

The Effect of Silver Nanoparticles on *Listeria monocytogenes* PCM2191 Peptidoglycan Metabolism and Cell Permeability

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Abstract

Listeria monocytogenes is Gram-positive bacterial pathogen, a causative agent of food poisoning and systemic disease – listeriosis. This species is still susceptible to several conventionally used antibiotics but an increase in its resistance has been reported. For this reason the search for new, alternative therapies is an urgent task. Silver nanoparticles seem to be the promising antibacterial agent. Minimal inhibitory concentration of silver nanoparticles was determined. Sublethal concentrations were used in study of nanosilver effect on cells lysis by estimation of the number of cells surviving the treatment with 0.25 or 0.5 of minimal inhibitory concentrations of silver nanoparticles. Autolysis of isolated peptidoglycan was studied by measuring the absorbance of preparation subjected to nanosilver treatment. Silver nanoparticles effect on *L. monocytogenes* envelopes permeability was determined by measuring the efflux of cF, DNA and proteins. It was demonstrated that nanosilver enhanced the lysis of *L. monocytogenes* cells and, to the lesser extent, autolysis of isolated peptidoglycan. The increase in the efflux of carboxyfluoresceine, DNA and proteins was also noted. The obtained results allow to postulate that *L. monocytogenes* peptidoglycan, constituting the main component of cell wall, is the target of silver nanoparticles activity against this pathogen.

Key words: *Listeria monocytogenes*, autolysis, peptidoglycan, permeability, silver nanoparticles

Introduction

An ionic form of silver has been used for centuries to cure several diseases which causative agents were bacteria such as *Staphylococcus aureus*, *Klebsiella* sp. and *Pseudomonas* sp. (Rai et al. 2009). It was shown that silver nanoparticles, AgNPs, have higher antibacterial activity than silver ions (Ingle et al. 2008). Nanoparticles are defined as the clusters of atoms of size from 1 to 10 nm with a large surface area to volume ratio, what is proportionally correlated with AgNPs antibacterial activity (Morones et al. 2005). Several studies demonstrated that antibacterial effect of silver nanoparticles is based on their interaction and subsequent damage of cell membranes and on the induction of reactive oxygen species (ROS), including free radicals. The interaction of AgNPs with cell membranes is promoted by their strongly positive zeta potential, which is a difference of an electric potential between the particle and the surrounding

solution (Stapsford et al. 2011). This interaction leads to membrane disruption, bacterial flocculation, efflux of cytoplasm, and as a consequence reduction in viability. Formation of ROS is responsible for oxidation, subsequent inactivation and damage of cellular proteins and DNA and peroxidation of lipids (Singh et al. 2008).

It was also proved that AgNPs are active against bacterial biofilms, which are complex bacterial communities resistant to antibiotics and the human immune system. Biofilm resistance is very important and now constitutes a medical challenge as recently the number of infections associated with antibiotic-resistant bacteria living in biofilms has been increased exponentially. These included infections caused by *Pseudomonas aeruginosa*, the causative agent of nosocomial respiratory tract pneumonia, infections of burn wounds, and chronic lung infections of patients with cystic fibrosis. Biofilm formed by *S. aureus* also constitutes a very important clinical problem being

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responsible of e.g. osteomyelitis, periodontitis and chronic wound infections. Well-defined diseases are also caused by biofilms formed by gastrointestinal tract pathogens belonging to Enterobacteriaceae family (for review see Markowska et al. 2013; Wolska et al. 2015). Beside their intrinsic antibacterial activity silver nanoparticles were proved to enhance the effect of conventional antibiotics, such as: vancomycin, amoxicillin, gentamycin, ampicillin, streptomycin in curing bacterial infections (Shahverdi et al. 2007; Birla et al. 2009).

