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Biocorrosion of dental alloys due to *Desulfotomaculum nigrificans* bacteria

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Purpose: Degradation processes of metallic biomaterials in the oral cavity limit the stability and reliability of dental materials. The influence of environment bacteria *Desulfotomaculum nigrificans* sulfate reducing bacteria on the corrosion processes of Co-Cr-Mo and Ti-6Al-4V alloys was assessed. *Methods:* After 28 and 56 days of contact of the materials with the bacterial environment, the surfaces of the biomaterials tested were observed by means of confocal scanning laser microscopy (CSLM), and their chemical composition was studied using X-Ray Photoelectron Spectrometry (XPS). *Results:* Corrosive changes and the presence of sulfur (with medium atomic concentration of 0.5% for Co-Cr-Mo and 0.3% for Ti-6AL-4V) were observed on the surface of the biomaterials. Image analysis conducted using Aphelion software indicated that corrosion pits took up approx. 2.3% and 1.8% (after 28 days) and 4.2% and 3.1% (after 56 days) of the total test surfaces of cobalt and titanium alloys respectively. The greatest number of corrosion pits had a surface area within the range of 1–50 μm^2. They constituted from 37% up to 83% of all changes, depending on the type of material. *Conclusions:* An evident influence of the SRB on the surfaces of cobalt and titanium alloys was observed. Significant corrosive losses caused by the activity of microorganisms were observed on the metallic surfaces under study. The results of this study have much cognitive and utilitarian significance.

Key words: *biocorrosion, sulfate-reducing bacteria, Desulfotomaculum nigrificans, XPS, Aphelion*

1. Introduction

Oral cavity is an environment where high humidity, constant temperature and source of food ingredients promote the development of complex and differentiated microorganisms, which can colonize, i.e., surfaces of dental materials. The composition of the oral microbiota can be stable over time or can change depending of the local environment, lifestyle, age, contact with prosthesis or dental implants, etc. [18]. Human saliva, which plays a significant role in oral mouth, i.e., provides optimal pH and buffer properties [6], is also responsible for forming special film, called biofilm [1].

In this environment, due to the contact of different microorganisms (bacteria and fungi) and products of their metabolic activity [27], the process of microbial corrosion of metallic biomaterials (prosthodontic elements, medical implants, etc.) can occur, which causes destruction of biomaterials, as shown in the work of Kameda et al. [11]. This process is a serious problem and still is little known. Information on this subject is scarce in the literature and insufficient for a meritorious description of these processes, especially as regards contact with anaerobic bacteria – sulfate-reducing bacteria (SRB), which have the ability to oxidize metal, and this finally leads to microbiologically influenced corrosion (MIC).

Numerous research works described several types of sulfate reducing bacteria [8], [14], which can occur in the oral cavity as transient flora, and can be involved in biocorrosion: *Desulfovibrio* (*desulfuricans, fairfieldensis*) and *Desulfotomaculum nigrificans* genera [17], [20].

One of the mechanisms of MIC shows that sulfate reducing bacteria exhibit the capability of inducing corrosion. Under anaerobic conditions, a metal surface

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acts as the anode in an electrochemical reaction and is oxidized, yielding Me^{2+} ions. Bacteria produce S^{2-} ions, which enter into a reaction with Me^{2+}, resulting in the formation of metal sulfide. In the cathode area, H^+ ions are produced and they react with hydroxyl groups present in aqueous environment. As a result, oxygen from sulfates is consumed for oxidizing metal, which leads to the formation of metal oxides. A study by Lata et al. [15] shows that the aqueous environment of the oral cavity additionally fosters adhesion of bacteria to metal surfaces. It was also stated [13] that the activity of SRB causes a difference in potential between surfaces in/not in contact with bacteria, which leads to the formation of local corrosion pits (corrosion characterized as deep localized penetration) on the surfaces of metals [28].

Corrosion of biomedical metals in the human body is critical, because it can adversely affect their biocompatibility and mechanical integrity, so studies in this area are very important [12].

The subject of this study was the evaluation of the influence of *Desulfotomaculum nigrificans* sulfate reducing bacteria on corrosion processes of commonly used metallic biomaterials for dental applications: Co-Cr-Mo and Ti-6Al-4V alloys.

The studies of bacterial proliferation and an analysis of the chemical composition of sample surfaces were conducted.

2. Materials and methods

Strains and growth conditions

The standard ATCC 7946 *D. nigrificans* strain, originating from the collection of the Department of Microbiology of the Medical University of Białystok (Poland), was used in this study.

