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Influence of silver and copper nanoparticles on *Staphylococcus aureus* biofilm formation

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Abstract: *Influence of silver and copper nanoparticles on* Staphylococcus aureus *biofilm formation.* The purpose of this study was to investigate the effect of silver and copper nanoparticles on biofilm formation by *Staphylococcus aureus*. The bacteria cells were treated with silver and copper nanoparticles in increasing concentrations (5, 12.5, 25 μg/mL). Characteristics of nanoparticles was defined by measurement of zeta potential, size distribution and TEM analysis. Biofilm formation by *S. aureus* was identified by crystal violet staining and SEM analysis. The results showed that both nanoparticles inhibited the ability to form biofilm by *S. aureus*. However, in all used concentrations, silver nanoparticles had stronger effect than copper nanoparticles.

Key words: *Staphylococcus aureus*, biofilm, copper nanoparticle, silver nanoparticle

INTRODUCTION

Biofilm is a complex multicellular structure formed by bacteria and other microorganisms. The biofilm structure is surrounded by a layer of extracellular matrix composed of extracellular polymeric substances produced by microorganisms. Cells in biofilms are adhering closely to each other and also to biological or abiotic surfaces (Stoodley et al. 2002, Hall-Stoodley et al. 2004). This structure may be formed by one or more bacterial species and the cells in biofilm are physiologically different from the planktonic cells of the same microorganism (Stoodley et al. 2002). Nowadays biofilms generated by bacteria are considered to be an important factor in the development of chronic endodontic (Choi et al. 2018) and skin diseases (Sonesson et al. 2017), as wells as neoplasms including gallbladder cancer (Di Domenico et al. 2017). Furthermore, close adhesion to each other and the surface make bacteria highly resistant to bactericides and antibiotics (LewisOscar et al. 2015), leading to chronic and acute illnesses (Habash et al. 2017).

Nanoparticles which are within the area of interest of nanobiotechnology are used in many fields of science (Pulit et al. 2011), including medicine (Sawosz Chwalibog et al. 2014) Nanoparticles are the particles in size from 1 to 100 nm, which have unique properties when compared to their parent material. This results from a different distribution of electrons on their surface, and therefore they are characterized by greater reactivity (Sawosz Chwalibog et al. 2014).

Many of the metallic nanoparticles showed a proven antibacterial effect. The most popular are nanoparticles of: platinum (Pt-NPs), copper (Cu-NPs), zinc (Zn-NPs), gold (Au-NPs) and silver (Ag-NPs) (Pulit et. al. 2011). Pt-NPs, Ag-NPs and Au-NPs had harmful effects on both yeast and bacterial strains for example *Staphylococcus aureus* (Chwalibog et al. 2010), while Zn-NPs generate reactive oxygen species (ROS) which damage the cell membrane of bacteria (Vimbela et al. 2017).

Many studies sought to establish a mechanism of action of antibacterial activity exhibited by silver in both colloidal and ionic form. Loss of membrane functionality resulting from interaction between released Ag^+ ions and the cell membrane, as well as extensive cell membrane damage caused by the formation of ROS, ultimately causes damage to the cell due to oxidative stress (Belluco et al. 2016). It has been proven that Ag-NPs inhibited growth of different types of microorganisms such as bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus*), yeasts (*Saccharomyces cerevisiae*) and algae (*Chlorella protothecoides*) (Dorobantu et al. 2015). It has been shown that they can bind to the cell wall of bacteria and then penetrate it causing damage leading to changes in cell membrane permeability and cell death (Prabhu and Poulose 2012). In addition, Ag-NPs may be the cause of free radicals formation (Kim et al. 2007). They can also be a source of free silver ions that inhibit crucial bacterial enzymes leading to the death of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria (Yoshinobu et al. 2003). Habash et al. (2017) suggested that Ag-NPs enhanced tobramycin's activity against the biofilm-forming *Pseudomonas aeruginosa* (Habash et al. 2017). Moreover, Ag-NPs inhibited *Klebsiella pneumoniae* biofilm formation. It was also proven that Gram-negative bacteria are more sensitive to Ag-NPs than Gram-positive (Qayyum et al. 2017). Qayyum et al. (2017) showed 80 and 75% inhibition of *E. coli* and *S. mutans* biofilm formation, respectively.

