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The influence of different composite mixtures (PLA/HA) manufactured with additive laser technology on the ability of *S. aureus* and *P. aeruginosa* to form biofilms

ANNA E. WOŹNA[1]*, ADAM F. JUNKA[2], PATRYCJA E. SZYMCZYK[1]

[1] Wrocław University of Science and Technology, Wrocław, Poland.
[2] Wrocław Medical University, Wrocław, Poland.

Purpose: Staphylococcus aureus (Gram-positive coccus) and *Pseudomonas aeruginosa* (Gram-negative bacterium) are the leading etiologic agents of biofilm-related, life-threatening infections in patients after orthopaedic implantations. The aim of the present paper is to estimate the ability of these two bacterial strains to form a biofilm on bioresorbable composites manufactured from polylactide (PLA) and hydroxyapatite (HA) with the use of Selective Laser Sintering (SLS) method. *Methods*: Microbiological tests were conducted on two variants of a solid specimen made with additive laser technology. Samples with different content of hydroxyapatite were made, with appropriate manufacturing parameters to ensure stability of both composite ingredients. The geometry of samples was obtained by technical computed tomography. Microbiological tests determined the number of bacterial cells after incubation. *Results*: The results indicate significantly decreased ability of *S. aureus* and *P. aeruginosa* to form biofilms on the surface of materials with higher content of hydroxyapatite ceramics. *Conclusions*: The data may be useful for future applications of SLS technology in the production of bioresorbable PLA/HA medical implants.

Key words: *biomaterials, biofilm, prototype additive manufacturing, biopolymers, bioceramic*

1. Introduction

Bone implants are a specific example of biomaterials exposed to the risk of infection. In the case of significant bone loss, the body is unable to regenerate on its own. Therefore, the loss needs to be replaced with an appropriate implant. Modern implants are highly biocompatible and have low cytotoxicity [16]. However, biomaterial-related infections still pose a significant threat for patients undergoing implantation procedure. Therefore, the ability of implants (including resorbable composites) to reduce or prevent microbial colonization is one of the key characteristics determining their possible use. Biofilms are sessile communities of microbial cells being able to develop on virtually all types of surfaces [17]. Microbes in a biofilm form are embedded in extracellular slime protecting them from unfavourable environmental factors (dry heat, low temperature, UV radiation, drying), but also from immune system components and antimicrobial substances [4]. The most common etiological factors for chronic osteoarthritis are primarily *Staphylococcus aureus* and *Pseudomonas aeruginosa* with infection rate of 30% and 15%, respectively [29]. These bacteria are responsible for about 33% of infections related with orthopaedic implantation [6], [8], [9]. The ability of microbes to adhere to the surface of osteoinductive materials has already been analyzed by numerous research teams, however more stress was put on the effectiveness of antimicrobial substances impregnating biomaterial than on the intrinsic ability of the microorganisms to form a biofilm [5], [14], [27]. The risk of biofilm development increases with the biocompatibility of the materials used [26], so the analysis of the rate of biofilm development in the case of

* Corresponding author: Anna E. Woźna, Wrocław University of Science and Technology, ul. Łukasiewicza 5, 50-371 Wrocław. Poland. Phone: 48503655331, e-mail: anna.wozna@pwr.edu.pl
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fully biocompatible and bioresorbable composites is of pivotal meaning. Studies into a conventionally produced PLLA composite with the addition of 50% hydroxyapatite have shown that the bacteria are more likely to form a biofilm on metallic and composite materials than on a pure bioresorbable polymer [26]. Research on ankle fixation proves that the use of a bioresorbable material does not increase the probability of post-operative complications resulting from the formation of a biofilm. At the same time, the use of such biomaterial corresponds with a lower risk of infection related with lack of re-surgery (which is necessary in the case of non-resorbable implants) [25]. The technology used in our experiment, namely SLS, is a free-form fabrication method of creating patterns with the use thermal fusing (sintering) of powdered materials. SLS machine produces models on a moveable platform by applying layers of material, which are merged by heat from a laser beam [28]. Manufacturing time and cost is reduced, compared to the stereolitography (SLA) technique. The precision of the SLS process has been reported to be within 10 μm [28].

