МИКРОБИОЛОГИЯ И ВИРУСОЛОГИЯ
MICROBIOLOGY AND VIROLOGY

RESISTANCE OF *RHODOCOCCUS RUBER* BIOFILMS TO CUO NANOPARTICLES DEPENDING ON EXOPOLYMER MATRIX COMPOSITION

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ABSTRACT

Background. *The widespread use of copper oxide nanoparticles (CuO NPs) increases their release into the environment, which leads to accumulation in trophic chains. Bacterial biofilms are more resistant to physico-chemical factors compared to planktonic cells due to an exopolymer matrix (EPM) consisting of polysaccharides, proteins, lipids and nucleic acids. Rhodococcus actinobacteria are promising for environmental biotechnology due to biodegradation of petroleum products, pesticides and other organic pollutants, as well as bioaccumulation of heavy metals.*

The aim. *To investigate effects of CuO NPs on the viability of Rhodococcus ruber IEGM 231 cells in biofilms and the dynamics of EPM components.*

Methods. *R. ruber biofilms were grown on microscopy cover glass with CuO NPs and EPM components were studied using confocal laser scanning microscopy (CLSM) by differentiating staining with LIVE/DEAD to determine the number of living and dead cells, Nile Red for lipids, FITC for proteins and Calcofluor White for beta-polysaccharides.*

Results. *It was found that R. ruber biofilms grown in a mineral medium with 1.0 vol.% n-hexadecane are more resistant to CuO NPs compared to biofilms growing in a rich culture medium (meat-peptone broth). This was due to more intensive EPM formation, which plays a major role in protecting cells from the bactericidal action of nanometals. A weak stimulating effect of a low (0.001 g/l) concentration of CuO NPs on biofilm formation was registered. Dynamics and localization of main EPM components were monitored during prolonged (24–72 h) biofilm cultivation with CuO NPs. When exposed to high (0.01–0.1 g/l) concentrations of CuO NPs, a consistently high lipid content and an increase in concentrations of polysaccharides and proteins were revealed.*

Conclusion. *Understanding the complex interaction mechanisms of nanometals and biofilms will contribute to the development of effective biocatalysts based on immobilized bacterial cells. Also, the obtained data can be used to combat unwanted biofilms with the help of metal nanoparticles.*

Key words: *biofilms, Rhodococcus, metal nanoparticles, confocal laser scanning microscopy, exopolymer matrix*

Received: 02.06.2022
Accepted: 21.09.2022
Published: 08.12.2022

For citation: Bayandina E.A., Glebov G.G., Kuyukina M.S., Ivshina I.B. Resistance of *Rhodococcus ruber* biofilms to CuO nanoparticles depending on exopolymer matrix composition. *Acta biomedica scientifica.* 2022; 7(5-1): 100-109. doi: 10.29413/ABS.2022-7.5-1.11

УСТОЙЧИВОСТЬ БИОПЛЁНОК *RHODOCOCCUS RUBER* К НАНОЧАСТИЦАМ CUO В ЗАВИСИМОСТИ ОТ СОСТАВА ЭКЗОПОЛИМЕРНОГО МАТРИКСА

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РЕЗЮМЕ

Введение. *Широкое использование наночастиц оксида меди (НЧ CuO) увеличивает их выброс в окружающую среду, что приводит к накоплению в трофических цепях. Бактериальные биоплёнки более устойчивы к физико-химическим факторам по сравнению с планктонными клетками благодаря экзополимерному матриксу (ЭПМ), состоящему из полисахаридов, белков, липидов и нуклеиновых кислот. Актинобактерии рода Rhodococcus перспективны для экобиотехнологии благодаря способности к биодеградации нефтепродуктов, пестицидов и других органических загрязнителей, а также биоаккумуляции тяжёлых металлов.*

Цель исследования. *Изучить влияние НЧ CuO на жизнеспособность клеток Rhodococcus ruber ИЭГМ 231 в биоплёнках и динамику основных компонентов ЭПМ.*

Материалы и методы. *Биоплёнки R. ruber выращивали на покровных стёклах в присутствии НЧ CuO, компоненты ЭПМ изучали с помощью конфокальной лазерной сканирующей микроскопии (КЛСМ) путём дифференцирующего окрашивания флурофорами: LIVE/DEAD – для живых и мёртвых клеток; Nile Red – для липидов; FITC – для белков; Calcofluor White – для бета-полисахаридов.*

Результаты. *Биоплёнки R. ruber, выращенные в минеральной среде с 1,0 об.% н-гексадекана, были более устойчивы к НЧ CuO по сравнению с биоплёнками, растущими в полноценной питательной среде (МПБ). Это обусловлено более интенсивным формированием ЭПМ, играющего основную роль в защите клеток от бактерицидного действия нанометалла. Показан слабый стимулирующий эффект низкой (0,001 г/л) концентрации НЧ CuO на биоплёнкообразование родококков. Изучены динамика и локализация основных компонентов ЭПМ биоплёнок при длительном (24–72 ч) культивировании с НЧ CuO. Выявлено повышенное содержание липидного компонента ЭПМ, увеличение относительного содержания полисахаридов и белков при воздействии высоких (0,01–0,1 г/л) концентраций НЧ CuO.*

