The study of the antimicrobial activity of colloidal solutions of silver nanoparticles prepared using food stabilizers

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Abstract The bactericidal effect of colloidal solutions of silver nanoparticles based on food stabilizers, gum arabic and chitosan, against bacterial cultures of microorganisms in food production is described. The antibacterial activity of nanotechnology products containing different amounts of stabilizing additives when applied to solid pH-neutral substrates is studied. For its evaluation a method making it possible to take into account the capability of nanoparticles to diffuse in solid media was applied. Minimal inhibitory concentrations of nanoparticles used against *Erwinia herbicola*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Sarcina flava* were found. A suggestion was made concerning the influence of the spatial structure of bacteria on the antibacterial activity of colloidal solutions of silver nanoparticles. The data concerning the antibacterial activity and minimal inhibiting concentrations of nanoparticles may be used for development of products suppressing activity of microorganisms hazardous for food production.

Keywords Silver nanoparticles · Gum arabic · Chitosan · *Erwinia herbicola* · *Pseudomonas fluorescens* · *Bacillus subtilis* · *Sarcina flava*

Introduction

In the last decade, the field of application of silver nanoparticles (SNP) in different industries has considerably enlarged.

Due to their unique physical, chemical and biological properties they are used in medicine, pharmaceutical, chemical, processing, and food industries (Zimon and Pavlov 2012; Popov et al. 2010). One of the most useful features of SNP is their clearly defined antimicrobial activity. Previous researchers showed that SNP colloidal solutions have an antibacterial action against gram-negative and gram-positive bacteria (Shrivastava et al. 2007; Yogesha et al. 2012, Zhang and Oyander-Craver 2012, Lara et al. 2009). It should be noted that efficiency of such an action depends on a number of factors that should be taken into account when studying colloidal solutions of nanoparticles.

The most important factor that determines the level of the antibacterial action of SNP is the size of the particles. According to the data of Lok et al. (2007), colloidal solutions of SNP 9.2 nm in diameter showed a greater antimicrobial activity than solutions of particles 64 nm in diameter. Moreover, the antibacterial activity of nanoparticle solutions decreased considerably when the experiments were conducted in a medium provoking aggregation of nanoparticles. According to the researchers, the difference in the bactericidal activity of SNP of different diameters is due to the fact that the size reduction of the particles results in the increase of the specific phase interface and, consequently, the increase of active silver concentration. This was shown by Sintubin et al. (2011), where SNP 10–11 nm in diameter obtained using the biogenic method, had a stronger action against the bacterial cultures *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus aureus* (*S. aureus*) than the particles 20–30 nm in diameter obtained using a chemical method. Moreover, the antibacterial activity of SNP decreases considerably, in cases when aggregation of the particles takes place under the influence of medium substances (Zhang and Oyander-Craver 2012).

To a large extent, the capability of nanoparticle colloidal solutions to maintain high dispersion depends on the

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stabilizing additives being included into the compound. To stabilize chemically obtained nanoparticles, polyvinylpyrrolidone (PVP) and sodium dodecylsulfate (SDS) are often used (Mikhienkova and Mukha 2011). However, PVP somewhat decreases the activity of SNP, whereas SDS, which is a strong detergent, exhibits its own bactericidal action, which complicates the direct evaluation of antibacterial activity of the nanoparticles. In other cases, a mixture containing D-glucose and hydrazine was added as a reductant (Shrivastava et al. 2007). Colloidal solutions of SNP obtained using this method exhibited a pronounced bactericidal action against bacterial microorganisms resistant to antibiotic substances. However, in this case we cannot eliminate the possibility that the test results were influenced by hydrazine, which possesses highly toxic properties (“Hazard assessment report. Hydrazine” 2007). SNP of relatively small size of 9.5 ± 0.3 nm and 8.7 ± 0.2 were obtained using fulvic and humic acids (Litvin et al. 2012). Another way to prepare the colloidal solution of SNP is to stabilize the particles using starch molecules (Yogesh et al. 2012). Starch has no bactericidal action against microorganisms and, thus, has no effect on the total antimicrobial activity of the colloidal solution. The main stabilizing additive used by Zhang and Oyanedel-Craver (2012) was casein, a milk protein, which does not inhibit activity of microorganisms. Thus, the use of non-toxic substances as stabilizers makes it possible to perform a more objective assessment of the antibacterial properties of SNP, and, moreover, expands the field of application of nanoparticle colloidal solutions for industrial production.

