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Coatings Based on Two-Dimensionally Ordered Linear Chain Carbon for Protection of Titanium Implants from Microbial Colonization

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Abstract

Purpose of the study – to evaluate the antibacterial activity and biological compatibility of alloy coatings based on two-dimensionally ordered linear chain carbon (TDOLCC). ***Materials and Methods.*** Coatings based on TDOLCC were synthesized using alloying additions like nitrogen (TDOLCC+N) and silver (TDOLCC+Ag) on the surfaces of titanium plates and polystyrene plates by the ion-stimulated carbon condensation in a vacuum. The authors examined the superficial bactericidal activity of the coatings and its resistance to mechanical effects. Coated plates were evaluated in respect of rate of microbial biofilms formation by clinical isolates with multiple and extreme antibiotic resistance. Specimens were colored with crystal violet solution to visualize the biofilms. Cytotoxic effect of coatings was evaluated in respect of primary culture of fibroblasts and keratinocyte cell line HaCaT. ***Results.*** The authors observed pronounced superficial bactericidal effect of TDOLCC+Ag coating in respect of microorganisms of several taxonomic groups independently of their resistance to antibacterial drugs. TDOLCC+Ag coating proved capable to completely prevent microbial biofilm formation by antibiotic resistant clinical isolates of *S. aureus* and *P. aeruginosa*. Silver-containing coating demonstrated mechanical resistance and preservation of close to baseline level of superficial bactericidal activity even after lengthy abrasion treatment. TDOLCC based coatings did not cause any cytotoxic effects. Structure of monolayers formed in cavities coated by TDOLCC+N and TDOLCC+Ag was indistinguishable from the monolayers in cavities of control plates.

Keywords: titanium implants, microbial biofilms, antibiotic resistance, antibacterial coating.

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Background

Bacterial infections of the surgical wound are the serious complication after internal fixation, and their treatment is significantly impeded by the implant, colonized by microbial biofilms, at the inflammation site. It's proven that formation of biofilms on the surfaces of various materials for internal fixation (pure titanium, titanium alloys, stainless steel, cobalt-chromium-molybdenum alloy) occurs rather fast within 2 to 4 hours [1]. Prevention of biofilms formation is the key task in the prophylaxis of implant associated infections [2].

One of the prospective ideas for treatment of patients with high risk of deep surgical wound infection is the application of metal implant with modified active microbicidal coatings. Various methods of physicochemical modification of the surfaces are known for creation of antibacterial properties. The "ideal" antibacterial coating applied on implants' surface used in traumatology and orthopaedics should be biocompatible, have no local irritating effect, should exhibit pronounced bactericidal properties in early postoperative period and maintain superficial bactericidal activity against a wide range of microorganisms — wound infection agents — during the whole period of implantation, as well as prevent bacterial adhesion to implants surface and inhibit formation of microbial biofilms [3].

The use of diamond-like carbon coatings for implants protection was suggested in the beginning of 1990s. Diamond-like carbon features chemical inertness, corrosion resistance and mechanical wear resistance. Diamond-like coatings contain a combination of sp^2- and sp^3- carbon of higher biding energy [4, 5]. Instability of diamond-like coatings in biologic fluids is the serious restricting factor for use in traumatology and orthopaedics, when instability is manifested by detachment or splitting of coating. Currently the application of diamond-like coatings is limited due to significant internal mechanical strain in their layer and rather weak adhesion to metal surfaces [4]. Mentioned disadvantages are missing in coatings based on two-dimensionally ordered linear chain carbon (TDOLCC). TDOLCC films produced on various carriers represent ordered quantum string ensembles positioned at distance of 0,4–0,5 nm and, thus, weakly interacting with each other [6]. TDOLCC based coatings have high adhesion to carriers, strength and elasticity as well as good biocompatibility [7–9]. In the process of film production TDOLCC is easily doped with various chemical elements which allows to give surfaces additional physical and chemical properties [10, 11].

Purpose of the study — to evaluate the antibacterial activity and biocompatibility of alloy coatings based on two-dimensionally ordered linear chain carbon (TDOLCC).

