

A. ROGUSKA<sup>##</sup>, A. BELCARZ<sup>\*\*</sup>, P. SUCHECKI<sup>\*\*\*</sup>, M. ANDRZEJCZUK<sup>\*\*\*</sup>, M. LEWANDOWSKA<sup>\*\*\*</sup>

## ANTIBACTERIAL COMPOSITE LAYERS ON Ti: ROLE OF ZnO NANOPARTICLES

Problem of post-operative infections of implant materials caused by bacterial adhesion to their surfaces is very serious. Enhancement of antibacterial properties is potentially beneficial for biomaterials value. Therefore, the metallic and metallic oxide nanoparticles attract particular attention as antimicrobial factors. The aim of this work was to create nanotubular (NT) oxide layers on Ti with the addition of ZnO nanoparticles, designed for antibacterial biomedical coatings. Antimicrobial activities of titanium, TiO<sub>2</sub> NT and ZnO/TiO<sub>2</sub> NT surfaces were evaluated against bacterial strain typical for orthopaedic infections: *S. epidermidis*. TiO<sub>2</sub> NT alone killed the free bacterial cells significantly but promoted their adhesion to the surfaces. The presence of moderate amount of ZnO nanoparticles significantly reduced the *S. epidermidis* cells adhesion and viability of bacterial cells in contact with modified surfaces. However, higher amount of loaded nanoZnO showed the reduced antimicrobial properties than the medium amount, suggesting the overdose effect.

*Keywords:* bone implants; TiO<sub>2</sub> nanotubes; ZnO nanoparticles; Staphylococcus epidermidis; antibacterial properties

### 1. Introduction

To date, studies of modified Ti surfaces for biomedical purposes focused on observations of morphology and on identifying their physicochemical properties which influence the biological response. However, as implant material, not only the biocompatibility, bioactivity or its physicochemical properties are important but also their antibacterial properties play a role. Infections caused by implant are one of the most serious and common complications in traumatic surgery [1,2]. They are characterized by bacterial colonization and biofilm formation on the implanted device and infection of the adjacent tissues. Bacteria within biofilms are far more resistant to antibiotics than planktonic cells and may cause a persistent infection despite aggressive antibiotic therapy.

Current strategies aimed at minimizing the incidence of bio-materials-associated infection involve the loading of antibacterial agents (e.g. antibiotics, specific polymers, toxic metal ions) onto the surface of implantable devices [3-7]. The primary advantage of such antibacterial coatings is the release of the antibacterial agent at the site of implantation, minimizing the risk of concentrations being reached that could cause harmful side effects in other parts of the body. Recently, nontraditional antibiotic agents have been of tremendous interest in overcoming resistance that is developed by several pathogenic microorganisms against most of the commonly used antibiotics [8,9]. Especially, silver nanoparticles have proven their effectiveness for preventing bacterial adhesion and biofilm formation [10]. Zinc oxide nanoparticles

are proposed as another metallic component of antibacterial properties. They are believed to destruct lipids and proteins of the bacterial cell membrane, resulting in a leakage of intracellular contents and eventually the death of bacterial cells [8]. ZnO-enriched surfaces are capable to inhibit the growth of bacteria [11]. They can also stimulate proliferation of osteoblasts, increase of alkaline phosphatase activity in these cells, synthesis and secretion of collagen and extracellular matrix mineralization [12].

In this work, we deposited ZnO nanoparticles on TiO<sub>2</sub> nanotubes fabricated on Ti surface. Such composite coatings, containing the ZnO nanoparticles inside and onto the nanotubes, were expected to release zinc ions slowly and to show a prolonged antibacterial activity. We evaluated the antibacterial properties of the composite coatings against free (planktonic) bacterial cells and also against bacterial adhesion to tested surfaces. *S. epidermidis* bacterial strain was selected for this pilot study as a model microorganism.

### 2. Experimental

The titanium oxide layers were fabricated by the electrochemical anodization of Ti samples (Ti foil, 0.25 mm-thick, 99.5% purity) in an optimized electrolyte: NH<sub>4</sub>F (0.86 wt.%) + deionized (DI) water (47.14 wt.%) + glycerol (52 wt.%) under a constant voltage of 20 V. After anodization, the samples were rinsed with DI water and dried in air. Subsequently, thermal an-