The *D. nigrificans* strain was cultivated on a TSI medium (Triple Sugar Iron Agar, BBL BD Biosciences Sparks) with the following composition (quantities given per 1 liter of purified water): 10 g enzymatic casein hydrolysate, 10 g peptone, 5 g sodium chloride, 10 g lactose, 10 g sucrose, 1 g glucose, 0.2 g ferrous ammonium sulfate, 0.2 g sodium thiosulfate, 0.025 g phenol red, 13 g agar at a temperature of 37 °C, for a period of 72 hours, under anaerobic conditions [7] with the use of a Genbox anaer generator (bioMerieux). In order to obtain semi-liquid medium, the primary medium was diluted three times. This medium provides an appropriate growth conditions for *D. nigrificans.* Next, 10 ml of culture was transferred to a thrice diluted TSI medium and incubated for another 72 hours at a temperature of 37 °C under anaerobic conditions, like in work [19].

Biomaterials

Samples of Co-Cr-Mo and Ti-6Al-4V alloys (SANDVIK, Sweden) were tested in the present study. For each test, twelve samples of each material, with a diameter of 8 mm and a height of 3 mm, were polished progressively with coarse to fine (up to 2000 grit) polishing papers. The polished samples were ultrasonically degreased in acetone/alcohol and sterilized in an autoclave at a temperature of 121 °C for 15 minutes. Six samples were tested in a bacterial environment, and six in environment without bacteria.

Biofilm formation

The *D. nigrificans* biofilm was formed as described in works [14], [17]. After sterilization process, alloy samples were put into containers, one disk per container, incubated in thrice diluted TSI medium, and inoculum was cultivated, adjusted to 0.5 on the McFarland scale (approximate cell density 1.5×10^8 cfu/ml). The negative control was also prepared. In this case steel samples were placed into containers with 3-fold diluted TSI medium without bacteria. Afterwards prepared samples were incubated for a period of 28 and 56 days in an incubator at a temperature of 37 °C under anaerobic conditions. After the set period of time passed, samples were removed from the solution and rinsed three times in sterile PBS (pH 7.2) in order to remove the bacteria making up the plankton suspension and rinse the surface which was held in an environment without bacteria. A dark color of the solution in contact with sulfate reducing bacteria, in which the test materials were held, was observed, as for stainless steel [19].

Confocal scanning laser microscopy

Confocal scanning laser microscopy (CSLM, LEXT OLS 4000, Olympus, KeyMed House Stock Road SS2 5QH Southend-on-Sea, U.K.), a non-destructive real-time imaging technique, was used for evaluation of sample surfaces, which was based on observation of adsorbed bacteria and the formation of corrosion centers after process of incubation in inoculum.

Samples were observed in two stages:

(a) the first stage – just after being rinsed three times in sterile PBS in order to remove free bacteria from the surface of steel,
(b) the second stage, before CSLM microscope observations, samples were additionally rinsed in an ultrasonic cleaner in an acetone/alcohol solution in order to remove adsorbed bacteria from the surface of the test materials.

The images obtained were analyzed using image analysis software (Aphelion 3.1, ADCIS, France). The

entire surface of the sample was examined, and images were taken from the representative area (three places) of the sample. The final results of area and amount of biocorrosion pits are the average of all measurements.

XPS

X-ray Photoelectron Spectroscopy (XPS) using a PHI 5000 VersaProbe – Scanning ESCA Microprobe™ (ULVAC-PHI, Japan/USA) was utilized to analyze chemical composition [9] at corrosive locations. The aim of this study was to evaluate the presence of sulfur on the surfaces of test materials after contact with the *D. nigrificans* strain.

Statistical analysis

In this study, all experiments were carried out using six samples (six in the environment of bacteria and six in the environment without bacteria) for tests. Collected data was statistically analyzed and differences were determined using the one-tailed Student's *t*-test. Statistical analyses were performed using Statistica 12. A p-value < 0.05 was considered statistically significant.

3. Results

Microscope observations of the surfaces of cobalt and titanium alloys after being held in an environment with/without sulfate reducing bacteria (positive and negative control) for 28 and 56 days were carried out using confocal scanning laser microscopy (CSLM). Figure 1 presents the surfaces of biomaterials tested as negative control after 28 days of contact with TSI medium only. The surface of Ti-6Al-4V alloy (Fig. 1b; CSLM, bars 20 μm) is more uniform without visible changes of structure in comparison to the surface of Co-Cr-Mo alloy (Fig. 1a; CSLM, bars 20 μm).

As mentioned earlier, microscope observations of surfaces treated with sulfate-reducing bacteria were performed in two steps. In the first step, samples were observed using CSLM without cleaning in an ultrasonic cleaner in order to present the quantity of *D. nigrificans* bacteria adsorbed onto surfaces and of corrosion products on the test surfaces. Figure 2 shows example photographs of the surfaces of Co-Cr-Mo (Fig. 2a) and Ti-6Al-4V (Fig. 2b) alloys. Adsorbed products of the biocorrosion reaction and non-uniformly distributed bacteria were observed. The amounts of bacteria attached to the surface were similar between the materials studied. As in other studies [19], an increased amount of biocorrosion products was observed on the surfaces of materials at locations where SRB colonies were present. Each of these products took up an area of about a few μm^2 on the surface, as shown in Fig. 2 (CSLM, bars, 10 μm).

