ASSESSMENT OF BIOLOGICALLY SYNTHESIZED Ag NANOPARTICLES TOXICITY AGAINST *E. coli, Staphylococcus aureus, Parachlorella kessleri* AND *Sinapis alba*

JANA KADUKOVÁ[1], OKSANA VELGOSOVÁ[1], ANNA MRAŽÍKOVÁ[1], RENÁTA MARCINČÁKOVÁ[1], EVA TKÁČOVÁ[2]

[1]*Technical University in Košice, Faculty of Metallurgy, Department of Material Science, Park Komenského 11, Košice, SK- 042 00, Slovak Republic*
[2]*Regional Office of the Public Health, Ipeľská 1, Košice, SK-040 11, detached office Department of Environmental Microbiology, Roosveltova 8, Košice, SK- 040 01, Slovak Republic*

Abstract: In general, Ag^+ ions and AgNPs are considered to be the most toxic for bacterial cells and less toxic for higher organisms. In the present work inhibitory effects of biologically prepared silver nanoparticles on the growth of bacteria *E. coli* CCM 3954 and *Staphylococcus aureus* CCM 3953, green microscopic alga *Parachlorella kessleri* LARG/1 and seed germination and root growth of plant *Sinapis alba* seeds were investigated. Surprisingly, silver nanoparticles showed much stronger inhibitory effects on plant seed germination and root growth than on the bacterial growth. At concentration of 75 mg/l AgNPs both seed germination and root growth of *Sinapis alba* was inhibited whereas inhibition of the growth of *E. coli* and *S. aureus* was observed at >195 mg/l. Growth inhibition of alga *Parachlorella kessleri* was recorded at 300 mg/l AgNPs concentration. The inhibitory effect of silver ions was much higher compared to silver nanoparticles. Even 20 mg/l concentration of Ag^+ ions inhibited the root growth and concentration > 45 mg/l inhibited germination of *Sinapis alba* seeds. Inhibition zones in both studied bacteria were found at concentration > 140 mg/l.

Keywords: silver nanoparticles, growth inhibition, germination tests, *Sinapis alba, Parachlorella kessleri, E. coli, Staphylococcus aureus*

1. Introduction

Engineered nanoparticles have received a lot of attention recently due to their rapidly increasing applications. Rapid developments in the manufacture and use of engineered nanoparticles have also led to an urgent need for assessing their possible risk to humans and the environment, but environmental risk assessment significantly lags behind invention and today's global consumption volumes (WANG *et al.,* 2014; MOOS *et al*., 2014). According to the Woodrow Wilson Database there were more than 1300 nanotechnological consumer products on the market by March 2011 and 313 of them contained nanosilver. Currently, nanosilver is perhaps the most preferred antimicrobial nanomaterial (IVASK *et al.,* 2014). However, release of biocidal NPs from consumer and household products into the waste streams and further into the environment may pose treat to non-target organism (WANG *et al.,* 2014).

Antimicrobial properties of silver nanoparticles are mostly studied using common tests with bacteria, rarely by tests involving algae or higher plants. But there is

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significant difference among toxic concentrations found by different scientists, while some of them declare as toxic concentrations in nanomoles others found that millimoles are effective against particular organisms (SHARMA *et al*., 2009). So there is still need to find what influence the toxicity of nanoparticles. Therefore, better knowledge of AgNPs toxicity mechanisms is required in order to evaluate the environmental risk of their toxicity (OUKARROUM *et al.,* 2012).

As there are some assumptions that biologically produced nanoparticles are less toxic against organisms the aim of the article was to focus on the toxic effects of biologically produced AgNPs on G^- and G^+ bacteria, microscopic algae and higher plants (seeds of *Sinapis alba*).

2. Materials and methods

2.1 Nanoparticle characterisation

Synthesis of AgNPs by the algae *Parachlorella kessleri* (syn. *Chlorella kessleri*) strain LARG/1, supplied by Institute of Botany, Slovak Academy of Sciences was carried out according to the method described previously (KADUKOVA *et al*., 2014). Algae were added into Erlenmeyer flasks containing 0.5 mM silver nitrate solution. Spherical silver nanoparticles in the 12 nm average size range were used in the testing. Prepared nanoparticles were characterised by UV-VIS spectroscopy (UNICAM UV/VIS Spectrometer UV4, CHROMSPEC Company) and TEM microscopy (JEOL model JEM-2000FX microscope operated at an accelerating voltage of 200 kV).