Nanoparticles of Cu-NPs are also widely used. Chatzimitakos and Stalikas (2016) showed that Cu-NPs disturbed some of the metabolic pathways of the Gram-positive strains such as *S. aureus* and Gram-negative strains such as *Escherichia coli*. Cu-NPs had also an inhibiting effect on *Pseudomonas aeruginosa* (LewisOscar et al. 2015) and *Listeria monocytogenes* (Ghasemian et al. 2015) biofilms. The same effect was observed against biofilm formation in Cooling Water Systems (Ogawa et al. 2016). In addition, *in vitro* analysis showed more than 60% inhibition of biofilm formation by *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* in presence of Cu-NPs (Chari et al. 2017). Beside the interference with metabolic pathways, Cu-NPs caused the damage of the bacterial cell membrane (Vimbela et al. 2017). Cu-NPs had also the negative effect on fungi such as *Aspergillus flavus* (Essa and Khallaf 2016).

The objective of this study was to determine the effect of Ag-NPs and Cu-NPs on *S. aureus* biofilm by measurement the cell viability and scanning electron microscope analysis (SEM). Characterization of the nanoparticles was obtained by survey of zeta potential, size distribution and also by determina-

tion of the shape and size of individual nanoparticles by transmission electron microscope (TEM).

MATERIAL AND METHODS

Bacteria culture

Bacteria strain *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) was obtained from LGC Standard (Łomianki, Poland). The strain was stored in 20% glycerol solution at –20°C. In next stage, cells were thawed and washed with distilled water to remove glycerol and next transferred to nutrient broth medium (Bio-Rad, Warsaw, Poland), sterilized in an autoclave (Classic 2100, Prestige Medical, Chesterfield, UK). Bacterial cultivation was conducted in strain specific conditions (aerobic atmosphere in 37°C) in incubator shaker (SI500, Stuart, Stafford, UK) with shaking speed set on 70 rpm for 24 h.

Preparation and characterization of copper and silver nanoparticles

Nanoparticles of Ag-NPs and Cu-NPs were obtained from Nano-Tech (Warsaw, Poland) and they were produced by electric nonexplosive patented method (Polish Patent 3883399) from high purity metals (99.9999%) and high purity demineralized water. The suitable amount of the powder of nanoparticles was suspended in ultrapure water to receive a concentration of 50 μg/mL.

Size distribution and zeta potential of nanoparticles in water (50 μg/mL) were measured by the dynamic light scattering and electrophoretic method using a Zetasizer Nano ZS, model ZEN3500 (Malvern Instruments, Malvern, UK). Each sample was measured in three replicates after 120 s of equilibration at 25°C.

Nanoparticles TEM analysis

The shape and size of individual nano_particles was determined by TEM. Amount of 10 μL of Ag-NPs and Cu-NPs suspension in ultrapure water (50 μg/mL) were placed onto formvar--coated copper grids and allowed to dry. Dried grids were placed in TEM and observed with the JEM-2000EX TEM at 80 keV (JEOL, Tokyo, Japan). The Morada 11 Mgpx camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) was used to capture the images.

Biofilm formation assay

Staphylococccus aureus cells stored in a refrigerator were used to set up a night culture of bacteria. Amount of 100 μL bacterial suspension were transferred to a flask with 10 mL of sterile nutrient broth (Bio-Rad, Warsaw, Poland) and placed in 37°C in Incubator Shaker (SI500, Stuart, Stafford, UK).

Amount of 10, 25 and 50 μl Cu-NPs and Ag-NPs suspensions were applied in triplicate on a 96-well plate. Then the plate was allowed to dry for 24 h on Mini-Shaker (PSU-2T, Biosan, Riga, Latvia) placed in the laminar flow chamber. After drying, 100 μL of the overnight bacterial culture suspension were added into the wells. Final concentrations of the nanoparticles were 5, 12.5, 25 μg/mL. The plate was placed in the incubator for 48 h. Simultaneously, wells containing no nanoparticle suspensions were prepared. Afterwards, planktonic bacterial cells

were removed by rinsing three times with distilled water. Subsequently, 200 µL of methanol (POCH, Gliwice, Poland) were added to the wells and incubated at room temperature for 15 min. After incubation, the alcohol was removed and the plates were allowed to dry. Then 200 µL of crystal violet staining (Sigma-Aldrich, Munich, Germany) were added to determine the number of biofilm forming bacteria. After 15 min incubation, the dye was rinsed under running tap water and the plates were left to dry. Amount of 200 µL of 33% acetic acid (POCH, Gliwice, Poland) solution was added to the dried wells. Afterwards, the absorbance of the solutions in each well was measured at 570 nm using a microplate reader (Infinite M200, Tecan, Durham, NC,USA).

