

The chemical digestion of Ti6Al7Nb scaffolds produced by Selective Laser Melting reduces significantly ability of *Pseudomonas aeruginosa* to form biofilm

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In our previous work we reported the impact of hydrofluoric and nitric acid used for chemical polishing of Ti-6Al-7Nb scaffolds on decrease of the number of *Staphylococcus aureus* biofilm forming cells. Herein, we tested impact of the aforementioned substances on biofilm of Gram-negative microorganism, *Pseudomonas aeruginosa*, dangerous pathogen responsible for plethora of implant-related infections. The Ti-6Al-7Nb scaffolds were manufactured using Selective Laser Melting method. Scaffolds were subjected to chemical polishing using a mixture of nitric acid and fluoride or left intact (control group). Pseudomonal biofilm was allowed to form on scaffolds for 24 hours and was removed by mechanical vortex shaking. The number of pseudomonal cells was estimated by means of quantitative culture and Scanning Electron Microscopy. The presence of nitric acid and fluoride on scaffold surfaces was assessed by means of IR and rentgen spetorscopy. Quantitative data were analysed using the Mann–Whitney test ($P \leq 0.05$). Our results indicate that application of chemical polishing correlates with significant drop of biofilm-forming pseudomonal cells on the manufactured Ti-6Al-7Nb scaffolds ($p = 0.0133$, Mann–Whitney test) compared to the number of biofilm-forming cells on non-polished scaffolds. As X-ray photoelectron spectroscopy revealed the presence of fluoride and nitrogen on the surface of scaffold, we speculate that drop of biofilm forming cells may be caused by biofilm-suppressing activity of these two elements.

Key words: SLM technology, Pseudomonas aeruginosa, scaffolds, surface modification, Ti-6Al-7Nb scaffolds

1. Introduction

Implant-associated infections are caused by adhered microorganisms growing in biofilm form. These structures are defined as microbial communities embedded within polymeric matrix. The biofilm displays elevated level of resistance against antimicrobial agents, immune system and environmental factors. Therefore, once settled, biofilm is extremely hard to remove or eradicate [8]. The first, among subsequent stages of biofilm formation, is referred to as the adhesion, which is attachment of microorganisms to implant surface. Thus, generation of surfaces reducing the number of adhering microbial cells is of para-

mount importance. The number of approaches, including manufacture of surfaces coated with antimicrobials or surfactants, was developed to obtain such result. However, these approaches did not match the expectations completely. Therefore, this field of biomaterial science is still in the process of intensive development and concern of healthcare environment [5].

The bone implants are specific example of biomaterials threatened with risk of infection. In case of significant bone loss, the organism is unable to regenerate by its own means. Therefore, the loss needs to be replaced with appropriate implant.

The advanced implants are manufactured from the spectrum of materials, such as pure metals and their alloys, plastics, ceramics and composites [11]. Mod-

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ern implants display high level of biocompatibility and low cytotoxicity. Other desired features are low weight and high durability. To obtain such features, modern manufacture processes are used. One of them is additive method, referred to as the Selective Laser Melting (SLM). The SLM technology is an additive manufacturing process based on topical fusion of metallic powder particles using high-power laser beam. The parameters of process are customized and performed layer by layer according to 3D CAD data. The subsequent layer is built directly on the previous layer providing durability of the whole model [19]. The latitude in the formation of geometry provides the possibility of manufacturing homogenous implants (scaffolds) of high manufacturing accuracy. The application of dimensional structures makes it possible to increase flexibility and global porosity of implants decreasing their density and weight [12], [18].