In the second step of studies, the bacteria and adsorbed products were removed in an ultrasonic bath. This process reveals the extent of biologically induced corrosion of alloys (Figs. 3–6). After 28 days of contact with sulfate-reducing bacteria, small dark points on the surfaces of Co-Cr-Mo (Fig. 3a; CSLM, bars, 400 μm) and Ti-6Al-4V (Fig. 4a; CSLM, bars, 400 μm) were revealed, and they appear to be similar. But when these surfaces are examined under higher magnification

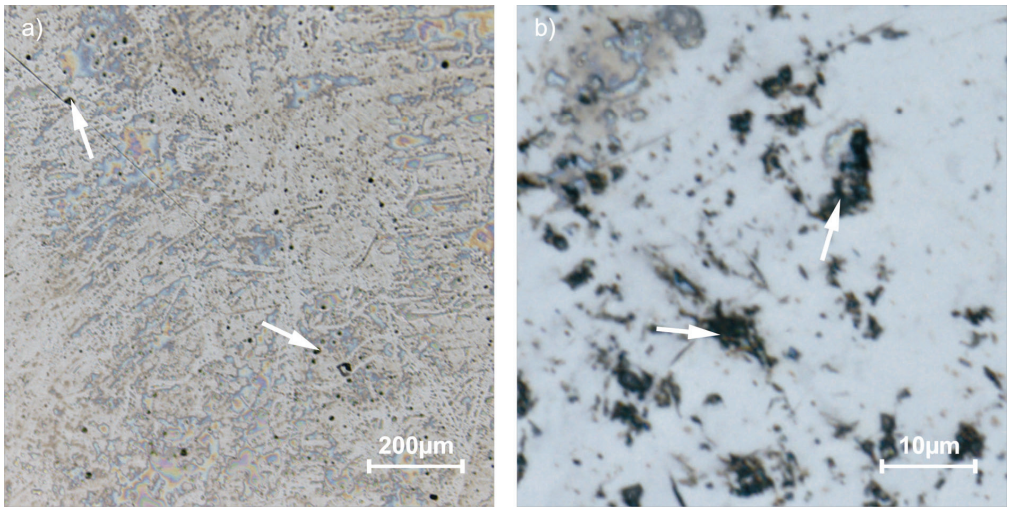

Fig. 1. Confocal scanning laser micrographs of: (a) Co-Cr-Mo, (b) Ti-6Al-4V after 28 days of contact with solution without bacteria (negative control); images represent typical field of view; bars, 20 μm

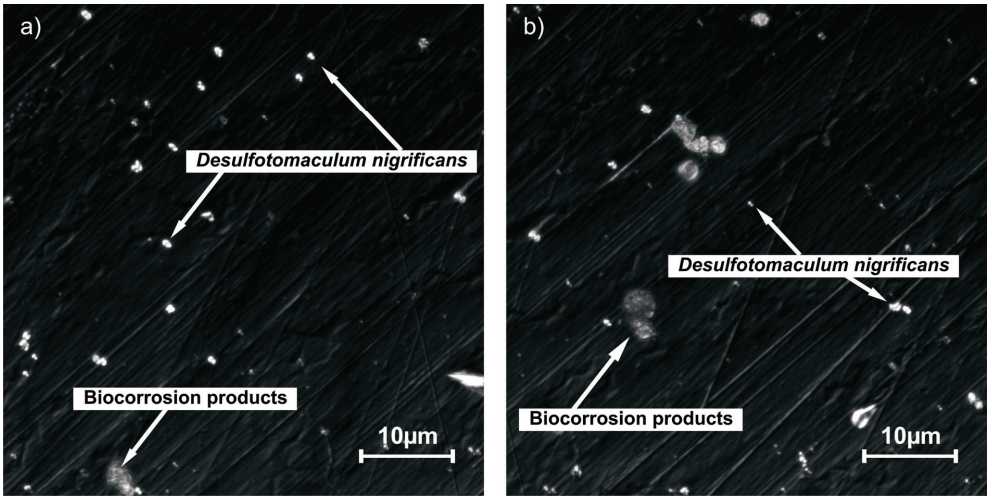

Fig. 2. Confocal scanning laser micrographs of *D. nigrificans* and biocorrosion products in the presence of bacterial inoculum. Biofilm was grown on the surface of Co-Cr-Mo (a) and Ti-6Al-4V (b) discs for 28 days, surfaces without cleaning in the ultrasonic cleaner. Image represents typical field of view. Bars, 10 μm

