

INFLUENCE OF SILVER NANOPARTICLES WITH DIFFERENT SURFACE COATING ON THE EXCISED FROG HEART

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Abstract

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The surface coating of silver nanoparticles is crucial to the nanoparticle properties as size and shape, stability, solubility and targeting, which are responsible to their biological effects. In order to study the influence of differently coated silver nanoparticles (AgNPs) on *in vitro* heart preparations of *Rana ridibunda* frog, we synthesized two types of AgNPs – nanoparticles coated with the polysaccharide starch (AgNPs/Starch, $D_{\text{mean}} = 15$ nm) and nanoparticles coated with the trisaccharide raffinose (AgNPs/Raff, $D_{\text{mean}} = 25$ nm). The aqueous dispersions of both types of AgNPs were introduced in concentrations of 1, 3, 10, 30, 50 and 100 $\mu\text{g/ml}$ by a cannula into the frog heart ventricle. The effects of AgNPs were estimated by using of as-synthesized nanoparticle dispersions and aqueous dispersions of nanoparticles purified by ultracentrifugation. Separate measurements of heart contractions were performed with the addition of supernatants obtained by ultracentrifugation of as-synthesized AgNPs samples to estimate the biological effects of the stabilizing agents. The nanoparticles were administrated alone or together with prazosin or propranolol, the widely used blockers of α_1 - and β -adrenoreceptors, respectively. The data obtained showed that as-synthesized AgNPs/Raff significantly increased the force of contraction of isolated frog hearts but was not affected by purified AgNPs with the same coating. Both as-synthesized and purified AgNPs/Starch did not influence the force of heart contraction. The positive inotropic effect of non-purified raffinose-coated AgNPs was partially abolished by 3 $\mu\text{mol/l}$ prazosin and 30 $\mu\text{mol/l}$ propranolol. It can be concluded that starch as a polysaccharide covers more tightly the metal core of silver nanoparticles and made AgNPs/Starch less reactive than the AgNPs/Raff. The results show that the purified AgNPs/Raff was less potent to affect the cardiac activity because of their higher ability to aggregate in the medium studied.

Key words: silver nanoparticles, carbohydrate stabilizing agents, adrenoreceptors, cardiac myocytes, inotropic effect, excised heart

Introduction

An understanding of the interactions between nanoparticles and biological systems is of significant interest. Nanoparticles are structures with at least one dimension of less than 100 nm and unique properties (Roco, 2003). These have found many applications in medicine, especially in can-

cer treatment and drug delivery (Lim et al., 2011; Talekar et al., 2011), industry and everyday life. In the area of water purification nanotechnology offers the possibility of an effective removal of pollutants and germs (Tiwari et al., 2008), especially silver nanoparticles are very effective against Gram-positive and Gram-negative bacteria (Furno et al., 2004; Morones et al., 2005).

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From another perspective, there is debate about the risks and benefits of using nanoparticles (Royal Society, 2004). The emerging ecotoxicological data shows toxic effects of low concentrations (mg/l) of nanoparticles on fish and invertebrates often (Handy et al., 2008). The literature on mammalian models gives information about respiratory toxicity of nanoparticles and significant lung damage (Lam et al., 2004). Elevated blood metal levels were also measured, suggesting movement of the particles throughout the body (Takenaka et al., 2004). There are significant gaps in the literature for other organisms, including amphibians. In addition there are almost no ecotoxicological data that have systematically investigated particle-size and coat effects although they will affect various physico-chemical properties.

So, the aim of this study is to investigate the relationship between surface coating of AgNPs, the composition of the media (and the consequent stability of the AgNPs) and their effects on frog heart activity.

Materials and Methods

Subjects

All experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Bulgarian Center for Bioethics. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the Institutional Animal Care and Use Committee, April 1997, Oakland University, USA.

