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Antibacterial activity and toxicity of silver - nanosilver versus ionic silver

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Abstract. The *in vitro* study of antibacterial activity of silver nanoparticles (NPs), prepared via modified Tollens process, revealed high antibacterial activity even at very low concentrations around several units of mg/L. These concentrations are comparable with concentrations of ionic silver revealing same antibacterial effect. However, such low concentrations of silver NPs did not show acute cytotoxicity to mammalian cells - this occurs at concentrations higher than 60 mg/L of silver, while the cytotoxic level of ionic silver is much more lower (approx. 1 mg/L). Moreover, the silver NPs exhibit lower acute ecotoxicity against the eukaryotic organisms such as *Paramecium caudatum*, *Monoraphidium* sp. and *D. melanogaster*. The silver NPs are toxic to these organisms at the concentrations higher than 30 mg/L of silver. On contrary, ionic silver retains its cytotoxicity and ecotoxicity even at the concentration equal to 1 mg/L. The performed experiments demonstrate significantly lower toxicity of silver NPs against the eukaryotic organisms than against the prokaryotic organisms.

1. Introduction

Fundamental and applied physico-chemical research in the field of nanomaterials has witnessed rather great boom in the last few years. Nanomaterials attract attention due to their unique physico-chemical properties that are rooted in their diameter, eventually in their large surface area. These unique properties cannot be additionally found for the chemically identical material in its bulk form. The nanoparticles (NPs) are not nowadays only the target of scientific research but they can be continuously more and more frequently found not only in scientific laboratories, industrial applications, and chemical technologies but also as a part of common life due to their usage in commercially available products.

Silver NPs represent material, which can be used as a potential antibacterial agent in medical applications and different commercial products due to its biological activity [1,2]. Recently, silver NPs as well as various silver-based compounds containing ionic silver (Ag⁺), exhibiting high antimicrobial activity, have been synthesized [3-11]. Especially silver NPs, with magnificent bactericidal activity against both gram-positive and gram-negative bacteria including multi-resistant strains, can be

considered as potential disinfection agents usable in variety practical applications [3,5,10-12]. Silver NPs can effectively eliminate bacteria and yeasts even at rather low concentrations in units of mg/L [5,7]. These low concentrations are not additionally toxic against higher organisms [7,13]. Furthermore, there has not been proved any bacterial resistance against silver NPs, which however represents rather crucial problem in the case of conventionally prescribed antibiotics [14,15]. The silver NPs can be, due to its high antibacterial activity, low toxicity against higher organisms and unproved bacterial resistance, considered one of the greatest antibacterial agents for the treatment of burns [12] or for the prevention of bacterial colonization on catheters, prosthetics and dental materials [3,16-21]. Since the beginning of the 21st century, silver NPs have been widely used as antibacterial agents in many devices of everyday usage, e.g. textile coating or various cosmetic or disinfectant products. However, toxicity of silver NPs against living organisms and/or environment still remains an unsolved question.

2. Experimental Part

2.1. Synthesis, Stabilization, and Characterization of Silver Nanoparticles

The modified Tollens process was used for the preparation of silver nanoparticles (NPs) [5,22]. This process is based on the reduction of the complex cation $[\text{Ag}(\text{NH}_3)_2]^+$ by a suitable reducing substance. Two disaccharides, D-(+)-maltose monohydrate (p.a., Riedel-de Haën), D(+)-lactose (p.a., Fluka), and two monosaccharides D-glucose (p.a., Fluka) and D(+)-galactose (p.a., Fluka), were used as the reducing agents. The concentrations of the reaction components were as follows: 10^{-3} mol/L for silver nitrate (99.9 %, Tamda), $5 \cdot 10^{-3}$ mol/L for ammonia (p.a., 25% [w/w] aqueous solution, Lachema), 10^{-2} mol/L for sodium hydroxide (p.a., Lachema) and 10^{-2} mol/L for the reducing agents. Sodium hydroxide was added to the reaction mixture for the initiation of the reduction and for the purpose of the reduction completed within few minutes. pH of the reaction system was established at the value 11.5. The reduction process was continually monitored by the measurement of turbidity of the colloid system as the formation proceeded. All the measurements were performed at laboratory temperature (23 °C). Subsequent stabilization of the as-prepared silver NPs was performed by an addition of the chosen stabilizer in the final concentration of 1% (w/w). The tested stabilizers involved sodium dodecyl sulfate (SDS; > 98%, Aldrich), polyoxyethylenesorbitan monooleate (Tween 80; > 98, Aldrich), and polyvinylpyrrolidone (PVP) of the average molecular weight 360,000 (PVP 360, Aldrich).

