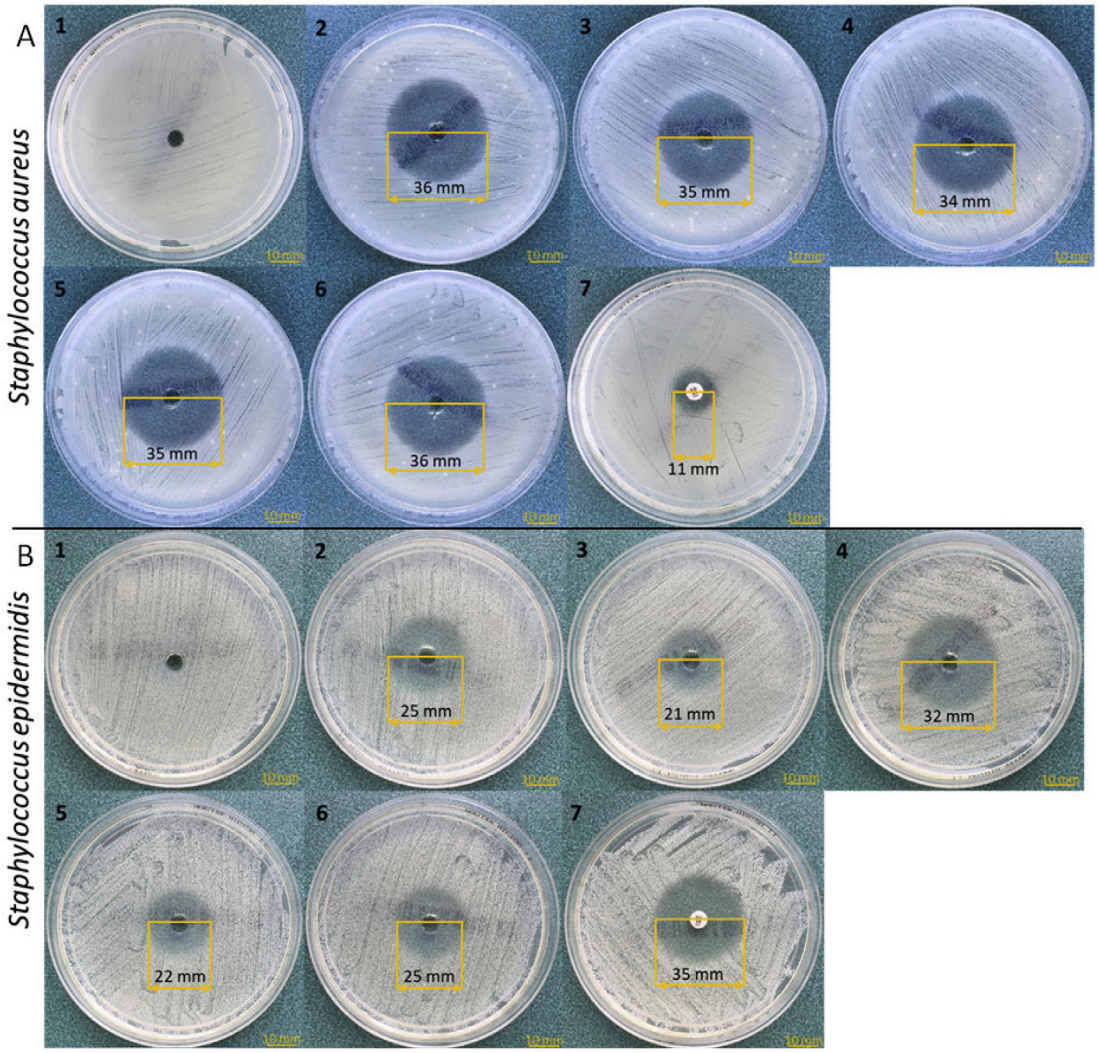

Fig. 8. Inhibition zones of reference (A) *Staphylococcus aureus* (DSM 24167) and (B) *Staphylococcus epidermidis* (ATCC 700296) after culture with artificial saliva (1) and with the doxycycline released from the coating after 1 h (2), 2 h (3), 4 h (4), 6 h (5), and 12 h (6) into the artificial body fluid.

Despite slight differences, the smallest size was obtained for the solution after 4 h of incubation (34 mm).

The antibacterial activity was also characterized by the number of bacteria colonies expressed using the McFarland scale. Their amount was determined after 18 h of incubation and then compared with the initial quantity, thus assessing the multiplication of bacteria. As shown in Fig. 9, a significantly larger increase in the number of colonies was observed in the case of *Staphylococcus aureus* than for the *Staphylococcus epidermidis* strain.

The number of colonies varied depending on the time of sample immersion, showing a tendency to decrease in the range of up to 6 h of immersion. In the case of samples that were immersed for more than 6 h, the number of bacteria again increased. However, the number of bacterial colonies ranged from 117 CFU/mL to 540 CFU/mL. In the case of *Staphylococcus epidermidis*, the inhibition of bacterial growth was already observed for samples obtained after 1 h of immersion. The number of bacteria that occurred during the 24 h of the experiment was below the limit of quantification. Doxycycline released from hybrid layers showed greater efficacy in inhibiting the growth of new bacterial colonies in the case of *Staphylococcus epidermidis* than in the case of *Staphylococcus aureus.*