Solutions and drugs

All substances were dissolved in modified Ringer solution, hereafter referred to as Ringer solution. It has the following composition – 100 mmol/l NaCl, 1.3 mmol/l KCl, 0.7 mmol/l CaCl₂ and 1.2 mmol/l NaHCO₃. 200 µl of this solution was introduced by a cannula into the frog heart ventricle. The applications of pure or AgNPs-containing Ringer solution were performed regularly at 15 min intervals during 120 min experiments. The sources of chemicals used were as follows: prazosin, propranolol and all salts were from Sigma-Aldrich Inc., St. Louis, MO, USA.

Synthesis of AgNPs

AgNPs were synthesized and characterized in the Laboratory of Nanoparticle Science and Technology, Department of General and Inorganic Chemistry, Faculty of Chemistry and Pharmacy, Sofia University “St. Kliment Ohridski”. AgNPs/Starch and AgNPs/Raff were prepared by one-step, one-phase “green” synthesis based on chemical reduction methods in aqueous solutions.

AgNPs/Starch were synthesized by reduction of Ag⁺ (AgNO₃, 0.001 M) using D-(+) glucose (0.1 M) as a reducing agent, soluble starch (0.2%, w/v) as a stabilizing agent, and sodium hydroxide (0.1 M) as a reaction catalyst (Vasileva et al., 2011). The reaction mixture was incubated in an ultrasonic environment at 30°C for 60 min. The colour of the solution changed immediately from colorless to pale brown and subsequently to light yellow, indicating nanoparticle formation.

AgNPs/Raff were obtained by reduction of Ag⁺ (AgNO₃, 0.1 M) using D-(+) raffinose (0.1 M) both as reducing and stabilizing agent, and sodium hydroxide (0.1 M) as a reaction catalyst. The reaction mixture was kept in an ultrasonic environment at 30°C for 60 min. A change of the solution color from colorless to pale brown and subsequently to yellow orange was observed, indicating nanoparticle formation.

Using the methods described above, AgNPs/Starch and AgNPs/Raff were obtained with Ag concentrations of 32 mg/l and 110 mg/l, respectively. AgNPs/Starch and AgNPs/Raff primary dispersions were centrifuged at 14 000 rpm for 90 and 60 min, respectively. The supernatants containing soluble components of the reaction mixtures were decanted and the nanoparticle pellets were re-dispersed using sonication in 10-fold and 6-fold less volume of double distilled water for AgNPs/Starch and AgNPs/Raff, respectively. As a result of the described procedures, concentrated stock dispersions of starch- and raffinose-stabilized silver nanoparticles were obtained with silver concentrations of 320 mg/l and 650 mg/l, respectively. This step ensured purification of the nanoparticles employed in the study by elimination of the reaction side products. The stock dispersions were kept in closed containers at room temperature and used after appropriate dilutions in the following experiments.

The zeta potential of the AgNPs were measured in 1 mM KCl at pH 6.8 by Zetasizer Nano ZS (Malvern) instrument. The morphology and particle sizes were examined using a transmission electron microscope (TEM, JEM-2100) operating at accelerating voltage of 200 kV. The average particle size were determined from the TEM images for at least 150 particles using imaging software (Image J). The aqueous dispersions of as-synthesized and purified AgNPs were characterized by UV–visible spectroscopy using an Evolution 300 spectrometer (Thermo Scientific, USA). Double distilled water was used as a reference for the base line.

Frog heart preparation *in vitro*

All experiments were performed at room temperature (20–22°C). Frog hearts were cannulated and excised. Afterwards, their hearts were connected to a force transducer. The volume of the cannula is approximately 500 µl. Contractions

were recorded and analysed on a computer using interface and TENZO1 software (Stocks, Sofia, Bulgaria) (the experimental procedure was described in detail (Sazdova et al., 2009; Sazdova et al., 2010). Separate time control measurements were performed with the addition of supernatant from AgNPs/Starch and AgNPs/Raff, respectively. The nanoparticle dispersions were homogenized by an ultrasonic bath UST2.4-150 (Siel, Bulgaria) for 30 min prior to each experiment then appropriate dilutions were made (1, 3, 10, 30, 50 and 100 $\mu\text{g/ml}$). Immediately before their introduction into the cannula were further vortexed.