Заключение. *Понимание механизмов взаимодействия нанометаллов и биоплёнок родококков будет способствовать созданию эффективных биокатализаторов на их основе и способов очистки окружающей среды. Полученные данные могут также использоваться для борьбы с нежелательными биоплёнками с помощью наночастиц металлов.*

Ключевые слова: *биоплёнки, Rhodococcus, наночастицы металлов, конфокальная лазерная сканирующая микроскопия, экзополимерный матрикс*

Статья поступила: 02.06.2022
Статья принята: 21.09.2022
Статья опубликована: 08.12.2022

Для цитирования: Баяндина Е.А., Глебов Г.Г., Куюкина М.С., Ившина И.Б. Устойчивость биоплёнок *Rhodococcus ruber* к наночастицам CuO в зависимости от состава экзополимерного матрикса. *Acta biomedica scientifica.* 2022; 7(5-1): 100-109. doi: 10.29413/ABS.2022-7.5-1.11

INTRODUCTION

Due to monoclinic structure, superheat conductivity, high stability and antimicrobial activity, CuO NPs are used as an active catalyst, gas sensor, highly efficient heat-conducting material, in magnetic recording media and solar panels, in agriculture in the form of fertilizers, as well as antimicrobial agents [1–3]. CuO NPs can accumulate in living organisms and exhibit a cytotoxic effect on animals, plants and microorganisms. Antimicrobial activity of nanometals usually includes adsorption on the cell wall, subsequent penetration into cells and damage to cellular compartments. The main mechanisms of nanometal toxicity are increased production of reactive oxygen species (ROS) and membranotropic action [4]. However, despite the growing use and accumulation of CuO NPs in the environment, the toxicological aspects of their effects on living organisms, in particular bacterial cells and their communities, have not been sufficiently studied.

Biofilms are complex, structurally organized communities consisting of microbial cells and associated exopolymer matrix (EPM), which consists mainly of polysaccharides, proteins, lipids and nucleic acids. Biofilm formation can be considered as a way of bacterial growth that protects cells, thereby allowing them to survive in a stressful environment (under strong ultraviolet radiation, dehydration, in the presence of antibiotics) [5–7], as well as to protect cells from predators and parasites [8]. Due to the protective properties of EPM, microbial biofilms are more resistant to physico-chemical stresses compared to planktonic cells, leading to a decrease in the effectiveness of antimicrobial agents and problems in medical practice. However, along with undesirable biofilms, the biofilm formation process has a positive value, for example, when developing heterogeneous biocatalysts consisting of microbial cells immobilized on solid carriers.

Actinobacteria of the genus *Rhodococcus* are known for their bioremediation potential due to their ability to degrade complex organic pollutants such as petroleum hydrocarbons, pesticides and other xenobiotics, accumulate and inactivate heavy metal ions [9]. Biocatalysts based on immobilized *Rhodococcus* cells are used for the treatment of oily wastewater and bioremediation of polluted ecosystems [10]. Previously, it was shown that treatment with nickel nanoparticles increased the efficiency of biopurification of water contaminated with jet fuel in a bioreactor with immobilized rhodococci [11]. Therefore, a fundamental understanding of the interactions between nanometals and *Rhodococcus* biofilms can contribute to the development of more effective biocatalysts and environmental purification strategies. In addition, some representatives of this genus are pathogenic – *R. corynebacterioides* and "*Rhodococcus fascians* assemblage", causing bacteriosis of fruit and berry plants, and "*Rhodococcus equi* (*Rhodococcus hoagii/Prescottella equi*)" causing human and animal diseases [12], which raises the question of combating unwanted *Rhodococcus* biofilms.

Fluorescence microscopy methods and, in particular, modern confocal laser scanning microscopy (CLSM), are widely used in biofilm studies, since they allow quantitative analysis of the components of the EPM without its destruction using differentiating fluorescent staining [13].

The purpose of this work is to study the effect of CuO NPs on the viability of *Rhodococcus ruber* cells in biofilms and the dynamics of the main (polysaccharides, proteins and lipids) components of EPM.

MATERIALS AND METHODS

The used strain *Rhodococcus ruber* IEGM 231 was from the Regional Specialised Collection of Alkanotrophic Microorganisms (acronym IEGM; WFCC/WDCM #768; www.iegmcol.ru). Cultivation of biofilms on microscopy 24 × 50 × 0.15 mm coverslips (Gerhard Menzel, UK) was carried out in parallel in meat-peptone broth (Nutrient Broth) (Biotechnology LLC, Russia) and liquid mineral medium K [14] with 1.0 vol.% *n*-hexadecane, in 150 ml Erlenmeyer flasks with constant stirring (130 rpm), 28° C for 24–72 hours. Biofilms were obtained under dynamic conditions for uniform cell adhesion to glass.

CuO NPs in the form of nano-powder (Equilibrium LLC, Saransk, Russia) were suspended in a phosphate buffer (PBS) and processed using Soniprep 150 ultrasonic homogenizer (MSE, UK) immediately before use. Ultrasound-treated suspensions of CuO NPs were introduced in tenfold concentrations (0.001–0.1 g/l) to daily growing biofilms and cultured under dynamic conditions (130 rpm; 28 °C) for 24–72 hours.

