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Antibiofilm properties of thin-film coating prototypes with copper oxide nanoparticles for orthopedic implants from titanium and its alloys: Experimental study

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Abstract:
Objective: to evaluate the bacteriostatic properties of the developed prototypes of thin-film coating for orthopedic implants made of titanium and its alloys.
Materials and Methods. Using scanning electron microscopy, we examined the morphology of Ti-6AL-4V (ASTM F1472) samples with a thin-film coating containing cupric oxide nanoparticles with a dispersion of 50-70 nm applied to their surface by plasma electrolytic oxidation. Then we assessed the impact of prototypes of thin-film coating on the propensity of clinical strains of microorganisms to adhere and form biofilms, and on their growth properties.
Results. The developed prototype of a thin-film coating caused a significant decrease in the mass of biofilms formed by clinical strains of various microorganisms by 11% (Staphylococcus aureus), 38% (Staphylococcus epidermidis) and 7% (Pseudomonas aeruginosa), along with a reduction in bacterial growth properties by 12.7 % (S. aureus), 13.3% (S. epidermidis) and 10% (P. aeruginosa).
Conclusion. The developed prototype of a thin-film coating for products made of titanium and its alloys reduced the virulence factors of clinical microbial strains due to its pronounced bacteriostatic effect via inhibiting bacterial adhesive activity and their ability to form biofilms.

Keywords: prototyping, thin-film coatings, copper oxide nanoparticles, orthopedic implant.

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Introduction

The abiotic surface of orthopedic implants made of titanium and its alloys may become the substrate for the formation of microbial biofilms, which is facilitated by the local conditions of the surgical wound formed in the course of a primary surgical intervention and by the response of periprosthetic tissues to the metal structure. It is for this reason that in modern materials science and biomedical engineering, there is a trend to develop metal coatings with ultimate bacteriostatic properties for all clinically significant strains of microorganisms including multidrug-resistant and pandrug-resistant strains of *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa and Escherichia coli*. At the same time, the bacteriostatic properties of the coatings, determined by their morphology and physicochemical properties, should provide a bactericidal effect during the entire stay of the implant in the body, prevent the colonization of microbial biofilms and remain intact, gradually releasing active components into the periprosthetic tissues [1].

To obtain bacteriostatic coatings for orthopedic implants, various methods are used: passive treatment of an abiotic surface with agents possessing anti-adhesive properties, followed by elution of antimicrobial drugs or development of biodegradable coatings providing a temporary effect. In order to implement a prolonged antimicrobial effect, the surfaces of orthopedic implants are physically or chemically modified with the purpose to change the crystalline phase of metal oxides formed as new layers. The new morphology of such coatings can reduce microbial colonization without affecting osseointegration [2, 3].

A promising current direction of development in the field of biomedical materials science for combating biofilm formation is the use of cupric oxide, CuO, for the so-called contact killing of microorganisms on surfaces, including the creation of surface nanocomposite coatings based on an amorphous film with copper (Cu) nanoparticles applied to coatings made of titanium (Ti) through a source of gas aggregation clusters. It was also described that on polyethylene surfaces with a diamond-like carbon coating with added Cu, the antimicrobial effect was more pronounced than on a similar coating without Cu [4]. The presence of a bacteriostatic effect of thin Ti-Cu films combined with the growth of osteoblastic cells was also described [5]. All such coatings were confirmed to have pronounced bacteriostatic properties without causing cytotoxicity [6].

To further intensify the antimicrobial activity of Ti-Cu alloy coatings, ultrasonic plasma electrolytic oxidation is

used, which, according to the literature, has a bactericidal effect on more than 99% of clinical strains of Staphylococcus spp. There are also coatings made of Ti and its alloys, including those containing cupric oxide obtained by electrochemical oxidation. The latter method is labor-intensive and time-consuming, and the coating formed in this way may contain toxic sulfur-containing impurities [7].

Hence, the development of thin-film coatings for orthopedic implants containing cupric oxide nanoparticles with prolonged biocidal effects on abiotic surfaces and periprosthetic tissues, as well as the assessment of their bacteriostatic and anti-biofilm properties, is a promising direction in traumatology and orthopedics research.