The aim of this paper is to evaluate the ability of common opportunistic pathogens that cause both acute and chronic nosocomial infections to form a biofilm on PLA/HA composites of various composition within individual samples.

2. Materials and methods

2.1. Fabrication of PLA/HA composites

Two variants of a solid specimen were prepared with additive laser technology. The main difference was the content of HA in the bioresorbable PLA/HA composites. Samples with 20% HA and 30% HA were made in a square shape (10 × 10 × 10 mm), with appropriate manufacturing parameters to ensure stability of both composite ingredients. Technological parameters of the process are shown in Table 1.

Table 1. Technological parameters used in the preparation of samples for microbiogical tests

Laser power	9.22 W
Layer thickness	500 μm
Scanning speed	2.2 m/s
Number of scan repetitions	5

2.2. Geometric analysis using CT

Technical computed tomography (CT) was used [23] to obtain the geometry of the composite samples. The samples were scanned using μCT system (Metrotom 1500, Carl Zeiss, Oberkochen, Germany) and the accurate volume of every single sample was obtained. The system consists of a flat panel detector with a resolution of 1024 × 1024 px (400 μm pixel size) and 16 bit grey scale, a rotary table and microfocus X-ray tube with maximum accelerating voltage 225 kV and maximum current 1000 μA. In order to achieve the maximum resolution [21], the tube voltage was fixed at 220 kV and the current at 120 μA. The number of projections carried out during the 360° rotation of the sample was 800, with 1 s integration time for each one. The data obtained were analyzed using software VG Studio MAX (Volume Graphics GmbH, Heidelberg, Germany).

2.3. Microbiological tests

2.3.1. Quantitative cultures

Two reference strains: *S. aureus* ATCC6538 and *P. aeruginosa* ATCC 15441 were cultured on the stable Columbia Agar (CA) medium (Biocorp). Next, strains were transferred to the liquid Enriched Broth (EB) medium and incubated at 37°C. After 24 hours' incubation in the aerobic conditions, the density of the bacterial suspension was measured with densitometer (Biomerieux) and then the suspension was diluted to reach 3×10^8 cells/ml solution by serial dilution method. Then, composite samples with 20% and 30% content of HA were incubated with bacterial suspensions at 37°C for 24 hours. After incubation, samples were thoroughly rinsed using a physiological saline solution to remove loosely-adhered bacteria and to leave a biofilm on the samples only. Subsequently, the samples were washed in 1 ml of a mild detergent (0.5% saponine, Sigma-Aldrich) and vortexed vigorously for 1 minute to free bacterial cells from the biofilm extracellular layers. After vortexing, the bacterial suspensions were serially diluted from 1:10 to 1:1 000 000 times. 100 μl of each dilution was cultured on the appropriate stable medium and incubated at 37 °C for 24 hours. After this time, bacterial colonies were counted and the number of bacterial cells forming a biofilm on the implants was assessed. All measurements were repeated three times. All experiments were performed in triplicate to calculate the

average value. Additionally, the numbers of cfu were normalized to the sample surface using the following equation: cfu per mm^2 surface = cfu per sample/ surface area mm^2.

2.3.2. The use of scanning electron microscopy to confirm the ability of *S. aureus* ATCC6538 and *P. aeruginosa* ATCC15441 strain to form a biofilm on the biomaterials tested

The results of quantitative microbiological tests (Section 2.3) were confirmed by visualization of bacterial cells with the use of electron microscopy as follows: samples with a bacterial biofilm were fixed using 3% glutarate (POCH) for 15 minutes at room temperature. Then, they were rinsed twice with phosphate buffer (Sigma-Aldrich) for fixative elimination. The next step was dehydratation with ethanol in increasing concentrations (25, 60, 95, 100%) for 5 minutes in each concentration. After alcohol was rinsed off, the samples were dried. Finally, samples were covered with Au/Pd (60:40, sputter current: 40 mA, sputter time: 50 sec) using QUORUM machine and examined with Scanning Electron Microscope Zeiss EVO MA25.

