The effect of biologically and chemically synthesized silver nanoparticles (AgNPs) on biofilm formation

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Abstract. Bionanotechnology has emerged up as integration between biotechnology and nanotechnology for developing biosynthetic and environmental-friendly technology for synthesis of nanomaterials. Different types of nanomaterials like copper, zinc, titanium, magnesium, gold, and silver have applied in the various industries but silver nanoparticles have proved to be most effective against bacteria, viruses and eukaryotic microorganisms. The antimicrobial property of silver nanoparticles are widely known. Due to strong antibacterial property silver nanoparticles are used, e.g. in clothing, food industry, sunscreens, cosmetics and many household and environmental appliances. The aim of the study was to compare the effect of silver nanoparticles (AgNPs) synthesized biologically and chemically on the biofilm formation. The biofilm was formed by the bacteria isolated from the water supply network. The commonly used crystal violet assay (CV) was applied for biofilm analysis. In this study effect of biologically synthesized Ag-NPs on the biofilm formation was evaluated.

1 Introduction

Nanoparticles of metals are currently an area of intense research due to a wide variety of their potential applications in many areas, for example in biomedical, agricultural, environmental fields. Nanoparticles have specific properties and chemical reactivity [6, 17, 19]. One of them is high surface area to volume. The antimicrobial activity of AgNPs seems to be a function of the surface area to effectively interact with microorganisms. Large surface area of nanoparticles enhances the interaction with microbes and results in a wide spectrum of antimicrobial activities. Silver nanoparticles (AgNPs) are the most popular material to using as the antimicrobial agent.

In the AgNPs production the physical, chemical and biological approaches are applied. Biological synthesis involves biochemical conversion of a substrate, whereas the chemical

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one is based on the reduction of chemical compounds. The formation of NPs by physical and chemical routes requires toxic chemicals which results in toxic side effects [6]. Green synthesis also provides the cost effective, non-toxic, large-scale, and high-output nanoparticle products [13]. In biologically mediated nanoparticle synthesis, the microbial enzymes or plant phytochemicals having reducing or antioxidant properties are usually responsible for the reduction of metal ions into respective metal nanoparticles [5].

Several reports have successfully demonstrated that AgNPs have antimicrobial activity against a broad range of Gram-negative and Gram-positive including the pathogenic bacteria [9]. To determine the antibacterial effectiveness of AgNPs and consequences of their release into environment, studying the effect of AgNPs on biofilms is important because bacteria are often present in biofilm communities. To survive in harsh environment, bacteria attach to surface, and produce extracellular polymeric substrates (EPS) to create more complex structures, called biofilms [11].

Most of the bacteria in drinking water distribution system is attached to surfaces of pipes as a biofilm. Biofilms in water distribution system can pose financial and functional problems (impact on taste, odour and colour of water) and can be harbour of pathogenic microorganisms. Moreover old mature biofilms can reduce effective pipe diameters. All these negative effects of the presence of biofilm cause associated with maintaining the water supply network. Bacteria from drinking water biofilms are more resistant to disinfectants than planktonic microbes. Also biofilms are hard to remove from the pipes surfaces without dismantling them which is an expensive and practically impossible to do in the drinking water distribution system [1, 23].

In this context, the effect of silver nanoparticles obtained chemically (chem-AgNPs) and biologically (bio-AgNPs) on antimicrobial and antibiofilm activities against bacterial strains isolated from the water supply network biofilms was investigated.

2 Materials and Methods

2.1 Bacterial strains used in the experiment

The high biofilm-forming strains isolated from biofilm samples collected from drinking water distribution system in Wroclaw were used in the study. Taxonomic identification and characterization of these strains have been described previously by Biedron et al. [12]. The strains were used to test the antibacterial and antibiofilm properties of AgNPs synthesized biologically and chemically. *Bacillus subtilis* strain was isolated form the petroleum contaminated soil in Czechowice- Dziedzice in Poland was used in the biological synthesis of Ag-NPs [3]. The strain was identified and characterized in previous studies [7].

2.2 Synthesis of silver nanoparticles

2.2.1. Growth of Bacillus subtilis

The bacterial cultures were grown aerobically in Luria-Bertani (LB) at 30ºC for 96 h with constant shaking (110 rpm). Then, the freshly grown bacterial culture was centrifuged and the supernatant was collected and filtered through sterile 0.22-µm filter to sterile flasks. Cell free supernatant was subjected to synthesis of AgNPs [9].