Objective – to evaluate the bacteriostatic properties of the developed prototypes of a thin-film coating for orthopedic implants made of titanium and its alloys.

Materials and Methods

The material for our study included thin-film coatings containing single-component powders of biocidal cupric oxide nanoparticles, which were manufactured in accordance with the certificate developed by Advanced Powder Technology LLC (Tomsk, Russia) in compliance with Technical Conditions 1791-003-36280340-2008, and were intended for covering orthopedic implants made of titanium and its alloys. The safety data sheet for cupric oxide complied with European Union Directive 91/155; the manufacturer and supplier of nanoparticles was Advanced Powder Technology LLC. N-acetylcysteine (NAC) and chymotrypsin (CHT) were also used in the thin-film coating as components reducing bacterial adhesion and accelerating biofilm destruction (RF patent application No. 2023117375, entry No. W23037127 of June 30, 2023, issued by the Federal Service on Intellectual Property at the Federal Institute of Industrial Property).

The prototype of a thin-film coating was formed stage-by-stage by preliminary sandblasting the surface of the metal substrate with aluminum particles of 150-400 μm; cleaning from technological contaminants in an aqueous solution of surfactants using an ultrasonic bath; plasma electrolytic oxidation in anodic mode at electric current densities of 2-2.5×103 A/m2 for 30 minutes in an aqueous alkaline electrolyte containing 3-4 g/L of NaOH with the addition of 10 wt.% of CuO at room temperature and air bubbling in bubble mode at a speed of 0.1-0.4 m/s; drying the coating and uniformly heating the substrate in an oven at a temperature of 600 °C for 30 minutes with forced convection.

The thickness of the prototype coating layers was determined using a desktop scanning electron microscope Explorer (Aspex Corp., USA) (ReestrInform, No. 13908, institution: Gagarin State Technical University of Saratov) in the Metallograph program for analyzing the geometric parameters of micro-objects.

Bacteriostatic properties of a coating prototype applied to a product made of titanium and its alloys, Ti6Al-4V (ASTM F1472), were studied using samples obtained mechanically with a piercer. The product surface layer was represented by a composition of a 5% aqueous solution of polyvinylpyrrolidone containing the active substance in the form of 0.5 wt.% nanoparticles of CuO with a dispersion of 50-70 nm, NAC (0.3 wt.%) and CHT (0.01 wt.%). For this purpose, disks with a diameter of 10 mm, a thickness of 2 mm and a weight of 0.016±0.001 g were formed from plates of 11×19 mm. The bactericidal properties of individual components of the coating prototype were also studied, viz., CuO nanoparticles (1), NAC (2) and CHT (3).

Our study involved 65 clinical strains of microorganisms causing periprosthetic infection (PPI) including 15 of S. epidermidis, 15 of S. aureus, 20 of P. aeruginosa and 15 of E. coli, isolated from 60 patients of both sexes aged 63.8±4.6 years who were treated at the Research Institute of Traumatology, Orthopedics and Neurosurgery of Razumovsky State Medical University of Saratov of the Russian Federation Ministry of Healthcare for complications associated with internal orthopedic prosthetic devices, implants and grafts: T84 according to the International Classification of Diseases, 10th revision (ICD-10). Identification of the isolated strains was carried out using a microbiological analyzer BBL Auto Reader (Becton Dickinson, USA). The reference strains of *S. epidermidis* (ATCC 12228), S. aureus (ATCC 25923), *P. aeruginosa* (ATCC 27853) and E. coli (ATCC 25922) from the Becton Dickinson collection, USA, were used as a comparison group.

The sensitivity of clinical and reference strains to the examined samples of thin-film coatings was studied by a modification of the conventional disk diffusion method (DDM) in accordance with the guidelines MUK 4.2.1890-04 (Determination of the Sensitivity of Microorganisms to Antibacterial Drugs), taking into account the latest recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [8]. By placing the disks equidistant from each other in Petri dishes with Mueller–Hinton agar (Becton Dickinson, USA), inoculated with the studied strains, diameters of growth inhibition zones (DIZ) were measured, followed by the calculation of means based on 20 measurements in three series of experiments.

The features of the effects of the studied product samples on the adhesive properties of microorganisms and the kinetics of biofilm formation were investigated using the methodology proposed by G.D. Christensen et al. [9]. The initial solution contained a concentration of elements corresponding to 1 mg/mL.