Two-phase alloy Ti-6Al-7Nb is more and more frequently used as material for implantation because of its good durability, high anticorrosive properties and increased tolerability compared to more routinely used Ti-6Al-4V alloy [3]. The results obtained in our previous report indicate that chemical digestion of Ti-6Al-7Nb scaffolds using HF and HNO₃ mixture correlates with drop in the number of biofilm forming cells of *Staphylococcus aureus*, the leading Gram(+) pathogen of orthopaedic infections [17]. The question we addressed in the present study was whether the aforementioned modification could possibly interfere with adhesive ability of leading Gram(-) pathogen of orthopaedic infections, referred to as the *Pseudomonas aeruginosa*. This widely distributed pathogen, responsible for a number of dangerous and life-threatening infections, displays different molecular and phenotypic mechanism of biofilm formation compared to *S. aureus*. Thus, the aim of this paper was to evaluate an impact of chemical polishing using HF and HNO₃ on the ability of *P. aeruginosa* to form biofilm on Ti-6Al-7NB scaffolds generated by means of Selective Laser Melting.

2. Materials and methods

2.1. Fabrication of scaffolds

The scaffolds were manufactured using Selective Laser Melting method (SLM) from Ti-6Al-7Nb alloy using parameters described earlier [17]. The columnar scaffolds of \varnothing 6 mm \times 4 mm size were manufactured

in accordance with 3D CAD data model. The scaffolds' pores were of 450 μ m in diameter. The thickness of struts was 250. Technological parameters used in SLM process are presented in Table 1.

Table 1. Technological parameters used in SLM process

Parameter	Value
Laser power	25 W
Layer thickness	50 μ m
Scan velocity	0.2 m/s
Scanning strategy	Double x-y

2.2. Modification of scaffold surface

After generative construction, scaffolds were subjected to preliminary cleaning in an ultrasonic cleaner containing distilled water and acetone. The process of chemical digestion, main scaffold surface modification, was also performed in ultrasonic bath (MKD-2, MKD-Ultrasonic). 2/3 of the bath was filled with water. The plastic box of 500 cm³ was put inside the bath. The box contained a slot, where the scaffolds were placed (Fig. 1).

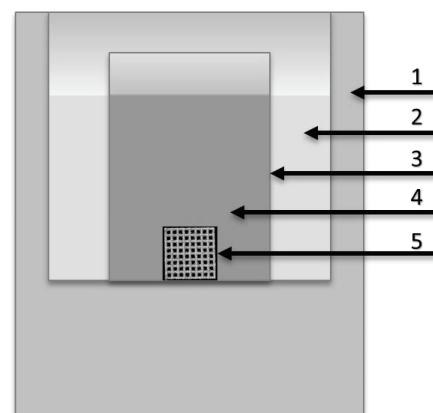


Fig. 1. Scheme of station for chemical digestion: 1 – case of ultrasonic bath, 2 – water, 3 – vessel, 4 – HF+HNO₃, 5 – scaffolds

The mixture of 5 ml HF(I) and 15 ml HNO₃(V) in 180 ml of ultrapure water was prepared for chemical digestion. The scaffolds were digested for 65 sec. Afterwards, the samples were rinsed in water (to neutralize acid) and dried in sterile airflow. Subsequently, the surfaces of scaffolds were assessed using micro-computed tomography (μ CT) as we described in our earlier work [17]. To verify the data obtained, analysis of scaffold surface was additionally performed using Scanning Electron Microscope (SEM).

2.3. Microbiological tests

2.3.1. Quantitative cultures of pseudomonal strains

For the experimental purposes, 18 clinical strains of *Pseudomonas aeruginosa* isolated from the different orthopaedic infections and 1 reference strain, ATCC15442 were used. The clinical strains are the part of Microbiology Department Strains Collection. The *P. aeruginosa* strains were cultured on McConkey agar plates and then incubated in the TSB liquid medium (Biocorp) overnight. Subsequently, the strain liquid culture was diluted to obtain suspension of 2×10^8 cells/mL. 2 mL of such suspension were introduced to the well of 24-well plate together with SLM scaffold. The samples were incubated in the bacterial presence for 24 h/37 °C in the aerobic conditions. After incubation, samples were vigorously rinsed using 0.9% NaCl and subjected to the vortex mixing for 1 min. Afterwards, the suspension was diluted and cultured on the McConkey agar plates. The next day, the number of colony forming units was counted. All measurements were repeated three times.

2.3.2. Statistical analysis

Statistical calculations were performed using Statistica 10 software and the Mann–Whitney test. Statistical significance was defined as $P \leq 0.05$.