2.2.2.Biological and chemical synthesis

In the extracellular production of AgNPs, a silver nitrate solution was added to 50 mL of the *Bacillus subtilis* culture supernatant to final concentration of 1 mM, and the mixture was kept for stirring at 200 rpm at 30°C for 48 h under dark conditions. The bioreduction of silver ions was monitored at regular intervals on a UV-Vis spectrophotometer (300–700 nm) for the formation of peaks to confirm the synthesis of silver nanoparticles. During the synthesis, the color change was observed from yellow to dark brown.

The chemical synthesis of AgNPs was performed as the control for biological synthesis. The chemical synthesis of AgNPs was carried out using ascorbic acid as reductant. 5 mL of ascorbic acid [2]. The reaction solutions changed from colorless to light yellow after 1 h of reaction. After 96 h of the reaction the precipitate appeared. The concentration of biological and chemical AgNPs measured by atomic absorption spectroscopy (AAS) was around 165 mg/L in solutions.

2.3 Evaluation of Ag-NPs effect on the biofilm formation

2.3.1 Growth of the bacteria

The strains isolated from the biofilm samples were grown on LB agar plates at 22°C for 48 hours. The single colony of bacteria was introduced to 150 mL of LB broth, and incubated at 22°C at 150 rpm for 48 hours. After incubation, the cultures were centrifuged and the pellet of bacteria was re-suspended in phosphate buffer saline (PBS). The each bacterial suspension was adjusted to 0.5 of McFarland's standard with PBS (approximately 10^7 CFU/ml) prior to use as an inoculum in experiments.

2.3.2. Biofilm inhibition assay

A volume of 120 µl of bacterial suspension was added to each well of 96-well plate. The biologically and chemically synthesized silver nanoparticles were added to the bacterial suspensions in the appropriate volume 15 µl of AgNPs to 105 µl of bacterial culture and incubated 72h at 22°C. The biofilm amount in a 96-well plate was quantified by crystal violet (CV) staining according to the method by Christensen et al. [8] with some modifications. The concentration of CV was determined by measuring the optical density of solution at 620 nm using a microtiter plate reader. The bacterial suspensions were used as controls. The experiment was performed in triplicates and average values were obtained.

2.4 Evaluation of antibacterial properties of chem- and bioAgNPs towards selected bacteria

2.4.1 Evaluation of antibacterial activity of chem- and bio-AgNPs by MT microplates method

The experiment was carried out in the MT2 MicroPlates™ (Biolog). They contain only mineral salts and redox dye to colorimetrically indicate the activity of bacteria. The bacterial cultures were prepared as described in 2.3.1. 105 µl of the strains suspensions were added to each microplate well, and 15 µl volumes of bio- or chem-AgNPs solutions. The microplates were incubated in the OmniLog Reader (Biolog) for 84 hours at 22°C. During the incubation, the colour development was recorded by camera every 15 minutes. We calculated from raw data the average value of color development and plotted a kinetic

curve for each tested variant. The bacterial suspensions without AgNPs were used as the control. The experiment was done in three replicates.

2.4.2 Determination of antibacterial activity of chem- and bio-AgNPs by disc diffusion method

Bacterial cultures were obtained as described in section 2.3.1. 100 µl of each bacterial suspension (0.4–0.5 McFarland's standard, approximately 10^7–10^8 CFU/ml) was spread onto Mueller-Hinton agar plates. The sterilized disks were put on these freshly prepared lawns, and then 10 µl chem- and bio-AgNPs solutions were added on the discs. The plates were incubated at 22°C for 72 h. The diameter of inhibition zone around each disc was measured. Experiments were conducted in triplicates and average values of inhibitory zone were determined.

3 Results and Discussion

The physical properties of AgNPs formed in bio- and chemical synthesis are discussed by Mendrek et al. [3]. The antibiofilm effect of bio-AgNPs and chem-Ag-NPs is presented on Figure 1. The antibiofilm activity was observed after 72 hours of incubation. The reduction in biofilm formation was observed in 10 tested strains as a result of biologically synthesized AgNPs.