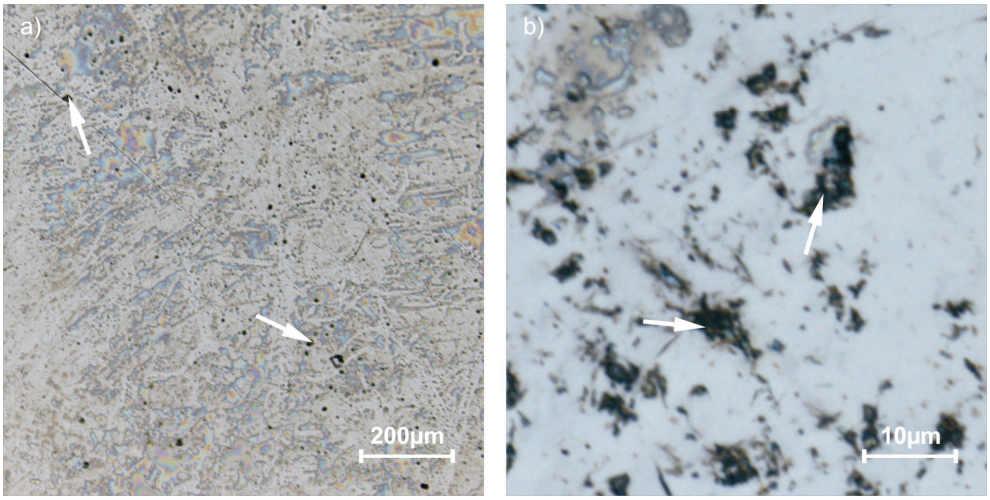

Fig. 3. Biocorrosion changes on the surface of Co-Cr-Mo (CSLM) after immersion in *D. nigrificans* for 28 days; (a) bars, 200 μm, (b) bars, 10 μm

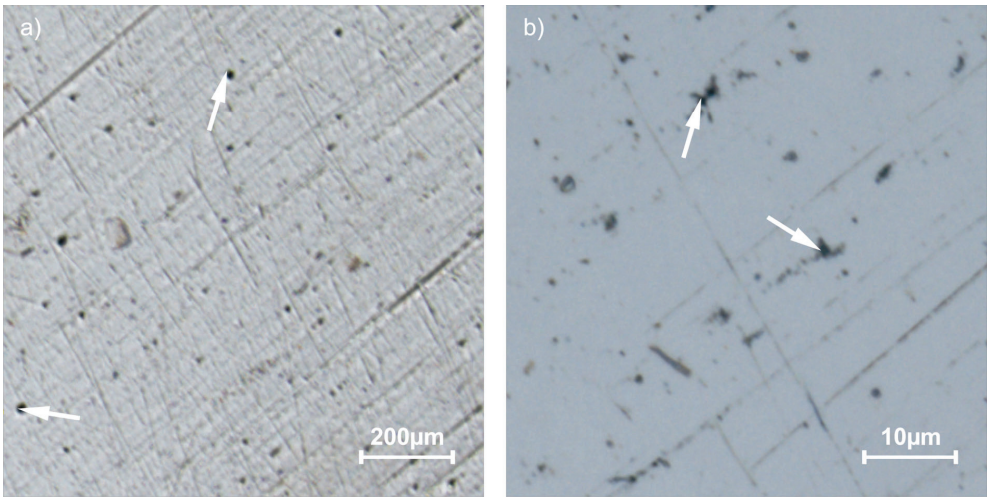

Fig. 4. Biocorrosion changes on the surface of Ti-6Al-4V (CSLM) after immersion in *D. nigrificans* for 28 days; (a) bars, 200 μm, (b) bars, 10 μm

(Fig. 3b, 4b), some differences between both materials are revealed.

Corrosion pits (marked with white arrows in the figures) were more evident in the case of cobalt alloy (Fig. 3b; CSLM, bars, 10 μm) in comparison to the titanium alloy (Fig. 4b; CSLM, bars, 10 μm). Also, some differences in their shapes were observed when both tested alloys were compared.

After additional 28 days (56 days in total) of contact biomaterials with SRB, the products of corrosion were more evident (Fig. 5b, Fig. 6b; CSLM, bars, 10 μm) with an increased amount of adsorbed corrosion products (Fig. 5a, Fig. 6a; CSLM, bars, 10 μm, surface without cleaning in ultrasonic bath) on the test materials.

From microscopic observations of the surfaces of biomaterials being tested (Fig. 5b, cobalt alloy; Fig. 6b, titanium alloy; CSLM, bars 400 μm) after 56 days of contact with sulfate – reducing bacteria, it can be concluded that titanium alloy is more resistant to biocorrosion. In the case of Co-Cr-Mo, larger corrosion pits were observed on the material's surface.

Image analysis of photographs taken after the biocorrosion process was performed with the aim of getting information about the magnitude of corrosive changes. The utilization of this method to evaluate SRB activity on the surfaces of biomaterials is a novelty.

Image analysis of biocorrosive changes on the surfaces of cobalt and titanium alloys, which were held in the inoculum of SRB for 28 and 56 days, conducted using Aphelion software provided information about the distribution, surface area and quantities of these changes (Figs. 7, 8).