2.2 Determination of the bacterial growth inhibition

Bacterial strains *Staphylococcus aureus* CCM 3953 and *Escherichia coli* CCM 3954 from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic were used. The supplied disk with bacteria was impaled on the sterile needle and transferred onto the agar slope in the tube. It was incubated for 1 – 4 hours at 37±2 °C. After that the culture was left to flow on the whole agar slope surface by the slow movement of the tube and it was consequently incubated for 24 – 48 hours. Pre-cultivated culture was inoculated on the sterile blood agar (M 144 A Columbia blood agar base 1 % from Himedia company with the following composition: peptone 23 g/l, corn starch 1 g/l, NaCl 5 g/l, agar 10 g/l, final pH 7.3) in Petri dishes. It was cultivated at 37±2 °C for 24 hours. The 25 µl of nanoparticle suspension was dropped in the centre of both upper and lower part of the inoculated agar plate. The silver ion and silver nanoparticle concentration of 20, 45, 75 and 300 mg/l were tested. The agar plates were incubated at 36±2 °C for 22±4 hours. The growth inhibition zone around the tested drop was measured in mm. If no inhibition zone was observed, the agar plates were incubated next 24 hours.

2.3 Determination of the algal growth inhibition

Algal strain *Parachlorella kessleri,* (syn. *Chlorella kessleri*) strain LARG/1 supplied by Institute of Botany, Slovak Academy of Sciences was used for the

experiments. Algae were inoculated on Milieu Bristol agar plates. The composition of Millieu Bristol solution is: 750 mg/l $NaNO_3$, 25 mg/l $CaCl_2.2H_2O$, 75 mg/l $MgSO_4.7H_2O$, 20 mg/l Fe-EDTA, 75 mg/l K_2HPO_4, 175 mg/l KH_2PO_4, 20 mg NaCl, 2,86 mg/l H_3BO_3, 0,22 mg/l $ZnSO_4.7H_2O$, 1,81 mg/l $MnCl_2.4H_2O$, 0,0306 mg/l MoO_3, 0,08 mg/l $CuSO_4.5H_2O$, 0,09 mg/l $CoSO_4.7H_2O$. The pH of the solution was adjusted to 7 before sterilization.

For the study of the AgNPs and Ag^+ ions effect on algal culture green alga *P. kessleri* was inoculated into 50 ml of sterile solutions containing silver nanoparticles or $AgNO_3$. Silver concentration in AgNPs solutions and Ag^+ solutions were 20, 45, 75 and 300 mg/l. The culture were incubated under continuous lighting (four 36 W, cool white fluorescent tubes) with average light intensity 3 720 lx at 20 – 24 °C for 14 days.

The 4 drops (25 µl) of tested nanoparticle suspension were placed on the plate surface. The procedure was repeated for each selected silver nanoparticle concentration (20, 45, 75 and 300 mg/l). The same procedure was also carried out with the silver ion solutions. The plates were incubated under continuous lighting (four 36 W, cool white fluorescent tubes) with average light intensity 3 720 lx at 20 – 24 °C for 7 days. Inhibition zone was measured in mm. All experiments were done in triplicates.

2.4 Determination of the germination inhibition

Inhibition of germination was performed using the seeds of *Sinapis alba* according to the standard procedure STN 83 8303. The seeds were kept in a dry and dark place at room temperature before use. Before the experiments the seeds were surface sterilized in a 10 % sodium hypochlorite solution for 20 min followed by rinsing thrice with deionised water. The tested samples of 10 ml AgNPs or $AgNO_3$ solutions at concentrations of Ag 20, 45, 75 and 300 mg/l were added into 90×10 mm Petri dishes. The bottom of the used Petri dishes was covered with two pieces of filter paper. After that the seeds were transferred onto the filter paper, with 30 seeds per dish at 1 cm or larger distance between each seed.

Deionised water was used in control samples. Petri dishes were covered and placed in the dark place at room temperature. Germination and root length were evaluated every day during 3 days. All studies were done in triplicates. Root growth inhibition expressed by IC_{50} was calculated as follows:

$$I_i = \frac{L_K - L_V}{L_K}.100 \quad (1)$$

where I_i is a growth inhibition [%], L_K is an average root length in control [cm], L_V is an average root length at tested concentration [cm]. From the graphical plot for I_i as a function of log c_i the value IC_{50} was calculated.