The formation of monospecific microbial biofilms was performed under static conditions in sterile polystyrene 96-well cell culture plates (Medpolymer, Russia, registration certificate for a medical product, 'Polystyrene Plate for ELISA according to Technical Conditions 9398-058-00480230-2009' of May 13, 2019, No. RZN 2015/2665). The results were assessed taking into account the Guidelines MR 4.2.0161-19, Identification Methods for Microbial Films on Abiotic Objects.

We used 18-hour bacterial culture suspensions of clinical isolates and reference strains of 5×106 CFU/mL, equivalent to 0.5 sensu the McFarland standards, in GRM broth with glucose, which were subsequently also used as a positive control. Sterile GRM broth was used as a negative control.

The coating elements contained in the GRM broth were mixed with a bacterial suspension in sterile tubes, after which 150 μL were added to the wells of a polystyrene cell culture plate and incubated at 37 °C for 24 hours. The control wells did not contain a bacterial suspension. Then the plates were rinsed three times with a 0.9% sodium chloride solution, after which a 0.1% aqueous solution of gentian violet dye was

added to each well and left for 30 minutes at a temperature of 22-25 °C. After rinsing the plate wells three times with a 0.9% sodium chloride solution, 200 μL of 95° ethylene was added to each of them for 30 minutes. At the end of the incubation, the optical density (OD) of the resulting eluates of the crystalline violet dye was measured on an Epoch (BioTech, USA, registration certificate dated November 3, 2010 No. FSZ 2010/08269) spectrophotometer at a wavelength of 620 nm. The results were presented in the form of arbitrary units of OD.

The growth properties of microorganism strains were studied after a 60-minute incubation of a bacterial suspension with coating elements at the same concentration, followed by seeding on solid media and incubation at 37 °C for 24 hours, with a subsequent counting of colony forming units in CFU/mL.

Statistical processing of the results was carried out using the STATISTICA 12.0 software. Measurement data were checked for normality of distribution using the Shapiro–Wilk and Kolmogorov–Smirnov tests. The distribution of the thickness values of the coating layers corresponded to normal; accordingly, the values were presented as M±SD, where M is the mean and SD is the standard deviation. The characteristics of the coating bacteriostatic properties did not correspond to the law of normal distribution, and therefore the data were presented in the form of a median (Me) and an interquartile range from 25% to 75%. To compare the results, the nonparametric Mann–Whitney U test was employed. Differences were considered statistically significant at $p<0.05$, which complied with the requirements for biomedical research.

Results

The prototype of a thin-film coating was formed stage-by-stage by preliminary sandblasting the surface of the metal substrate with aluminum particles of 150-400 μm; cleaning from technological contaminants in an aqueous solution of surfactants using an ultrasonic bath; plasma electrolytic oxidation in anodic mode at electric current densities of 2-2.5×103 A/m2 for 30 minutes in an aqueous alkaline electrolyte containing 3-4 g/L of NaOH with the addition of 10 wt.% of CuO at room temperature and air bubbling in bubble mode at a speed of 0.1-0.4 m/s; drying the coating and uniformly heating the substrate in an oven at a temperature of 600 °C for 30 minutes with forced convection.

Measurements of the coating layers of prototypes yielded the following values: 6±1 μm for TiO2 and 6±1 μm for TiO2+CuO.

As for the bacteriostatic properties of individual components in the prototype of a thin-film coating in relation to reference strains, we revealed that the maximum values of the DIZ were achieved when using options of the thin-film coating that included cupric oxide nanoparticles stabilized by polyvinylpyrrolidone (*Table 1*).

The growth inhibition zones in the reference strains of S. aureus, S. epidermidis and E. coli when using the full-component prototype of a thin-film coating were slightly less pronounced than when using a coating option containing exclusively CuO and polyvinylpyrrolidone, but the differences did not reach the level of statistical significance.

Features of the bacteriostatic effect of the full-component prototype of a thin-film coating on clinical strains are presented in *Table 2*.

Our results demonstrated that cupric oxide nanoparticles had a bacteriostatic effect on clinical strains of bacteria.