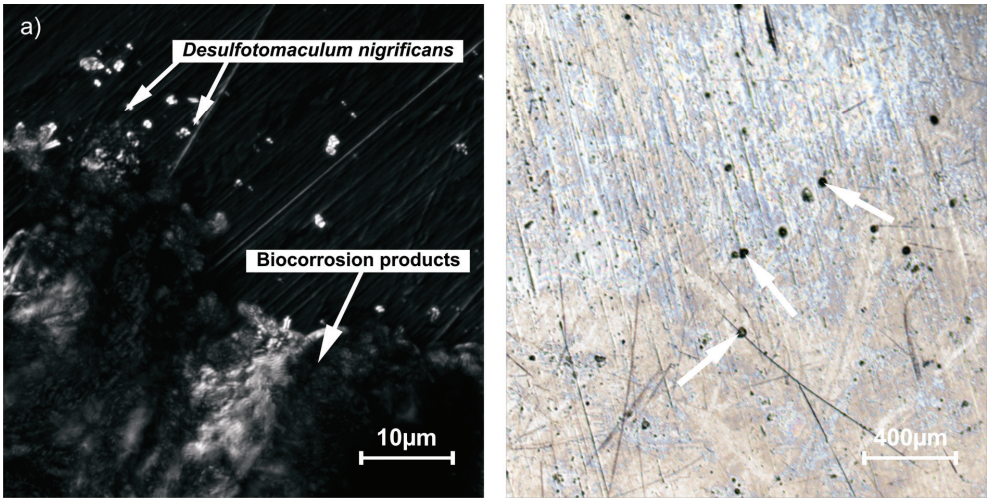

Fig. 5. Biocorrosion changes on the surface of Co-Cr-Mo (CSLM) after immersion in *D. nigrificans* for 56 days; (a) surface without cleaning in the ultrasonic cleaner; bars, 10 μm, (b) surface after cleaning in the ultrasonic bath; bars, 400 μm

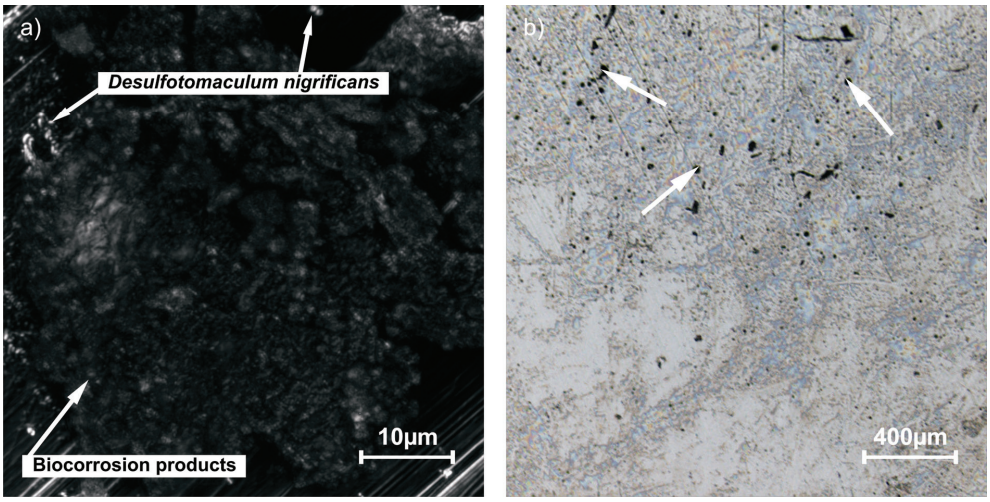

Fig. 6. Biocorrosion changes on the surface of Ti-6Al-4V (CSLM) after immersion in *D. nigrificans* for 56 days; (a) surface without cleaning in the ultrasonic cleaner; bars, 10 μm, (b) surface after cleaning in the ultrasonic bath; bars, 400 μm