Listeria monocytogenes is a Gram-positive pathogen able to survive in a variety of environments including food, soil and humans. It constitutes very severe problem for food industry because it can survive and multiply even in low temperature; moreover, it forms biofilm and therefore is hard to be eradicated from food production lines. This species is characterized by a unique life mode; it grows in the cytoplasm of the host cell and spreads between cells utilizing actin-based motility (Gray et al. 2006). This pathogen has an ability to cross three human barriers: intestinal, blood-brain and fetoplacental. *L. monocytogenes* is a causative agent of listeriosis, which can be a fatal infection especially for elder people, immunocompromised individuals, and pregnant women (Alleberger and Wagner 2010). The fatality rate achieves 30%, so this disease represents a major public health concern. Listeriosis in neonates is one of three principal causes of bacterial meningitides. The infections of central nervous system are also described in adults with the mortality rate reaching even 60% (Vázquez-Bolland et al. 2001). *L. monocytogenes* produces several virulence factors; the major one is listeriolysin O (LLO), a pore-forming toxin belonging to the family of cholesterol-dependent cytolysins that is crucial for escape from vacuole after entry to the mammalian cell (Hamon et al. 2012). The activity of LLO is vital for inducing diarrhea and an inflammatory response after reaching intestinal tract (Barbuddhe and Chakraborty 2009).

The peptidoglycan (murein) constitutes the main compound of *L. monocytogenes* cell wall and plays a major role in *L. monocytogenes* pathogenesis (Boneca 2005). Its structure is unusual for Gram-positive bacteria, remaining this of Gram-negative species (e.g. *E. coli*) because of the presence of partially deacetylated *N*-acetylglucosamine residues (Boneca et al. 2007). Another exceptional feature of this pathogen is its ability to encode a high number of surface proteins what reflects the ability of *L. monocytogenes* to survive in a range of diverse environments (Bierne and Cossart 2007). *L. monocytogenes* is still susceptible to a variety of antibiotic but it should be mentioned that it is intrinsically resistant to a broad spectrum of cephalosporins commonly used in the therapy of many bacterial infections. The resistance to cephalosporins is based on

several mechanisms including multidrug transporters and envelope proteins with a detoxification function (Krawczyk-Balska and Markiewicz 2016). In view of expanded resistance to antibiotics, the search for alternative therapies seems to be an urgent task.

The aim of the present study was to investigate the antibacterial effect of silver nanoparticles towards *L. monocytogenes* in order to identify the cellular target and mechanism of their activity.

Experimental

Materials and Methods

Bacterial strain, growth conditions and reagents used. Reference strain of *L. monocytogenes* PCM2191 was obtained from Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy in Wrocław, Poland). The strain was cultivated in tryptone soy yeast extract broth (TSYEB) medium (BTL, Poland) with constant shaking at 37°C. When required, the medium was supplemented with AgNPs and/or solidified with agar (15 g/l). Bacterial stock was stored in freezing solution containing 10% dimethyl sulfoxide (DMSO; v/v). All reagents were ultrapure and were purchased from Sigma, Germany.

Silver nanoparticles. Colloidal water solution of AgNPs was obtained from Nano-Tech (Warsaw, Poland). It contains nanosilver 4N in a concentration of 50 mg/kg, i.e. 50 ppm. The diameter of spherical nanoparticles varied from 2 nm to 35 nm, 70–75% of nanoparticles was within a range of 2–5 nm; their zeta potential was equal to 9.2 mV. Nanoparticles were synthesized by physical method according to the Polish Patent No. 3883399, starting from metallic silver (99.999%) and demineralized water. The detailed characteristics of the preparation used in all experiments was described previously (Chwalibóg et al. 2010).

