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Antibacterial Properties of Superhydrophilic Textured Copper in Contact with Bacterial Suspensions

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The method of pulsed laser processing with a nanosecond pulse duration was employed to obtain a nanotexture on the surface of copper alloys. The effect of the obtained micro- and nanotexture on the bactericidal properties of the surface upon its contact with suspensions containing of *E. coli* K12 C600 or *K. pneumoniae* 811 cells in a nutrient medium were studied. The evolution of cell morphology after on the nanotextured surface was analyzed using scanning electron microscopy, and changes in biological fluid during this contact were studied by mass spectrometry. It was shown that massive death of bacterial cells both in the suspension and on the nanotextured surface was determined by combined toxic effects of the hierarchically textured surface and high concentration of Cu^{2+} ions in the medium.

Key Words: *antibacterial surfaces; laser surface modification; biocorrosion; nanoparticles; extreme wettability*

Antimicrobial activity of copper is confirmed by clinical studies and is described in detail [6,11]. The studies evaluated the effectiveness of the use of copper alloys for fabrication of surfaces with which patients and medical personnel most often come in contact (door handles, headboards, handrails in the corridors, flush keys in toilets, switches, *etc*.) [6,7]. More recently, the use of copper vessels has been proposed as a low-cost alternative to antimicrobial treatment of water to solve the problem of providing drinking water to the population in developing countries [10].

One of the most promising ways to more effectively exploit the bactericidal properties of copper is based on the use of extreme wettability of contact surfaces. Extreme wetting regimes correspond to either superhydrophilic or superhydrophobic state of the surfaces. Our recent studies showed that antibacterial activity of superhydrophilic surfaces significantly exceeds activity of superhydrophobic samples of the same surface morphology and shape of texture elements [2,4].

The purpose of this work was to study in more detail various aspects of the death/survival of bacteria in contact with a superhydrophilic coating on the surface of M1M soft copper alloy obtained using nanosecond laser texturing and characterized by hierarchical micro- and nanomorphology.

EXPERIMENTAL METHODOLOGY

Antibacterial activity of M1M copper alloy plates with the superhydrophilic coating was analyzed. The plates of two sizes were used. Bacterial contamination in droplets of a bacterial suspension spreading over the surface was studied on 10×10×1 mm plates. In addition, bacterial contamination of a suspension of bacterial cells in 15 cm^3 nutrient medium was monitored upon contact with a 25×25×1 mm plate. The samples were prepared by treatment of the surface with a pulsed nanosecond infrared laser at irradiation power exceeding the ablation limit. A detailed laser processing technique was described previously [3].

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The selected treatment mode led to the formation of a highly porous superhydrophilic surface into which the drops of the studied bacterial suspension are completely absorbed within few seconds. In this work, we studied bactericidal activity of superhydrophilic copper plates against *E. coli* or *K. pneumoniae* cells.

E. coli K12 C600 (B-7158; GKPM-Obolensk) and *K. pneumoniae* 811 (B-7707; GKPM-Obolensk) were used as non-pathogenic and pathogenic cultures, respectively. Meat-peptone broth (MPB; Medgamal) was used as a suspension medium. To prepare bacterial suspensions, daily bacterial culture was introduced into MPB and incubated at 37°C for 18 h; bacterial suspensions with an initial titer of 10^7 CFU/ml were used in the experiments.

Bacterial contamination was monitored and analyzed using 2 different research protocols. According to the first protocol, a drop of the bacterial suspension (0.01 ml) was carefully placed on a superhydrophilic copper plate located inside a weighing bottle with 100% humidity. Saturation with water vapor prevented water evaporation and maintained wet contact mode between the suspension and textured copper surface. Bacterial contamination of the plate was measured after certain time intervals (30 min, 1, 2, 5, 24 h, 2, 4, and 6 days). For quantitative assessment of the bactericidal effect, the plate after contact for a certain time with absorbed drop of suspension was transferred in a test tube with 1 ml sterile saline and shaken on a shaker at 250 rpm for 10 min. After that, 0.5 ml of the obtained bacterial suspension was taken from the tube, and 10-fold dilutions were prepared. A 0.1-ml aliquot from each dilution was applied to Petri dish with Muller—Hinton agar (HiMedia Laboratories Pvt. Limited) and evenly distributed over the surface. After 24-h incubation at 37°C, the colonies were counted and the titer of bacteria on the plate was determined. For each bacterial culture and each time interval, a special superhydrophilic plate was used and each experiment was repeated at least 2 times.