3. Results and discussion

3.1 Bacterial growth inhibition

No growth inhibition of bacteria *E. coli* and *Staphylococcus aureus* were observed at AgNPs concentrations 20 mg/l after 24 and 48 hours, as well. The 12 mm inhibition

zones were observed in *E. coli* culture as well as *S. aureus* culture at Ag nanoparticles concentration of 195 mg/l. At concentration 75 mg/l growth inhibition in the location of the drop for *S. aureus* and partial growth inhibition for *E. coli* (visibly less colonies in the drop location) but no inhibition zone was observed. Some authors (SARAVANAKUMAR *et al.*, 2015) reported stronger inhibition effects on G^- bacteria *E. coli* in comparison with G^+ *S. aureus* but we did not observed it.

Results showed that silver ions caused stronger growth inhibition of the bacteria than silver nanoparticles. The silver ions concentration at which the 10 mm inhibition zone was observed at both studied bacteria was 140 mg/l. At 300 mg/l Ag^+ ion concentration 11 mm inhibition zone was present.

According to CHOI *et al.* (2008) IC_{50} of silver nanoparticles for *E. coli* was found to be 4 μM (0.43 mg/l). Growth inhibition of 55 % was found at the Ag nanoparticle concentration of app. 4.2 μM (corresponding to 0.5 mg/l). At the same silver ion (Ag^+) concentration the growth inhibition of *E. coli* culture was 100 %. Independently of silver form no growth was observed, when 1 mg/l of Ag (9.3 μM Ag) was added (CHOI *et al.,* 2008). According to PANACEK *et al.* (2006) and VIMALA *et al.* (2009) with the increase of the nanoparticle sizes their bactericidal effects decrease. *S. aureus* was more sensitive than *E. coli*. Toxic silver nanoparticle concentrations with the size of 44 nm for *E. coli* and *S. aureus* were 27 and 6.75 mg/l, respectively, and the nanoparticles at the size of 50 nm were toxic for *S. aureus* at the concentration of 54 mg/l. Silver nanoparticle and silver ion concentrations causing growth inhibition in selected bacteria are listed in the Table 1. On the other hand some authors (SHARMA *et al*., 2009; MIAO *et al*., 2009) claim that silver nanoparticle toxicity is significantly higher than silver ion toxicity and whereas the toxic concentration of silver nanoparticles is at the range of nanomoles the silver ions is at micromoles. RAI *et al.* (2008) found that the silver nanoparticles have 1.4 – 1.9 stronger antibacterial effects than silver ions.

It was supposed that the shape of nanoparticles is also important for silver nanoparticle toxicity - the most toxic are triangular nanoparticles in comparison with the spherical ones and the least toxic are rod shaped (PAL *et al.,* 2007). MORONES *et al.* (2005) found that the toxic concentration of cubooctahedral, icosahedral, decahedral silver nanoparticles with the size from 1 to 100 nm for *E. coli* was 75 mg/l.

3.2 Algal growth inhibition

During microscopic observation of algal culture no significant changes in the cell morphology were observed at the concentrations of 20, 45 and 75 mg/l. However, the colour of cells cultivated at the concentration of 300 mg/l turned from green to brown and the formation of aggregates occurred. The large aggregate formation was also observed by OUKARROUM *et al.* (2012), however, at much lower nanoparticle concentration (10 mg/l). The authors suggested that aggregate formations were caused by the presence of large nanoparticles (over 50 nm) which cannot directly enter the cell but can act as binding agents between algal cells resulting in algal cells growth inhibition. However, the size of our nanoparticles was up to 18 nm. Authors mentioned above studied the effects of chemically prepared Ag nanoparticles on the

Chlorella vulgaris cells and found that nanoparticles at concentration of 1 mg/l and 10 mg/l reduced the presence of viable cells by 33 and 88 %, respectively.

Table 1. Inhibitory concentration of silver nanoparticles and silver ions for the growth of selected bacteria.