\* INSTITUTE OF PHYSICAL CHEMISTRY, POLISH ACADEMY OF SCIENCES, 44/52 KASPRZAKA, 01-224 WARSAW, POLAND

\*\* CHAIR AND DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY, MEDICAL UNIVERSITY OF LUBLIN, LUBLIN, POLAND

\*\*\* FACULTY OF MATERIAL SCIENCE AND ENGINEERING, WARSAW UNIVERSITY OF TECHNOLOGY, WARSAW, POLAND

<sup>#</sup> Corresponding author: aroguska@ichf.edu.pl

nealing was performed at 650°C for 3 h to transform the TiO<sub>2</sub> nanotubes structure from amorphous (after anodic oxidation) to crystalline (mixture of anatase and rutile). ZnO was electrodeposited from 0.005 M Zn(NO<sub>3</sub>)<sub>2</sub> aqueous solution at a potential of -2.0 V vs SCE at 60°C. The duration of the deposition was 1, 3 or 5 min. After the deposition, the samples were rinsed with DI water and annealed at 300°C for 15 min.

The morphological characterization of the fabricated samples was carried out with a scanning electron microscope (SEM, Hitachi S-5500) at the accelerating voltages of 5 kV. A Thermo Noran X-ray energy dispersive spectrometer (EDS) coupled with SEM (Hitachi S-5500) was used for chemical characterization. Analysis was performed at the accelerating voltage of 8 kV. The release of zinc from modified titanium samples was measured by inductively coupled plasma mass spectrometry (ICP-MS, Elan 9000 Perkin-Elmer). The samples were incubated in distilled water (15 ml) for 1.5 and 3 h at room temperature without stirring. The amount of zinc released was determined in resulting solution.

Evaluation of antimicrobial activity of tested titanium-based samples against free *S. epidermidis* ATCC 12228 bacterial cells was performed according to standard method JIS Z 2801:2000 [13]. Bacterial cell suspension was incubated with tested surfaces for 1.5 h and 3 h at 37°C. Afterwards, the bacteria surviving in the suspension were counted and presented as a percent of CFU (colony forming units) in control bacterial suspension (incubated without titanium surfaces). Controls were evaluated for each time point separately. Results (from three independent) experiments were calculated as means ± SD (standard deviations).

Evaluation of inhibition of cells adhesion was performed on samples defatted and cleaned in acetone bath (10 min., twice) followed by chloroform (10 min., twice) and ethanol (10 min., twice). The samples, placed in 24-well plates (Costar, Corning Inc., USA), were sterilized by ethylene oxide method in paper/plastic peel pouch (1 h at 55°C, followed by 20 h aeration). 1 ml

of sterile suspension of bacterial cells (approx.  $1.0 \times 10^8$  cells/ml) in Mueller-Hinton broth, standardized to the McFarland Equivalence Standards using Phoenix Specnephelometer (Becton Dickinson, USA) was added to each well. After 1.5 h incubation of plates at 37°C, without shaking, non-adhered bacteria were gently washed away with 0.9% NaCl (50 ml, 3 times). Next, the samples were incubated with Viability/Cytotoxicity Assay Kit for Bacteria Live & Dead Cells (Biotium, USA) in 0.9% NaCl (according to manufacturer instructions). The bacteria were visualized on the surfaces by fluorescence microscopy (Olympus BX41 with UC 12 Soft Imaging System camera). Bacterial cells were counted using CellSens Dimension program (Olympus, Japan). The results are the means ± SD of two independent experiments using 10 randomly chosen samples from each repeat.

### 3. Results and Discussion

The optimized anodization conditions resulted in the formation of TiO<sub>2</sub> nanotubes (NT) perpendicular to the substrate and separated from each other. SEM examinations revealed that the nanotubes are open at the top and closed at the bottom. An average diameter of the obtained nanotubes was about 100 nm and height of about 1 μm. Figure 1 shows SEM images of typical TiO<sub>2</sub> nanotube layers after electrodeposition of ZnO NPs for different time. After 1 min. of electrodeposition, only some single spherical nanoparticles could be observed on the nanotube edges (Fig. 1a). Electrodeposition of ZnO NPs for 3 min. led to the formation of spherical nanoparticles tightly covering the nanotube edges and uniformly distributed over the nanotubular layer (Fig. 1b). Size of these nanoparticles was about 10 nm. The increase of electrodeposition time led to the formation of heterogeneous coatings with some areas of the TiO<sub>2</sub> nanotube surface completely covered by the agglomerated ZnO deposits (Fig. 1c).