Determination of MIC for AgNPs and their effect on *L. monocytogenes* growth and survival. For determination of minimal inhibitory concentration (MIC), the overnight culture of *L. monocytogenes* was diluted in fresh medium to the density of 2×10^6 /ml colony forming units (cfu). The test was performed in 96-well polystyrene plates. To each well the equal volumes of 2-fold concentrated AgNPs suspension and bacterial inoculum were added and the plates were incubated at 37°C for 24 h in static condition. The MIC was determined within the concentration range of AgNPs from 0.5 g/ml to 12 µg/ml at 0.5 intervals. The sample without AgNPs constitutes an experimental control. The MIC value was considered as the lowest concentration entirely inhibiting bacterial growth, according to Clinical and Laboratory Standards Institute (CLSI) instruction. Three

independent repetitions were performed. To determine growth and survival curves, the overnight culture of *L. monocytogenes* was diluted in the fresh medium and incubated until the density of 10^7 cfu/ml was reached. Then the culture was divided into three equal volumes, AgNPs were added to two of them to a concentration of 0.25 MIC and 0.5 MIC, respectively. The third sample without AgNPs constituted experimental control. The samples were incubated for 24 h, the aliquots were taken for the first 5 h in 1-hour intervals, and finally after 24 h. Their absorbance (A_{600}) was read. Additionally, 0.1 ml aliquots appropriately diluted in saline were plated on solid media and after 24 h of incubation the colonies were counted.

Measurement of autolysis/lysis of *L. monocytogenes* cells. Overnight culture of *L. monocytogenes* was diluted in 50 ml of fresh medium to A_{600} equal to 0.1 and incubated until A_{600} equal to 0.6 was reached. Bacteria were centrifuged (10 min, $8000 \times g$) and the pellet, after washing twice in phosphate buffered saline (PBS), was resuspended in Tris-HCl buffer pH 8.0 containing Triton X-100 (0.1%) or lysozyme (20 $\mu\text{g/ml}$). Both suspensions were divided in three parts, one served as a control without AgNPs, to the reminding two the nanosilver suspensions in a concentration of 0.25 MIC and 0.5 MIC was added. After 60 min of incubation 0.1 ml aliquots appropriately diluted in saline were plated on solid media and after 24 h the colonies were counted.

Measurement of *L. monocytogenes* peptidoglycan autolysis rate. Overnight culture was divided in three parts, to two of them the suspension of AgNPs in a concentration of 0.25 MIC or 0.5 MIC were added, the third without AgNPs was left as a control. The procedure of peptidoglycan isolation and its absorbance measurement precisely followed those previously described (Kurek et al. 2010).

Estimation of AgNPs effect on permeability of *L. monocytogenes* envelopes. Overnight culture of *L. monocytogenes* was diluted to A_{600} equal to 0.1 and incubated further to A_{600} equal to 1.0. Then bacteria were spun down, the pellet was rinsed twice with 20 mM phosphate buffer, pH 7.1, and dissolved in this buffer to A_{600} equal to 0.8. A fluorescent dye, carboxyfluorescein (cF), was added to the final concentration of 0.54 μM and after incubation at 40°C for 3 min three samples were prepared: the negative control without AgNPs and probes containing AgNPs at a concentration of 0.25 MIC or 0.5 MIC. The additional referential, positive control with 10% DMSO was also included. The samples were incubated at 37°C for 10 min, then the cells were spun down and the fluorescence of supernatants was measured in black 96-wells titration plates using fluorescence reader at wavelengths 490 nm (excitation) and 515 nm (emission), according to Johansen et al. 1997. To measure DNA and proteins release, the cell suspension pre-

pared as described above was split into four parts: the negative control without AgNPs, samples containing AgNPs at a concentration of 0.25 MIC or 0.5 MIC, and positive control containing 100 $\mu\text{g/ml}$ lysozyme. The samples were incubated at 37°C for 1 h, then cells were removed by centrifugation and the absorbance of supernatants at 260 nm (the released DNA) and 280 nm (the released proteins) was measured. This protocol was the modification of the procedure described previously (Markowska et al. 2014).

Statistical analysis The experiments were performed at least three times and every measurement was done in triplicate. The means \pm standard deviations were calculated. Statistical significance of the difference between experimental samples was estimated using Student's test with Graphpad prism (ver. 6.0). p value < 0.05 was considered as statistically significant.