Bacteria	**Inhibitory concentration of silver [mg/l]**	**Form of Ag/Size of particle [nm]**	**Source**
Staphylococcus aureus MRSA	3	Nano Ag	SHARMA *et al.* (2009)
	14.4	Nano Ag[1]/9	GUZMAN *et al*. (2012)
	28.8	Nano Ag[2]/14	GUZMAN *et al*. (2012)
	258.9	Nano Ag[3]/24	GUZMAN *et al*. (2012)
	215.7	Nano Ag[4]/30	GUZMAN *et al*. (2012)
Staphylococcus aureus CCM3953	5	Nano Ag	SHARMA *et al.* (2009)
Staphylococcus aureus	6.75	Nano Ag/25	PANACEK *et al.* (2006)
	6.75	Ag^+	PANACEK *et al.* (2006)
	3.125	Nano Ag	SARAVANAKUMAR *et al.* (2015)
	14.4	Nano Ag[1]/9	GUZMAN *et al*. (2012)
	28.8	Nano Ag[2]/14	GUZMAN *et al*. (2012)
	258.9	Nano Ag[3]/24	GUZMAN *et al*. (2012)
	215.7	Nano Ag[4]/30	GUZMAN *et al*. (2012)
	195	Nano Ag/12	this work
	140	Ag^+	this work
E. coli	3.38	Nano Ag/25	PANACEK *et al.* (2006)
	1.69	Ag^+	PANACEK *et al.* (2006)
	1.8	Nano Ag	SHARMA *et al.* (2009)
	14.4	Nano Ag[1]/9	GUZMAN *et al*. (2012)
	28.8	Nano Ag[2]/14	GUZMAN *et al*. (2012)
	258.9	Nano Ag[3]/24	GUZMAN *et al*. (2012)
	215.7	Nano Ag[4]/30	GUZMAN *et al*. (2012)
	10	Nanotriangles Ag	PAL *et al.* (2007)
	125	Nanospheres Ag	PAL *et al.* (2007)
	1	Nano Ag	CHOI *et al.* (2008)
	0.5	Ag^+	CHOI *et al.* (2008)
	3.125	Nano Ag	SARAVANAKUMAR *et al.* (2015)
	195	Nano Ag/12	this work
	140	Ag^+	this work

[1]prepared by sodium dodecyl sulphate + hydrazine; [2]prepared by sodium dodecyl sulphate + hydrazine + sodium citrate; [3]prepared by hydrazine + 1 mM sodium citrate; [4]prepared by hydrazine + 2 mM sodium citrate

The inhibition of algal growth on the agar plates was clearly visible only if solution with 300 mg/l nanoparticle concentration was dropped on the plate surface. The inhibition zone of 6.6 mm was observed. At lower nanoparticle concentration

(75 mg/l) the inhibition zone was not observed but in the drop location slower cell growth was observed within first 6 days. After 6 days algal cells grew on the whole agar surface. At the lowest studied nanoparticle concentration (45 mg/l) no differences were observed on the agar surface.

3.3 Determination of the germination inhibition

Germination assay is a basic procedure to determine toxic effects on plants. Seed germination and the early seedling growth are more sensitive to metal ions toxicity in comparison with mature plants because some of the defense mechanisms have not been developed yet (XIONG and WANG, 2005).

Dependence of inhibition of *Sinapis alba* seed germination on silver nanoparticle or Ag^+ ions concentration is shown in Fig. 1. At concentrations of 20 and 45 mg/l slight inhibition effects were observed. The concentration of 300 mg/l almost entirely inhibited the germination. At concentrations of 75 and 300 mg/l dark zones surrounding the seeds were observed. The Ag^+ ions showed significant inhibitory effect on seed germination compared to the silver nanoparticles. The germination inhibition was 65 % at concentration of 45 mg/l and at the 300 mg/l the inhibition was 100 %. But the concentration of 20 mg/l did not have such detrimental effects. Although, it caused slowing of the germination processes, the inhibition of germination was only 20 %.

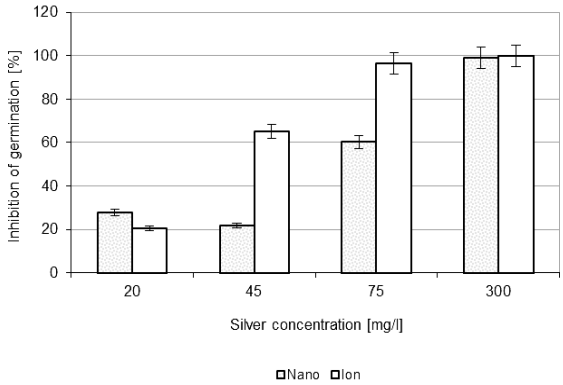

Fig. 1 Inhibition of seed germination by silver nanoparticles and Ag^+ ions at concentration of 20, 45, 75 and 300 mg/l Ag NPs and Ag^+ ions in the form of $AgNO_3$.