The effects of the coating prototype on the adhesive properties and biofilm formation in reference and clinical strains had a number of features (*Table 3*).

The ability to form biofilms among the reference strains of microorganisms was more pronounced in S. aureus ATCC 25923 vs. E. coli ATCC 25922 and P. aeruginosa ATCC 27853, while in S. epidermidis ATCC 12228 it was higher than in P. aeruginosa ATCC 27853. Differences were noted between clinical strains of S. aureus and P. aeruginosa.

Incubation of the examined strains with a prototype of a thin-film coating led to inhibition of their adhesive abilities and biofilm formation (*Table 4*).

According to our results d, incubation of clinical strains with a prototype of a thin-film coating yielded a significant reduction in the adhesive properties and ability to form biofilms in the following clinical strains: S. aureus (by 10.6%), S. epidermidis (by 37.9%) and P. aeruginosa (by 6.8%).

A 60-minute exposure of the prototype components of a thin-film coating to the studied microbial strains had an inhibitory effect on their growth, which was confirmed by their subsequent seeding on solid nutrient media (*Table 5*).

The thin-film coating prototype inhibited the growth of reference strains of S. aureus ATCC 25923, E. coli ATCC 25922 and P. aeruginosa ATCC 27853 by 6.6%, 22.5% and 7.3%, respectively; and also reduced the growth capabilities of S. aureus by 12.7%, S. epidermidis by 13.3% and P. aeruginosa by 6.3%.

Discussion

The chief material of choice in the manufacture of implants is titanium with its strength properties, corrosion resistance and high biocompatibility [10].

The increasing incidence of postoperative infectious complications during implantation of metal structures contributes to the development of research in the field of developing modification of their surface via applying various preparations with a wide antimicrobial spectrum, which could significantly increase the duration of a proper functioning of implants [11-13].

The complexity of developing approaches to the prevention and treatment of PPI is caused by the formation of bacterial biofilms at the interface of interaction between abiotic and biotic environments in the implantation zone. Traditional methods for combatting such biofilms demonstrated relatively low efficacy [14, 15].

The clinical isolates of infectious agents we studied, obtained from orthopedic patients, exhibited enhanced adhesive abilities regarding abiotic surfaces, as well as increased kinetics of biofilm growth compared with similar reference strains, as reported by other researchers [16].

A promising research field involves the use of nanotechnology for targeted medicinal drug delivery with programmed release of active components [17]. The additive manufacturing technique of laser sintering of Ti3Al2V alloy using 10 wt.% of tantalum (10Ta) and 3 wt.% of copper (3Cu) is known, which increases the bacteriostatic activity of the product by 78-86% against P. aeruginosa and S. aureus. The effectiveness of this method was proven in an experiment on rats with a femur fracture complicated by an infectious process [18].

Table 1. Diameter of inhibition zones of reference strains of microorganisms by the prototype of a thin-film coating and its individual components, mm

Coating option	Coating composition/ reference strain	*S. aureus* ATCC 25923	*S. epidermidis* ATCC 12228	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
1	CuO	13 (12; 13)	14 (13; 14)	14 (13; 14)	9 (9.0; 10.0)
2	NAC	4 (3; 4) $^{*}p_{1-2}$= 0.00034	5 (4; 5) $^{*}p_{1-2}$=0.00078	4 (4; 5) $^{*}p_{1-2}$=0.00062	1 (1; 1) $^{*}p_{1-2}$=0.00078
3	CHT	4 (3; 4) p_{1-3}=0.0039	4 (3; 4) p_{1-3}=0.0017	3 (2; 3) p_{1-3}=0.0017	4 (3; 4) p_{1-3}=0.0039
4	CuO+NAC+CHT	12 (11; 12) p_{2-4}=0.0090 p_{3-4}=0.0017	12 (11; 12) p_{2-4}=0.00078 p_{3-4}=0.0028	13 (13; 14) p_{2-4}=0.00073 p_{3-4}=0.0021	10 (10; 11) p_{2-4}=0.00068 p_{3-4}=0.0049

Results are presented as median (*Me*), 25 and 75% quartiles; **p*, level of statistical significance of differences between the bacteriostatic effect of various components of the prototype of a thin-film coating and reference strains of microorganisms at $p<0.05$; CuO, cupric oxide; NAC, N-acetylcysteine; CHT, chymotrypsin.