The prepared silver NPs were characterized by means of dynamic light scattering (DLS) at Zeta Plus analyzer (Brookhaven, USA). The average values of the particle diameters were determined from the DLS measurements. Microscopic TEM images of the silver particles were obtained at JEM 2010 (Jeol, Japan) transmission electron microscope. UV/VIS absorption spectra of the silver NP dispersions were obtained at Specord S600 (Analytic Jena AG, Germany) spectrophotometer.

2.2. Antibacterial and Antifungal Assay

The antibacterial and antifungal activity of the synthesized silver NPs were tested using a standard dilution micromethod, determining the minimum inhibitory concentration (MIC) leading to inhibition of bacterial and yeast growth. The prepared silver NP dispersions were diluted 2-2048 times with 100 µL of Mueller-Hinton broth Difco (Bedston Dickinson) inoculated with the tested bacteria or yeast at a concentration of 10^5 to 10^6 CFU/mL. The obtained concentrations of silver in the dispersion ranged from 54 mg/L to 0.05 mg/L. The minimum inhibitory concentration (MIC) was read after 24 h (antibacterial assay) and after 36 h (antifungal assay) of incubation at 37 °C. The silver NP dispersions were used in the form, in which they had been prepared and modified. In addition, reference antibacterial tests with the solution of ionic silver and the used modifiers were also performed. As the standard reference strains (labeling according to the Czech Collection of Microorganisms, Czech Republic) there were used the following ones: *Staphylococcus aureus* CCM 3953, *Enterococcus faecalis* CCM 4224. Further, the following bacterial strains isolated from human clinical material at

the University Hospital in Olomouc were used: *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus faecium* (VRE). Antimycotic activity was tested using *Candida albicans* I, *Candida tropicalis* and *Candida parapsilosis* strains isolated from the blood of those patients at the University Hospital Olomouc who had confirmed candida sepsis.

2.3. Cytotoxicity and Ecotoxicity Assay

In vitro cytotoxicity of the silver NPs and ionic silver was determined using the BJ cell lines (human fibroblasts). The cells, cultured in Dulbecco's Modified Eagle's Medium (supplemented with 10% fetal calf serum, 4 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin) in a humidified CO₂ incubator at 37°C, were redistributed into 96-well microtiter plates at the appropriate densities for their respective cell sizes and growth rates. After 12-hour preincubation, the solution of ionic silver and dispersion of the silver NPs were added in order to get the final concentrations ranging from 0.3 mg/L to 60 mg/L. Following 72 hours of incubation, the cells were treated for 1 hour with Calcein AM, and live-cell fluorescence was measured at 485 nm/538 nm (ex/em) with the Fluoroskan Ascent microplate fluorometer (Labsystems). LC100 values, the concentrations of silver lethal to 100% of the cells, were determined from the dose-response curves. The cytotoxic effects of surfactant-stabilized silver NPs were not determined because the pure solutions of the SDS itself proved to be highly cytotoxic. As the used cell lines had no cell walls they could not adequately resist changes in the surface activity caused by the presence of the used surfactants.

Environmental toxicity of the silver NPs and ionic silver was tested also on the chosen aquatic organisms such as *Paramecium caudatum* (supplied by Faculty of Medicine, Masaryk University in Brno, Czech Republic), algae *Monoraphidium* sp. (obtained from Faculty of Science, Palacky University kindly provided by dr. P. Hasler) and also soil organism such as *Drosophila melanogaster* (wild type laboratory strain of *Drosophila melanogaster* was obtained from the *Drosophila* strains' collection of Masaryk University kindly provided by prof. J. Relichova). The silver NPs in the form of aqueous dispersion were added in to the cultivation medium required by the particular organism. The cultivation medium involved 5g of yeast per 1L of distilled water was used for *Paramecium caudatum* cultivation, standard cornmeal medium was used for *Drosophila melanogaster* cultivation, and *Monoraphidium* sp. was cultivated in BBM medium (Bold-Basal/Bristol Medium). The silver concentrations, which impact was tested, were in its final value in the interval from 0.3 mg/L up to 60 mg/L. The values of LC100, i.e. the values of lethal concentration leading to death of 100% of the tested organisms, were determined from the dependency of the toxic effect on the dose (dose-dependent study). The toxic effect against *Monoraphidium* sp. was determined on the decrease of the amount of chlorophyll a in the dependency of silver concentration. Chlorophyll a was extracted into acetone and its concentration was determined using spectrophotometry. The toxic effect of silver NPs against *D. melanogaster* was determined on the bases of the number of hatched adults in the dependency on the silver concentration. In the case of ecotoxicity against *P. caudatum*, the toxic index LT50 (i.e. the time in which 50% of the tested organisms died) was determined.