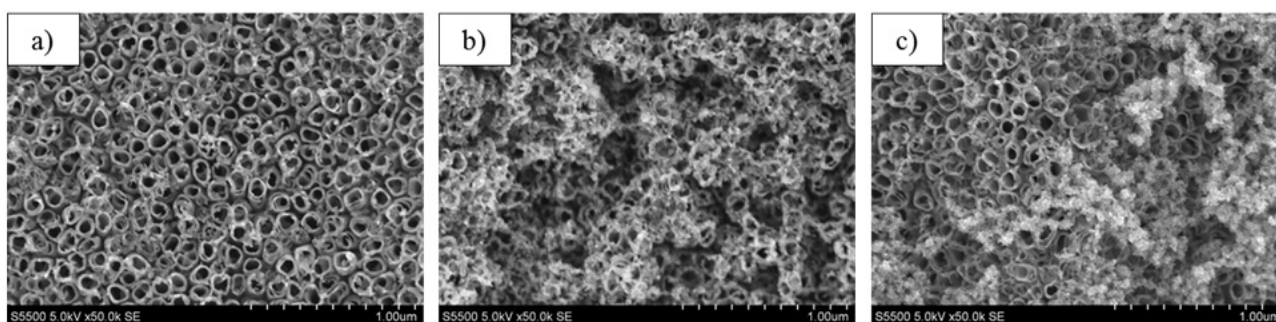


Fig. 1. SEM images of ZnO nanoparticles electrodeposited on TiO<sub>2</sub> nanotubes-modified titanium surfaces for 1 min. (a), 3 min. (b) or 5 min. (c).

The chemical composition of the modified surfaces was analyzed using EDS technique. In general, increasing the time of ZnO electrodeposition led to the higher concentration of Zn at the TiO<sub>2</sub> NT surface (Table 1). The highest concentration of Zn – 16.2% was observed for the longest electrodeposition time (5 min.), and is approximately 4 times greater than for 3 min electrodeposition, and more than 8 times greater than for 1 minute of electrodeposition

TABLE 1

Zn weight content in fabricated composite layers on Ti

Sample	Zn; wt. %
TiO <sub>2</sub> NT + 1 min ZnO	2.1
TiO <sub>2</sub> NT + 3 min ZnO	4.1
TiO <sub>2</sub> NT + 5 min ZnO	16.2

Figure 2 shows the profile of zinc release from tested coatings. The concentration of released Zn increased with time for all samples. Surprisingly, for nanoZnO deposited for 5 min. (the largest amount), Zn was released at the lower level than for nanoparticles deposited for 1 min. and 3 min. Also, the increase of the amount of the released zinc between the tested time points (1.5 h and 3 h) of solubilization was much smaller for 5 min. deposition when compared with those for 1 min. and 3 min. deposition.

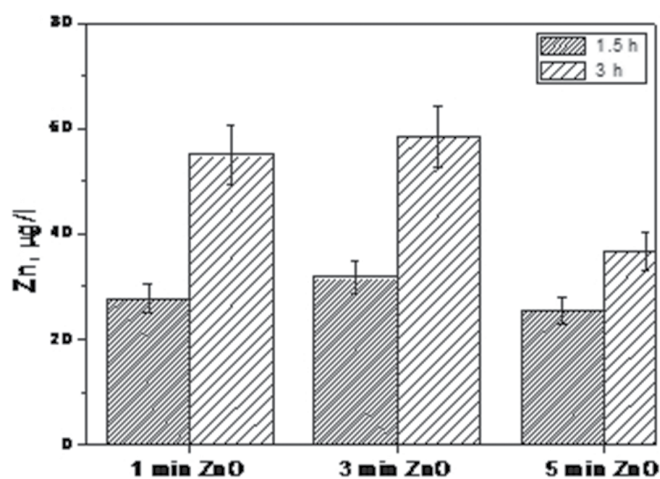


Fig. 2. The release of zinc from modified titanium samples after 1.5 h and 3 h incubation in distilled water.

The survival of *S. epidermidis* cells in the suspension, tested for two different periods of time, showed the time-dependent character of antibacterial action of tested samples. Amount of surviving cells was evaluated and calculated as a percent of that in control bacterial suspension. Results, presented in Table 2, were compared with reference samples: pure titanium and titanium modified with

TiO<sub>2</sub> nanotubes. For 3 h time point, the bactericidal effect was similarly strong (almost complete lack of surviving bacteria for all nanoZnO-loaded surfaces). It should be noted, however, that single cells survived on samples with ZnO deposited for 5 min., which was not observed for surfaces with nanoZnO deposited for 1 min. or 3 min., containing much less ZnO (Table 1). For 1.5 h incubation of bacterial cells with modified titanium plates, the differences between the tested samples were observed. Samples loaded with ZnO nanoparticles for 3 min. were the most effective at *S. epidermidis* killing: only 30.4% of surviving cells (Table 2). Lower (1 min.) and higher amount (5 min.) of loaded ZnO nanoparticles caused a decreased bactericidal activity of tested surfaces (54.7 % and 68.7% of surviving cells, respectively). Moreover, we observed that TiO<sub>2</sub>NT themselves also exhibited a bactericidal activity (74.8% survival of bacterial cells) in comparison with pure titanium (82.9% survival of bacterial cells).