2.3.3. Scanning Electron Microscopy of biofilm formed on Ti6Al7Nb alloy

The samples of *P.aeruginosa* biofilm on the scaffold were cultured as is described in Section 2.3.1. Subsequently, samples were carefully rinsed using saline and then fixed using 3% glutarate (POCH) for 15 minutes. Afterwards, the sample was subjected to dehydration procedure by incubation with increasing concentration of ethanol (25%, 60%, 95%, 100%) for 5 minutes in every solution. The samples were air-dried after dehydration. Next, the samples were covered with Au/Pd (60:40, sputter current: 40 mA, sputter time: 50 sec) using Quorum machine and examined using Scanning Electron Microscope (Zeiss EVO MA25).

2.4. Analysis of chemical composition of manufactured scaffolds

For analysis of the chemical composition of scaffold, the coal and sulphur analyser CS600 with resis-

tance and induction furnace (18 MHz, 2.2 kW) was applied. The sample was combusted in pure oxygen. Value of IR absorption of SO₂ and CO₂ was analysed. Analysis of oxygen, hydrogen and nitrogen was performed using high temperature extraction device TCHEN 600 Leco.

2.5. The rentgen spectroscopy of photoelectrons (XPS)

For a thorough analysis of the chemically etched samples, aiming to identify the nature of the resulting changes in the chemical composition (surface or volume), X-ray photoelectron spectroscopy was used. The surfaces of the samples were analyzed after chemical treatment and after a subsequent cleaning of the surface of argon ions (Ar⁺, 3 keV, 5–6 μA/cm²) in order to determine the degree of diffusion of elements. XPS studies were performed using Phoibos 100 spectrometer.

3. Results

The additive SLM technology allowed to manufacture scaffolds of high homogeneousness (Fig. 2). The subsequent chemical digestion reduced digested scaffold surfaces compared to non-digested ones by 28.5% (519.8 mm² vs. 726.34 mm², respectively). When subjected to the incubation with *Pseudomonas aeruginosa*, chemically digested scaffolds appeared to be colonized by bacteria to the lower degree compared to the non-digested scaffolds. This difference was statistically significant, ($p = 0.0133$, Mann–Whitney test) (Fig. 3). The results of quantitative cultures were additionally confirmed by means of Electron Microscopy, as presented in Fig. 4a, b. As one can observe (Table 2), the level of nitrogen detected on the surface

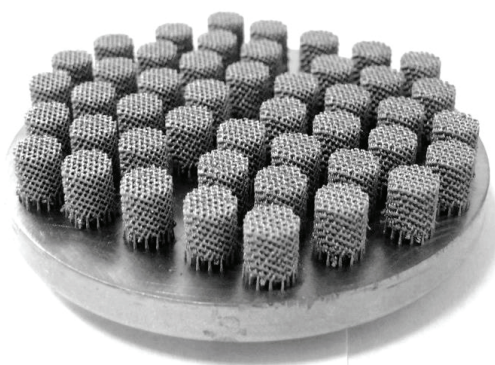


Fig. 2. Ti-6Al-7Nb scaffolds of complex geometry manufactured using Selective Laser Melting technology [17]

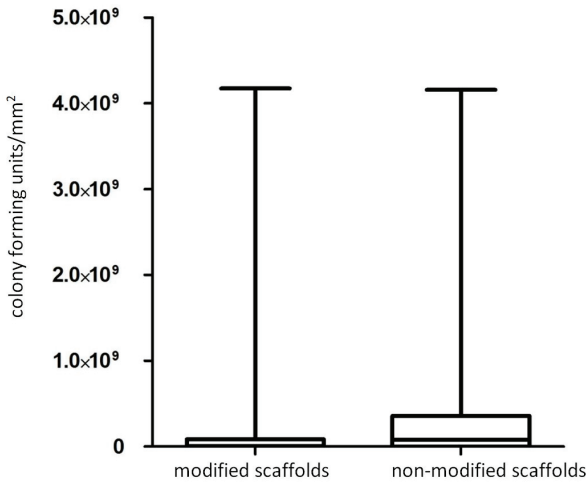


Fig. 3. Difference in the number of *P. aeruginosa* forming biofilm cells on digested vs. non-digested scaffolds. M-W test ($p = 0.0133$)