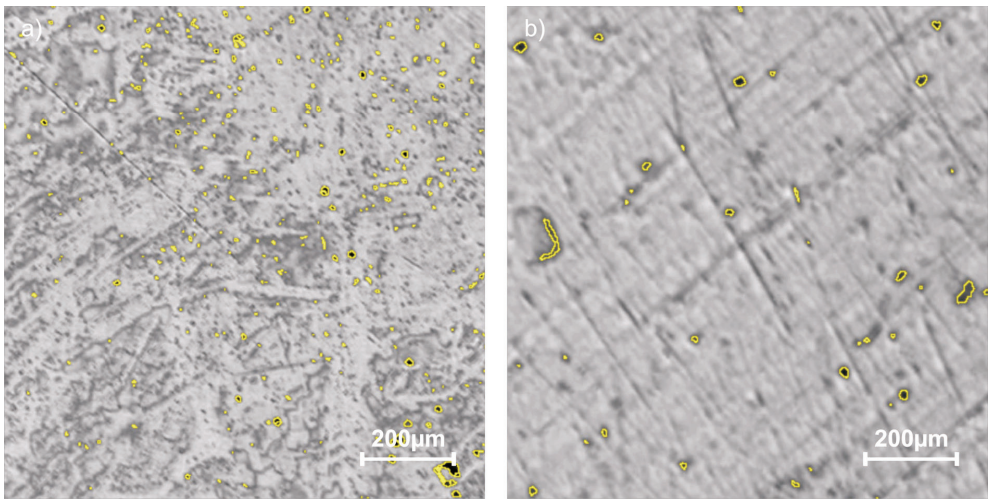

Fig. 7. Image analysis of example surfaces of (a) Co-Cr-Mo and (b) Ti-6Al-4V after 28 days of the biocorrosion process, Aphelion 3.1; bars, 200 µm

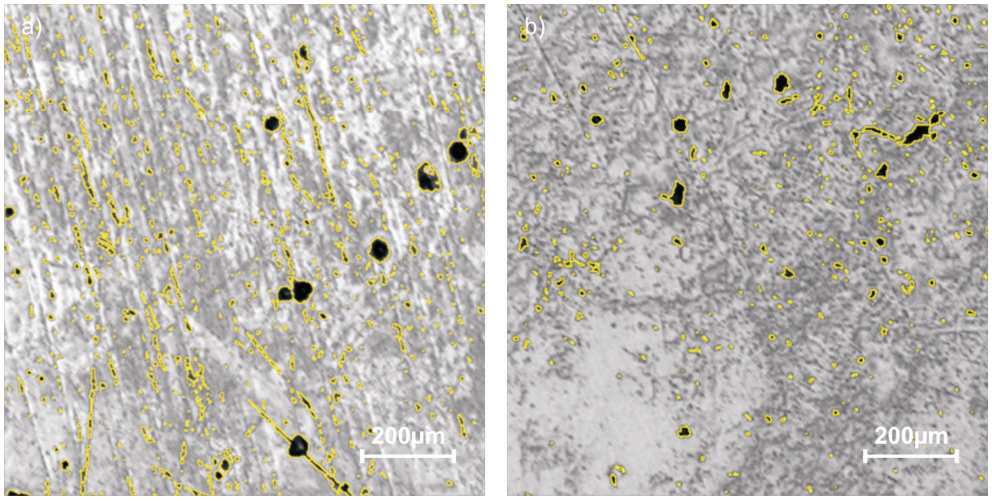

Fig. 8. Image analysis of example surfaces of (a) Co-Cr-Mo and (b) Ti-6Al-4V after 56 days of the biocorrosion process, Aphelion 3.1; bars, 200 µm

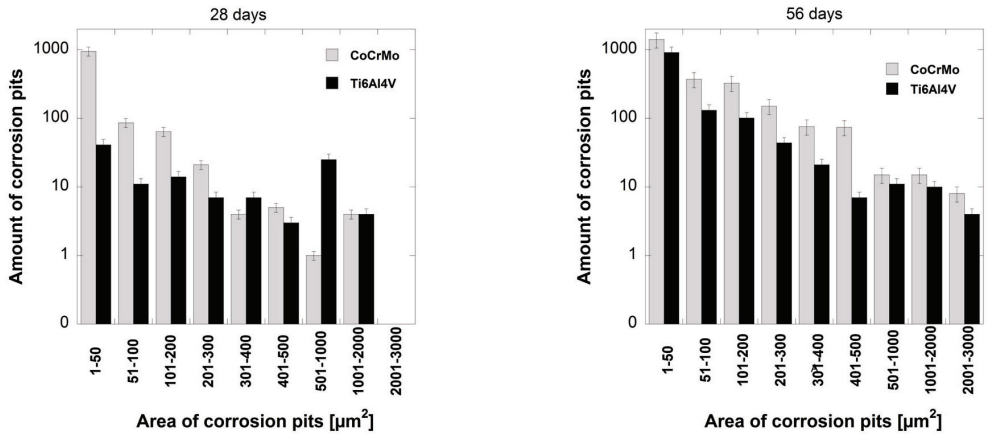

Fig. 9. The amount and area of biocorrosion pits on surfaces of test materials; (a) 28 days; (b) 56 days of contact with inoculum

From Figs. 7 and 8 it can be concluded that the area of corrosion pits on materials after 28 days of contact with the bacterial inoculum was smaller compared to materials after 56 days of contact. It is also shown that the cobalt alloy was characterized by a more intensive process of surface corrosion, with a higher amount of small biocorrosion changes in comparison to the titanium alloy.