CLSM scanning was performed on a FluoView 1000 microscope (Olympus Corporation, Japan) using an immersion lens (×100, numerical aperture 1.4). The cover glass with the grown biofilm was dried in air for 10–15 minutes, then the fluorescent dye LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, USA) was added and left in a dark place for 15–20 minutes. The sample was washed with deionized water to remove dye residues, media and planktonic cells. To excite the fluorescence of SYTO9 and Propidium Iodide (PI), which are part of the LIVE/DEAD dye, an argon laser (λ = 488 nm) with a 505/525 nm barrier filter and a helium-neon laser (λ = 543 nm) with a 560/660 nm barrier filter were used. Image analysis was performed using the FV10-ASW 3.1 program (Olympus Corporation, Japan).

Visualization and quantitative analysis of EPM components were performed by three-component staining of samples with the following fluorescent dyes: Nile Red (Invitrogen, USA), FITC (Invitrogen, USA), Calcofluor White (CFW) (Sigma, USA), staining lipids, proteins and beta-polysaccharides, respectively. Staining was performed sequentially: first, 1 mg of Nile Red was dissolved in 1 ml of dimethyl sulfoxide (DMSO), 15 µl of dye was applied to cover glass with a grown biofilm and kept in a dark place for 30 minutes. Then 1 mg of FITC was dissolved in 100 µl of DMSO, 15 µl of dye was applied to the biofilm preparation and kept in a dark place for 30 min. Finally, 15 ml of the commercial CFW solution was applied to the preparation and kept in a dark place for 2–3 minutes. The preparation was washed with deionized water to remove the remnants of dyes, plankton cells and the medium, and a CLSM scanning was per-

formed under the same conditions. To excite the fluorescence of Nile Red, FITC and CFW, an argon and helium-neon laser with a wavelength of 405, 488 and 543 nm and a barrier filter of 430/470, 505/525 and 560/660 nm, respectively, were used. The scans were processed in the FV10-ASW 3.1 program (Olympus Corporation, Japan). The biofilm growth was estimated by the total fluorescence intensity of its components. For quantitative analysis of EPM components (lipids, proteins and beta-polysaccharides), 20 similar ROI (regions of interest) were isolated on the scan and the fluorescence intensity of the isolated sites was determined. The adhesion of bacteria to the cover glass was evaluated in dynamics by the average number of attached cells per 1 mm^2. The parameters of each sample were measured using 5 fields of view.

The obtained data were subjected to statistical analysis using a standard Excel program, calculating the average value and standard deviation (m ± SD). The reliability of the differences between the mean data was determined using the Student's unpaired t-test.

RESULTS AND DISCUSSION

According to our data (Fig. 1), the viability of *R. ruber* IEGM 231 cells in biofilms grown in Nutrient Broth decreased by 60–90 % when exposed to elevated (0.01–0.1 g/l) concentrations of CuO NPs. Moreover, an increase in the duration of biofilm cultivation in the presence of 0.1 g/l CuO NPs enhanced their toxic effect. The results obtained are consistent with the data of other studies [15–17], reporting a pronounced inhibitory effect of copper nanoxide against microbial biofilms and planktonic cells.

However, when growing biofilms in a mineral medium with *n*-hexadecane, a higher resistance of *R. ruber* to CuO NPs was observed, especially noticeable at elevated concentrations of nanometal (the number of viable cells was 5.5–14 times higher compared to biofilms grown in Nutrient Broth). Thus, after 48–72 h cultivation in the presence of hydrocarbon and CuO NPs, the viability of cells in biofilms decreased by only 4–30 % compared to control samples. Earlier, a similar increase in the resistance of *Rhodococcus* to antibiotics was revealed after the transfer of the culture from a predominantly carbohydrate (Nutrient Broth) medium to a hydrocarbon substrate [14], although this fact with a higher biofilm tolerance to nanometal was shown for the first time in the present study. It should be noted that the control biofilms grown on *n*-hexadecane were formed by a large accumulation of cells, of which 80–90 % were viable (Fig. 2). The appearance of dead cells in the biofilm under optimal growth conditions is natural during its formation [18].

Biofilm formation is a complex dynamic process involving several stages: cell adhesion and distribution on the substrate surface, active cell division to form cell colonies or clusters and, ultimately, the formation of EPM [19]. The adhesive activity of bacteria is largely determined by the properties of the cell surface, primarily by the degree of its hydrophobicity [20], which is significantly influenced by culture conditions. *Rhodococcus* cells co-adhere better when growing on hydrophobic substrates [12]. This effect is due to the increased content of lipids, in particular saturated fatty acids and neutral phospholipids, in the cells grown in the presence of *n*-hexadecane [12, 14]. Also, when growing on hydrocarbons, rhodococci synthesize glycolipid biosurfactants that promote cell adhesion both to the substrate and to each other (co-adhesion) [21]. Co-adhesion, the formation of cellular aggregates, is a physiological adaptation of bacteria to protect against adverse environmental conditions [12]. The obtained CLSM scans of *R. ruber* biofilms (Fig. 2) show that when exposed to high concentrations of CuO NPs, viable cells are embedded in a matrix formed by dead cells and biomolecules – EPM components. According to our data (Fig. 2, Table. 1), the number of *R. ruber* in growing biofilms was 2–5 times lower at the maximum concentration of CuO NPs compared to the control, which indicates the inhibition of physiological processes of bacterial adhesion and co-adhesion by nanometal. Interestingly, the lowest (0.001 g/l) of the studied concentrations of CuO NPs stimulated the biofilm formation in the first 24 h of cultivation (Table 1).