Results and Discussion

MIC of AgNPs was 8 $\mu\text{g/ml}$, and susceptibility of Gram-positive *L. monocytogenes* to AgNPs was higher than that observed for one of Gram-negative pathogens *Pseudomonas aeruginosa* ATCC 10145, for which MIC value was equal to 1 $\mu\text{g/ml}$ (Markowska et al. 2014). The results presented in Fig. 1 showed that neither growth

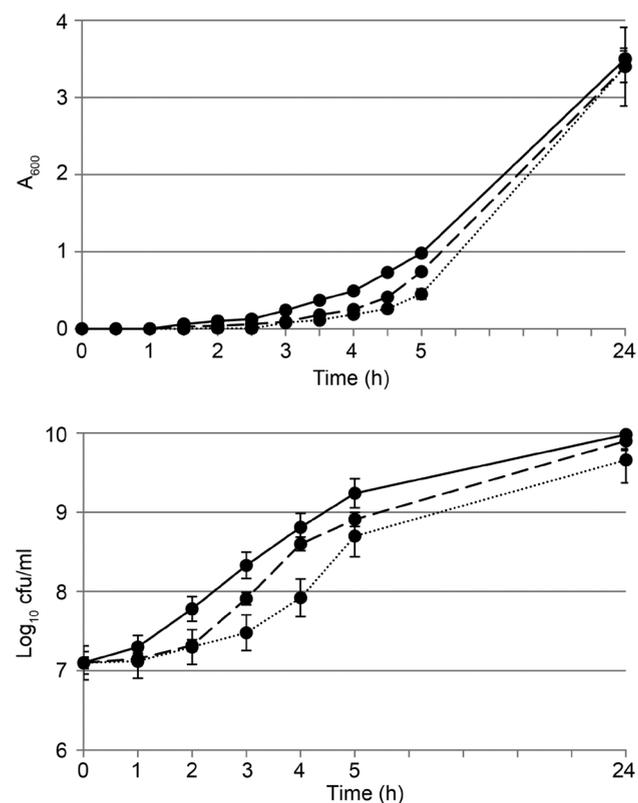


Fig. 1. Effect of AgNPs on *L. monocytogenes* growth (A) and survival (B).

Solid lines – control; dashed lines – 0.25 MIC AgNPs; dotted lines – 0.5 MIC AgNPs. Mean \pm SD values of triplicate cultures were shown.

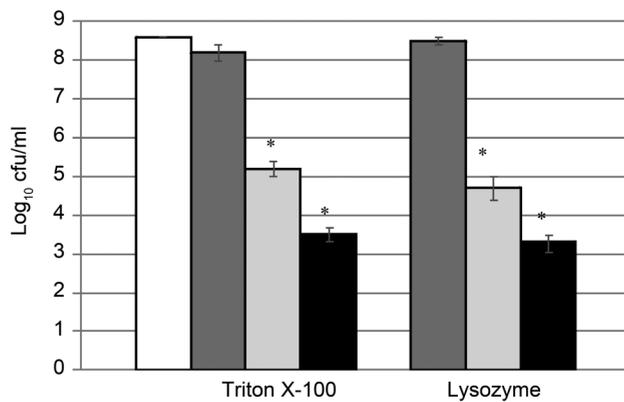


Fig. 2. Influence of AgNPs on lysozyme- or Triton X-100-induced autolysis/lysis of *L. monocytogenes*.

White bar – control, buffer only; dark grey bar – lysozyme or Triton X-100; light grey bar – lysozyme or Triton X-100+0.25 MIC AgNPs; black bar – lysozyme or Triton X-100+0.5 MIC AgNPs. The results are mean of three independent experiments with every measurement done in triplicate \pm SD. Statistically relevant differences ($p < 0.05$) were marked with asterisks.

nor survival of *L. monocytogenes* was substantially diminished in the presence of AgNPs in a concentration of 0.25 MIC or 0.5 MIC. In the subsequent experiments, AgNPs were used in these concentrations.