Statistical analysis

All data are presented as means \pm SEM. The n in the text refers to the number of experiments i.e. frog heart preparations *in vitro*. Statistical analysis including Student's t test for independent samples was carried out using Microsoft Excel (Professional Plus 2013) and R statistical package (v.2.15.2, Copyright © 2012 The R Foundation for Statistical Computing). $P < 0.05$ was considered statistically significant.

Results and Discussion

TEM micrographs showed that the resulting products mainly consisted of quasi-spherical nanoparticles with a relatively narrow size distribution and average size of 15 ± 4 nm for AgNPs/Starch, and of 25 ± 7 nm for AgNPs/Raff (Figure 1). In addition to the nanospheres, some typical polyhedral nanoparticles (multiple twinned nanocrystals) can be easily observed. UV-Vis absorption measurements of the as-synthesized solutions of starch- and raffinose-stabilized

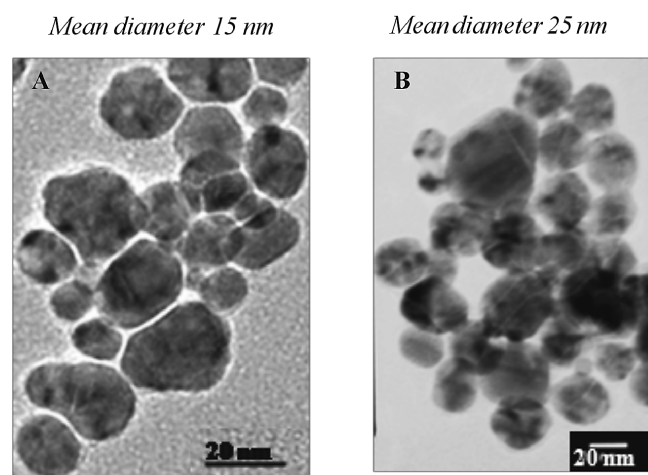


Fig. 1. Representative TEM images of (a) AgNPs/Starch dispersion and (b) AgNPs/Raff dispersion

AgNPs showed UV-visible spectra with characteristic surface plasmon resonance (SPR) bands at λ_{max} of 409 and 412 nm, respectively (Figure 2A). The preservation of the average size of the concentrated AgNPs was checked through minor changes in the position of the surface resonance plasmon peak in the recorded UV-Vis spectra (Figure 2B). This is a further proof that the suitably adjusted centrifugation-based concentration method represents a powerful approach to the preparation of several times more concentrated AgNPs dispersions that retain almost the same size characteristics. These dispersions are stable for several months. The registered reproduction of the optical characteristics (SPR maximum and full band width at half maximum, FWHM) of the nanoparticles in the concentrated aqueous dispersions demonstrated high colloidal stability of both types of nanoparticles after the centrifugation. The colloidal stability