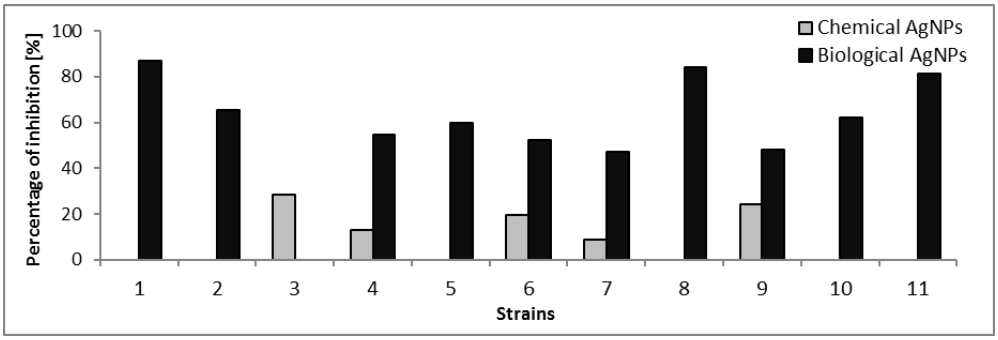


Fig. 1. The inhibition in biofilm formation by selected strains. Strains: 1. *Advenella incenata*, 2. *Moraxella osloensis* or *Enhydrobacter aerosaccus*, 3. *Kocuria rhizophila* or *Psychrobacter* sp., 4. *Microbacterium oxydans*, 5. *Bacillus thuringiensis*, 6. *Advenella incenata*, 7. genus *Advenella*, 8. *Micrococcus luteus*, 9. genus *Micrococcus*, 10. genus *Micrococcus*, 11. genus *Pseudomonas*.

However, the potency of bio-AgNPs biofilm inhibition was different. The maximum antibiofilm activity of biological AgNPs were observed for *A. incenata* (87% reduction), *M. luteus* (84% reduction) and strain belong to the genus *Pseudomonas* (81% reduction). No effect of biosilver nanoparticles was observed only for bacteria identified as *K. rhizophila* or *Psychrobacter sp*. The reduction of biofilm formation as an impact of chemically synthesized AgNPs were observed in five strains. In the case of these strains the reduction in biofilm formation were relatively low and ranged from 9% to 29%. The highest precentage of biofilm inhibition by chem-AgNPs were observed for *K. rhizophila* or *Psychrobacter* sp. and *Micrococcus* sp., 29% and 24%, respectively.

3.1 Antibacterial activity of chem- and bio-AgNPs

In Table 1 the results from the agar diffusion method are presented. The inhibition zone was observed for bio-AgNPs against almost all selected strains. However, there was no bio-AgNPs activity against *M. oxydans* (Fig. 3B). The highest antibacterial effect was observed against *A. incenata* and strain belong to the genus *Micrococcus* with inhibition zone of 16 mm for both strains (Fig. 3A). Biological Ag-NPs showed also high antibacterial effect against *A. incenata, B. thuringiensis, Pseudomonas* sp., and *K. rhizophila (or Psychrobacter* sp.), with inhibition zone of 15, 15, 14, 13 mm, respectively.

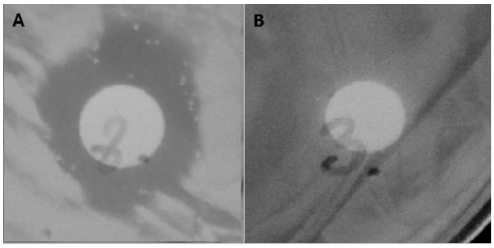


Fig. 3. Antibacterial activity: A. *Advenella incenata* and bio-AgNPs; B. *Microbacterium oxydans* and bio-AgNPs.

Table 1. Antibacterial activity spectrum of silver nanoparticles synthesized biologically and chemically obtained from disc diffusion method.

Strains	Diameter of inhibition zones (average values., mm)		
	Chem Ag- NPs	**Bio- AgNPs**	**Control**
1. *Advenella incenata*	0	15	0
2. *Moraxella osloensis* or *Enhydrobacter aerosaccus*	0	10	0
3. *Kocuria rhizophila* or *Psychrobacter* sp.,	0	13	0
4.*Microbacterium oxydans*	0	0	0
5. *Bacillus thuringiensis*	0	15	0
6. *Advenella incenata*	0	16	0
7. genus *Advenella*	0	9	0
8. *Micrococcus luteus*	0	9	0
9. genus *Micrococcus*	0	16	0
10. genus *Micrococcus*	0	10	0
11. genus *Pseudomonas*	0	14	0