Table 2. Diameter of inhibition zones of clinical strains of microorganisms by the prototype of a thin-film coating and its individual components, mm

Coating option	Coating composition/ clinical strain	*S. aureus* *n*=15	*S. epidermidis* *n*=15	*E. coli* *n*=15	*P. aeruginosa* *n*=20
1	CuO	13 (12; 13)	13 (13; 14)	14 (14; 15)	10 (9.0; 10.0)
2	NAC	3 (2; 3) $^{*}p_{1-2}$= 0.0039	4 (3; 4) $^{*}p_{1-2}$= 0.0039	4 (4; 4) $^{*}p_{1-2}$= 0.0018	2 (1; 2) $^{*}p_{1-2}$<=0.00042
3	CHT	3 (3; 4) $^{*}p_{1-3}$= 0.0018	5 (4; 5) $^{*}p_{1-3}$=0.0040	3 (2; 3) $^{*}p_{1-3}$= 0.00078	2 (2; 3) $^{*}p_{1-3}$=0.00078
4	CuO+NAC+CHT	16 (15; 16) $^{*}p_{2-4}$= 0.00078 p_{3-4}= 0.0008	15 (15; 15) $^{*}p_{2-4}$= 0.0018 p_{3-4}= 0.0017	13 (12; 13) $^{*}p_{2-4}$= 0.00078 $^{*}p_{3-4}$=0.0061	14 (14; 15) $^{*}p_{2-4}$=0.00035 $^{*}p_{3-4}$=0.00058

*R*esults are presented as median (*Me*), 25 and 75% quartiles; **p*, level of statistical significance of differences between the effects of individual components of the prototype of a thin-film coating on clinical isolates of periprosthetic infection pathogens at $p<0.05$; CuO, cupric oxide; NAC, N-acetylcysteine; CHT, chymotrypsin.

Table 3. Optical density values of gentian violet dye in clinical and reference microbial strains before exposure to the prototype of a thin-film coating, a.u.

Strains	Optical density values of gentian violet dye, a.u.	*P*-value
S. aureus ATCC 25923	0.091 (0.089; 0.091)	–
S. epidermidis ATCC 12228	0.088 (0.079; 0.092)	p_{1-2}=0.712
E. coli ATCC 25922	0.084 (0.073; 0.089)	p_{1-3}=0.694 p_{2-3}=0.911
P. aeruginosa ATCC 27853	0.071 (0.066; 0.073)	$^{*}p_{1-4}$=0.045 $^{*}p_{2-4}$=0.047 $^{*}p_{3-4}$=0.049
S. aureus clinical	0.604(0.565; 0.683)	$^{*}p_{1-5}$=0.0039 $^{*}p_{2-5}$=0.00077 $^{*}p_{3-5}$=0.00068 $^{*}p_{4-5}$=0.00051
S. epidermidis clinical	0.578 (0.564; 0.599)	$^{*}p_{1-6}$=0.0044 $^{*}p_{2-6}$=0.00089 $^{*}p_{3-6}$=0.00074 $^{*}p_{4-6}$=0.00062
E. coli clinical	0.584 (0.561; 0.596)	$^{*}p_{1-7}$=0.0055 $^{*}p_{2-7}$=0.00096 $^{*}p_{3-7}$=0.00082 $^{*}p_{4-7}$=0.00070
P. aeruginosa clinical	0.473 (0.441; 0.535)	$^{*}p_{1-8}$=0.00084 $^{*}p_{2-8}$=0.00024 $^{*}p_{3-8}$=0.00035 $^{*}p_{4-8}$=0.00041 $^{*}p_{5-8}$=0.039

Results are presented as median (*Me*), 25 and 75% quartiles; **p*, level of statistical significance of differences in adhesive properties and abilities to form bacterial biofilms between reference strains and clinical isolates of periprosthetic infection pathogens at $p<0.05$; a.u., arbitrary units.

Table 4. Optical density values of gentian violet dye after completion of incubation in clinical and reference strains of microorganisms in the presence of the full-component prototype of a thin-film coating, a.u.