Material and methods

Carbon coatings formation technology

Titanium plates were used as the carrier for TDOLCC based coating: plates with dimensions of 50×50×0,5 mm (to determine superficial bactericidal activity) and 12,5×50×0,5 mm (to examine formation of microbial biofilms). Coatings were synthesized in a PVD Coating Machine "URM 3.279.070 Diamond" by ion-stimulated carbon condensation in a vacuum. Carriers simultaneously were impacted by carbon flow obtained in the process of thermal evaporation or ion sputtering, and by argon ions flow, where TDOLCC coatings were formed in result of carbon flow condensation.

For production of TDOLCC silver alloyed coatings (TDOLCC+Ag) the authors drilled holes in graphite cathode where silver pins were inserted. Film was produced with simultaneous ion-plasma evaporation of silver in the same pulsed cathode discharge. Nitrogen alloying of coatings (TDOLCC+N) was achieved by supplying nitrogen to the vacuum chamber together with argon.

Evaluation of antibacterial activity of coatings

Bilayer agar method was used to detect antibacterial activity and study release rate of antibacterial factors from coatings [12]. Control and study samples (titanium plates 50×50×0,5 mm without coatings and coated by TDOLCC, TDOLCC+N and TDOLCC+Ag) were kept in distilled water for 15 minutes at room temperature to remove rapidly soluble components and then air sterilized at 160°C for 60 minutes. After cooling until room temperature the samples were moved by sterile tweezers to the surface of Mueller Hinton agar (Mueller Hinton II Agar, BD BBL, USA) in 90 mm polystyrene Petri dishes.

Mueller Hinton agar was melted and cooled up to 45°C in volume of 8,3 ml; 14,6 ml and 27,6 ml was filled as second layer on the surface of the plate. Estimated agar layer height above plate surface was 1, 2 and 4 mm respectively. Dishes were kept on level horizontal surface until full solidification of medium and then thermostat dried for 15 minutes.

Control strain of *P. aeruginosa* ATCC 27853 from American typical culture collection was used as test-culture. Daily cultured grown on nutrient agar (Nutrient Agar, M001, HiMedia, India) was used to prepare the suspension in sterile isotonic solution of sodium chloride with an optical density of 0.5 according to MacFarland's turbidity standard. Dishes were inoculated by bacterial suspension and incubated for 18 hours at 35°C. Then presence and growth pattern of microorganisms were evaluated on the surface of Mueller Hinton agar in the area of projection of control titanium plates and titanium plates with carbon coatings of various composition.

Evaluation of superficial bactericidal activity of coatings

A series of experiment samples was prepared each of which included 3 samples (titanium plates of 50×50×0,5 mm) with uniform coatings as well as a series of control samples without coatings. Control and experiment samples were kept in distilled water for 15 minutes at room temperature to remove fast soluble substances, then samples were dried, placed in glass Petri dishes and air sterilized at 160°C for 60 minutes. *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were used as test cultures. An extremely antibiotic-resistant isolate of *Pseudomonas aeruginosa* P-142 obtained from a patients with post-traumatic osteomyelitis was included into the study. Superficial bactericidal activity of samples was examined in accordance with Japanese industrial standard JIS Z 2801: 2010*. The number of vital bacterial cells for the series of samples was calculated by the formula:

$$N = \frac{(C_1 + C_2 + C_3)\,D}{3},$$

where N is the mean quantity of microbial cells for sample series, C_1, C_2, C_3 — quantity of colonies in the dish for each of samples in the series, D — dilution factor.

Rate of antimicrobial activity for experimental sample series was calculated by the formula:

$$R = \log(N_K/N_T),$$

where R is the rate of antimicrobial activity, N_K — average quantity of microbial cells for control sample series, N_T — average quantity of microbial cells for experiment sample series.

Bactericidal index was calculated by the formula:

$$I = \frac{N_K - N_T}{N_K} \times 100\%,$$

where I is bactericidal index, N_K — average quantity of microbial cells for control sample series, N_T — average quantity of microbial cells for experiment sample series.

Evaluation of antibacterial coatings resistance to mechanical actions

Plates with various types of coating were washed in distilled water with abrasive, namely filler for tumbling OTEC H0/050 (OTEC, Germany). Coated samples were placed in vials adding 15–20 g of abrasive component and 100 ml of distilled water. Incubation was performed during 96 hours at 35°C with continuous 150 rpm orbital shaking in the orbital shaker-incubator ES-20 (BioSan, Latvia). Following abrasive processing the samples were washed twice in distilled water, air sterilized at 160°C for 60 minutes and examined for bactericidal activity on the surface in accordance to JIS Z 2801: 2010.

