Antibacterial Properties of Copper Nanoparticle Dispersions: Influence of Synthesis Conditions and Physicochemical Characteristics

A Godymchuk\textsuperscript{1,2,a}, G Frolov\textsuperscript{2}, A Gusev\textsuperscript{2,3}, O Zakharova\textsuperscript{3}, E Yunda\textsuperscript{1}, D Kuznetsov\textsuperscript{2} and E Kolesnikov\textsuperscript{2}

\textsuperscript{1} Tomsk Polytechnic University, 30, Lenina ave., Tomsk, 634050, Russia
\textsuperscript{2} National Research Technological University "MISIS", 2, Leninsky ave., Moscow, 119049, Russia
\textsuperscript{3} Tambov State University named after G.R. Derzhavin, Internatsionalnaya str., 33, Tambov, 392000, Russia
\textsuperscript{a} E-mail: godymchuk@tpu.ru

Abstract. The production of bactericidal plasters, bandages and medicines with the inclusion of copper nanoparticles and copper ions may have a great potential in terms of their biomedical application. The work considers the influence of the synthesis conditions, size, aggregation status, and charge of nanoparticles in aqueous solutions as well as the type of microorganisms to the antibacterial properties of water suspensions of electroexplosive copper nanoparticles in the conditions in vitro in relation to strains \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa}, \textit{Staphylococcus aureus}, and \textit{Bacillus cereus}. Water dispersions of copper nanoparticles were shown to inhibit the growth of test cells for both G+ and G- microbacteria but the degree of such an influence strongly depended on the type of a test strain. The authors have demonstrated that use of deeply purified water and alcohol-containing stabilizers at the synthesis of nanoparticles via metals electric erosion in the liquid prevents the copper nanoparticles coagulation and significantly influences on their physicochemical characteristics and, consequently, antibacterial properties.

1. Introduction
The treatment and cleaning of medical instruments and devices, water purification, food industry are closely connected with the development of effective, environmentally friendly, available and low cost bactericide compositions for pathogenic microorganism control. Due to a wide presence in nature, implementation of different functions within the majority of living organisms, relatively low cost and environmental safety, copper compounds (Cu) have a high potential for their application as antibacterial agents being capable to replace silver and composites of different precious metals [1].

Thus, it has been demonstrated that Cu nanosized particles are able to show antimicrobial properties in relation to a wide range of microorganisms including pathogenic bacteria [2]. On the one hand, according to [3], Cu nanoparticles are hypotoxic. On the other hand, they show a high antibacterial effect in relation to the cells of test cultures of G+ and G- microbacteria, which makes reasonable their application as part of wound-healing preparations. At the same time, Cu nanoparticles are demonstrated to have high efficiency for application in bactericide plasters or bandages due to high antimicrobial activity to pathogenic microorganisms and the illegible sensibility of human tissues to copper compounds [4,5]. Other authors have shown that Cu nanoparticles may significantly increase the antibacterial activity of sorbents exposed to \textit{Escherichia coli} cultures owing to the copper capability to disrupt metabolism of a microbial cell while interacting with microorganisms [6]. In other
words, the production of bactericidal plasters, bandages and medicines with the inclusion of copper nanoparticles and copper ions may have a great potential in terms of their biomedical application. However, the lack of experimental data in the literature on antibacterial properties linked to physicochemical parameters of nanoparticles in water suspensions prevents forecasting their antibacterial activity and, respectively, preparing widely used antibacterial suspensions.

The purpose of this work was to evaluate the influence of synthesis conditions and physicochemical characteristics on antimicrobial properties of water suspensions of copper nanoparticles in relation with gram-positive and gram-negative bacteria.

2. Materials and methods

**Nanoparticle dispersions.** Dispersions of Cu nanoparticles were produced via electro-erosion synthesis (Table 1). The process of electric erosion of copper cathodes was implemented according to approach described in [7]. The arc chamber was filled with liquid, and electric impulses of the capacity of 6.5 kW were fed to the copper electrodes. As a result of appearance of electric sparkles in the places of granule contact destruction, melting and evaporation of the surface took place which accompanied by water saturation with the products of electric erosion and formation of colloidal solution of copper in the liquid. The given method allowed receiving stable dispersions of nanoparticles, stabilization of which occurred due to the formation of hydrate (solvent) multilayer shells around the particle of a solid phase. Moreover, this method is defined with a high environmental friendliness, expressivity and possibility to control the size of particles due to the control of the explosion parameters.