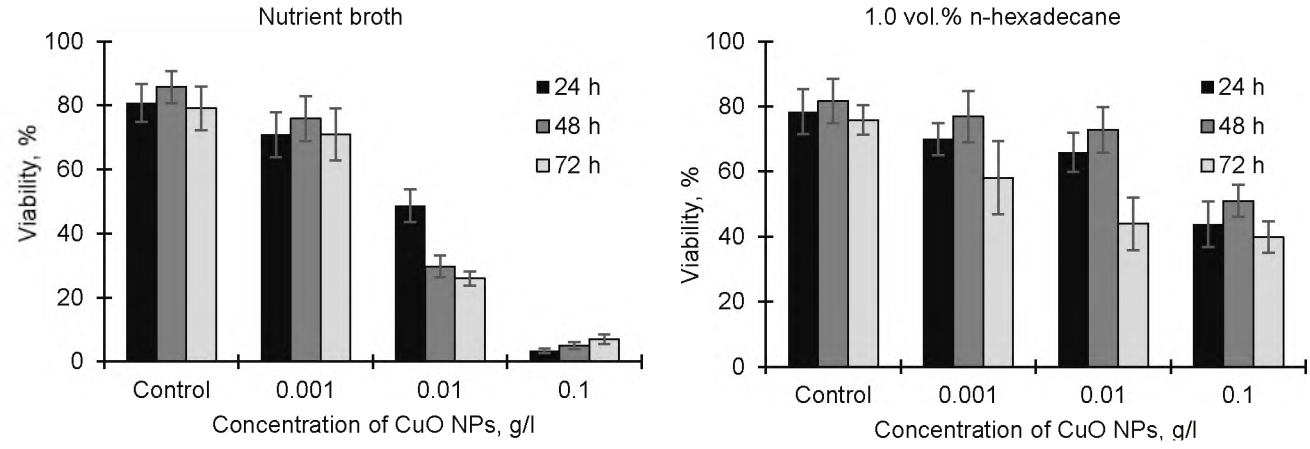


FIG. 1.
The effect of CuO NPs on the viability of R. ruber IEGM 231 cells in biofilms grown on different media