The effect of AgNPs on the autolysis/lysis of *L. monocytogenes* cells was studied in the cultures treated with nonionic surfactant Triton X-100 or lysozyme. The nonionic detergent Triton-X100 and lysosome are the commonly used agents for induction of autolysis or lysis of *L. monocytogenes* cells, respectively (Smith et al. 1991; Popowska et al. 2009). When used in the moderate concentration, they can be applied to study the effect of various substances on their activity. The results of the experiments are presented in Fig. 2. In the control cultures, treated only with lysosome or Triton X-100, 49% and 89% of cells survived, respectively. The observed killing effect of lysosome was much weaker than that of Triton X-100 as it had already been presented by Kurek and coauthors (2010). The observed result can be due to the low level of glucosamine acetylation in *L. monocytogenes* peptidoglycan (Amano et al. 1977). The addition of AgNPs at a concentration of 0.25 MIC resulted in very high, over 1000-fold increase of the lysis of cultures treated with lysozyme or Triton X-100. AgNPs added in a concentration of 0.5 MIC caused further drop in the number of living cells.

The addition of nanosilver to peptidoglycan also caused the enhancement of peptidoglycan autolysis; however, this effect was not as pronounced as the effect observed for the whole cells. The drop in absorbance (A_{600}) of the control peptidoglycan sample was 33% after 2 h incubation in the buffer. In samples treated with AgNPs at the concentration of 0.25 MIC or 0.5 MIC the observed drop was 49% and 53%, respectively. Only the last value was statistically relevant, as it was shown in

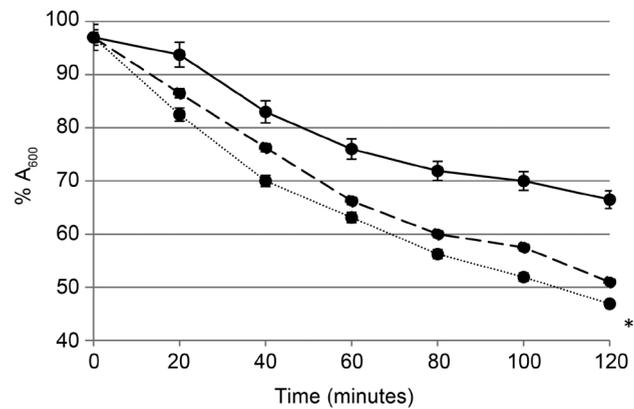


Fig. 3. Autolysis of isolated *L. monocytogenes* peptidoglycan in the presence of AgNPs.

Absorbance A_{600} at time 0 was considered as 100%. Solid line – control; dashed line – 0.25 MIC AgNPs; dotted line – 0.5 MIC AgNPs. The results are mean of three independent experiments with every measurement done in triplicate \pm SD. Statistically relevant difference ($p < 0.05$) was marked with an asterisk.

Fig. 3. It can be speculated that the AgNPs enhanced the ability of autolysins – peptidoglycan hydrolyzing enzymes which catalyse polymer destruction (Rice and Bayles 2008). Five *L. monocytogenes* autolysins have been identified (Popowska 2004); however, the analysis of the bacteria genome revealed the presence of more than twenty proteins with the putative peptidoglycan hydrolase domains (Bierne and Cossart 2007).

The effect of AgNPs on *L. monocytogenes* cells permeability was estimated by two methods. First, the efflux of cF dye was measured and after 10 min of treatment with AgNPs at a concentration of 0.5 MIC the efflux of cF was enhanced by 14% in comparison to the control sample, which was 79% relative to the total leakage determined after cell lysis with DMSO (Table I). In the second set of experiments the efflux of macromolecules, DNA and proteins, was studied by measuring respectively A_{260} and A_{280} of the supernatants of cultures treated with AgNPs at a concentration of 0.25 MIC or 0.5 MIC, in the control culture, and in the culture treated with lysozyme (positive control).