The amount of biofilm-forming bacterial cells was expressed as an optical density (OD), which is correlated to the number of bacterial cells in biofilm.

Biofilm SEM analysis

Amount of 2 mL of bacterial culture (10^6 CFU/ml) were incubated on sterile cover glass coated with Ag-NPs and Cu-NPs, or untreated bacteria were deposited on the surface of a cover glass without nanoparticles and incubated for 24 h at 37°C inside Petri dish. All samples were dried and covered with a gold. Finally, the samples were imaged with SEM (FEI Quanta 200, Tokyo, Japan) at an acceleration voltage of 15 kV.

Statistical analysis

Obtained data were analyzed using one-way ANOVA with STATGRAPHICS® Plus 4.1. Differences with $P < 0.05$ were considered statistically significant. Results were presented as means with standard deviations.

RESULTS AND DISCUSSION

The analysis of size distribution of Cu--NPs and Ag-NPs (the table) showed that both materials occurred as aggregates. An average diameter of the aggregates of Cu-NPs was 839.7 nm while average diameter of Ag-NPs – 211.1.nm. This results showed that Cu-NPs form more prominent aggregates than Ag-NPs.

The zeta potential measurements (the table) presented that both Ag-NPs and Cu-NPs had a negative potential but were not stable. The zeta potential for Cu-NPs and Ag-NPs was –14.3 and –25.6 mV, respectively, and it indicates that Ag-NPs has less ability to generate aggregates and therefore greater stability and better quality.

TABLE. The size distribution and values of zeta potential of copper nanoparticles (Cu-NPs) and silver nanoparticles (Ag-NPs) in concentration 50 µg/mL

Tested material	Size range; average size of aggregates (d.nm)	Average zeta potential (mV)
Cu-NPs	3–15; 839.7	–14.3
Ag-NPs	10–40; 211.1	–25.6

Size of individual nanoparticles was determined on the basis of TEM images and it was 10–40 nm for Ag-NPs and 2–15 for Cu-NPs (Fig. 1), what confirmed that only Cu-NPs were forming aggregates.

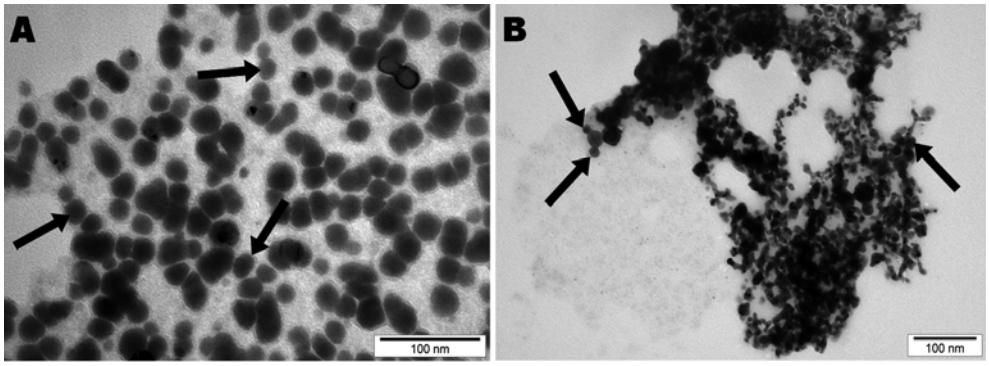

FIGURE 1. A transmission electron microscopy (TEM) image of A – Ag-NPs, B – Cu-NPs, in concentration 50 µg/mL. Nanoparticles were indicated by arrows

Previous preliminary studies proved the toxic effects of Ag-NPs on a various microorganisms. Qayyum et al. (2017) showed that Gram-positive bacteria are more resistant to Ag-NPs than Gram--negative. They designated a minimum inhibitory concentration of 16 and 8 µg/mL, respectively. In our study we showed that Ag-NPs in concentration 5 µg/mL suspension inhibit the formation of *S. aureus* biofilm by more than 70% (Fig. 2). The most significant inhibition of biofilm formation was recorded for the highest concentration of Ag-NPs suspension (25 µg/mL). The inhibitory activity may be due to the ability of Ag-NPs to damage the cell membrane (Prabhu and Poulose 2012) or to generate ROS (Kim et al. 2007). This effect has also been observed for other Gram-positive bacteria including *S. mutans* (Qayyum et al. 2017). We indicated that using the lowest concentration of Ag-NPs caused a significant decrease in