According to the second protocol, for each bacterial culture, test superhydrophilic plates were placed in separate sterile weighing bottles with 15 ml bacterial suspension with an initial titer of 10^7 CFU/ml for both strains. In the control, bottles with the same bacterial cultures but without copper plates were used. The bottles were stored at room temperature for 6 days. To evaluate the bactericidal effect of the plates in contact with the suspension, 0.5 ml bacterial suspension was taken from each bottle, and 10-fold dilutions were prepared. A 0.1-ml aliquot from each dilution was applied to a Petri dishes with Mueller—Hinton agar and uniformly distributing over the surface. After 24-h incubation at 37°C, the bacterium titer (CFU/ml) was determined.

The morphology of bacterial cells in contact with the superhydrophilic surface and the texture of the copper alloy plates were examined under a SUPRA 40 VP scanning electron microscope (Carl Zeiss). Changes in copper concentration in MPB depending on the plate contact time with the bacterial medium were determined by mass spectrometry using inductively coupled plasma on an Agilent 7500ce spectrometer (Agilent Technologies).

RESULTS

The cytotoxicity of copper manifesting upon contact of copper and its alloys with bacterial suspensions significantly depends on surface composition and morphology [5,8,11,12]. In addition, the toxic effect during contact of copper-containing surfaces with biological fluids is determined by both for copper/copper oxide nanoparticles located on the metal surface and copper ions passing into solution as a result of corrosion processes.

Pure copper produces most pronounced antibacterial effect, but the effect of copper alloys and oxides is also essential. Our previous studies [3] showed that the selected laser treatment mode led to the formation of a porous layer of copper (II) oxide. This layer is formed during ablation as a result of aggregation of copper oxide nanoparticles deposited on the surface of the copper substrate from the laser plume. The size of nanoparticles is tens of nanometers, while the formed aggregates of particles are several hundred nanometers in size. The hierarchical texture formed by copper oxide on the surface plays an important role in the antibacterial activity of copper-containing plates.

The analysis of cell survival on a superhydrophilic copper substrate after impregnation with a bacterial suspension showed the absence of living cells after 30-min contact for both studied strains. Analysis of the morphology of bacterial cells upon contact with the textured substrate showed that death of bacterial cells adsorbed on textured surfaces occurred via disintegration of the outer membrane due to perforation with nanoparticles (Fig. 1), followed by cell deformation, loss of intracellular fluid, and bacteriolysis.

The results of the survival of bacterial cells in suspension in contact with a superhydrophilic copper plate (protocol 2) for different times of suspension contact with the surface of the plate for both strains are shown in Figure 2. In the presence of nutrient medium in the bacterial suspension, the number of CFU increased significantly and reaching stationary level in 1 day for both *E. coli* (Fig. 2, curve *1*) and *K. pneumoniae* (curve *3*) suspensions. On the contrary, the contact of suspension with a superhydrophilic copper plate led to almost complete death of bacterial cells in

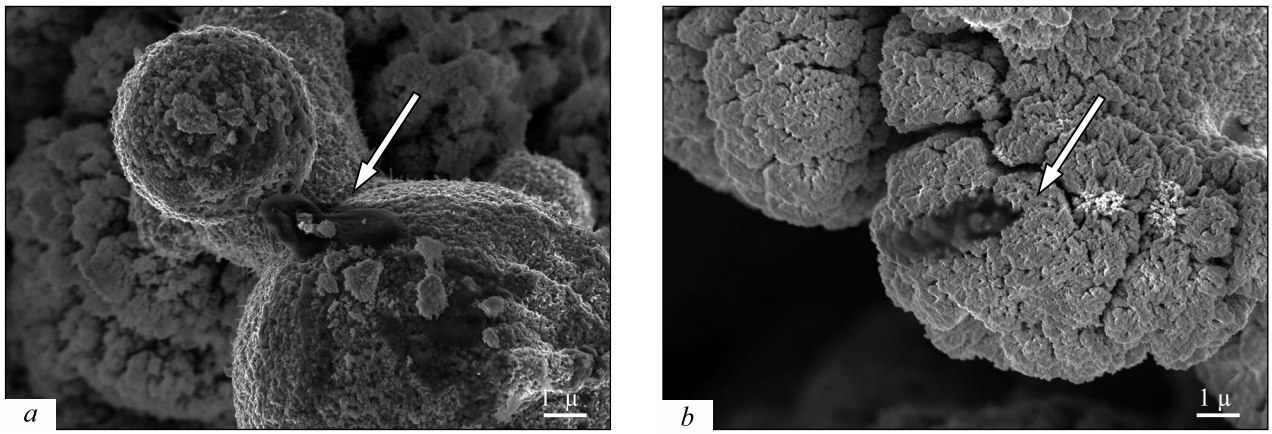

Fig. 1. *K. pneumoniae* (*a*) and *E. coli* (*b*) cells deposited on the surface of the textured superhydrophilic copper plate after 24-h contact of the droplet of bacterial suspension with the plate. Electron microscopy. Arrows show bacterial cells.

the entire volume within 5 h for *E. coli* (curve *2*) and within 24 h for *K. pneumoniae* (curve *4*).