R. Misra et al. reported that entrapment doxycycline in polymeric PLGA-PCL nanoparticles improves the antibacterial properties of the drug against *E. coli* and that increasing the PLGA content has a beneficial effect on the efficiency of encapsulation. Although the drug has high antibacterial activity, the use of a polymeric system results in a decrease in minimum inhibitory concentration and minimum bactericidal concentration. The authors claim that this outcome may be caused by the easier penetration of small molecules of the drug into the interior of the bacteria cells [26]. The antibacterial properties of doxycycline against *Staphylococcus epidermidis* were also confirmed by R. Xing et al. The bacterial growth was stopped, and the inhibition zones on agar plates were observed. The higher the drug concentration is, the greater the degree of inhibition of biofilm formation at the titanium alloy surface [27]. Doxycycline is a broad-spectrum antibiotic and may also be used for other bacterial strains. F. Tamimi et al. tested the antibacterial activity against gram-negative *Actinobacillus actinomycetemcomitans* and *Bacteroides frosthytus*, which are periodontal pathogens responsible for many periodontal diseases. A wide inhibition zone was observed for *Actinobacillus actinomycetemcomitans,* but the strongest effect of the antibiotic was demonstrated in the case of *Bacteroides frosthytus*, where practically 100% of the bacteria were killed [28]. The antibacterial activity of doxycycline differs depending on the bacteria type and becomes higher as the drug concentration in the system increases [29]. The minimum inhibitory concentration (MIC) was evaluated using four strains: *K. pneumonia*, *P. aeruginosa*, *S. aureus*, and *E. coli.* The highest value was measured for *P. aeruginosa* (25 μg/mL), and the lowest, for *S. aureus* (1 μg/mL). Doxycycline shows the ability to

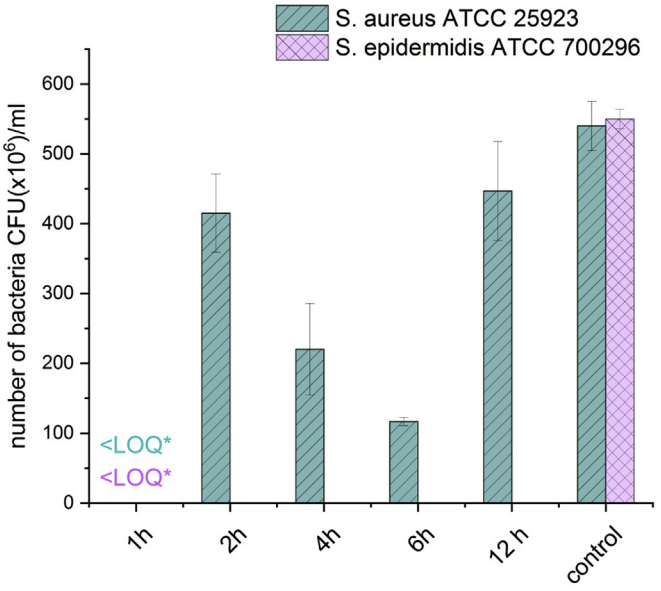

Fig. 9. Number of bacteria determined after 18 h of bacterial culture with the solution containing doxycycline released after various times.
* < LOQ: limit of quantification means that changes in the solution were observed and that the bacteria grown was inhibited; however, the number of bacteria could not be calculated according to the McFarland scale. For all the samples with *S. epidermidis,* the optical density was slightly increased; however, the number of bacteria was below the LOQ.

inhibit bacteria growth for all of the tested strains [30]. In our case, the concentration of the drug released after 30 min of coatings immersion in artificial body fluid was higher, equal 91.67 μg/mL. This concentration was enough to inhibit bacteria (*S. aureus* and *S. epidermidis*) and large inhibition zones were obtained (36 mm and 25 mm, respectively). When concentration of drug increased up to 110.63 μg/mL no significant difference was observed in the inhibition of *S. aureus*, where the S. epidermidis was more sensitive and the inhibition zone of bacteria increased. After 4 h of bacteria culture, the activity of the drug slightly decreased. After 4 h the pH of the solution where the drug released also was changed, and it might influence on the doxycycline activity. When the concentration of the drug increased due to longer time of the coating immersion, the activity of the drug againt bacteria increased, as well. It was reported, that bacteriostaticity is achieved by stopping protein biosynthesis. Furthermore, the drug has the ability to reduce collagenase activity. These phenomena are caused by the formation of a permanent connection between the antibiotic and the 30S ribosomal subunit and, rarely, 50S. This results in the properties of the aminoacyl-tRNA – mRNA – ribosome complex changing by blocking the connection [8].

4. Conclusions

This research confirmed the effectiveness of using plasma electrolytic oxidation and the dip-coating technique for the formation of a hybrid oxide – poly (lactide-*co*-glycolide) layer, which may additionally contain a biologically active substance. The differences in the pH of the solution strongly affect the stability of the doxycycline and the concentration of the doxycycline released from the coating. The polymer layer will degrade in more than 4 weeks, however, the doxycycline released from the coatings after 1 h inhibits the growth of bacteria. For medical applications, this result indicates that the potentially implanted surface might be protected and that the risk of septic inflammation decreases. On the modified surfaces of the Ti–15Mo alloy, the adhesion and proliferation of osteoblast-like cells occurred, which indicated that the surfaces were cytocompatible. Investigations carried out using *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 700256) strains confirmed that the amount of loaded doxycycline was sufficient to inhibit bacterial growth. These results confirmed the utility and efficiency of using hybrid oxide-polymer layers as potential biomaterials for implantology, especially for dental implants. This kind of coating may reduce the risk of postoperative infections and complications.

CRediT authorship contribution statement

Alicja Kazek-Kęsik: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Agnieszka Nosol:** Investigation, Data curation, Writing - original draft. **Joanna Płonka:** Validation, Formal analysis. **Monika Śmiga-Matuszowicz:** Investigation, Visualization. **Sebastian Student:** Software. **Monika Brzychczy-Włoch:** Supervision. **Małgorzata Krok-Borkowicz:** Investigation. **Elżbieta Pamuła:** Supervision. **Wojciech Simka:** Supervision.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2020.04.009.

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