3. Results

3.1. Composite fabrication

Specimens were made of polylactide (PLA) and hydroxyapatite (HA) spherical powders with particle size of up to 100 microns.

Two different solutions of feedstock with different percentage of HA content were prepared. In Fig. 2 the SLS-produced PLA/20%HA and PLA/30%HA composites are shown.

Differences in the topography of the samples are caused by different concentrations of HA particles. Increasing HA content up to 30% increases their surface porosity.

3.2. CT reconstruction

The manufacturing method used and its accuracy result in each sample being unique, so it is necessary to choose an appropriate measurement method enabling the verification of the manufactured specimens

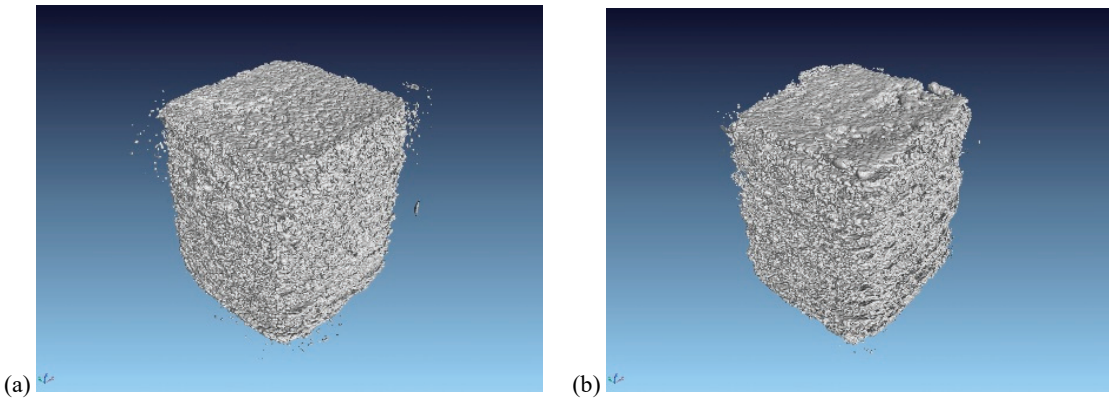


Fig. 1. Composite specimens: (a) composite with 20% HA content, (b) composite with 30% HA content

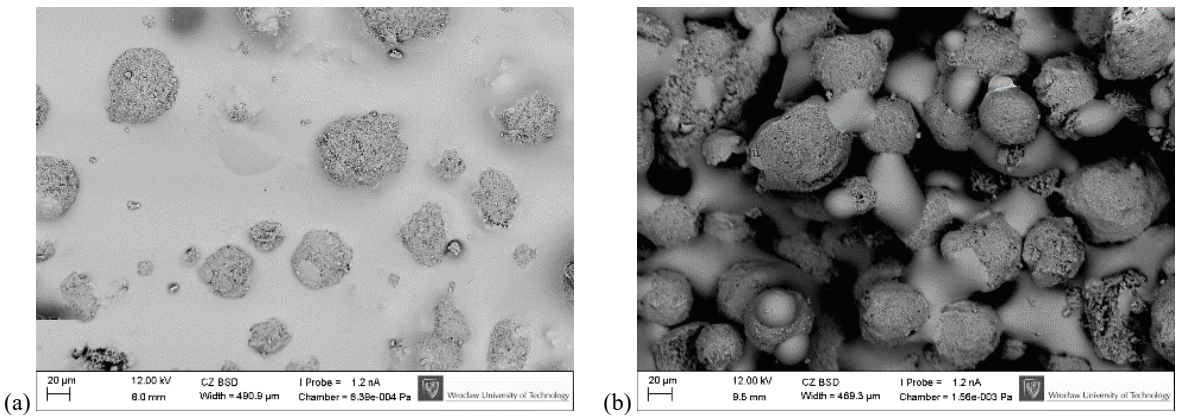
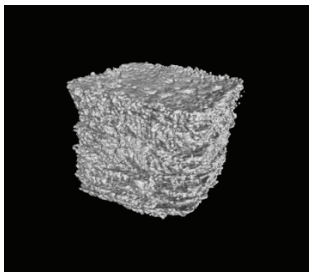
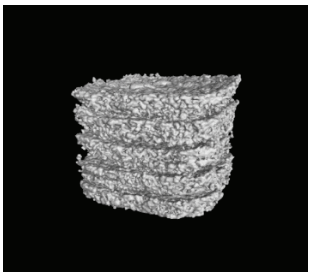