**Examination of physicochemical properties of nanoparticles.** Information about dislocation structure of nanoparticles was obtained by means of transmission electronic microscopy (TEM) using a microscope JEM 2100 (Jeol, Japan). In this experiment studied suspensions were dropped on TEM copper nets with amorphous carbon substrate. All the measurements were conducted with a speeding voltage of 200 kV; cathode LaB6 was used as a source of electrons.

| Dispersions        | WD-Cu                                                                 | WDF-Cu                                                                 | WAD-Cu                                                                 |
|--------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|
| Name of the dispersion | Water dispersion of copper nanoparticles                              | Water dispersion of copper nanoparticles additionally purified with filtration | Water-alcoholic dispersion of copper nanoparticles                      |
| Characteristics (TEM-analysis) |                                                                 |                                                                 |                                                                      |
| Notes              | Synthesis of nanoparticles was implemented in distilled water (pH = 6.11 ± 0.2, conductivity – 0.2 µS) | Synthesis of nanoparticles was implemented in distilled water (pH = 6.11 ± 0.2, conductivity – 0.2 µS), additionally purified with reverse-omosis filtration | Synthesis of nanoparticles was implemented in water solution of ethanol [8], synthesized suspension of copper nanoparticles was diluted in 100 times with distilled water (pH = 6.11 ± 0.2, Conductivity – 0.2 µS) |

Sedimentation stability of particle suspensions was evaluated with the spectrophotometry method measuring the alteration of a light transmission coefficient (%). Synthesized dispersions were treated with ultrasonication for 5 min and poured into quartz cuvettes (width – 1 cm, volume – 4 ml). The spectrum of distilled water (pH = 6.11 ± 0.2, conductivity – 0.2 µS, electric distillatory DE-4, Russia) was used as a standard to compare the obtained spectra. Coefficient of light transmission was measured using a spectrophotometer Helios Alpha (Thermo Scientific, USA) with the wavelength of 540 nm. The measurements were conducted every 60 minutes during 24 hours.
To control the aggregation stability particle size distribution and zeta-potential in the dispersions were determined using dynamic light scattering technique (Nano Zetasizer, Malvern, USA). To run measurements a general-purpose capillary U-shape polystyrene cuvette was filled with 1 ml of dispersion avoiding the formation of bubbles. Triplcate samples of each nanopowder were measured at 25 °C (He-Ne laser, laser capacity – 4 mW, wavelength – 633 nm).

The change in the composition of the particle surface in water dispersions was defined with the IR-Fourier spectroscopy. The particle dispersions were centrifuged for 60 minutes with the speed of 12,000 r/min and dried up in the air within 72 hours. The received films were examined on the Fourier Spectrometer Nicolet 5700. Spectral resolution of the device was 0.09 cm\(^{-1}\). The received spectra of reflection were compared with the known spectra of the chemical substances [9] and water.

**Nutritional media preparation for microorganisms cultivation.** Strains of the following bacteria were used to evaluate antibacterial activity: coliform bacterium *E. coli*, *Pseudomonas aeruginosa* *P. aeruginosa*, *Staph. aureus* *S. aureus*, soil bacterium *B. cereus*. According to various references, e.g. [10,11], the aforementioned bacteria are the most frequently used test-objects for studying nanoparticle ecotoxicity. Once entered the body, some of the listed bacteria propagate quickly and become the cause of infectious inflammatory diseases as well as contribute to the development of purulent complications in the post-surgical period.

Solid nutritional medium (agar) was made on the base of Hottinger broth (protein base used for nutritional media preparation for microorganisms, PanEco, Moscow,). Combination of the medium components provides the necessary nutritional elements for the growth of cultures in the form of certain colonies on the solid medium surface. Hottinger agar was melted on a boiling water bath and cooled to 45–50 °C. Then, 25 ml of agar was dispensed into sterile 100 mm Petrie dishes (RUSHIMSET, Tambov) with the resulting layer of 4.0 ± 0.5 mm. After setting the Hottinger agar was dried at 37 ± 1 °C for 45-60 min. The prepared agar was kept for not more than 10 days at 2–8 °C (thermostat TC1/80, Russia). pH of the agar was 7.3 ± 0.2 [12].