On the surface areas of the test alloys (each total area tested was approximately 1.3 mm^2), most corrosive changes had a surface area within the range of 1–50 μm^2, which constituted from 37% up to 83% of all changes, depending on the type of material. The least changes had a surface area within the range of 2001–3000 μm^2, which constituted 0.3% of all changes and was observed only after 56 days of contact with SRB. A large percentage was also noted for corrosion pits with a surface area within the range of 51–100 μm^2 (8–16% of all changes). Thus, after comparing the total surface of these changes to the total surface of the image subjected to analysis, it can be concluded that corrosion pits took up approx.: 2.3% for Co-Cr-Mo (after 28 days), 1.8% for Ti-6Al-4V (after 28 days), 4.2% for Co-Cr-Mo (after 56 days), 3.1% for Ti-6Al-4V (after 56 days) of the total surface of the test materials.

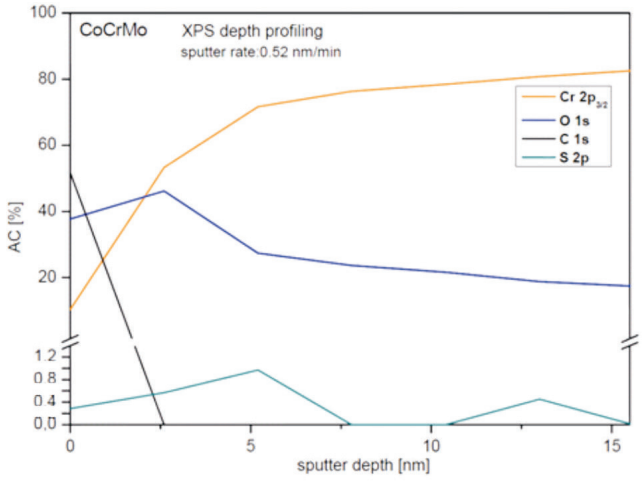

Fig. 10. XPS analysis results:
on the surface of Co-Cr-Mo, green line-sulfur,
after 28 days of contact with inoculum

In addition, to confirm SRB activity on the surfaces of biomaterials, chemical composition analysis was performed at points of corrosive changes. XPS analysis (an example figure of Co-Cr-Mo surface analysis is given in Fig. 10) indicated the presence of sulfur on both materials tested, probably in the form of chemical compounds, which explains the dark color of the contact solution in which biomaterial samples were held.

4. Discussion

Numerous metal alloys have been employed as materials for treatment of dental disorders. The major materials used as dental implants, prosthetic or orthodontic materials are: 316 stainless steel, cobalt/titanium alloys. Among these metals, titanium alloys are characterized by the highest corrosion resistance and passivity in biological media due to the presence of titanium oxide (TiO_2) film (naturally, chemically or electrochemically formed) on their surfaces [21], [22]. The rutile-type tetragonal structure of this oxide is less reactive in biological media due to its stronger structure and has better biocompatibility with surrounding tissues. As mentioned in the work of Hsu et al. [10], the cobalt alloy (Co-Cr-Mo) also exhibits attractive properties such as biocompatibility and corrosion resistance due to the presence of a passive oxide film on its surface. Reduction of the loss of dental materials arising from corrosion phenomena can increase the long-term success of, i.e., dental implant systems. Most corrosion tests on these alloys are performed electrochemically. However, oral cavity also contains microflora composed of different bacteria strains, which can exacerbate corrosion of metallic biomaterials. Kameda et al. [11] studied the influence of representative indigenous oral bacteria, *Streptococcus mutans* and *Streptococcus sanguinis*, on orthodontic metallic appliances. In the work of Wilson et al. [25], it was stated that bacteria induce corrosion by several mechanisms, such as: their presence on the metal surface can establish cathodic or anodic regions, which can result in the generation of corrosion currents, and a wide range of metabolic products, such as organic acids, can react directly with the metal.

The results of Kameda et al. [11] show that microbiologically induced corrosion of dental materials by *Streptococcus mutans* and *Streptococcus sanguinis* was found in stainless steel materials but not in titanium materials. However, the work of Souza et al. [23], involving electrochemical tests (polarization resistance of the passive titanium film), shows that the presence of *S.mutans* colonies on the titanium surface negatively affected corrosion resistance.

The process of biocorrosion in the environment of sulfate-reducing bacteria [17] was investigated for stainless steel materials. However, no literature data was found regarding the impact of sulfate-reducing bacteria (SRB) on dental alloys (cobalt or titanium alloys). Thus, the aim of this work was to study the influence of the environment of *Desulfotomaculum nigrificans* bacteria, which, as mentioned earlier, can

also be found in the oral cavity as transient flora, on the corrosion resistance of two different dental alloys.