Examination of microbial biofilms formation on the surface of antibacterial coatings

To evaluate protective effect of carbon based coatings of various composition against microbial biofilms the authors used clinical isolates of *S. aureus* and *P. aeruginosa* with multiple antibiotic-resistance and high ability for films formation which were obtained from patients with bone infections. Experiment samples with various coatings and control samples without coating were placed into glass centrifuge tubes and air sterilized at 160°C for 60 minutes. Suspensions of test cultures were prepared in sterile isotonic solu-

* JIS Z 2801: 2010 Antibacterial products — test for antibacterial activity and efficacy. Tokyo, Japan: 2010.

tion of sodium chloride (optical density of 0.5 according to MacFarland's turbidity standard). 10 ml of tryptic soy broth (Tryptic Soy Broth, BD BBL, USA) was added to each tube with samples as well as 50 mcl of test culture suspension (estimated starting concentration of microbial cells in medium 5×10^5 ml^{-1}). Tubes with samples were incubated in the shaker for 24 hours at 35°C with 100 rpm orbital shaking. Water solution of crystal violet (0,1%) was used to stain samples for biofilms visualization after incubation. To quantify rate of biofilm formation stained samples were placeв into tubes with 10 ml of 96% ethanol and underwent alcoholic extraction of crystal violet sorbed by biofilm for 24 hours at 44°C. Concentration of crystal violet in obtained extracts was measured on Infinite M200 plate reader (TECAN, Switzerland) with 540 nm wavelength. Mass of biofilm was presented as mass of staining sorbed by biofilm, and was calculated by the formula:

$$m = \frac{V(C_1 + C_2 + C_3 - F_1 - F_2 - F_3)}{3},$$

where m is the mass of crystal violet sorbed by biofilm, mcg; V — volume of washing solution, ml; C_1, C_2 и C_3 — concentration of staining in washing solutions of experimental sample series, mcg/ml; F_1, F_2 и F_3 — concentration of staining in washing solutions of control sample series, mcg/ml.

Examination of biocompatibility of coatings with cell cultures

The authors used epithelial human cells of the HaCAT line (keratinocytes). Cell samples frozen at -80°C were transferred to vial with water of 37°C. After defrosting the tube was processed with alcohol, content was resuspended and moved to sterile polypropylene tubes (15 ml) which contained 10 ml of complete incubation medium (DMEM/F-12, 11039 GIBCO; 100 U/ml penicillin; 100 mcg/ml streptomycin; 0,25 mcl/ml amphotericin B; 10% inactivated fetal calf serum, HiCloneInc). After 5 min of centrifugation (4°C, 200 g) the liquid phase was discarded, the cell sediment was resuspended in 5 ml of full incubation medium and used for inoculation. Cell concentration was determined in Gorjaev›s chamber. Primary culture of skin fibroblasts was isolated from the dorsal areas of the skin of Wistar rats by the method of primary explants. Then, the cells were cultured for 5–7 passages, the passage was carried out when 70% confluence was reached, the dilution ratio during the passage was 1:5. Culturing mode: complete incubation medium (DMEM/F-12, 11039 GIBCO; 100 U/ml enicillin; 100 mcg/ml streptomycin; 0,25 mcl/ml amphotericin B; 10% inactivated fetal calf serum, HiCloneInc), 37°C, 5% CO_2 in the atmosphere and 90% relative humidity.

To study cytotoxic properties of the coating the cells at 70% confluence were removed from the surface of culture vial by T-75 phosphate buffered saline (containing 0.05% trypsin and 0.5 mM EDTA), then introduced 3 ml of cell suspension (350 thousands of cells) in complete incubation medium into each cell of 6-well polystyrene plates (Tissue culture Plate 6-Well Flat Bottom Cell+, Sarstedt, Germany) with surfaces coated by TDOLCC+N and TDOLCC+Ag. Similar culture plate without coatings was used as control. 3 wells were inoculated for each type of coating. Coated table were additionally sterilized by ethylene oxide prior to study. In 24 hours after incubation (37ºC, 5% CO_2) the authors examined cells morphology and monolayer structure using inverted microscope Leica DM IL (Leica Microsystems, Germany) at magnitude of ×150.