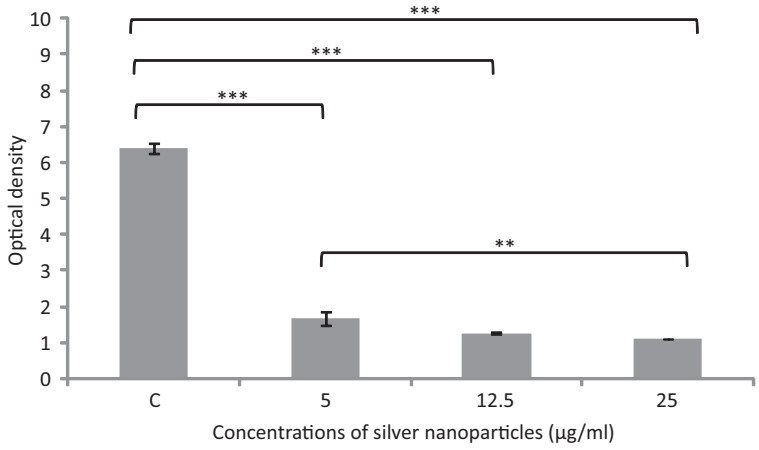

FIGURE 2. Effect of silver nanoparticles on *S. aureus* biofilm formation: C – control, 5, 12.5, 25 – concentration of solution of silver nanoparticles. Statistically significant differences were indicated as: $^{**}P < 0.01$, $^{***}P < 0.001$

the number of biofilm forming bacteria. We observed statistically significant differences between control group and all tested concentrations and the effect caused by the concentration of 25 µg/mL was the most prominent. It suggests that the relationship between the concentration of Ag-NPs and *S. aureus* ability to biofilm formation is not linear.

Nanoparticles of Cu-NPs have shown an antibacterial activity (Pulit et al. 2011). Essa and Khallaf (2016) showed that Cu--NPs had a better effect on Gram-positive than Gram-negative bacteria in concentration 50 µg/mL. In addition, Cu-NPs influenced *S. aureus* cells by induction of a negative effect on some bacterial metabolic pathways (Chatzimitakos and Stalikas 2016). In our study, Cu-NPs also had an observable and statistically significant inhibitory effects on *S. aureus* bacteria in all tested concentrations. The most prominent effect was observed at concentration 25 µg/mL of Cu-NPs – almost 70% inhibition of *S. aureus* biofilm formation. It can be related with ability of Cu-NPs to damage bacterial cell membrane (Vimbela et al. 2017). This effect of Cu-NPs was also observed against *Listeria monocytogenes* biofilm formation (Ghasemian et al. 2015). We observed a directly proportional effects between biofilm formation and the concentration of nanoparticles (Fig. 3). We indicated that there is a noticeable inhibitory effect on *S. aureus* biofilm formation which is also dependent on used concentration of Cu-NPs and there are statistically significant differences between tested groups.

Biofilm SEM analysis has shown that both nanoparticles inhibit *S. aureus* ability to form biofilm. In control group large number of bacteria cells were forming compact biofilm structure, in contrast to experimental groups (Fig. 4). In group with Ag-NPs we observed less number of bacteria cells comparing to Cu-NPs, which is in accordance to Ruparelia et al. studies (2008). In addition, in our study

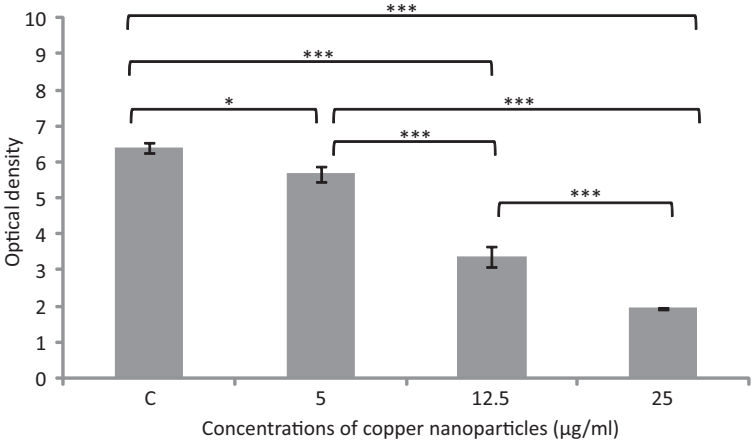

FIGURE 3. Effect of copper nanoparticles on *S. aureus* biofilm formation: C – control, 5, 12.5, 25 – concentration of solution of copper nanoparticles. Statistically significant differences were indicated as: **P* < 0.05, ****P* < 0.001