3. Results and discussion

3.1. Synthesis of Silver NPs Using the Modified Tollens Process

The silver NPs were prepared using the modified Tollens process, which is based on the reduction of the $[Ag(NH_3)_2]^+$ by a suitable reducing agent. Using D-maltose the silver NPs of spherical morphology and with the diameter of 25 nm were prepared. The silver NPs with the diameter of 35 nm, 44 nm, and 50 nm were also prepared using the modified Tollens process. However in this case different reducing sugars with different redox potential were used – lactose, glucose, and galactose [23]. The decreasing reducing power of the saccharides was reflected in larger particle diameter. The as-prepared silver NPs were characterized by the following methods: dynamic light scattering (DLS) and transmission electron microscopy (TEM). The images of the as-prepared silver NPs are listed in figure 1. The

images only confirmed that the tested particles were of the following diameters: 25nm (D-maltose) and 50 nm (D-galactose). The increase in the average diameter of the silver NPs was indirectly confirmed by the UV/VIS absorption spectra. The absorption peak, characteristic for metal silver, is called surface Plasmon and is shifter towards higher wavelength and broadens with the growing diameter of the silver NPs. Such changes in the absorption spectrum are characteristic for the silver NPs with increasing particle diameter (figure 1).

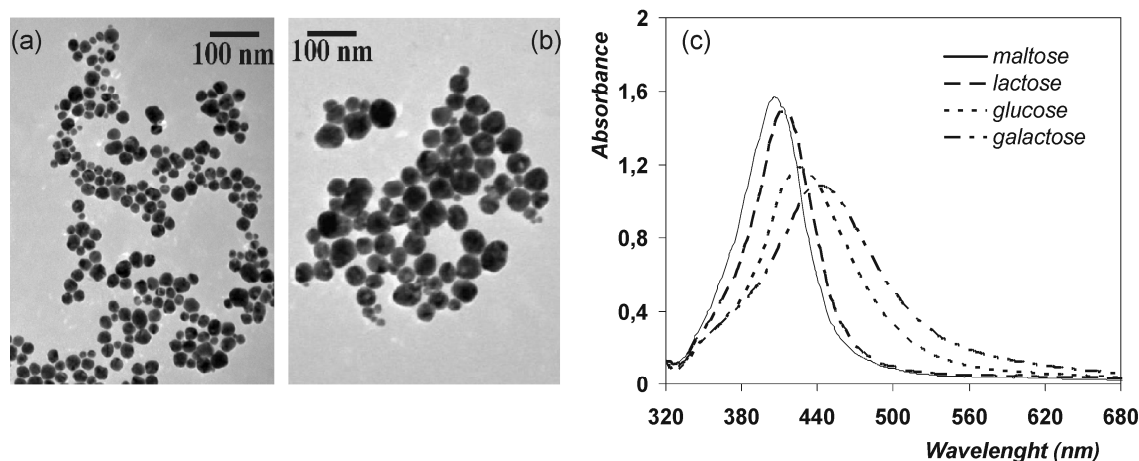


Figure 1. TEM images of silver NPs prepared by reduction via (a) maltose and (b) galactose and (c) absorption spectra of silver NPs prepared by various reducing substances.

3.2. Antibacterial and Antifungal Activity of Silver NPs

The silver NPs, with different diameters, prepared via the modified Tollens process were used for the determination of the dependency of the antibacterial effect on their diameter. The obtained values of MICs are listed in table 1. Based on these results it can be concluded that the antibacterial effect decreases with the growing particle diameter. The greatest antibacterial effect was gained with the smallest tested particles, having the diameter of 25 nm, which were prepared using D-maltose as the reducing agent. On contrary the lowest antibacterial effect was achieved with the particles prepared using D-galactose as the reducing agent. These large particles did not even reveal any antibacterial effect against *Enterococcus faecalis* CCM 4224 and *Enterococcus faecium* (VRE). The decrease in the antibacterial effect is evidently connected with the decreasing surface area, which is also reflected in reduced effective surface, which can come into contact with the present bacteria. Additionally, the 50 nm silver NPs cannot pass through the cell wall as easily as the particles having the diameter of 25 nm. Even though the antibacterial activity of the silver NPs, with the diameter equal to 25 nm, was rather high and their MICs were in the interval from 3.38 mg/L to 6.75 mg/L, in comparison with the antibacterial activity of ionic silver was slightly lower.