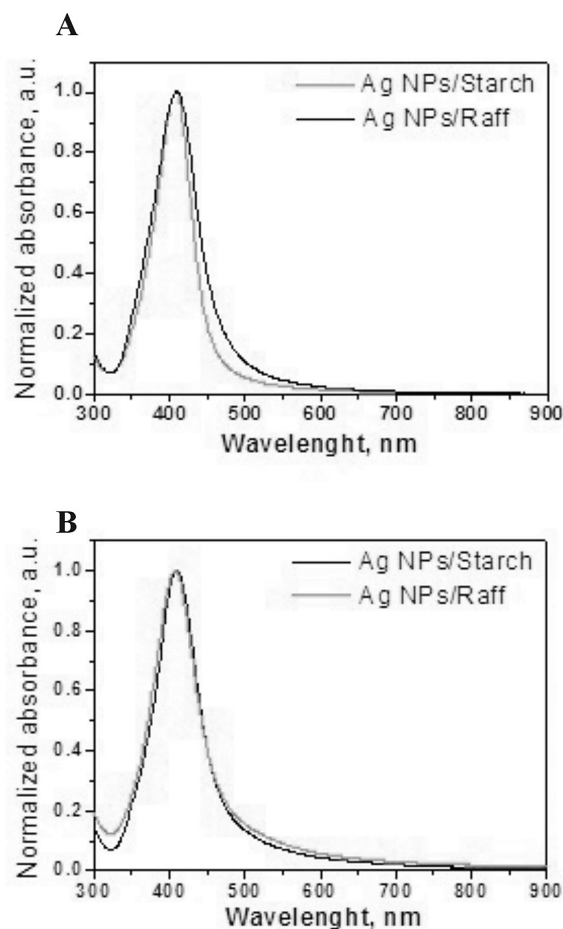


Fig. 2. A: UV-vis absorption spectra of the as-prepared silver NP dispersions B: Corresponding spectra after the concentrating procedure

of AgNPs/Starch and AgNPs/Raff was also confirmed by the ζ potential values of -25.3 ± 1.3 mV and -47.2 ± 1.1 mV, respectively. Nanoparticles have two key properties that make them attractive (i) on a mass base, they have much larger surface area and (ii) various chemical groups can be bounded to increase their affinity towards target compounds (Tiwari et al., 2008).

Frog heart preparations *in vitro* develop regular contractions with stable pattern and force. That is why these preparations are used in various physiological and pharmacological studies. Under our experimental conditions the spontaneous contractions of excised frog hearts slightly declined during the experiment, preceded by an initial moderate decrease of the force lasting during the first 15–20 min (Figure 3A, \blacklozenge). Therefore, we decided to apply AgNPs half an hour after the start of the experiment. Inhibitors were introduced 15 min before the first concentration of AgNPs. We applied increasing concentrations of AgNPs (1, 3, 10, 30, 50 and 100 $\mu\text{g/ml}$) which were introduced with a 15 min period between each.

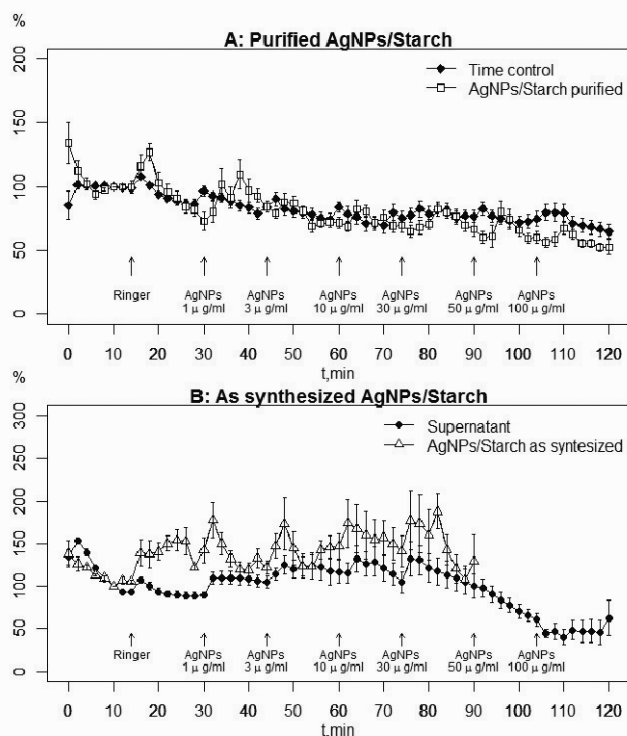


Fig. 3. Effect of AgNPs/Starch on the maximal force of contractions of frog heart preparations *in vitro*.