During long-term contact of bacterial suspension with superhydrophilic copper, a small fraction of bacteria was sedimented onto the copper substrate, which led to their death due to perforation, deformation of the membrane, and loss of intracellular fluid. The fact that almost all cells in the medium died without direct contact with the substrate indicates the existence of an additional mechanism of the bactericidal action of copper plates. The cytotoxic effect of copper ions in solutions was mentioned not once. Cytotoxicity of copper ions can be associated with the generation of radical compounds in the cell and binding of copper ions with sulfhydryl groups of proteins [1]. Although in the process of evolution and adaptation to increased content of copper ions, some bacteria developed mechanisms of protection against heavy metal ions [9], an increase in concentration above a certain critical level should still cause cell death. For *E. coli*, we analyzed the correlation between the content of copper ions in the medium and the titer of live bacteria. It should be emphasized that copper ions are released in the suspension as a result of corrosion processes at the interface superhydrophilic plate/bacterial suspension. In this case a significant role is played by both electrolyte corrosion initiated by chlorine ions present in MPB and biocorrosion associated with vital activity of cells deposited on the surface of the copper plate. The dynamics of copper ion content in the suspension of *E. coli* during contact with a superhydrophilic copper plate is shown in fragment *b* of Figure 2. Our findings suggest that massive death of *E. coli* cells was observed after attaining the concentration of ≈0.03 mg/liter

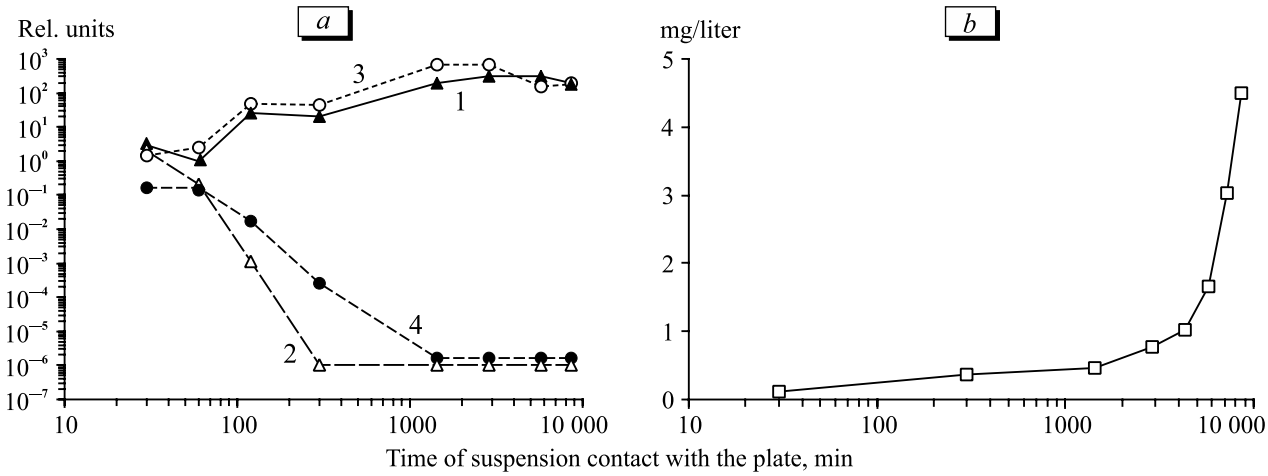

Fig. 2. Changes in standardized titer of bacterial cells (*a*) and copper ion concentration in the suspension of *E. coli* in contact with the superhydrophilic copper plate (*b*). *1*, *2*) *E. coli* cells; *3*, *4*) *K. pneumoniae* cells; *2*, *4*) bacterial suspension contacted with the superhydrophilic copper plate; *1*, *3*) control suspension stored in the glass bottle without contact with the metal. The data are presented as standardized titer equal to the ratio of the titer of bacterial cells in suspension contacted with copper for certain time to the bacterial titer in the initial suspension.

copper ions in suspension medium. It is worth of note that the increase in the contact area between the superhydrophilic copper plate and bacterial suspension due to the hierarchical roughness of the copper surface leads to intensification of corrosion and, as a result, enhances the bactericidal effect.

Thus, the superhydrophilic state of the surface of a copper plate promotes the antibacterial effect not only with respect to bacterial cells located on the surface, but also for bacteria dispersed in medium contacting with the copper. As the main mechanisms of bactericidal action, one can figure out death of cells due to their interaction with texture elements and surface nanoparticles and toxic effect of copper ions passing into solution as a result of corrosion processes.

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