Fig. 2. Composite surfaces: (a) composite with 20% HA content, (b) composite with 30% HA content

in a repeatable and quantitative manner. The internal and external geometry of the fabricated composites was evaluated by technical computed tomography (CT) reconstruction.

Based on 3D volumetric data obtained as a result of reconstruction, it was possible to determine the surface of the whole volume of the sample. The surface area was measured automatically using the VG Studio MAX software. The results of this study are presented in Table 2.

Table 2. Results of the measurements of the composite samples' surfaces [mm^2] performed with VG Studio MAX software

PLA/20% HA	PLA/30% HA
	
Surface [mm^2]	
724.78	728.73

The surface area determined by CT analysis was similar for specimens of both HA contents. The total porosity of the fabricated structures was between 11 and 13%. The results indicate that differences in morphology do not significantly increase the internal structure of this material. However, it should be noted that HA particles are distributed homogeneously in the polymer matrix regardless of the analyzed sample.

3.3. Microbiological tests

The results presented in Table 3 show that *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15441 strains were able to form a biofilm on bioresorbable PLA/HA composites, however the number of cells that adhered to the surface of the materials under study was dependent on HA content in the material.

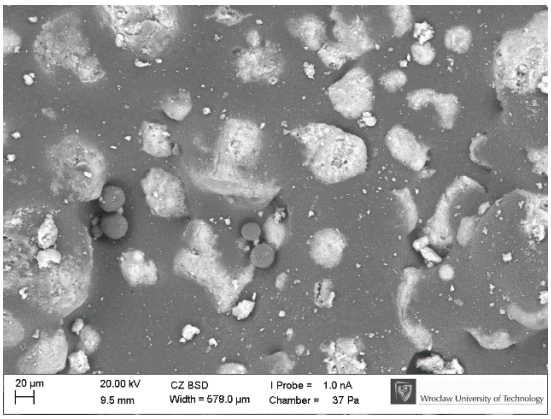
Both staphylococcal and pseudomonal cells easily formed a biofilm on both groups of samples. The differences in the number of bacterial cells depend on the content of hydroxyapatite in the samples. Increased HA content corresponded with the increase of bacterial cell number (2× and 15× increase for *S. aureus* and *P. aeruginosa*, respectively).

Table 3. Ability of *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 15441 to form a biofilm on the investigated bioresorbable composites

Composite		Number of bacterial cells/mm^2 [cfu/mm^2]	
		Staphylococcus aureus ATCC 6538	*Pseudomonas aeruginosa* ATCC 15441
Type	Surface [mm^2]	Average value	
20% HA	724.78	$3.9 \pm 0.4 \times 10^5$	$7.0 \pm 9.6 \times 10^5$
30% HA	728.73	$8.6 \pm 2.5 \times 10^5$	$109.8 \pm 97.0 \times 10^5$

To evaluate the growth of bacterial cells on the tested samples and to confirm the results obtained using quantitative methods described above, SEM analysis was conducted. As can be seen in Fig. 3, the surfaces of the examined materials are covered with bacterial colonies. Bacterial cells form small clusters rather than a dense layer.

The biofilm, irrespective of bacterial species, formed more easily on specimens with higher HA amount.