Results

Morphology and structure of carbon based coatings

The authors suggest the following atomic model of sp^1-carbon coating (Fig. 1).

Coatings have multilayer structure and every layer is represented by chains of carbon atoms in the sp^1 hybridization normally oriented towards

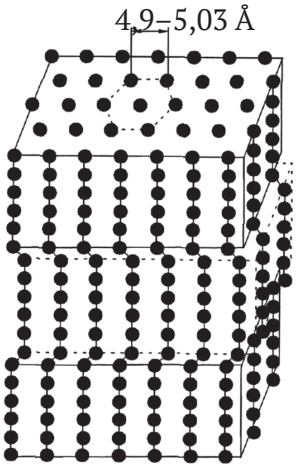


Fig. 1. Model of atomic structure for sp^1-carbon film

layer surface. Chains are packed in a hexagonal grid. The film consists of carbon chains with zigzags randomly oriented in the azimuthal direction with respect to the axis of the chains.

Antibacterial activity of carbon based coatings

The examination allowed to detect antibacterial activity related to diffusion and distribution of antibacterial components in the thickness of nutrient medium only for TDOLCC+Ag coating and only for cases of the minimum thickness of the layer of agar nutrient medium (1 mm) on its surface. With the introduction of a larger volume of nutrient medium (layer thickness of 2 mm and 4 mm) due to an increase in the volume of distribution for the antibacterial component, its bacteriostatic concentrations were not achieved and microorganisms' growth was observed on the medium surface. Two-layer agar method is intended for exposure of remote antibacterial effects by diffusion components of the coating and for creation of bacteriostatic concentrations in the volume and on the surface of nutrient medium. Additionally, for antibacterial components with known minimum inhibition concentrations for control strains, one can indirectly estimate their release rate from the coating to the medium. Values of the minimal inhibition concentration of silver cations and silver nanoparticles for the control strain of *P. aeruginosa* ATCC 27853 are reported from 3 to 8 mcg/ml according to various publications [13, 14]. Considering known values of minimal inhibition concentration of silver compounds for *P. aeruginosa* and obtained experimental data one can suggest that release rate of microbiologically active silver forms from surface of TDOLCC+Ag coating do not exceed 0,6–1,6 mcg/cm^2 per day. Calculated data requires clarification in direct kinetic experiments for identification of silver concentration diffusion from the coating to the experiment medium. At the same time absence of cytotoxic effects on peri-implant area as well lack of antibacterial effect in the distance over 1–2 mm from implant surface can be predicted for medical devices with similar coatings due to negligible volume of active silver released from the coating surface.

Superficial bactericidal activity for carbon coatings of various composition

Measurements of superficial bactericidal activity in accordance with JIS Z 2801: 2010 standard are presented in Table 1 and on Figure 2.

TDOLCC and TDOLCC+Ag coatings exhibit moderate superficial bactericidal activity against control strains of *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 (bactericidal indices 59,7–79,5%). TDOLCC+Ag coating exhibited pronounced superficial bactericidal activity against all microorganisms included into the study (bactericidal indices 98,7–99,0%).

Table 1

Parameters of superficial bactericidal activity of carbon coatings

Microorganism	TDOLCC		TDOLCC +N		TDOLCC +Ag	
	R	I, %	R	I, %	R	I, %
S. aureus ATCC 25923	0,42	61,8	0,69	79,5	1,88	98,7
E. faecalis ATCC 29212	0,39	59,7	0,45	64,5	2,01	99,0
P. aeruginosa P-142	0,00	0,0	0,00	0,0	1,88	98,7

R — level of antimicrobial activity; *I* — bactericidal index.