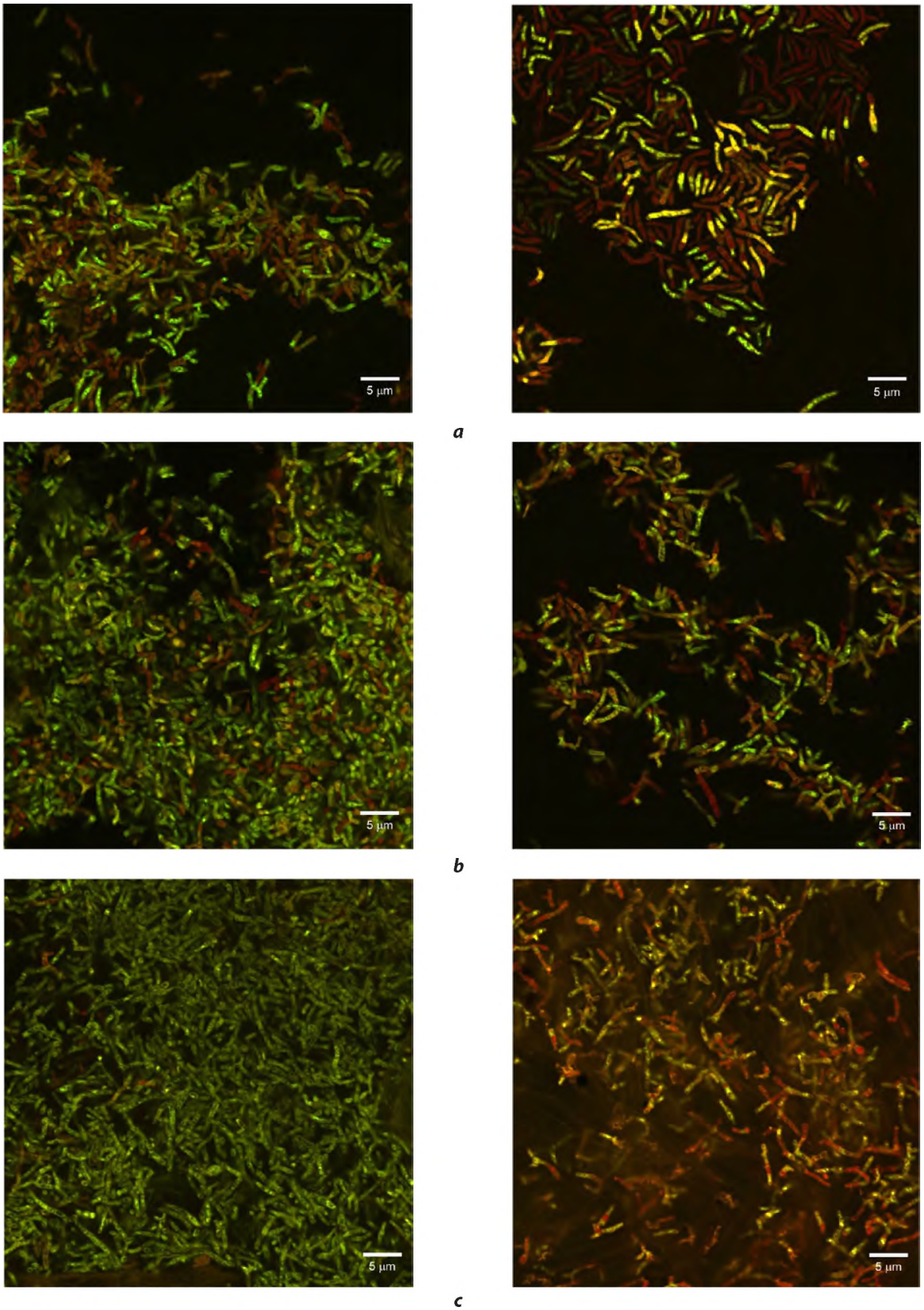


FIG. 2.

*CLSM-images of R. ruber IEGM 231 biofilms grown in a mineral medium with 1 vol.% n-hexadecane for 24 (**a**), 48 (**b**) and 72 (**c**) hours and stained with LIVE/DEAD® fluorescent dye to detect live (green) and dead (red) cells. On the left panel – control biofilms; on the right panel – biofilms cultivated in the presence of 0.1 g/l CuO NPs*

According to [22], the interaction of metal nanoparticles with biofilms can be described by three sequential mechanisms: transfer of NPs to the biofilm – liquid interface, attachment to the biofilm surface (outer region), and migration inside the biofilm matrix. Upon NPs contact with the polymer compounds of the matrix, a "biomolecular corona" is formed, which leads to a change in the surface properties of the nanometal [23] and, consequently, partial neutralization of its toxic properties. It is possible that biosurfactants released by *Rhodococcus* when growing on a hydrophobic substrate are able to bind to metal NPs, similar to their metal-chelating action against heavy metal ions [24], and neutralize their toxic effects.

TABLE 1

DYNAMICS OF *R. RUBER* IEGM 231 NUMBERS (CELLS/MM^2) IN BIOFILMS GROWN IN THE PRESENCE OF VARIOUS CONCENTRATIONS (G/L) OF CUO NPS

Time, h	Control	0.001	0.01	0.1
24	$(4.17 \pm 0.3) \times 10^4$	$(5.71 \pm 0.5) \times 10^4$	$(4.07 \pm 0.42) \times 10^4$	$(1.76 \pm 0.37) \times 10^4$
48	$(8.12 \pm 1.04) \times 10^4$	$(6.94 \pm 0.98) \times 10^4$	$(0.23 \pm 0.29) \times 10^4$	$(2.69 \pm 0.14) \times 10^4$
72	$(10.4 \pm 1.3) \times 10^4$	$(4.84 \pm 0.42) \times 10^4$	$(3.86 \pm 0.17) \times 10^4$	$(3.94 \pm 0.41) \times 10^4$