Table 1. Minimum inhibitory concentrations of silver (MICs), in the form of NPs with different particle diameter, against the tested bacteria.

Tested strain	Minimum inhibitory concentration (mg/L Ag)				
	Ionic silver	Size of Ag NPs/reducing agent			
		25 nm (Maltose)	35 nm (Lactose)	44 nm (Glucose)	50 nm (Galactose)
<i>Enterococcus faecalis</i> CCM 4224	1.69	6.75	54	-	-
<i>Staphylococcus aureus</i> CCM 3953	1.69	3.38	6.75	6.75	54
<i>Pseudomonas aeruginosa</i>	0.84	3.38	13.5	13.5	27
<i>Staphylococcus aureus</i> MRSA	0.84	3.38	27	27	54
<i>Enterococcus faecium</i> VRE	1.69	6.75	54	-	-

The silver NPs, with 25 nm diameter, were considered as the most effective and therefore all of the consequential experiments considering antifungal activity were performed just with these particles. Comparably with the antibacterial effect, also the antifungal effect of these silver NPs was reasonably high. It was proved by the obtained MICs values tested against the selected yeast. The MICs were equal to approx. 1 mg/L (table 2). The MICs values against the yeasts are lower than against the tested bacterial strains, which only confirmed that the tested yeasts are more sensitive on the presence of silver NPs. Interestingly, the antifungal activity of silver NPs and ionic silver equals. Yeasts revealed similar sensitivity against silver without any dependency on its form - nanoparticulate or ionic.

Although the antibacterial and antifungal activity of the silver NPs, with the average particle diameter equal to 25 nm, is reasonably high, it can be even enhanced. The enhancement of the aggregation stability of the silver NPs represents one of the ways. The silver NPs are highly sensitive on the presence of electrolytes, which can under the “suitable concentration” reflected in partial of complete aggregation of the originally well-dispersed particles that would be followed by sedimentation of the solid part of the dispersion. Partial aggregation proceeds also in the case when the dispersion of silver NPs is added to the bacterial cultivation medium in the course of the dilution test. The used cultivation medium involved a variety of electrolytes that are necessary for the optimal growth of the bacterial strains but on the other hand cause the unwanted aggregation of the tested particles. Partial aggregation of the silver NPs is then reflected in the existence of larger aggregates of the primarily prepared silver NPs and subsequently also in the decrease in the antibacterial effect. The problem of partial aggregation can be prevented by a suitable surface modification of the silver NPs, which can be performed by surfactants or polymers. In this study, the modification was performed by anionic (SDS) and non-ionic (Tween 80) surfactants and by polymer PVP 360. Majority of the differently modified silver NPs revealed lower MICs against strains than the unmodified silver NPs (table 2). Only in four cases the MICs of the modified silver NPs corresponded with the values determined for the unmodified ones. The antibacterial activity of the stabilized NPs was not negatively influenced by their partial aggregation, which was then reflected also in enhanced antibacterial activity of these particles. The unmodified silver NPs revealed the MICs in the interval from 3.38 to 6.75 mg/L; in the case of the modified particles the MICs values decrease a ranged from 1.69 mg/L to 3.38 mg/L. Comparable effect was achieved also in the tests evaluating the toxic effect of silver NPs against yeasts. The stabilized silver NPs showed antifungal effect in the concentration range from 0.052 mg/L to 0.84 mg/L, with respect to the tested sort of yeast and the type of the used stabilizer. On contrary, the unmodified silver NPs suppress the yeast growth in the concentrations interval from 0.42 mg/L to 1.69 mg/L.

The solutions of pure stabilizers (SDS, Tween 80, and PVP 360) did not reveal any antibacterial effect in the tested concentration which was equal to 1% (w/w). In the case of yeasts, the solution of SDS revealed partial suppressive effect. The SDS modified silver NPs can therefore exhibit better antifungal effect due to the synergic effect of the silver NPs and SDS.

Table 2. Minimum inhibitory concentrations (MICs) of the non-stabilized and stabilized silver NPs against the tested bacteria and yeasts.