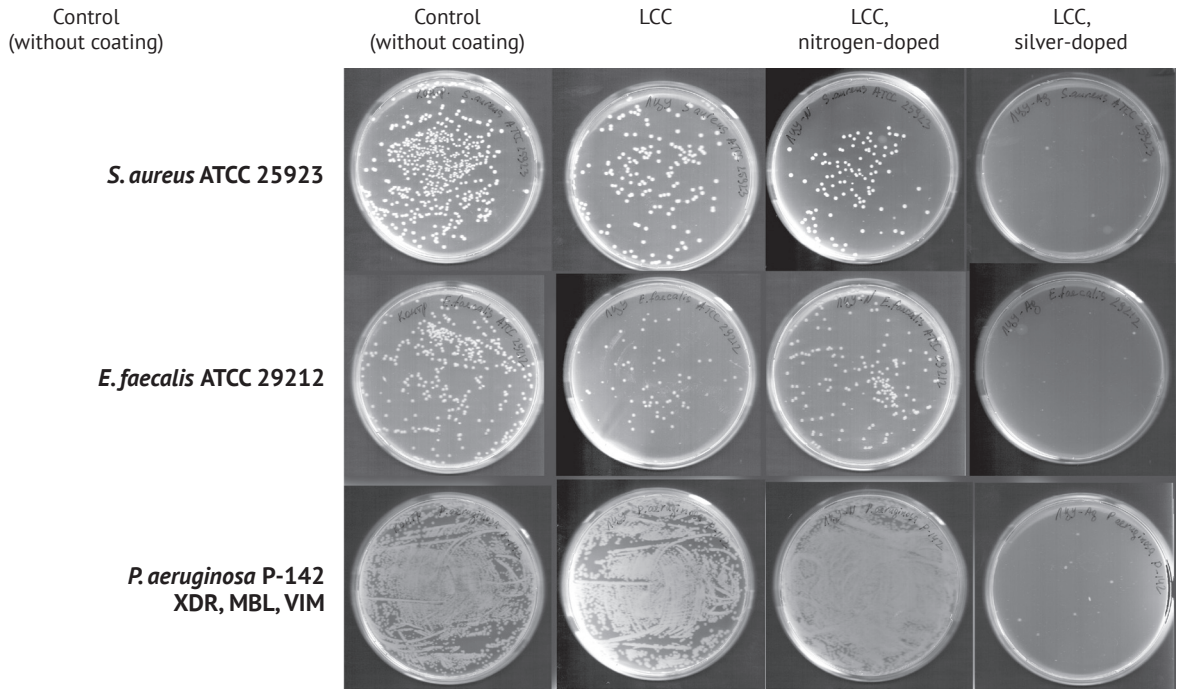


Fig. 2. Superficial bactericidal activity of carbon coatings based on TDOLCC (JIS Z 2801: 2010)

Mechanical stress resistance of coatings

Examination results for superficial bactericidal activity measured after 96 hours abrasive treatment are presented in Table 2. TDOLCC+N lost superficial bactericidal properties after washing which can be related to rapid wash out of nitrogen-containing components. The authors observed maintenance of superficial bactericidal activity of TDOLCC+Ag coating (no less than 98% from baseline) against all microorganisms included into the research.

The rate of microbial biofilms formation on the surface of carbon coatings

TDOLCC based coating did not inhibit formation of microbial biofilms of microorganism test cultures. Rate of biofilms formation by *P. aeruginosa* P-142 strain was significantly higher as compared to MRSA strains, the biofilms stained by crystal violet were visualized by the naked eye. TDOLCC+Ag coating almost completely suppresses biofilms formation. The quantification results of the intensity of microbial biofilms formation by *P. aeruginosa* P-142 strain on the surface of the samples are presented in Table 3.

Table 2

Parameters of superficial bactericidal activity of carbon coatings after 96 hour abrasion processing

Microorganism	TDOLCC +N		TDOLCC +Ag	
	R	I, %	R	I, %
S. aureus ATCC 25923	0,13	26,3	1,80	98,4
E. faecalis ATCC 29212	-0,01	-3,2	1,89	98,7
P. aeruginosa P-142	0,00	0,0	1,78	98,3