TABLE 2

Release-killing capability of tested samples in relation to incubation time. Bacterial strain: *S. epidermidis* ATCC 12228. Control – bacterial suspension without contact with titanium surface.

Sample	CFU (% of CFU in control)	
	1.5 h	3 h
Control	100 ± 9.6	100 ± 14.7
Ti	82.9 ± 8.1	53.8 ± 5.4
TiO <sub>2</sub> NT	74.8 ± 5.4	17.5 ± 1.0
TiO <sub>2</sub> NT + 1 min ZnO	54.7 ± 1.8	0 ± 0
TiO <sub>2</sub> NT + 3 min ZnO	30.4 ± 4.9	0 ± 0
TiO <sub>2</sub> NT + 5 min ZnO	68.7 ± 0.9	0.2 ± 0.065

The results of the test of bacterial adhesion (for 1.5 h incubation) were summarized in Figure 3. The bacteria adhered

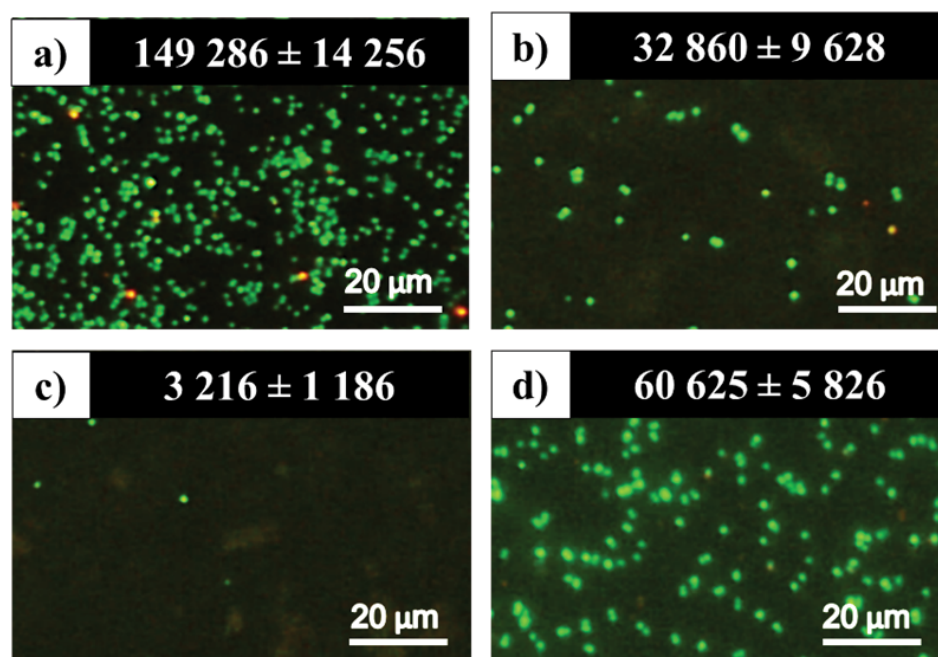


Fig. 3. Fluorescence microscopy images of *S. epidermidis* cells adhered to tested surfaces after 1.5 h incubation with bacterial cell suspension. TiO<sub>2</sub> NT (a); TiO<sub>2</sub> NT + 1 min. ZnO (b); TiO<sub>2</sub> NT + 3 min. ZnO (c); TiO<sub>2</sub> NT + 5 min. ZnO (d). In insets: number of adhered cells per 1 mm<sup>2</sup>

to tested surfaced were mainly live ones, as indicated by green fluorescence. It was found that bacteria adhered abundantly ( $\approx 149\,000$  cells/mm<sup>2</sup>) to titanium surface modified with nanotubes but without nanoZnO. Much less adhered cells were detected on titanium/TiO<sub>2</sub>NT surfaces loaded with ZnO nanoparticles. We observed that samples with medium ZnO content (deposition for 3 min.) exhibited much higher antibacterial capacity (only 3 216 adhered cells/mm<sup>2</sup>) than samples with the lowest (1 min. deposition) and the highest (deposition for 5 min.) ZnO content: 32 860 and 60 625 cells/mm<sup>2</sup>, respectively (Figure 3). These observations are in agreement with the results concerning the killing of free bacterial cells in the suspension (Table 2) and profile of Zn release (Figure 2). The explanation of this phenomenon may be supplied by SEM observations which revealed that, for samples deposited for 5 min., the ZnO deposit was relatively thick, nonhomogeneous and irregularly shaped. Such a thick metallic multilayer is do expected to show a reduced (as confirmed by Zn release profiles) solubility when compared with samples with ZnO nanoparticles deposited for 1 min. and 3 min. (as presented in Figure 2).