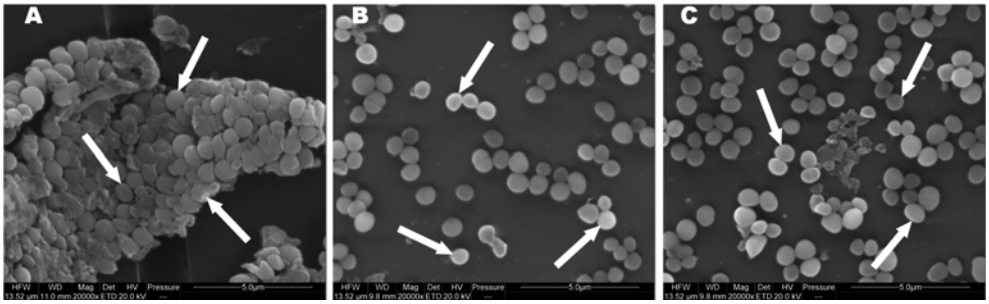

FIGURE 4. A scanning electron microscopy (SEM) image of *S. aureus* biofilm formation: A – control, B – Ag-NPs, C – Cu-NPs. Nanoparticles were in concentration 25 µg/mL. Bacteria cells were indicated by arrows

showed that Ag-NPs have better anti-biofilm activity than Cu-NPs.

We proved that both Ag-NPs and Cu-NPs decreased the number of biofilm forming bacteria *S. aureus*. However, Ag-NPs had a stronger effect on bacteria than Cu-NPs at all tested concentrations (5, 12.5, 25 µg/mL). We obtained similar effect of biofilm formation inhibition for the lowest used concentration of Ag-NPs (5 µg/mL) and the highest concentration of Cu-NPs (25 µg/mL).

Even though the effect of Ag-NPs was noticeable between control and tested groups, the differences between tested concentrations were not so prominent as it was observed for Cu-NPs. It can be related to different mechanism of action of this nanoparticles or results from differences in size distribution. Therefore, further studies are necessary to explain this obtained effect.

CONCLUSION

In our study, we showed that both Cu-NPs and Ag-NPs inhibit biofilm formation by *Staphylococcus aureus*. The effect of Ag-NPs was stronger than that of Cu-NPs, without significant differences between used concentrations of Ag-NPs. Our results indicate the potential use of Cu-NPs and Ag-NPs in medicine as a coating to prevent biofilm formation on surgical instruments or endoprostheses. In addition, such coatings could be used in the food and pharmaceutical industries where it is important to harness microbiological purity. However, due to the lower efficiency of Cu-NPs, application of higher concentrations is required.

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Streszczenie: *Wpływ nanocząstek srebra i miedzi na tworzenie biofilmu przez* Staphylococcus aureus. Celem pracy było zbadanie wpływu nanocząstek srebra i miedzi na formowanie biofilmu przez gronkowca złocistego. Komórki bakteryjne zostały potraktowane wzrastającymi stężeniami (5; 12,5; 25 μg/mL) nanocząstek srebra i miedzi. Charakterystyki nanocząstek dokonano przez pomiar potencjału zeta, rozkładu wielkości oraz analizy z wykorzystaniem TEM. Tworzenie biofilmu przez *S. aureus* zostało określone przez barwienie fioletem krystalicznym. Wyniki wykazały, że zarówno nanocząstki srebra, jak i miedzi hamują zdolność do tworzenia biofilmu przez *S. aureus* i analizę z wykorzystaniem SEM. Nanocząstki srebra mają jednak silniejszy efekt we wszystkich zastosowanych stężeniach od działania nanocząsteczek miedzi.

Słowa kluczowe: gronkowiec złocisty, biofilm, nanocząstki srebra, nanocząstki miedzi

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