A: Effect of purified AgNPs/Starch (\blacklozenge) is compared with time control (\square). B: Experiments of frog heart preparations with as-synthesized AgNPs/Starch (\bullet) are compared with time control data with supernatant (\triangle). Data are means \pm SEM of 6 experiments.

The reaction mixture contained sodium and nitrate ions, which might compromise the observed effects. On the other hand, AgNPs dispersed in double distilled water and in presence of salts had higher ability to aggregate or to increase in size and affected particle behaviour (Handy et al., 2008). Therefore we studied the influence of purified (aqueous dispersion) and as synthesized (dispersed in the reaction mixture) AgNPs.

Purified AgNPs/Starch showed no significant effect on the force of contraction of frog heart preparations within the whole concentration range of AgNPs (Figure 3A). The dilution of purified AgNPs in Ringer solution led to a colour change of the solution, which indicated changes in size of AgNPs. We tested the influence of as synthesized AgNPs/Starch to avoid changes in the size and aggregation. The data was compared to results from experimental group treated with corresponding supernatant obtained by ultracentrifugation and diluted in the same manner as AgNPs. They increased the amplitudes of the force of contraction insignificantly (Figure 3B) but preparations became unstable at the application of the lowest concentration of AgNPs and none of them did not keep vitality for more than 90 minutes (Figure 3B, r 90–120 min). Even small increases in salinity above that of freshwater can dramatically decrease colloid concentration by aggregation and precipitation processes (Stolpe and Hasselov, 2007) and increase their toxicity (Kashiwada, 2006).

Further, we studied the effect of AgNPs/Raff (aqueous dispersion and as synthesized). Raffinose is a trisaccharide and compared with starch did not cover so tightly the silver core of the nanoparticles and leave more active sites. The application of purified AgNPs/Raff with increasing concentration (Figure 4A), caused a minimal increased effect of the force of heart contractions, but they are statistically insignificant. They also showed instability in aqueous solution with a colour change. We also tested the influence of as synthesized AgNPs/Raff. Their effects were compared with the corresponding time control with supernatant (Figure 4B). A significant positive inotropic effect was observed after introduction of as synthesized AgNPs/Raff at all tested concentrations.

The observed positive inotropic effect may result from stimulation of the sympathetic nervous system or due to the influence of the hormone adrenaline released from adrenal glands. Because the experiments were performed on excised heart preparation, the increase of the amplitude of the heart contraction cannot be a result of hormonal secretion. In order to estimate which of the adrenoreceptors were activated by nanoparticles, prazosin (a blocker of α_1 -adrenoreceptors) and propranolol (a blocker of all subtypes of β -adrenoreceptors) were used.