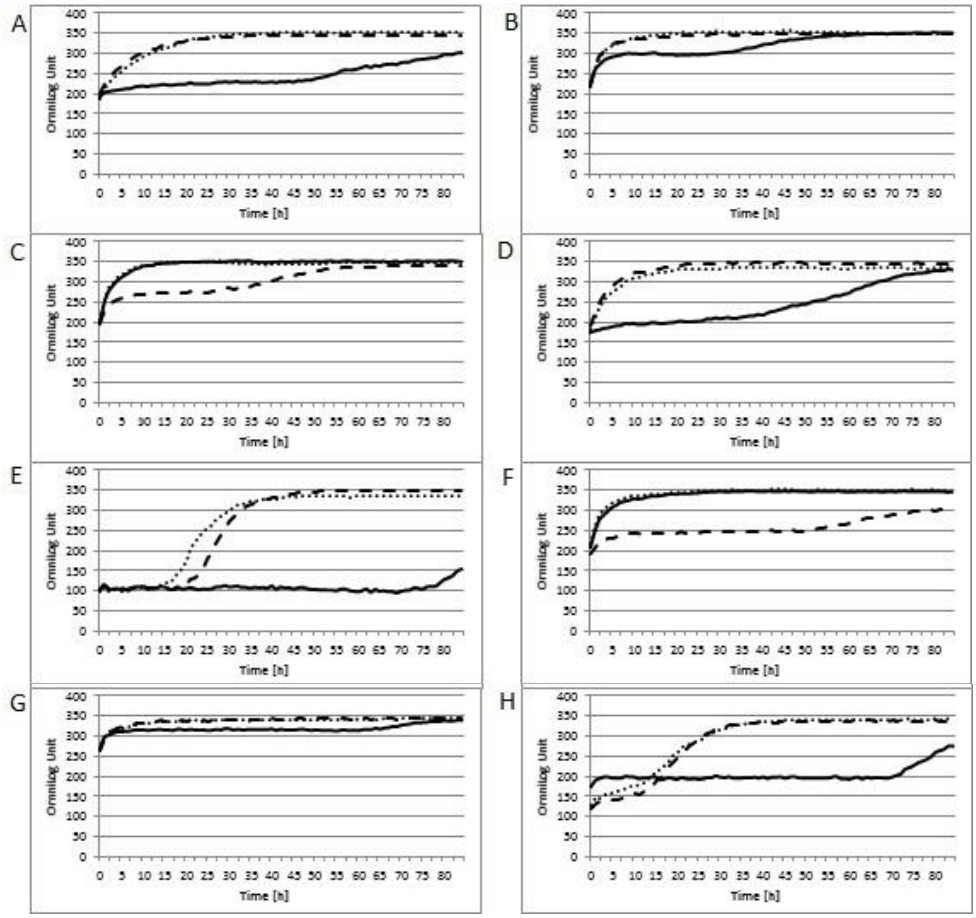
The results obtained from the MT assay are presented on Figure 2. Based on the results obtained from the analysis of the kinetic curve was found that all strains in control samples have reached the plateau phase between 5 and 35 hour of experiment (Fig. 1A–K). Based on kinetic curves there were no differences between the control and samples incubated with bio- AgNPs for strain having the sequence nearest *M. osloensis* or *E. aerosaccus* (Fig. 1 B), *K. rhizophila* or *Psychrobacter* sp.(Fig. 1C), *A. incenata* (Fig. 1F), strains belong to the genus *Advenella* (Fig. 1G) and genus *Micrococcus* (Fig. 1I). A clear effect of the chem-AgNPs was observed for strain belongs to the genus *Micrococcus*. Based on the kinetic curve analysis it can be concluded that the microbial growth is inhibited (Fig. 1J). In other cases, although the initial growth inhibition were observed, inhibitory effect of nanoparticles has been overcome for *M. oxydans* after 42 hours of incubation with chem-AgNPs (Fig.1D), for *A. incenata* after 55 h (Fig.1A), in case of *M. luteus* and strain belongs

to the genus *Pseudomonas* after 70 h (Fig. 1.H, K) and for *B. thuringiensis* after 75 hour of experiment (Fig.1E).

The kinetic curves obtained after incubation with a bio-AgNPs show very weak effects of bio-AgNPs on the tested strains. Based on kinetic curves of eight strains there were no differences between the control and samples incubated with bio- AgNPs (Fig.1 A, B, D, E, G, H, J and K). In the case of the strain identified as *K. rhizophila* or *Psychrobacter sp* in the first 27 hours it was observed inhibition effect, after this time there has been growth of the activity of the bacteria and the inhibitory effect of nanoparticles has been overcome (Fig. 1 C). A similar trend was observed in the case of *A. incenata* (Fig. 1 F), and strain belongs to genus *Micrococcus* (Fig. 1 I) where the increase in activity was observed, respectively 55 and 60 hours of incubation with bio-Ag-NPs.