Practical use of the colloidal solutions of silver requires specification of particular use rates of nanoparticles stipulated by technological and economical expediency and safety standards. Solutions of SNP obtained using different methods make a bactericidal effect against a number of microorganisms, if the minimal concentration of nanoparticles in the substrate is within the limits of 0.004–0.3 g/l (Mikhienkova and Mukha 2011, Sintubin et al. 2011). However, it should be noted that colloidal solutions of nanoparticles of a rather small diameter exhibit stable antibacterial activity when the concentration of SNP in the substrate is within the range of 0.016–0.08 g/l (Petica et al. 2008).

Introduction of colloidal solutions of SNP into the food industry draws special attention to the possibility of food products with nanoparticles. It is also important that colloidal solutions contain no accessory substances harmful for people. Therefore, we have conducted research in order to study the influence of colloidal solutions of SNP on microorganisms in food products. We used SNP products prepared with non-toxic natural polymers as stabilizers: gum arabic (E414) and chitosan. A number of microorganisms causing food product spoilage were utilized in this study. In order to test the SNP bactericidal effect on food microorganisms with different

morphology, cell wall structure, and ability to produce endospores we selected 5 different bacterial species. We used the following gram-negative bacteria: *Escherichia coli*, rod-shaped bacteria commonly involved in the spoilage of fresh vegetables, meat, poultry, fish, and eggs (Jay et al 2006; Sperber and Doyle 2009; Morris and Potter 2013), *Erwinia herbicola* (*E. herbicola*), rod-shaped bacteria that cause vegetable and fruit rotting (Ibrahim and AL-Saleh Emir 2010, Ray and Bhunia 2013), *Pseudomonas fluorescens* (*P. fluorescens*), rod-shaped bacteria, primary spoilage microorganisms in fresh meat, poultry, seafood, and eggs (Doyle 1989, Speranza et al. 2013). Two gram-positive bacteria were also tested: *Bacillus subtilis* (*B. subtilis*), rod-shaped bacteria which have the ability to form protective endospores, often found in pasteurized milk, bakery products, and canned food (Fratamico et al 2005; Morris and Potter 2013), and *Sarcina flava* (*S. flava*), cocci bacteria which contaminate brewing products and yeast (Hough et al 1982; Priest and Campbell 1996; Ray and Bhunia 2013). Assessment of the antibacterial activity was made based on the minimal inhibitory concentration of the product in the medium (MIC) and on the rate of the antibacterial activity (K_a), which shows the ability of the nanoparticles to diffuse in solid media and to affect microorganisms.

Materials and methods

Colloidal solutions of SNP

For this study we chose 4 samples of colloidal solutions of SNP provided by “Sentosa factoring NP”, SPE, Ltd. The solutions were produced using the method of chemical reduction of a water-soluble silver salt in the water medium by ascorbate or sodium citrate with consequent addition of a food stabilizer (Koshelev et al. 2010). Factory tests were conducted in order to estimate the size of synthesized particles using a spectrophotometer SF-56. According to the test results provided by the manufacturer, plasmon resonance absorption bands maxima of produced colloidal solutions reached 405 nm. Concurring with data obtained by Krutyakov et al. (2008), such a value corresponds to the size of SNP 10–15 nm. Gum arabic is used as a stabilizer in three products (G12, G8, and G4), and the fourth (H12) contains the stabilizing additive chitosan (Table 1). Before the tests all the solutions passed a microbiological purity check.

The research was conducted using axenic cultures of microorganisms of gram-negative bacteria *E. coli*, *E. herbicola*, *P. fluorescens* and gram-positive bacteria *S. flava*, *B. subtilis*. These types of bacteria were cultivated on the indicator and elective media at 37 °C and 28 °C.

Table 1 Products composition

Product name	Stabilizer	Concentration of silver nanoparticles in the initial solution (g/l)	Ratio between the stabilizer and silver nanoparticles (respectively)	Extra additives
G4	Gum arabic	3	4:1	–
G8	Gum arabic	3	8:1	glycine
G12	Gum arabic	3	12:1	glycine
H12	chitosan	3	12:1	–

Bactericidal activity test

The bactericidal activity test was based on standard agar-well diffusion method (Tang et al. 2014; Abolhassani 2010; Sequeiros et al. 2010). The sterile substrate was poured into a petri dish, the layer being 0.5 to 0.8 cm thick. After consolidation of the substrate microorganism slurry containing 10^6 CFU was placed on its surface using a sterile pipette. Then, the cells of the microorganism were thoroughly spread over the surface of the substrate using a Drigaski spatula. Next, a hole 12 mm in diameter was cut out in the center of the Petri dish, where 0.15 ml of the examined product diluted to SNP concentration of 0.03–0.06 g/l was introduced. The prepared Petri dishes were incubated for 48 h, with observations being made every 12 h. This method is discussed in further detail in another article (Podkopaev et al. 2014). The rate of the antibacterial activity K_a , which makes it possible to compare efficiency of different products with the same concentration of nanoparticles, was calculated according to the formula:

$$K_a = \frac{D_i - d_0}{D_i}$$

Where D_i is the diameter of the bacterial-biomass-free (inhibition) zone, d_0 is the diameter of the hole (Fig. 1).