Table 3

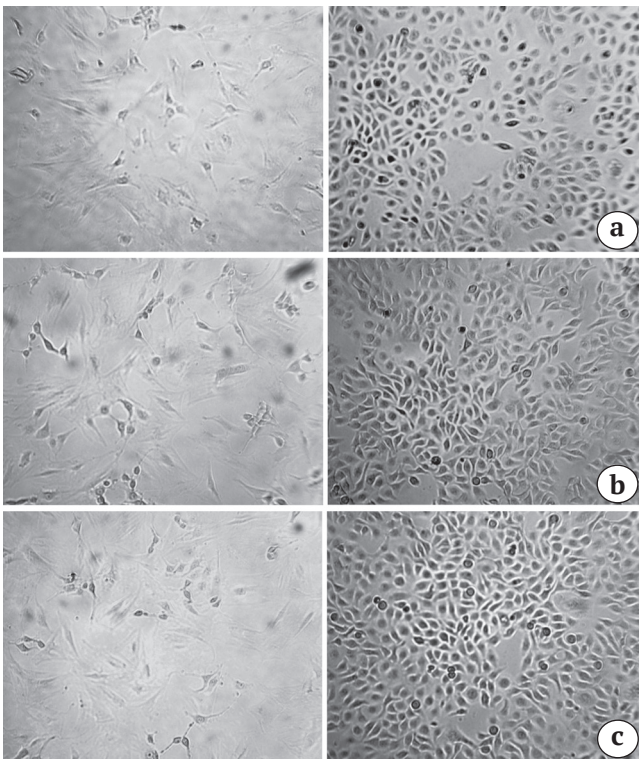
Rate of microbial *P. aeruginosa* P-142 biofilms formation on the surface of specimens with carbon coatings

Coating type	Staining mass sorbed by biofilm, mcg/cm^2
TDOLCC	2,75
TDOLCC +N	0,63
TDOLCC +Ag	0,06

Results of microscopy for clinical isolates *S. aureus* included into the study demonstrated reduced intensity of biofilms formation on TDOLCC+N coating as compared to TDOLCC. TDOLCC+Ag completely suppressed biofilms formation of *S. aureus*.

Adhesion and cells morphology on the surfaces with TDOLCC coatings

In 24 hours of incubation the monolayer of primary cutaneous fibroblasts with surface density no less than 70–95% was formed both in control plate wells and in wells of plates coated by TDOLCC +N and TDOLCC +Ag (Fig. 3).



Structure of the monolayer formed in the wells with TDOLCC +N and TDOLCC +Ag coatings was indistinguishable from the monolayer in the wells of control plates. No possible cytotoxic effects (vacuolation of cells, loss of typical morphology with decreased size, rounding and detachment from surface) were observed. Similar results were obtained during examination of coatings influence on the morphology of human HaCaT keratinocytes line.

Thus, analysis of cells morphology demonstrated that TDOLCC based coatings did not have pronounced effects on their ability to spread on the surface and form monolayer as well as did not cause cytotoxic effects.

Discussion

Creation of coatings that would ensure long-term sustenance of local effective antibiotics concentration or other biocides against the main pathogens of periprosthetic infections is the severe limitation for wide introduction of implants with antibacterial properties into clinical practice. Many carriers used for coatings creation continue release antibiotics in concentrations lower than minimal inhibition concentration during an indefinite period of time which promotes additional selection of antibiotic resistance. It's suggested that the "ideal" antibacterial coatings should release antibiotics in the volumes ensuring sufficient concentrations to in-

Fig. 3. Type of culture cells of primary rat fibroblasts (left row) and HaCaT (right row) after 24 hour culturing on plates with various coating:
a — no coating;
b — coating TDOLCC+N;
c — coating TDOLCC+Ag

hibit microorganisms over a long period of time, and afterwards the release should stop quickly to eliminate the risk of developing resistant bacteria [15].

The method of ion-stimulated carbon condensation in a vacuum proposed by the authors allows to apply two-dimensionally ordered linear chain carbon coatings with antibacterial alloys on the surface of titanium implants and features relative simplicity of implementation and simultaneously high performance.

Earlier a variety of methods have been suggested to apply "passive" coatings to steel and titanium implants, such coatings did not possess own antibacterial activity though slow down microbial adhesion by changing physical properties. Such methods included application of crystalline titanium oxide, polymethacrylic acid, polyethylene oxide, polyethylene glycol as well as creation of hydrophobic nanostructured surfaces [16, 17]. Many of the above technologies allow significant reduction of bacterial adhesion, however, until present there is no convincing data proving their clinical effectiveness while even singular bacteria adhesion leads to microbial biofilm formation in time and implants can remain in the human body for months and years. Besides, the surface modification inhibiting microbial adhesion often deteriorates osteointegration properties of implants [18, 19].