of digested scaffold was over 3 times higher compared to un-digested sample. Similarly, the level of fluorine on the surface of digested sample was significantly increased compared to non-digested sample (Fig. 6a). The relation of fluorine to titanium after ionic cleaning of the samples decreased from 0.15 to 0.10, which additionally supports a thesis that the presence of fluorine results from chemical digestion.

Table 2. Mass content of selected elements in the digested and non-digested scaffolds

Element	% mass content		ASTM content F1295-11 % mass
	non-digested scaffold	digested scaffold	
C	0.017	0.021	<0.08
H	0.0046	0.0071	<0.009
N	0.049	0.18	<0.05
O	0.21	0.28	<0.20

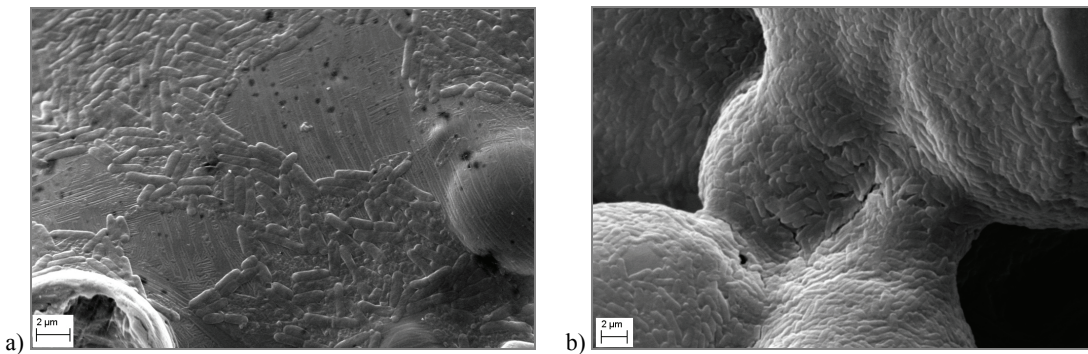


Fig. 4. Pseudomonal biofilm formed on chemically digested (a) and non-digested surface of the scaffolds (b). Differences in the number of cells can be seen

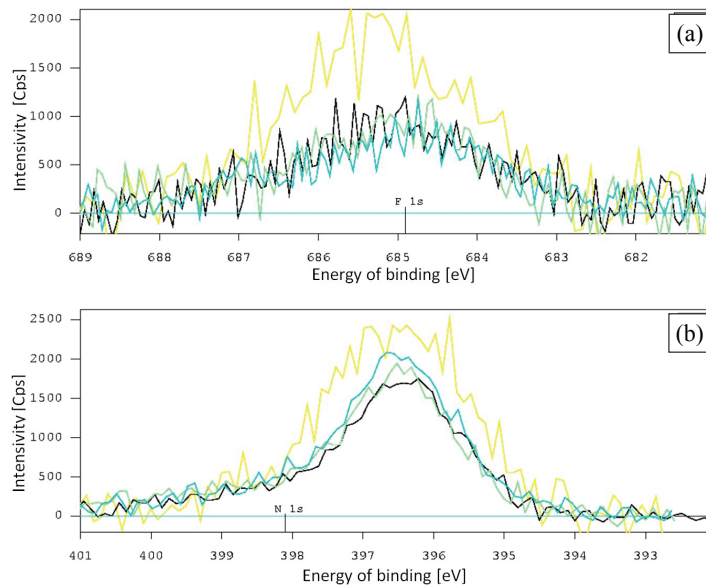


Fig. 5. (a) Fluorine: spectrum F1s of the surface of the chemically digested scaffold; (b) Nitrogen: spectrum of the surface of the chemically digested scaffold. Green curve – scaffold surface after digestion; black, green, blue curve – surface of sample after ionic cleansing in time of 3, 6, 9 min., respectively