Tested strain	Minimum inhibitory concentrations (mg/L Ag)				
	Ionic silver	Non-stabilized silver NPs	Stabilized silver NPs		
			SDS	Tween 80	PVP 360
<i>Staphylococcus aureus</i> CCM 3953	1.69	3.38	1.69	3.38	3.38
<i>Pseudomonas aeruginosa</i>	0.84	3.38	3.38	1.69	1.69
<i>Staphylococcus aureus</i> MRSA	0.84	3.38	1.69	3.38	1.69
<i>Enterococcus faecium</i> VRE	1.69	6.75	3.38	3.38	3.38
<i>C. albicans</i> I	0.42	0.42	0.052	0.1	0.1
<i>C. parapsilosis</i>	1.69	1.69	0.84	0.84	0.84
<i>C. tropicalis</i>	0.84	0.84	0.42	0.42	0.42

3.3. Cytotoxicity and Ecotoxicity of Silver NPs

Cytotoxicity of the silver NPs, with the diameter of 25 nm, in different concentrations was performed *in vitro* against human fibroblasts. The cytotoxicity was evaluated as the number of living cells using the fluorescent spectroscopy. The obtained dependency of the toxic effect of silver NPs on their concentrations can be found in figure 2a. It can be seen that the silver NPs cause death of the human fibroblasts at the concentrations higher than 60 mg/L. On contrary ionic silver cause death of all the tested fibroblasts already at the concentration equal to 2 mg/L.

Comparable results were gained also for the case of ecotoxicity tests of silver NPs against the organisms of *Monoraphidium* sp. and *D. melanogaster*. The silver NPs cause death of all the tested organisms at the silver concentration higher than 60 mg/L (figure 2b and 2c). On contrary ionic silver revealed to be more toxic against *Monoraphidium* sp., when all of the tested cells were death at the concentration equal to 3 mg/L of silver. Ecotoxicity of ionic silver against *D. melanogaster* was not monitored. The organism of *P. caudatum* was more sensitive to silver NPs. For this organism, the silver NPs were toxic at the concentrations equal to 30 mg/L and higher. However, in comparison with ionic silver, the silver NPs were further less toxic. Ionic silver cause death of the organisms of *P. caudatum* nearly immediately even at such low concentrations as 0.4 mg/L (figure 2d).