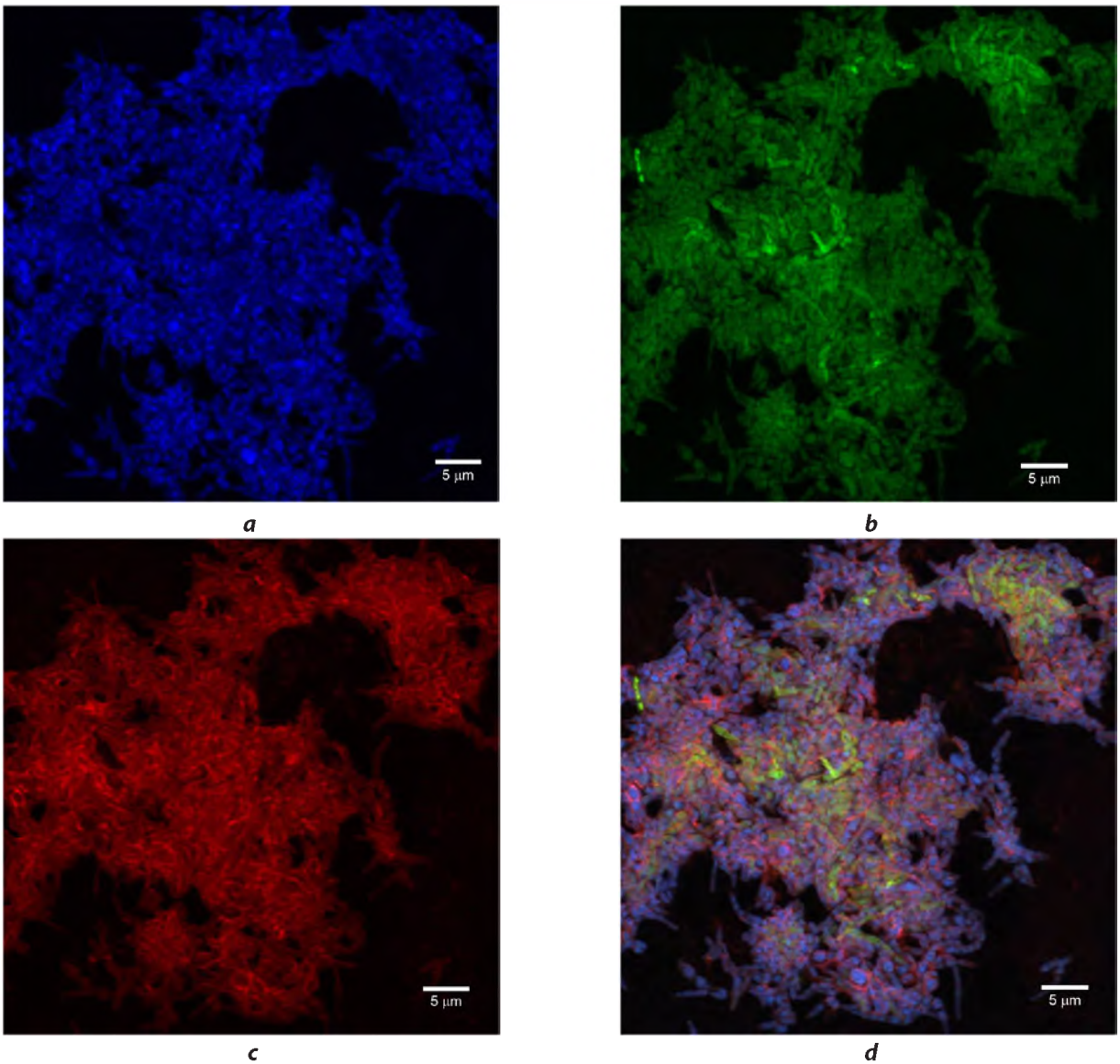


FIG. 3.

*CLSM-images of R. ruber IEGM 231 control biofilms grown in a mineral medium with 1 vol.% n-hexadecane for 24 hours and stained with fluorescent dyes: FITC (**a**), CW (**b**), NileRed (**c**), and their combined image (**d**)*

Using a modified method of sequential multicolored staining [25], clear differential fluorescence signals of CFW, FITC and Nile Red dyes were obtained with *R. ruber* IEGM 231 biofilms, which allowed visualizing and quantifying the EPM components: beta-polysaccharides, proteins and lipids (Fig. 4).

It is known [13, 26] that proteins and polysaccharides provide binding of EPM molecules and restrict the movement of newly dividing bacterial cells inside the matrix, keeping them in close contact with each other, which contributes to the maturation of the biofilm. In addition, polysaccharides and proteins are involved in the initial stages of colonization and temporary immobilization of bacterial cells on surfaces [27].

Analysis of the intensity dynamics of fluorescent signals revealed (Fig. 4) a gradual uniform increase in the content of all three components of EPM (polysaccharides, proteins and lipids) in control *R. ruber* IEGM 231 biofilms throughout the entire cultivation period. The introduction of low (0.001–0.01 g/l) concentrations of CuO NPs led only to a slight increase in the content of matrix components by 24 hours of cultivation, which is consistent with insignificant effects of these nanometal concentrations on cell viability in biofilms and even stimulating the biofilm formation on the first day of exposure to the minimum CuO NPs concentration (Fig. 2, Table 1).

In the next two days, a significant difference was revealed in the dynamics of EPM accumulation in biofilms at relatively low concentrations of nanometal. Thus, when exposed to a minimum (0.001 g/l) concentration of CuO NPs, the relative content of polysaccharides, proteins and lipids remained stably low, whereas the effect of a 10-fold higher (0.01 g/l) concentration of CuO NPs was a sharp increase in all EPM components after 72 h of cultivation. A similar increase in the quantitative composition of EPM was detected in the first 24 h of biofilm cultivation in the presence of the maximum (0.1 g/l) studied concentration of CuO NPs, and these dynamics was maintained throughout the experiment. The obtained data correlate with the revealed higher resistance of *R. ruber* biofilms to copper nanoxide when grown in a medium with hydrocarbon, and clearly indicate the increasing protective role of EPM with an increase in stress exposure.

Since the development of biofilms on the first day of exposure to heavy metals is crucial for the formation of a stress reaction by bacterial communities [28], a comparative analysis of the EPM composition was carried out after 24 hours of cultivation in the presence of CuO NPs. This analysis revealed (Fig. 5) a relatively high lipid content, which remains high when biofilms are exposed to increasing concentrations of nanometals. Importantly, CLSM scans showed (Fig. 6) that lipid components are localized in the extracellular matrix of mature biofilm, possibly they are represented by glycolipid biosurfactants [21]. The relative amount of polysaccharides remained the lowest at low (0.001–0.01 g/l) concentrations of CuO NPs and sharply increased at the maximum concentration of nanometals. Interestingly, an increased content of polysaccharides was observed on the periphery of the cell conglomerates in a growing biofilm (Fig. 6). A similar dynamic was characteristic of the protein component of EPM, while the protein content increased by 30 % under the influence of nanometal and had a maximum value after 72 h of exposure with a maximum (0.1 g/l) concentration of CuO NPs. Unlike lipids and polysaccharides, an increase in protein content was observed associated with bacterial cell surfaces, suggesting that proteins promote cell co-adhesion.