BARRENA *et al*. (2009) and EL-TEMSAH and JONER (2012) reported that silver nanoparticles (size 29 nm) exerted visible inhibitory effects on the germination of cucumber and lettuce seeds, nanoparticles with the size < 100 nm inhibited germination of ryegrass and barley, however, no other toxicity effects were observed.

Regarding the root growth the similar inhibitory influence of nanoparticles as that in germination was observed, as well (Fig. 2). Significant difference of the inhibitory effect between the concentration of 20 and 45 mg/l was not found. However, a further increase of the concentrations up to 300 mg/l led to the significant increase of root

growth inhibition. The inhibition of root growth by Ag^+ ions was even stronger. At concentration > 45 mg/l it was above 90 %.

The colour of seeds treated with the 75 and 300 mg/l of AgNPs solutions turned to dark brown. However, no root colour changes as was mentioned by LEE *et al.* (2012) were observed in our experiments. Interesting was the formation of darker zones around the seeds (at the highest concentration almost black). Accumulation of Ag NPs could cause the zone formation which could be a part of detoxifying mechanisms.

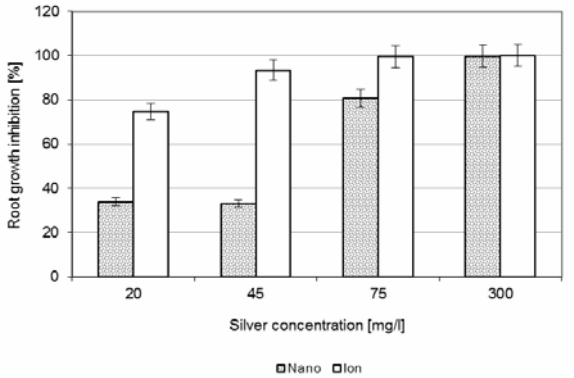

Fig. 2 Inhibition of root elongation by silver nanoparticles and Ag^+ ions at concentration of 20, 45, 75 and 300 mg/l Ag NPs and Ag^+ ions in the form of $AgNO_3$.

LEE *et al.* (2012) observed that the growths of *Phaseolus radiatus* seedlings and *Sorghum bicolor* seedlings growing on agar plates were adversely affected by increasing the AgNPs exposure concentrations from 5 to 40 mg/l. The growth reduction was from 35 – 80 % and 40 – 53 % for *P. radiatus* and *S. bicolour*, respectively. The authors found the EC_{50}s of the AgNPs of *P. radiatus* and *S. bicolor* were 13 and 26 mg/l, respectively. Brown tips and necrosis were detected in the exposed roots of both plants. In their study the citrate-coated nanoparticles (size 5 – 25 nm) were used.

In the case of silver ion solution, brown colours of seeds or brown spots on their surfaces were more often present in comparison with AgNPs. On the other hand, zones around seeds had lighter colour. Not only root elongation but also development of seed leaves was strongly affected by silver ion presence as seed leaves were observed only at Ag^+ ions concentrations of 20 and 45 mg/l.

In both studied parameters (germination and root length) significant inhibition was observed in the case of silver ions. According to the standard procedure STN 83 8303 the preliminary IC_{50}s 5.2 mg/l and 42.8 mg/l for Ag^+ ions and silver nanoparticles, respectively, were calculated.

4. Conclusion

In general, Ag^+ ions and AgNPs are considered to be the most toxic for bacterial cells and less toxic for higher organisms. However, our results revealed that AgNPs

expressed much stronger inhibitory effects on plants than bacteria. At concentration of 75 mg/l AgNPs both seed germination and root growth of *Sinapis alba* was inhibited whereas inhibition of the growth of *E. coli* and *S. aureus* was observed at >195 mg/l. Silver ions had stronger inhibitory effects on bacterial growth as well as *Sinapis alba* seeds germination and root growth than AgNPs. In the case of the green alga *Parachlorella kessleri* slight algal growth inhibition on agar plates was observed at AgNPs concentration of 75 mg/l and visible inhibition zones were reported at 300 mg/l.

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