Strains	Optical density values of gentian violet dye, a.u.	*P*-value
S. aureus ATCC 25923	0.086 (0.082; 0.090)	–
S. epidermidis ATCC 12228	0.083 (0.076; 0.089)	p_{1-2}=0.865
E. coli ATCC 25922	0.073 (0.069; 0.080)	p_{1-3}=0.794 p_{2-3}=0.780
P. aeruginosa ATCC 27853	0.071 (0.066; 0.073)	p_{1-4}=0.034 p_{2-4}=0.042 p_{3-4}=0.047
S. aureus clinical	0.546 (0.528; 0.567)	*p_{1-5}=0.00035 *p_{2-5}=0.00029 *p_{3-5}=0.000088 *p_{4-5}=0.000070
S. epidermidis clinical	0.419 (0.385; 0.462)	*p_{1-6}=0.00054 *p_{2-6}=0.00042 *p_{3-6}=0.00017 *p_{4-6}=0.00011
E. coli clinical	0.560 (0.521; 0.578)	*p_{1-7}=0.00022 *p_{2-7}=0.00014 *p_{3-7}=0.000065 *p_{4-7}=0.000051
P. aeruginosa clinical	0.443 (0.417; 0.468)	*p_{1-8}=0.00050 *p_{2-8}=0.00037 *p_{3-8}=0.00010 *p_{4-8}=0.00006

*R*esults are presented as median (*Me*), 25 and 75% quartiles; *p, level of statistical significance of differences in adhesive properties and abilities to form bacterial biofilms between reference strains and clinical isolates at $p<0.05$; a.u., arbitrary units.

Table 5. The effect of the full-component prototype of a thin-film coating on the growth properties of reference strains and clinical isolates of periprosthetic infection pathogens, CFU/mL

Strain/Experimental options	Before exposure	After exposure to thin-film coating prototype
S. aureus ATCC 25923	2119 (2017; 2165)	1988 (1964; 1990) *p=0.0035
S. epidermidis ATCC 12228	2014 (1973; 2028)	1824 (1792; 1980) p=0.079
E. coli ATCC 25922	1216 (1182; 1234)	992 (976; 1000) *p=0.0039
P. aeruginosa ATCC 27853	1228 (1201; 1256)	1139 (1113; 1155) *p=0.015
S. aureus clinical	2241 (2227; 2269)	1988 (1964; 2001) *p=0.0078
S. epidermidis clinical	2113 (1985; 2134)	1865 (1841; 1904) *p=0.0019
E. coli clinical	1276 (1259; 1292)	1219 (1185; 1263) p=0.822
P. aeruginosa clinical	1249 (1217; 1277)	1174 (1159; 1188) *p=0.0042

*R*esults are presented as median (*Me*), 25 and 75% quartiles; *p, level of statistical significance of differences in the growth properties of microbial reference strains and clinical isolates of periprosthetic infection pathogens before and after exposure to the prototype of a thin-film coating at $p<0.05$; CFU, colony forming units.

Our study confirmed the bacteriostatic properties of the developed products made of titanium and its alloy with a thin-film coating (with cupric oxide nanoparticles with a dispersion of 50-70 nm) applied to its surface in relation to clinical isolates of S. aureus, S. epidermidis and P. aeruginosa.

G. Karabulut et al. (2023) also used CuO nanoparticles with a similar dispersion to provide a bacteriostatic effect for medical products made of stainless steel and reported positive experimental results [19].

The prototype of a thin-film coating developed by us made it possible to achieve an inhibitory effect on the adhesive properties and ability to form biofilms by clinical strains of S. aureus, S. epidermidis and P. aeruginosa collected from orthopedic patients, which confirmed the prospects for its implementation in clinical practice.

Conclusion

The developed prototype of a thin-film coating on products made of titanium and its alloys reduces the virulence factors of clinical microbial strains due to its pronounced bacteriostatic effect via inhibiting bacterial adhesive activity and ability to form biofilms.

Author contributions: all authors contributed equally to the manuscript preparation.

Conflict of interest: None declared. The study was carried out within the framework of the Government Procurement by the Russian Federation Ministry of Healthcare, Development of Agents Effective Against Biofilm-Forming Microorganisms in the Treatment of Infectious Complications of Joint Replacement, state registration number of R&D 121032300172-2.

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