It should be noted that when exposed to toxic factors, cells localized in the upper layers of the formed biofilm die and their contents exit. This leads to an increase in EPM along with its supersynthesis by surviving cells, which also contributes to the protection of cells inside the matrix from the toxic effects of NPs. Persistent cells, which make

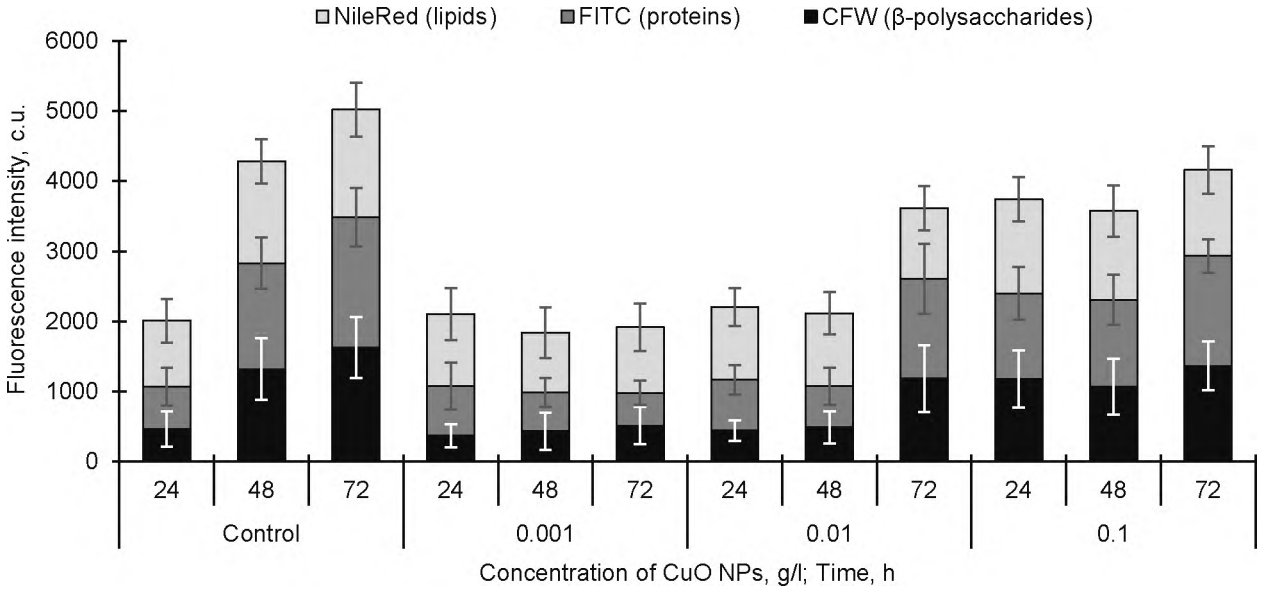


FIG. 4.

Dynamics of EPM components in R. ruber IEGM 231 biofilms grown in a mineral medium with 1 vol.% n-hexadecane, depending on the cultivation time (h) and the concentration of NPs CuO (g/l)

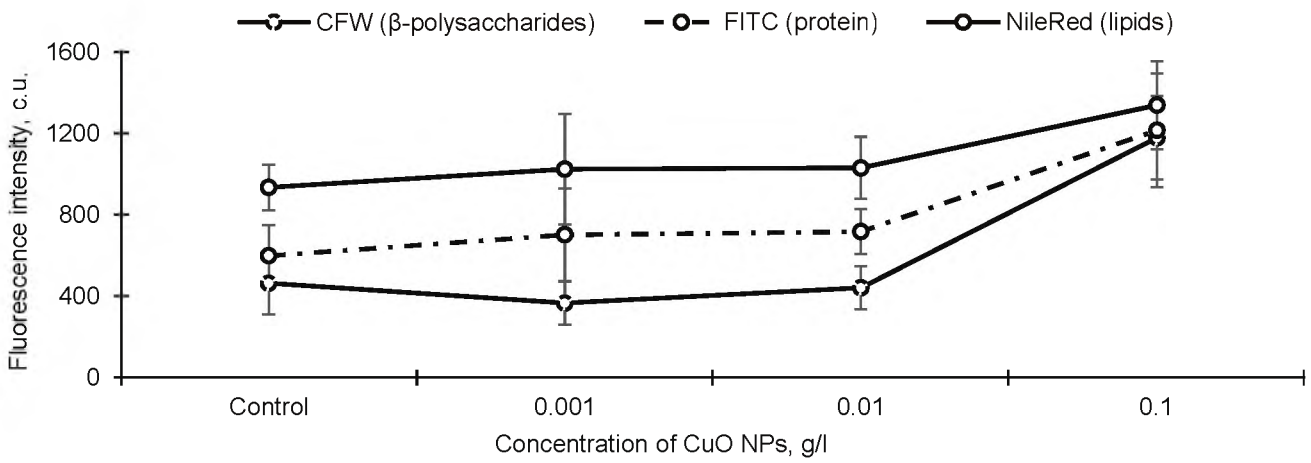
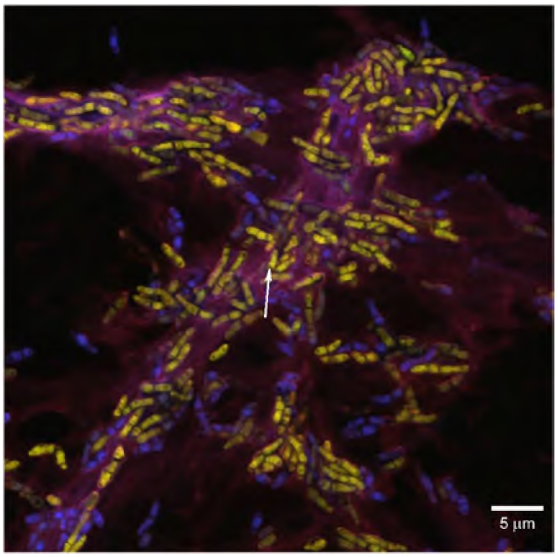
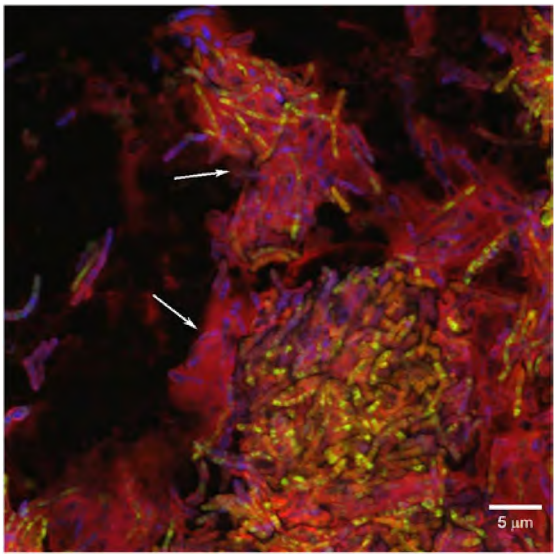