**Preparation of the test strain suspensions (inoculation).** Two days prior to carrying out the experiment the test culture was reinoculated from the storage medium onto slant nutritional agar in test tubes. The next day the obtained agar culture was used to produce suspension of the test strain employing turbidity standard. Sterile dilutant with the concentration of 10\(^{-3}\) kl/ml was used, and tenfold serial dilutions (till 8th dilution inclusive) were prepared.

To control dilution 0.1 ml of suspension from 6th and 7th dilutions of culture was plated to the dishes with nutritional agar by method of direct surface inoculation. Three such inoculations were made out of each culture. After inoculation test tubes with culture were immediately placed in a refrigerator. Dishes with cultures were incubated in the thermostat at 37 ±1 °C within 18…24 hours.

On the day of the experiment the average number of colonies grown in three dishes was calculated for each series of inoculations.

Before the measurement the inoculate was mixed thoroughly and, then, 2 ± 0.2 ml was applied with a pipette to the surface of a Petrie dish with nutritional medium and evenly distributed over the surface with roll motion, the excess was removed with the pipette. Petrie dishes filled with nutritional medium and test strain were installed onto a horizontal surface. Before the inoculation the absence of condensate on the internal surfaces of the caps was controlled.

The laboratory glassware was treated with 3-% water solution of phenol or 2% chloramine solution for disinfection [13].

**Examination of antibacterial properties of nanoparticles.** Disk diffusion test was used to assess antibacterial properties of the studied dispersions. It represents the measurement of the inhibition zone around the paper carrier of antibacterial preparation. The inhibition zone forms as a result of the preparation diffusion from the carrier into the media. Within certain limits the inhibition zone is reversely proportional to the minimum suppressing concentration (MSC). The result of the measurement is the definition of the microorganism as one from the sensitivity categories (sensitive, intermediate or resistant).

For the experiment small batches of disks (wafers) were made from filtering paper with the following properties: white band, filtering capability of not more than 45 seconds, neutral aqueous extract pH, ash content of the filter – 0.00042…0.00062 at the temperature not higher than 18 °C [14]. Wafers of 5 x 5 mm were made before the experiment under sterile conditions. They were soaked 10 min in the tested aqueous dispersion and then dried up in a suspended state to remove the excess liquid. Laboratory tools were thoroughly disinfected. Laboratory glassware was washed twice in 2% HNO\(_3\) solution and rinsed 7 times with distilled water. All the surfaces including inner surfaces of air hoods and tables were treated with disinfecting compositions of sodium bicarbonate. After the experiment the wafers were disposed.
Incubation of nanoparticle dispersions in test strains. Application of the discs was implemented using sterile tweezers. The distance from the disc to the edge of the dish and between disks was 15…20 mm. Thus, one dish with a diameter of 100 mm had not more than 6 discs saturated with particle suspensions. The discs were thoroughly pressed to the agar by tweezers so that the discs evenly contacted the surface of agar. In the center of the plate a paper disc with gentamicin (control) was located. After application of the discs the Petrie dishes were placed in the thermostat (thermostat TC 1/80-SPU, Russia) upside down and incubated at the temperature of 35 ± 0.5 °С during 18 hours. As soon as the process of incubation was finished the dishes were placed upside down to the dark mat surface with the light falling down onto them under the angle of 45°. The diameter of the zones of growth inhibition (DZGI) of bacteria around the discs was measured with a ruler. The accuracy up to 1 mm was checked with trammel.

While measuring the DZGI parameter the zone of a full suppression of the visible growth was taken into consideration without taking into account very little colonies revealed within the zone of growth inhibition at specific conditions and hardly noticed deposit at the edge of the zone.