MIC was accepted as a component of SNP in the introduced product which makes it possible to identify the bacterial-biomass-free (inhibition) zone around the hole 24 h after the beginning of the experiment.

Statistical analysis

The antibacterial activity tests were replicated several times. The results were assessed using a Student's *t*-test. *P*-values below 0.05 were considered significant. K_a values below 0.073 were considered insignificant and, thus, were ignored when specifying MIC.

Fig. 1 The effect of silver nanoparticles solution on the growth of *Escherichia coli* cultivated in solid medium. D_i shows the diameter of the bacterial-biomass-free (inhibition) zone. d_0 shows the diameter of the hole, containing the silver nanoparticles solution

Results

Antibacterial activity of nanoparticles

The experiment showed that colloidal solutions of SNP have an antimicrobial effect against the following microbial strains: *E. coli*, *E. herbicola*, *P. fluorescens*, *B. subtilis*, and *S. flava*. 12 h after the beginning of the experiment it was possible to clearly delimit the inhibition zones. Eventually, the inhibition zone did not expand or shrink, and after 48 h from the beginning of the experiment it kept its form.

As a result of the research, average antibacterial activity rate values of the products containing nanoparticles in different concentrations acting against the microorganisms studied were calculated (data not shown). Based on the data obtained, graphs were constructed, which reflected the influence of the concentration of SNP in the product on K_a for a number of bacterial strains (Fig. 2). As is evident from the graphs, the product based on gum arabic G12 produces the most pronounced bactericidal action. This product is the only one of the four that can inhibit growth of all the test cultures within the range of the concentrations studied. Meanwhile, G12 produces the smallest effect against *S. flava*, where, SNP concentration being 0.06 g/l, K_a is on average 60 % lower than in the experiments with other cultures at a similar concentration. The H12 product on the basis of chitosan provided no significant effect against the microorganisms studied. Product on the basis of gum arabic G8 within the range of the concentrations studied inhibited growth of *E. herbicola* and *B. subtilis*. Product on the basis of gum arabic G4 inhibited the growth of *B. subtilis*, a significant effect of the product against other organism strains was not identified.

Minimal concentrations of the SNP colloidal solutions inhibiting growth of the bacterial microorganisms are represented in Table 2. Growth of *E. herbicola* was inhibited by the

Fig. 2 Rates of antibacterial activity of silver nanoparticles solutions with different concentrations on **a** *E. herbicola*, **b** *S. flava*, **c** *P. fluorescens* and **d** *B. subtilis*. All the results below 0.073 were considered insignificant

products G8 and G12 at the minimal concentration of nanoparticles of 0.05 and 0.04 g/l correspondingly. MIC against *P. fluorescens* was found only in case of G12 and amounted to 0.03 g/l. All the products on the basis of gum arabic produced a pronounced effect against *B. subtilis* (gram-positive bacillus) at the minimal concentration of nanoparticles within the range of 0.03 to 0.04 g/l. MIC against *S. flava* under the effect of SNP products within the required range of concentrations was specified for G12, where it amounted to 0.06 g/l.

Discussion

It should be noted that colloidal solutions of gum-arabic-stabilized SNP showed better antibacterial properties than

the solution based on chitosan. Such results may be explained by the influence of pH of the medium on the stability of the colloidal system of the product. Elective and indicative substrates Endo and plain agar-agar used in the course of the experiments have pH from 7.2 to 7.4. However, as shown in previous research (Saini et al. 2011), in the media with pH values from 6.5 to 10 generation of non-central-symmetric aggregates of chitosan-stabilized nanoparticles results from neutralization of chitosan amino groups. Thus, the surface of the colloidal particles shrinks, as it was specified by Petica et al. 2008, and efficiency of the bactericidal effect of the nanoparticles is negated. All the while, gum arabic amino groups at a neutral pH are less susceptible to neutralization (Pantelglou et al. 2010), which enables colloidal solutions of nanoparticles to preserve stability in neutral media.