Besides adsorbed *D. nigrificans* bacteria and biocorrosion products (Fig. 2), corrosion pits (Figs. 3, 5 and Figs. 4, 6 for cobalt and titanium alloys after 28 and 56 days respectively) were also observed on the test surfaces of biomaterials used to make elements of dental prostheses or dental implants [10], [12], as the main causes of destruction of materials of this type. As shown in Figs. 3–6, changes in the structure of the test alloys were observed on their surfaces after contact with a solution containing sulfate-reducing *D. nigrificans* bacteria compared to a surface not treated with SRB (Fig. 1). CLSM analysis indicated the presence of biocorrosion centers just after 28 days of contact of the materials with the environment of sulfate-reducing bacteria. After additional 28 days (56 days in total), these changes were more evident. What is more, biocorrosion products and corrosion pits were greater on the cobalt alloy surface (Fig. 3 and Fig. 5 after 28 and 56 days, respectively) in comparison to the titanium alloy surface (Fig. 4 and Fig. 6 after 28 and 56 days, respectively). This phenomenon confirms the higher corrosion resistance of titanium alloy when compared with other metallic biomaterials, including cobalt alloy [22].

Also, changes in the structure of the material were observed, being manifested, above all, as discolorations of the surface of the biomaterial and small corrosion pits, which are the effect of biocorrosion occurring (Fig. 3b, 4b). A longer time of contact (56 days) of the test biometals with sulfate-reducing bacteria led to the exacerbation of these processes, and as a result, to the formation of larger corrosion pits on surfaces being tested (Fig. 5b, 6b). This may indicate an intensively progressing process of microbiologically induced corrosion, which occurs in the structure of biofilm, as indicated also in work [26]. Observed corrosion pits were non-uniformly distributed over the surfaces of the materials and had surface areas ranging from 1 μm^2 to 3000 μm^2.

The phenomenon of biocorrosion of metals is explained in many ways, e.g., in the work of Lata et al. [14] it was stated that the activity of corrosive bacteria creates a difference of electrical potential between the area attacked by micro-organisms and the area free from bacterial activity, which leads to the formation of local pits on the surface of metals. Additionally, when combined with organic substances, their metabolic activity may lead to the formation of aggressive corrosion products, such as incomplete metabolic organic acid [2].

One of the mechanisms of MIC is cathodic depolarization, as suggested by Kuhr and Vlugt in 1934 [16]. Also, according to work of Bryant [4] the role of hydrogenase was considered as a unique mechanism of MIC by SRB metabolic activities. The utilization of hydrogen in their cells can be expressed as follows [16]

$$NAD^+ + H_2 \text{ (hydrogenase activity)} = NADH + H^+ \quad (1)$$

The XPS analysis shown in Fig. 9 indicates that the corrosion film on cobalt alloy's surface was composed of Cr, O, C, S. The dark color of the contact solution and the presence of sulfur (probably in the form of compounds) on the surface of the materials is a sign of bacterial corrosion [19]. This corrosion product may be deposited and become a cathode with a large surface area relative to the unreacted cobalt alloy, accelerating the dissolution of the surface. As a possible energy source, hydrogen was an important electron donor for sulfate reduction. It was shown in the work of Chen and Clayton [5] that the passive film on steel surface may be deteriorated by bacterially induced sulfides and by the removal of alloying elements. A similar effect can occur in the case of metal alloys, where the increased amount of oxygen near the surface of the material may be an effect of the formation of a layer of oxides, including titanium or cobalt oxides. These oxide films are protective layers that may contribute to reduction of corrosion. However, the dissolution of TiO_2 may occur in certain media, such as those containing high fluoride concentrations, hydrogen peroxide (H_2O_2) and lactic acid, e.g., it can occur in the oral cavity [23].

Corrosive changes are one of the main causes of destruction of materials used in dentistry in the oral cavity. This process causes the release of metal ions into the surrounding medium and deterioration of the metal, which in fact reduces the functional properties of the material and its mechanical strength [3]. Corrosion of metallic appliances in the oral cavity by bacteria releases metal ions, which may migrate within the entire organism, be deposited in organs of the human body, stimulate an initial inflammatory response, and act as allergens or carcinogens [11], [24].

The image analysis applied for evaluation of the size and quantity of formed corrosion pits is a novelty in this work, compared to other works dealing with biocorrosion. The results of this analysis provide valuable information about the proportion of the surface area of these changes to the total surface area of the dental alloys. Biocorrosion covered between 1.8% and 4.2% of the total surface of the test materials, which gives a global perspective on this process

in terms of material destruction and allows its intensity to be estimated depending on the type of alloy.

An evident influence of the alloy type on bacterial proliferation was observed. Significant corrosion losses caused by the activity of microorganisms were observed on the metallic surfaces under study. The materials studied exhibited varying resistance to biocorrosive destruction. Based on data found in the literature, it was concluded that Ti6Al4V was the most suitable material for implant applications in the human body [22] when considering its corrosion resistance. Similar results were obtained in this work, however resistance to biocorrosion was assessed. Although the cobalt alloys do not passivate as titanium alloys do, they also have good resistance against corrosion in a biological environment, compared, i.e., to stainless steel [19]. The results of this study have much cognitive and utilitarian significance.

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