(a) 

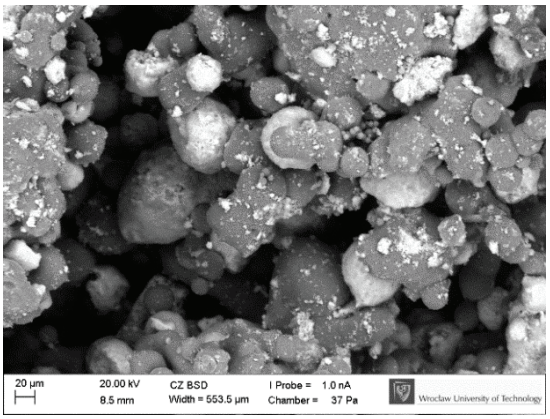
(b) 

Fig. 3. Surface of (a) 20% HA material and (b) 30% HA material with a biofilm

We hypothesize that more extensive surface of specimens with higher HA content was a more attractive surface for bacteria to adhere to and subsequently develop into a biofilm form. Moreover, HA, which is a natural inorganic component of the bone, is recognized by pathogens as target surface. It fully confirms the results obtained by quantitative culturing and SEM analysis.

4. Discussion

Dynamic development of biomaterials (growing need for new biomaterials), methods of their processing and innovative manufacturing technologies of bone replacements motivates researchers to constantly seek new solutions that combine various sciences and technologies. Bioresorbable materials produced by generative technologies significantly extend the capabilities of modern implant surgery. However, as shown by numerous authors, inflammatory reactions following implantation due to the presence of microorganisms still remain a major challenge in the context of biomaterial application [15], [22], [25]. Materials based on such hydrophobic polymers as polyurethane or polyethylene are covered with the host's protein layer in a very short time after implantation, significantly reducing access of microorganisms [25]. In the case of hydrophilic polymeric materials such as polylactide, acidic environment starts to form shortly after implantation, promoting development of inflammation. However, the major advantage of this type of materials is their ability to resorb due to their hydrophilic properties [16]. This feature, important from the clinical point of view, motivates research for solutions that would reduce the risk of complications.

Numerous methods of surface modification, such as lamination, use of low-temperature plasma or application of active surface additives were developed in order to reduce the formation of a biofilm on the surface of polymeric implants. It was observed that a 7-day-old biofilm is 1.000 to 5.000 times more resistant to antibiotics than the so-called "free-swimming", not grouped planktonic cells, however this challenge may be overcome by application of antibiotic therapy coupled with electrical field of low intensity [22]. Gollwitzer et al. showed that the use of coatings with poly(d, l) lactide implants made of stainless steel and titanium reduces the number of bacteria adhering to these surfaces by over two times on the stainless steel implants and almost 15 times on titanium implants. Better results were obtained after using antibiotics additions [15]. The comparison of the number of bacteria forming a biofilm on the samples made from poly(methyl methacrylate) (PMMA) and bioresorbable composites studied in this paper suggests that in the case of bioresorbable composites, the number of bacteria is significantly reduced [10]. It is also possible to limit the number of adherent bacterial cells using various methods of post-treatment, including using a properly selected chemical bath [12], [20]. Bagno et al. [1] concluded that osteoblasts are more likely to adhere to polystyrene surfaces pre-treated with peptides and that it can be also a method of limiting inflammatory environment on the biomaterial during resorption. However, it is also suggested that HA effect on bone metabolism in the first few months after surgery may be favourable but may cause complications in the long-term [24]. The present study is the first preliminary analysis of PLA/HA composites manufactured with additive laser technology and further studies are needed to assess the suitability of the produced composites for replacement of bone loss.

5. Conclusion

The results of our studies indicate that *S. aureus* and *P. aeruginosa* strains are able to adhere to the bioresorbable composites made with Selective Laser Sintering method. We observed that the number of bacterial colonies of both types extend along with the increase of hydroxyapatite content (2× and 15× for *S. aureus* and *P. aeruginosa*, for 20 and 30%, respectively), which was proven using quantitative cultures and scanning electron microscopy. However, the application of powder bed additive manufacturing technology opens up new possibilities for the design of bone replacement implants. The results of the study discussed in this article are a promising starting point for further experiments with various antibacterial additives implemented to reduce complications caused by bacterial infections in the course of implantation procedure.

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