In this study, biogenic silver nanoparticles had potent antibiofilm effects. Antibacterial effects of AgNPs have been previously studied [14, 16], but there are a few studies on effects of AgNPs against bacterial biofilm. In most of the research the pathogenic bactera was applied [18].

While, the study reported by Sheng et al. [22] describes the effect of chemically AgNPs on intact wastewater biofilms from a local wastewater treatment plant. The results indicate that AgNP treatment decreased microbial community diversity but did not significantly affect the microbial community function. This provides direct evidence for the functional redundancy of microbial community in engineered ecosystems such as wastewater biofilms.



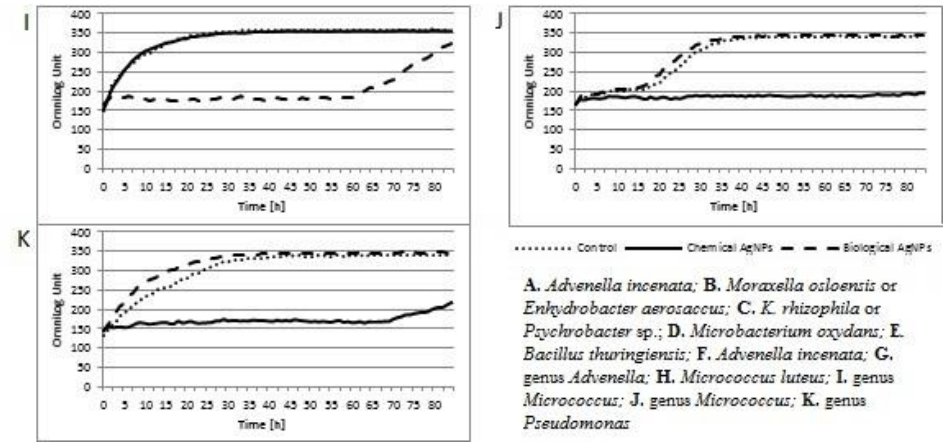


Fig. 2. The scheme of bacteria sensitivity profile to chem- and bio-AgNPs obtained by using the MT microplates. Bacteria sensitivity profile displayed in the form of kinetic graphs of the their growth.

Also, inhibition of nitrification byAgNPs has been relatively well-characterized in the previous research. It was presented that nitrification can be inhibited by AgNPs even at concentrations lower than1mg Ag/l [23]. Ammonia oxidizing bacteria (AOB) are more sensitive to AgNPs than nitrite oxidizing bacteria (NOB) [26]. The effects of AgNPs on other microbial functional groups in biological wastewater treatmentsystems are far less well studied. Biofilms are difficult to break due to their extracellular matrix. Grun et al. reported that highly reactive Ag+ ions bind to biological molecules, like proteins and polysaccharides within the EPS. Probably, AgNPs or released from them free Ag+ ions may be involved in blocking the formation of some compounds of EPS [1]. Kalishwaralal et al. said that exopolysachharide synthesis is arrested by AgNPs, and microorganism cannot form biofilm [15]. Which may explain the impact of nanoparticles to inhibit the formation of the biofilms. Low antimicrobial effects on the selected strains observed in our study might by associated with low concentration of AgNPs in our experiment. The penetration rate of the biofilm may also differ between strains. The ability of silver nanoparticles to agglomerate may also have meaning to their the activity [17]. Therefore, Nookola et al. [19] demonstrated antibacterial and antifungal properties of chemical silver nanoparticles on environmental strains. The nanoparticles were coated by citrate, which would prevent aggregation [19]. Rai et al. shown that silver nanoparticles can be used as effective antimicrobial agents for Gram-negative and Gram-positive bacteria, including antibiotic-resistant bacteria [16]. However, in our study AgNPs are not so effective in killing bacterial strains (the results from MT microplates), but we observed promising activity biol-AgNPs on the inhibition of biofilm formation. From the results from our study the biological silver nanoparticles can be involved in biofilm prevention. Silver nanoparticles, as opposed to chemical disinfectants, are not strong antioxidants and these are chemicals that behave indifferently in water [20].

4 Conclusions

1. No relations of results obtained from two applied antibacterial methods was noted.
2. Biologically synthesized AgNPs was more active against tested bacteria than chemically synthesized AgNPs in traditional disc diffusion method.
3. Higher antibacterial activity and reduction of biofilm formation was observed for biogenic AgNPs.

4. The further research and development are necessary to complete the knowledge on bio-AgNPs effects towards environmental bacteria and biofilm formation process.

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