3. Results and discussion

Characteristics of nanoparticles in dispersions. The Table 2 shows the average size and size distribution both for particles and aggregates of Cu dispersions based on the TEM-images analysis (figure 1a, 1b, and 1c). It can be seen that particles connect to each other mostly due to colloidal interparticle interaction and rarely with phase contacts that confirms high aggregative stability of synthesized copper suspensions.

Table 2. Morphological and dispersity characteristics of the dispersions (TEM data)

| Dispersion | Particle size, nm | Average particle size, nm | Aggregate size, nm | Particle shape |
|------------|-------------------|---------------------------|-------------------|---------------|
| WD-Cu      | 10 … 40           | 38                        | 70 … 150          | spherical     |
| WDF-Cu     | 10 … 40           | 26                        | 100 … 200         | spherical     |
| WAD-Cu     | 10 … 20           | 20                        | 100 … 1000        | spherical     |

Figure 1. TEM-images of the dispersions: WD-Cu (a), WDF-Cu (b), WAD-Cu (c)

Since zeta-potential value is proportionate to the aggregate stability of sols [15]. WDF-Cu-dispersion has the highest stability (Table 3). The suspension was synthesized in a disperse phase with a higher degree of purification and, respectively, with a lesser ion power. Adding electrolytes is known to contribute to coagulation of hydrophobic colloids that causes surface charge neutralization. This fact explains lesser stability of the WD-Cu, received at the explosion of metal in distilled water. For nanoparticles of the WAD-Cu, synthesizing in the ethanol solution contributed to neutralization of the particle charge (Table 3).
Table 3. Average size and zeta-potential of the dispersions (DLS data)

| Dispersion | Average size of agglomerates, nm | Zeta-potential of the particles, mV |
|------------|----------------------------------|-----------------------------------|
| WD-Cu      | 4780.2 ± 120.1                   | −12.00 ± 2.8                      |
| WDF-Cu     | 279.3 ± 22.3                     | −39.80 ± 3.1                      |
| WAD-Cu     | 435.0 ± 16.4                     | −1.74 ± 0.5                       |

Evaluation of nanoparticles stability in suspensions. The figure 2 demonstrates a change of light transmission coefficient (%) of the synthesized water dispersions. WD-Cu-dispersion obtained at the explosion of copper granules in the distilled water had a minimal stability: it had the highest light transmission in the commence time (40 %), the particles were completely deposited within 24 hours. Whereas WDF-Cu suspension (synthesis in the additionally purified water) was characterized with a low light transmission (10 %), and sedimentation of particles started only in 15 hours.

It was experimentally proven that WAD-Cu-dispersion had the highest stability: change of the light transmission coefficient within 24 hours was 7 %. The most probable is chemisorptive stabilization by functional groups of ethanol. Due to the formation of stable micelles the aggregate stability of such a hydrosol may be kept for a long time.

![Figure 2. 24-h change of the light transmission coefficient of the dispersions](image_url)

According to the experimental data a peak of the main fluctuations of hydroxyl group in the area of 3250 nm in the IR–spectra of the dispersions is presented (Figure 3). Thus, it was probably the formation of hydroxides of metals on the particle surface, which were not separated by centrifuging at a high speed.
Apparently, in suspension saturated with oxygen a partial dilution of copper nanoparticles is possible with formation of hydroxide in accordance with the data given in the article [16].

**Antibacterial properties of copper nanoparticles.**

*Influence of nanoparticles dispersions on gram-negative microorganisms.* For the line of G−-bacteria a tendency to a toxic action of the synthesized nanoparticles dispersions weakly depended on the type of the microorganism (Table 4). Thus, WD-Cu-dispersion was characterized with the highest DZGI value. The received result may be explained by the bigger size of dispersion particles (> 4 μm). The more the size of the particles was the more probable was the formation of cavities, hollows, and cracks between the disc penetrated with antibacterial particles and the surface of bacteria containing agar. As a result, it was quite possible to suppose the simplification of the diffusion of the smaller particles out of the limits of the disc that contributed to the increase of the DZGI (Figure 4).