The following components were used as antibacterial agents in the "active" coatings: metal nanoparticles or cations (silver, copper, zinc), antiseptics (chlorhexidine, iodine, polyhexamethylene biguanidine), antibiotics (aminoglycosides, cephalosporins, vancomycin, linezolid) [20, 21]. Animal experiments were used to evaluate microbiological effectiveness of various antibacterial coatings applied on bone screws, pins and intramedullary fixators, with that authors observed stable reduction of infection complications rate. The key issues were short-term effect of antimicrobial agent, usually not exceeding 48-96 hours, as well as loss of acting substance at implant insertion [22, 23].

Some techniques for synthesis of antibacterial coatings on metal implants for trauma and orthopaedic surgery passed medical trials and have been introduced into clinical practice. It's known that biological inertness and good biocompatibility of titanium implants results from titanium oxide on their surface. Electrochemical technologies have been developed to create oxide layers with a developed surface and porous structure on the surface of titanium devices to promote good osteointegration. Adding povidone-iodine to the electrolyte composition for anodizing surface of titanium implants resulted in creation of titanium oxide coatings containing iodine in combination with a water-soluble polymer in its porous structure [24]. Use of such iodine oxide coatings on hip and knee prostheses, screws and plates for internal fixation, transpedicular spine systems for prophylaxis of periprosthetic infection and for infection termination during revisions proved good clinical efficiency [25].

Intramedullary Ti-6Al7Nb nails with antibacterial coating (UTN PROtect, Synthes) intended for treatment of femur fractures were certified in 2005 for clinical use in the EU and have a thin layer of poly-D,L-lactide impregnated with gentamycin on their surface. Medical trials of UTN PROtect nails demonstrated good clinical, laboratory and roentgenological outcomes in 6 months after implantation and allowed to recommend this implant for prophylaxis of infection and for termination of infectious process during revisions [26, 27].

Inorganic biocides (first of all silver cations and nanoparticles) are a good alternative to antibiotics as a part of antibacterial coatings while they feature high long-term bactericidal activity, good biocompatibility, stability and very lengthy resistance formation in bacteria [28, 29]. *In vitro* and *in vivo* studies demonstrate good biocompatibility of silver coatings without cytotoxicity and genotoxicity [30]. Silver can be included in composition of various carriers, including polymers, bioceramics, bioglass, carbon films [30, 31]. Silver doped implant surfaces efficiently inhibit bacterial adhesion and formation of microbial biofilms, and at the same time do not substantially impact osteoblasts and epithelial cells activity [18, 22].

Pronounced superficial bactericidal effect of TDOLCC+Ag based coatings against antibiotic sensitive and antibiotic resistant microorganisms was observed in the present study. Bactericidal activity was also demonstrated by the ability to prevent formation of microbial biofilms on metal surfaces with applied coatings. High mechanical stress resistance of silver coatings to abrasive treatment and its biocompatibility makes above suggested technology prospective for adding antibacterial properties to the wide range of medi-

cal implants. Low release rate of active silver forms from coating surface stipulates absence of cytotoxic effects on the peri-implant area. At the same time the authors predict long-term (no less than 1-2 weeks) implant surface protection from colonization by microorganisms and formation of microbial biofilms.

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Authors' contribution

D.V. Tapalski — analysis and interpretation of the data and the writing of the manuscript.

N.S. Nikolaev — contributed to the study concept and design, analysis and interpretation of the data and the writing of the manuscript.

A.V. Ovsyankin — contributed to the study concept and design, analysis and interpretation of the data and the writing of the manuscript.

V.D. Kochakov — analysis and interpretation of the data and the writing of the manuscript.

E.A. Golovina — analysis and interpretation of the data and the writing of the manuscript.

M.V. Sukhorukova — analysis and interpretation of the data and the writing of the manuscript.

M.V. Matveenkov — analysis and interpretation of the data and the writing of the manuscript.

R.S. Kozlov — coordination of study, manuscript preparation.

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