It was found that the bactericidal activity of products G4, G8 and G12 increases when gum arabic content in the product increases. Thus, product G12 is the most efficient inhibitor of growth of bacterial microorganisms within the specified range of concentrations. This may be referred to the fact that when gum arabic concentration increases in the colloidal solution the amount of gum arabic absorbed on the surface of nanoparticles by way of formation of non-covalent interactions between negatively charged carboxylate groups of gum arabic and positively charged SNP increases (Roque et al. 2009). These results concur with the data obtained by Gils et al.

Table 2 Minimal inhibitory concentrations (MIC) of silver nanoparticles in colloidal solution products

Bacteria	MIC value of silver nanoparticles (g/l)			
	G4	G8	G12	H12
<i>E. herbicola</i>	> 0.06	0.05	0.04	> 0.06
<i>P. fluorescens</i>	> 0.06	> 0.06	0.03	> 0.06
<i>S. flava</i>	> 0.06	> 0.06	0.06	> 0.06
<i>B. subtilis</i>	0.04	0.03	0.03	> 0.06

(2009) in a study of SNP stabilization using hydrogel on the basis of gum arabic.

Colloidal solutions of SNP inhibited the growth of both gram-negative and gram-positive bacilli uniformly well. MIC against product G12 when used against *E. herbicola*, *P. fluorescens*, *B. subtilis* is within the range of 0.03 to 0.04 g/l and is comparable to the results of previous researchers (Petica et al. 2008, Lara et al. 2009, Yogesha et al. 2012, Sintubin et al. 2011). In the context of the morphology, these microorganisms are bacilli, which spatially represent either single cells or cell chains (Wheelis 2008). Having free access to the bacteria cell wall, SNP attach to its surface disturbing its barrier and transport functions (Guzman et al. 2012). It is assumed that nanoparticles are also capable of penetrating into the bacterial cells and inactivating cell enzymes, which leads to their death (Raffi et al. 2008).

However, as the results show, colloidal SNP solutions exhibit less antibacterial activity against circular shaped bacteria *S. flava*. Spatially, these microorganisms form cuboidal arrangements of eight or more cells (Wheelis 2008). Literature offers little data on the effect of SNP against such bacteria as *Sarcina*. Nevertheless, a great deal of experiments are made with the use of *c Staphylococcus aureus* (*S. aureus*), a circular shaped bacteria, cells of which spatially represent grape-like clusters (Wheelis 2008). In a number of works (Shrivastava et al. 2007; Petica et al. 2008) it is noted that MIC of colloidal solutions of SNP, when used against *S. aureus* exceeds MIC values for gram-negative bacilli *E. coli* and *Pseudomonas aeruginosa* by several times. At the same time, an assumption is made that the process of attaching of SNP to the surface of the cell wall and its penetrating into the cell becomes difficult because of peptidoglycan contained in the cell walls of gram-positive bacteria. Later works (Lara et al. 2009, Litvin et al. 2013), contain no data that could confirm these assumptions. On the contrary, the authors were unable find any considerable differences in the rate of the bactericidal action of SNP against gram-negative (*E. coli*, *P. aeruginosa*) and gram-positive (*Streptococcus pyogenes*) microorganisms. The authors Yogesha et al. (2012) who studied *P. aeruginosa*, *E. coli* and *B. subtilis* hold to the same opinion. It should be noted that spatially the bacteria of *Streptococcus* group are like the other tested microorganisms form cell chains (Wheelis 2008), which in no way influences the access of the silver nanoparticles to the surface of the cell walls. Sintubin et al. (2011) do not distinguish gram-positive or gram-negative cell walls as a factor affecting the capability of SNP to inhibit growth of microorganisms. However, according to their data, MIC of the silver nanoparticles against *S. aureus* is also several times higher than MIC against *E. coli* and *P. aeruginosa*. Based on the above, it may be assumed that the spatial location of the cells, which represent densely packed cube-like structures (*S. flava*) or grape-like clusters (*S. aureus*), impedes the access of SNP to the whole surface of each bacterial cell, creating a

part of the structural formation. This possibly leads to a decrease in the antibacterial action of SNP and increases the value of MIC. Supposedly, to increase the efficiency of the bactericidal action of colloidal SNP solutions it is required to ensure maximum access of nanoparticles to the surface of the bacteria cell walls. For this purpose it may be necessary to destroy the structural formations of the bacteria to separate the cells from one another.

Thus, the findings outlined in this work will make it possible to expand the field of application of colloidal SNP solutions in the food industry. A food nanoparticle stabilizer is selected, which enables maximum preservation of high dispersion ability of colloidal solution in solid media with a normal pH. The assumption made concerning the influence of the spatial cooperation of the bacteria on the antibacterial activity of SNP will help to apply colloidal solutions of SNP in order to inhibit growth of bacterial microorganisms more efficiently.

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