FIG. 5.
Comparative dynamics of EPM components of R. ruber IEGM 231 biofilms grown in a mineral medium with 1 vol.% n-hexadecane for 24 hours, depending on the concentration of CuO NPs



a



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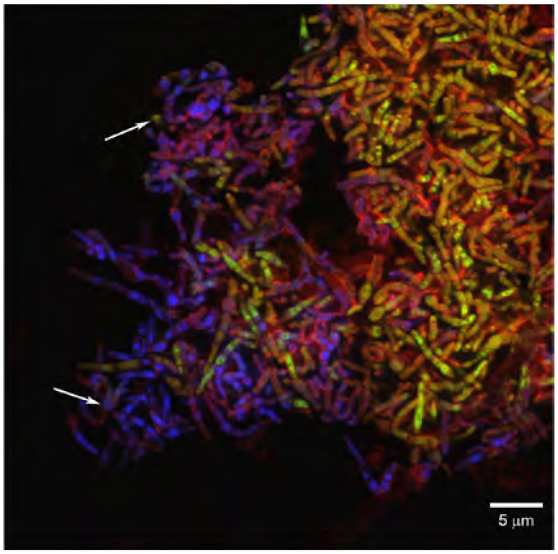

c

FIG. 6.
*Combined CLSM images of R. ruber IEGM 231 biofilms grown in a mineral medium with 1 vol.% n-hexadecane in the presence of 0.01 g/l CuO NPs. Biofilms were stained with fluorescent dyes: FITC (proteins), NileRed (lipids) and CFW (beta-polysaccharides). Arrows show the localization of cell-associated proteins (**a**), extracellular lipids (**b**) and polysaccharides at the periphery of cell conglomerates (**c**)*

up about 1 % in the stationary phase, are protected by both the polymer matrix and the surface cells themselves. Due to the ability of bacteria to horizontal gene transfer, persistors develop resistance to toxic effects, which contributes to the subsequent restoration of biofilm [29]. Our study of the long-term (up to 72 h) dynamics of EPM components helps to clarify the mechanisms of *Rhodococcus* resistance to metal nanoparticles – from young to mature biofilm.

CONCLUSIONS

The present study showed that *R. ruber* IEGM 231 biofilms grown in a hydrocarbon-containing medium were more resistant to CuO NPs compared to biofilms growing in a rich culture medium (nutrient broth). This higher resistance of the biofilm correlated with a more intensive formation of EPM, which plays an important role in protecting cells from the bactericidal action of nanometals. In addition, a weak stimulating effect of a low (0.001 g/l) concentration of CuO NPs on the *R. ruber* biofilm formation was detected on the first day of cultivation. The dynamics of the structural EPM components using CLSM with differentiated fluorescent staining revealed a high lipid content, an increase in the polysaccharide and protein components when exposed to elevated (0.01–0.1 g/l) concentrations of CuO NPs. The revealed dynamics and localization of these components in the biofilm matrix indicated a complex adaptation mechanism of *Rhodococcus* to stress caused by nanometals and require further study. The results obtained can be used in the development of strategies to combat unwanted biofilms using metal nanoparticles, as well as for the possible functionalization of bacterial cells with low concentrations of nanometals in order to increase their biocatalytic potential.

Funding

This study was funded by the Ministry of Science and Higher Education of the Russian Federation (AAAA-A20-120081990069-3, AAAA-A19-119112290008-4) and Russian Science Foundation (18-14-00140).

Acknowledgments

The research work was carried out using the equipment of the Core Facilities Centers "Specialized cabinet of atomic force and confocal microscopy" (PSU) and "Regional Specialized Collection of Alkanotrophic Microorganisms" (IEGM-PFRC UB RAS).

Conflict of interests

The authors of this article report that there is no conflict of interest.

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Статья опубликована в рамках V Всероссийской научно-практической конференции молодых учёных с международным участием «Фундаментальные и прикладные аспекты в медицине и биологии».