4. Discussion

The infections related to the presence of biofilm on biomaterials might stay in latent form even for a year after implantation. The symptoms include at least one of the following: pus from the soft tissues, localized pain/fever, abscess formation and osteomyelitis [16]. Treatment of such symptoms is hard, which is related to the poor penetration of antibiotics through bone structure, disturbed blood circulation and biofilm formation having detrimental impact on surrounding bone and soft tissue surfaces. In many cases, localized infection might turn into systemic one, leading to bacteraemia and dissemination of microorganisms to the distant organs by blood flow [4]. The most common etiological factor of osteomyelitis among G(+) bacteria is *Staphylococcus aureus*, whereas *Pseudomonas aeruginosa* is one of the hardest to eradicate bacteria among Gram(-) pathogens [13]. This dangerous bacteria displays intrinsic resistance against many antibiotics, including some of penicillins, aminoglycosides or tetracyclines and their glycylocykline derivative – tigecycline [10]. When in biofilm form, pseudomonal resistance to antibiotics might be even 1000× higher compared to its planktonic form. Biofilm tolerance to antibiotics might include those antimicrobials which are effective when acting against planktonic form of microorganisms [2]. Taking into consideration all the above-mentioned factors, it seems to be obvious that procedure of implantation should be performed in rigorous aseptic manner to avoid biofilm formation. Moreover, the implants themselves should be made from materials displaying anti-adhesive properties in terms of biofilm formation with simultaneous high adhesive properties for bone-forming cells, such as osteoblasts. These desired properties might be achieved by application of new manufacturing processes and novel alloys implants are made of.

The generation of individually matched bone implants with diversified structure that can fill bone loss resulting from injuries or diseases, becomes more and more popular as it provides satisfactory therapeutic results due to its better anatomical matching compared to implants made using traditional manufacturing techniques [6], [14]. Selective Laser Melting is an additive manufacturing technology, capable of producing parts based on their CAD designs or geometries captured by reverse engineering anatomic shapes. The biggest advantage of the additive technologies is unrestricted capabilities of shape formability due to melting cross-sections on consecutive layers. This allows one to create even closed and empty pockets inside parts, or spatial structures such as lattice structures. Different part design with

appropriate lattice structures could lead to modification of the mechanical properties, such as stiffness and weight of part. From among additive technologies, especially SLM allows us to obtain melted materials of comparable mechanical properties to standard ones. Applied in tissue engineering, the SLM can provide cell scaffolds made of biocompatible materials, e.g., Ti-6Al-7Nb alloy, with sufficient dimensional accuracy. The individually matched bone implants are frequently submitted to procedures of surface modifications in order to get a still greater increase of their suitability and biocompatibility [9], [15].

As the chemicals applied in this work were the only factor differentiating two types of the scaffolds described, it was logical to assume that the diminished ability of *Pseudomonas aeruginosa* to colonize the scaffolds and form biofilm on it was the result of digestion process. We hypothesized that the presence of nitrate and fluorine may be the cause explaining the phenomenon observed. Indeed, the content of the aforementioned elements was elevated in the modified scaffold, as is presented in Table 2, Fig. 5. In 2006, Barraud et al. [1] described involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. Fluorine, the other element used in the study presented for chemical digestion of samples, is well known for its potential to reduce biofilm, especially the cariogenic one. Therefore, this elemental is used in the polymeric form in experimental resins [7]. Herein, we show correlation between the presence of nitric acid and fluorine and reduced ability of *P. aeruginosa* to form biofilm on the surface of Ti-6Al-7Nb scaffolds. These two compounds were used in digestion process to remove some alloy particles loosely attached to the scaffold. The presence of these elements led to a decrease in the number of pseudomonal cells adhered to the scaffold. This data, however of preliminary nature, is promising in the light of severity of bone infections caused by biofilm-forming microorganisms. Moreover, the results obtained show the necessity of combined approach to biofilm treatment and advantages of cooperation between scientists from different fields of knowledge, such as microbiologists and engineers.

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