As recently reported, metal nanoparticles (eg. nanoAg) exhibit higher antibacterial efficacy than the metals in a solid mass [10]. In fact (as showed for nanoZnO), smaller dimensions induce the increased antibacterial capacity of metallic nanoparticles [14]. On a base of results obtained in this study and available literature it may be concluded that the dose of antibacterial metal nanoparticles loaded on surfaces of biomedical implants should be adequate to the expectations. It cannot be too large because otherwise the nanoparticles may be transformed into solid multilayer of a reduced solubility. Moreover, which is especially important for Zn<sup>2+</sup> ions, it should not be too small because bacteria may metabolize zinc as an oligo-element [15].

#### 4. Conclusions

In this work the antibacterial properties of composite coatings on Ti consisting of TiO<sub>2</sub> nanotubes loaded with ZnO nanoparticles were evaluated. TiO<sub>2</sub> NT alone (without nanoZnO) showed noticeable bactericidal properties but did not inhibit the adhesion of live bacterial cells. ZnO nanoparticles, deposited on TiO<sub>2</sub> NT-modified titanium, killed bacteria very efficiently (just after 1.5 h of contact) and reduced the adhesion of *S. epidermidis* cells. This confirmed the high potential of nanoZnO-loaded

TiO<sub>2</sub> nanotubes-modified surfaces against bone implants-related infections. An important observation concerned the quantity of loaded nanoparticles. They suggested that the overdose of nanoZnO particles may be a limiting factor and reduce the efficacy of antibacterial surfaces, probably due to the reduced solubility of aggregated nanoparticles. More extensive study should be performed in future to confirm these important conclusions.

#### Acknowledgments

This work was financially supported by the National Science Center (DEC 2011/03/N/ST5/04388) and was developed using the equipment purchased within the agreement No. POPW.01.03.00-06-010/09-00 Operational Program Development of Eastern Poland 2007-2013, Priority Axis I, Modern Economy, Operations 1.3. Innovations Promotion.

#### REFERENCES

- [1] A.G. Gristina, Clin.Orthop.Relat. **R 298**, 106 (1994).
- [2] A.D. Hanssen, J.A. Rand, J. Bone, Joint Surg. **80A**, 910 (1998).
- [3] M. Sygnatowicz, K. Keyshar, A. Tiwari, JOM **62**, 65 (2010).
- [4] C.K. Popat, M. Eltgroth, J.T. La Tempa, A.C. Grimes, A.T. Desai, Biomaterials **28**, 4880 (2007).
- [5] P.H. Chua, K.G. Neoh, E.T. Kang, W. Wang, Biomaterials **29**, 1412 (2008).
- [6] Y.Z. Wan, G.Y. Xiong, H. Liang, S. Raman, F. He, Y. Huang, Appl. Surf. Sci. **253**, 9426 (2007).
- [7] J. Xu, G. Ding, J. Li, S. Yang, B. Fang, H. Sun, Y. Zhou, Appl. Surf. Sci. **256**, 7540 (2010).
- [8] A.J. Huh, Y.J. Kwon, J. Control. Release **156**, 128 (2011).
- [9] E. Weir, A. Lawlor, A. Whelan, F. Regan, Analyst **133**, 835 (2008).
- [10] M. Rai, A. Yadav, A. Gade, VTT SYMP **27**, 76 (2009).
- [11] M. Premanathan, K. Karthikeyan, K. Jeyasubramanian, G. Manivannan, Nanomed.-Nanotechnol. **7**, 184 (2011).
- [12] G. Jin, H. Cao, Y. Qiao, F. Meng, H. Zhu, X. Liu, Colloid Surface **B. 117**, 158 (2014).
- [13] JIS Z 2801:2000. Antimicrobial products – test for antimicrobial activity and efficacy. Japanese Industrial Standard, Japanese Standard Association.
- [14] S. Nair, A. Sasidharan, R.V. Divya, D. Menon, K. Manzoor, S. Raina, J. Mater, Sci.-Mater. **M.20**, 253 (2009).
- [15] R. Brayner, R. Ferrari-Illiou, N. Brivois, S. Djediat, M. Benedetti, F. Fiévet, NanoLett. **6**, 866 (2006).