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...
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, bloodstream 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-Bollard 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 bacteria (e.g. E. coli) because of the presence of partially deacetylated N-acetylgalactosamine residues (Boneca et al. 2007). Another exceptional future 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 (Bieren 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 cephaplosporins 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 (TSYE) 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 × 10⁶/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
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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 at a concentration of 0.25 MIC and 0.5 MIC, respectively. The third sample without AgNPs constituted the 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 x 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 µg/ml). Both suspensions were divided in three parts, one served as a control without AgNPs, the remaining 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, carboxyfluoresceine (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% DSMO 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 prepared 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 µg/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 ± standard deviations were calculated. Statistical significance of the difference between experimental samples was estimated using Student’s t-test with Graphpad prism (ver. 6.0). *p* value < 0.05 was considered as statistically significant.

**Results and Discussion**

MIC of AgNPs was 8 µg/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 µg/ml (Markowska et al. 2014). The results presented in Fig. 1 showed that neither growth...
nor survival of \textit{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 \textit{L. monocytogenes} cells was studied in the cultures treated with nonionic surfactant Triton X-100 or lysozyme. The nonionic detergent Triton-X100 and lysozyme are the commonly used agents for induction of autolysis or lysis of \textit{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 lysozyme or Triton X-100, 49% and 89% of cells survived, respectively. The observed killing effect of lysozyme 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 \textit{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. It can be speculated that the AgNPs enhanced the ability of autolysins – peptidoglycan hydrolyzing enzymes which catalyse polymer destruction (Rice and Bayles 2008). Five \textit{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 \textit{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).

| AgNPs concentration | % efflux of cF at time (min) |
|---------------------|-----------------------------|
| 0 (negative control)| 72 ± 2                      |
| 0.25 MIC            | 73 ± 2                      |
| 0.5 MIC             | 74 ± 2                      |
| 72 ± 1              | 84 ± 2                      |
| 93 ± 2              | 93 ± 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 ± standard deviations are shown). Statistically relevant difference ($p<0.05$) was marked with an asterisk.

Fig. 2. Influence of AgNPs on lysozyme- or Triton X-100-induced autolysis/lysis of \textit{L. monocytogenes}.

Fig. 3. Autolysis of isolated \textit{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 ± SD. Statistically relevant difference ($p<0.05$) was marked with an asterisk.
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 the 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 which can result in the enhancement of cell permeability (Chwalióbóg 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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**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 ± SD. Statistically relevant difference (p < 0.05) were marked with asterisks.
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