Table I
Increase in cF efflux from *L. monocytogenes* caused by AgNPs.

| AgNPs concentration | % efflux of cF at time (min) | |
|----------------------|------------------------------|-------------|
| | 0 | 10 |
| 0 (negative control) | 72 \pm 2 | 79 \pm 1 |
| 0.25 MIC | 73 \pm 2 | 84 \pm 2 |
| 0.5 MIC | 74 \pm 2 | 93* \pm 2 |

Efflux values are given as percentages relative to the total leakage determined after cell lysis with DMSO (means of three independent experiments with every measurement done in triplicate \pm standard deviations are shown). Statistically relevant difference ($p < 0.05$) was marked with an asterisk.

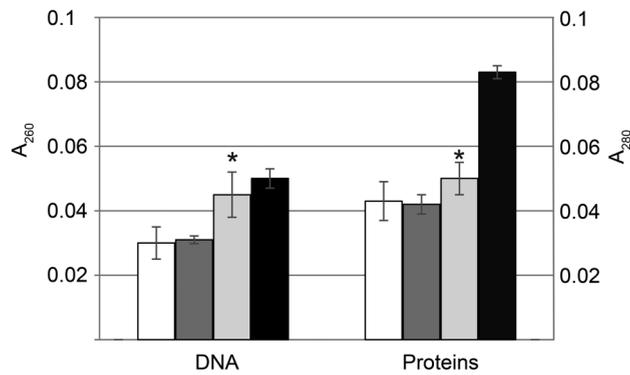


Fig. 4. Effect of AgNPs influence on the efflux of DNA and proteins from *L. monocytogenes*.

White bars – control, no AgNPs added; dark grey bars – 0.25 MIC AgNPs; light grey bars – 0.5 MIC AgNPs; black bars – 100 µg/ml lysozyme (positive control). The results are mean of three independent experiments with every measurement done in triplicate \pm SD. Statistically relevant difference ($p < 0.05$) were marked with asterisks.

It was demonstrated that AgNPs in a concentration of 0.5 MIC enhanced DNA efflux by 48% after 60 min of treatment with AgNPs in comparison to control culture (Fig. 4). The efflux of proteins was enhanced by 30% after 60 min exposure to AgNPs at a concentration of 0.5 MIC. The maximal efflux of DNA and proteins caused by 60 min exposure to lysozyme amounted to 171% and 191% of the control sample without AgNPs. The observed enhancement of cF, DNA and proteins efflux as a result of AgNPs treatment points to the damage of cell wall. It was previously demonstrated that AgNPs are able to cover cells surface and to induce the formation of the hollows in cell envelopes what can results in the enhancement of cell permeability (Chwalibóg et al. 2010). In turn, AgNPs, when adsorbed on the cell surface, modify membranes potential what stimulates nanoparticles transport to the cytoplasm (Morones et al. 2005; Marambio-Jones and Hoek 2010).

Until now there have been only few papers describing the effect of silver nanoparticles on *L. monocytogenes* membranes. Microscopic analysis demonstrated deformation, disintegration and decrease in cell surface roughness of *L. monocytogenes* treated with silver nanoparticles synthesized by *Jatropha curcas* (Chauhan et al. 2016). It has also been demonstrated that AgNPs released from nanocomposites can penetrate the cell wall and plasma membrane of *L. monocytogenes* what results in separation of the cytoplasmic membrane from the cell wall (Tamayo et al. 2014). The results of our group demonstrated that AgNPs caused the decrease in *L. monocytogenes* cell length even by 50% what may also indicate their interaction with the cell wall (data not shown) (Milczarek 2015). It has been postulated recently that the activity of AgNPs against *L. monocytogenes* and the other foodborne pathogens make

them useful in food industry, particularly in food packaging and food preservation (Patra and Baek 2017).

In conclusion, the original results presented here show that *L. monocytogenes* peptidoglycan is the target of AgNPs activity. This effect is demonstrated by the increase of cell autolysis and autolysis of the extracted peptidoglycan and also by the enhancement in cell permeability. Interference with *L. monocytogenes* cell wall integrity and functionality constitutes the important mechanism of nanosilver antibacterial activity towards this Gram-positive pathogen.

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