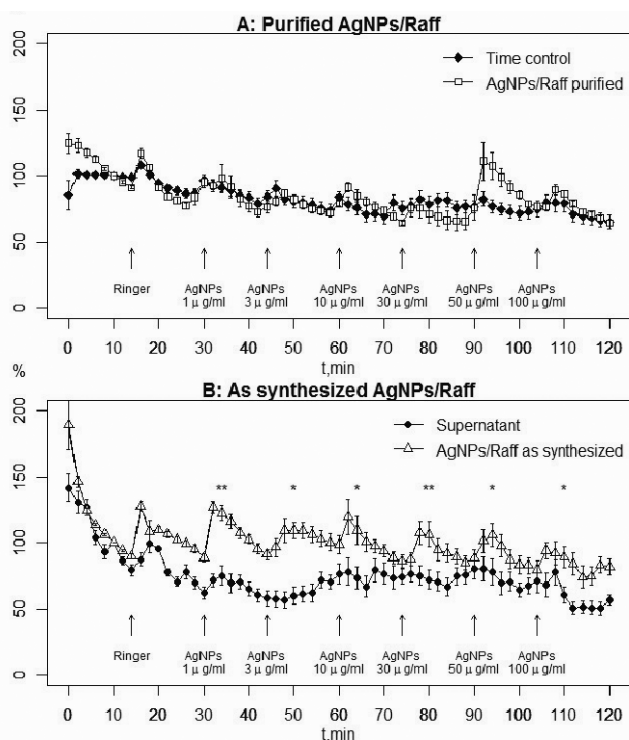


Fig. 4. Effect of AgNPs/Raff on the maximal force of contractions of the frog heart preparations. **A:** Experiments of frog heart preparations with purified AgNPs/Raff (◆) are compared with time control (□). **B:** Experiments of frog heart preparations with as-synthesized AgNPs/Raff (●) are compared with supernatant as a control condition (△). Data are means ±SEM of 6 experiments. ** $P < 0.01$, * $P < 0.05$.

The positive inotropic effect of as synthesized AgNPs/Raff in the presence of prazosin (Figure 5A) was significantly reduced, but not at all tested concentrations. In the presence of propranolol (Figure 5B) the positive inotropic effect of as synthesized AgNPs/Raff was removed only at concentrations of 30 and 50 μg/ml AgNPs. In accordance with obtained results studied AgNPs activate adrenoceptors non-specifically by influence on heart cell membranes.

The data obtained can be explained with increasing ionic strength by addition of salts to the media with Ringer solution. AgNPs start to be affected by attractive forces as van der Waals or electrostatic attraction due to surface charge. It is also possible to achieve aggregation by adding multi-charged polymers, which forms bridges between them. Multiple charged cations as Ca^{2+} and anions may show the same effect of promoted aggregation (Handy et al., 2008). Thus,

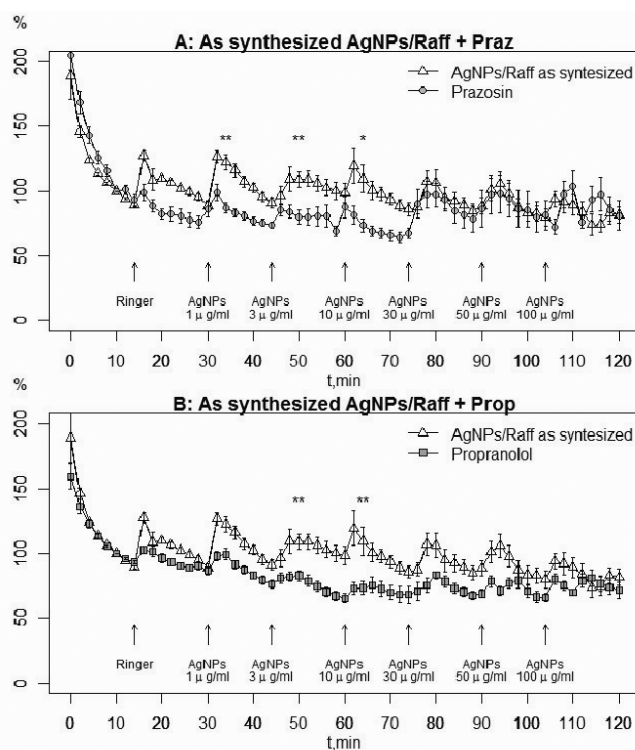


Fig. 5. Effect of as-synthesized AgNPs/Raff on the maximal force of contractions of the frog heart preparations added in the presence of blockers of α_1 - and β -adrenoreceptors. Experiments of frog heart preparations with 3 μM Praz (●, A) and that pretreated with 30 μM Prop (■, B) are compared with the effect of alone as-synthesized AgNPs/Raff (△). Data are means ±SEM of 6 experiments.

AgNPs/Starch had a higher capability to aggregate, especially in reaction mixture wherein probably has free starch molecules, and a higher toxicity. As synthesized AgNPs/Raff did not reveal the same toxicity but dramatically increase heart activity. This can have severe environmental consequences of their incorporation in the food chain of aquatic ecosystems. So, in the area of water purification and other industrial applications of AgNPs more appropriate and safe is the use of AgNPs/Starch compared with AgNPs/Raff and purified than as synthesized.

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