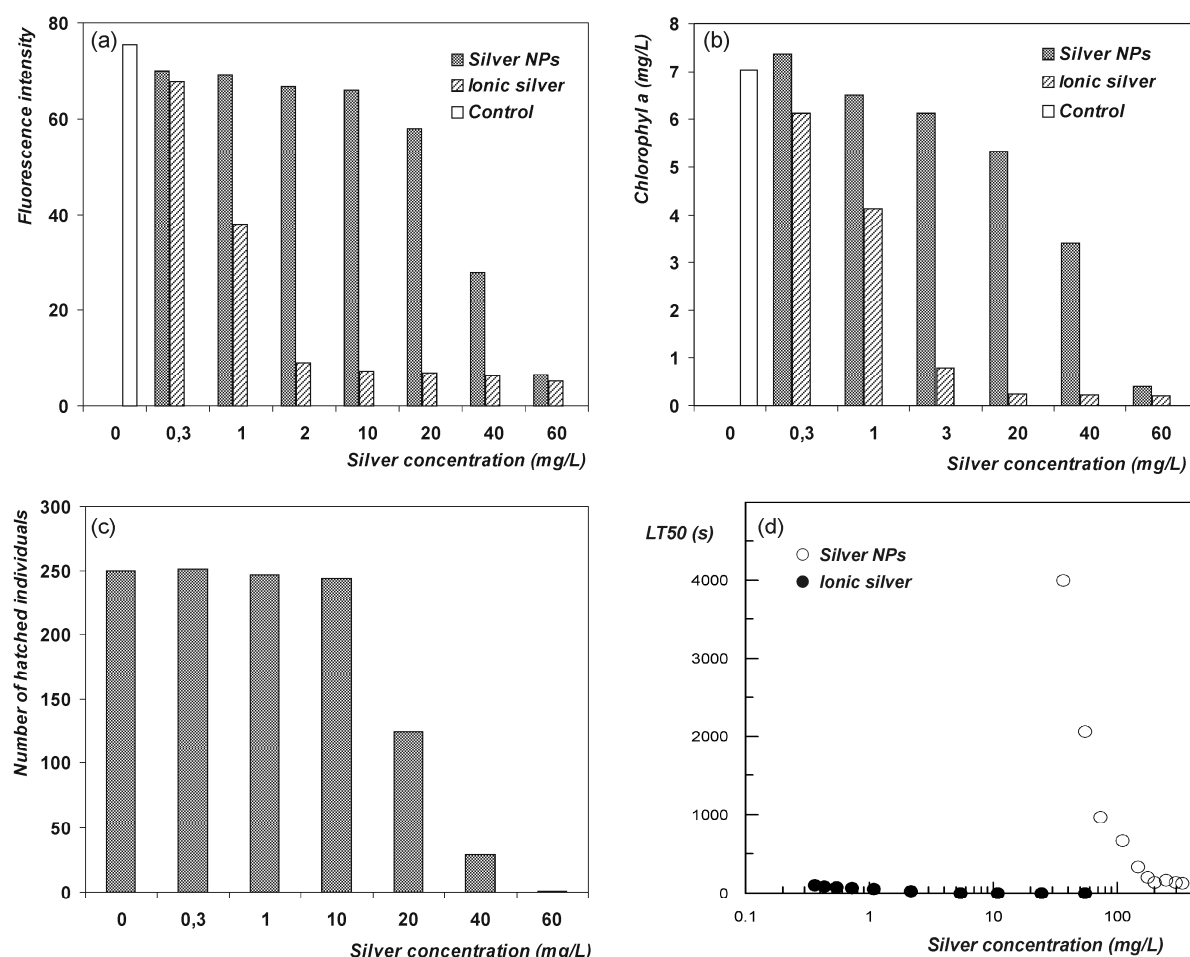


Figure 2. Toxic effect of silver NPs and ionic silver against (a) human fibroblasts, (b) *Monoraphidium* sp., (c) *D. melanogaster*, and (d) *P. caudatum*.

3.4. Comparison of Antimicrobial Activity and Toxicity of Silver NPs and Ionic Silver

The results of the antibacterial activity and toxicity of both of the silver forms, i.e. ionic and nanoparticulate, intermediate comparison of concentrations of silver leading to growth inhibition of pathogenic organisms with the concentrations evoking toxic effect against higher organisms (table 3). Due to this comparison it is possible to pronounce, which of both of the silver forms is more effective against the pathogenic organisms with respect to the low cytotoxicity and ecotoxicity. The silver NPs suppress the bacterial and yeast growth at the concentration interval from 1 mg/L to 3 mg/L with respect to the tested microorganism and to the applied modifier. Ionic silver inhibits the bacterial and yeast growth at the concentration approx. equal to 1 mg/L, which is the concentration comparable with the inhibition concentration of silver NPs. However, the cytotoxic (against human fibroblasts) and ecotoxic (against *Monoraphidium* sp., *D. melanogaster*, and *P. caudatum*) concentrations of ionic silver leading to the death of all of the tested organisms are significantly lower than the toxic concentrations of silver in the form of NPs. Therefore it can be concluded that silver NPs represent more significant antimicrobial agent than silver in its ionic form because silver NPs suppress the growth of microorganisms at such low concentrations that are not toxic against human cells and that do not also represent any risk for the environment.

Table 3. Comparison of antimicrobial activity and toxicity of silver NPs and ionic silver.

	Antimicrobial effect (MICs; mg/L)		Cytotoxicity and ecotoxicity (LC100; mg/L)			
	bacteria	yeasts	Human fibroblasts	<i>Monoraphidium</i> sp.	<i>Drosophila</i> <i>melanogaster</i>	<i>Paramecium</i> <i>caudatum</i>
Silver NPs	1-3*	1	60	60	60	30
Ionic silver	1	1	2	3	-	0.4

*MICs of modified silver NPs

4. Conclusion

Silver NPs has proved to be further more suitable antibacterial agent than the substances on the bases of ionic silver especially because of its significantly lower toxicity. The silver NPs, comparably with ionic silver, suppress bacterial and yeast growth at comparable concentrations equal to approx. 1 mg/L - 3 mg/L. Nevertheless, in the case of ionic silver these concentrations are also toxic against eukaryotic organisms including human cells. On contrary silver NPs effectively suppress bacterial and yeast growth at these concentrations that are not toxic to the tested human fibroblasts and also against *P. caudatum*, *D. melanogaster* or *Monoraphidium* sp. Based on these findings, the silver NPs do not represent any risk for human beings, when used in medical applications and commercially available products, but only under the condition that the silver concentration is retained at units of mg/L, which is sufficient for the suppression of bacterial and yeast growth.

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