![IR-spectra of the dispersions](image)

**Figure 3.** IR-spectra of the dispersions

![Photos of the visual control of inhibiting zones](image)

**Figure 4.** Photos of the visual control of inhibiting zones of the *E. coli* C 600 exposed to WD-Cu (a), WFD-Cu (b), WAD-Cu (c)

*Influence of nanoparticles dispersions on gram-positive microorganisms.* It is known that the main difference of G−-bacteria from G+-ones is the presence for the first and absence for the second of the outer membrane that prevents penetration of antibodies inside the cell. That is why it was considered that due to its more capacious and impenetrable cell wall, G−-bacteria were more resistant to antibodies than G+-ones. We logically supposed that action of nanoparticles for G+-microorganisms would be the opposite. However, at the incubation of the
cells in the dispersions there was no identical tendency: toxicity of nanoparticles depended not only on the method of their preparation but also on the type of the test organism.

We revealed that the G+ wax-like stick *B. Cereus* was instable to a larger extent in relation with WDF-Cu-dispersions than to WD-Cu. The increase of toxic effect may be related to the particle size and aggregation degree of the synthesized dispersions: the particle with less size are characterized with more probable migration activity, and, consequently, the probability of its penetration and impact on the cell may be increased. At the same time experimental results showed that the staphylococcus *S. aureus* was more sensitive to suspensions with a larger disperse phase as in WD-Cu-dispersion.

On the other hand, it was obvious that the maximal values of DZGI (4.2 mm) is typical for the WD-Cu-dispersions having the maximal size of the disperse phase (> 4 μm, Table 3). At that, this result was found for both G– (*E. coli*) and G+-bacteria (*B. Cereus*).

Thus, the studied Cu nanoparticles dispersions showed bactericidal action on all the test strains but the degree of such an influence depended on the method of preparation of the nanoparticles dispersion and the type of a test strain (Table 4).

**Table 4. Diameter of diffusional zone of the cultures growth inhibition (DZGI) exposed to nanoparticles dispersions**

| Dispersion | Diameter of diffusional zone of growth inhibition of the test strain, mm |
|------------|-------------------------------------------------------------------------|
|            | *E. coli*                  | *P. aeruginosa*               | *S. aureus*                | *B. cereus*               |
| WD-Cu      | 4.2                       | 3.0                          | 3.1                        | Only below the disk       |
| WDF-Cu     | Only below the disk        | Only below the disk           | Only below the disk        | 4.4                       |
| WAD-Cu     | Only below the disk        | Only below the disk           | Only below the disk        | 1.1                       |

When describing the mechanism of toxicity of water nanoparticle dispersions of copper and its compounds it is important to note that copper-containing preparations cause breakage of ferments and disturbance of metabolism of microorganisms, i.e. they act as strong ferment poisons even in small concentrations (so called “oligodynamic action”). Either in the form of salts (CuCl₂), or in the form of organic compounds (such as a p-hydrozimercurybenzoat) cooper connects SH-groups and, thus, dramatically changes tertiary and quaternary structure of the ferment proteins.

Speaking about the toxicity of water-alcohol suspensions it is to be said that in addition to the action of copper the presence of alcohol molecules in suspension may not only cause breakages of the surface structures or layers of the cell, but also a coagulation of proteins. Consequently, presence of alcohol provokes bactericide effect of the suspensions.

4. Conclusion

The conducted studies allowed receiving dependencies which describe the link between the synthesis conditions, physicochemical characteristics and toxic properties of copper nanoparticles in water suspensions in the conditions *in vitro* in relation to strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus cereus*. Water dispersions of copper nanoparticles were shown to inhibit the growth of test cells for both G+ and G- microbacteria but the degree of such an influence strongly depended on the type of a test strain. The authors have demonstrated that use of deeply purified water and alcohol-containing stabilizers at the synthesis of nanoparticles via metals electric erosion in the liquid prevents the copper nanoparticles coagulation and significantly influences on their physicochemical characteristics and, consequently, antibacterial properties.

Experimental data obtained using disc-diffusion method of strains sensitivity in relation to water-base dispersions of copper nanoparticles allowed making two main conclusions. First, when choosing antimicrobial agent for such cells a defining factor will be the dispersity of the synthesized suspensions. Secondly, bactericidal activity of copper nanoparticles increases due to a partial metal release and emissions of copper ions to the surface with their further diffusion into a cell which actively leads to a toxic effect (suppression of the strain growth).

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