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RESEARCH ARTICLE

In vitro assessment of the toxicity of small silver nanoparticles and silver ions to the red blood cells

Zhenxing Chi[1,2] · Hongwei Lin[1] · Weiguo Li[1] · Xunuo Zhang[1] · Qiang Zhang[1]

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Abstract

This work reports the toxicity of small silver nanoparticles (nanoAg, 20 nm) and silver ions (Ag^+) to the red blood cells with the silver concentration level of 10^{-6} g/mL. Results show that red blood cells (RBCs) start hemolysis when treated by nanoAg of 1.5×10^{-5} g/mL or Ag^+ of 2.9×10^{-7} g/mL. A low ATPase activity of 30% has been observed after RBCs being treated with Ag^+ of 2.6×10^{-7} g/mL, while the nanoAg does not obviously affect the ATPase activity. In molecular level, Ag^+ is more toxic to the amino acid residues than nanoAg according to the change of fluorescence characteristics of hemoglobin (Hb). However, the nanoAg has been found to be more toxic than Ag^+ to the secondary structure of Hb in terms of the loss of α-helix content.

Keywords Silver nanoparticles · Silver ions · Toxicity · Red blood cells · Hemoglobin · ATPase

Introduction

Over the years, silver nanoparticles have been widely used in biomedical fields including antibacterial applications (Ahmed et al. 2016), chemical sensing and imaging (Schäferling and Resch-Genger 2017), molecular delivery (Levi-Polyachenko et al. 2016), gene therapy (Gopinath et al. 2008), and cancer treatments (Reshi et al. 2016). With the suddenly increased demand of using silver nanoparticles, silver in the forms of nanoparticle and ion (Beer et al. 2012) has largely accumulated in the body of patients. Non-patients exposed to silver in manufacturing facilities (Park et al. 2009) and contaminated environments (Duruibe et al. 2007) also face the problem of silver accumulation in human body. However, many reports reveal that certain concentrations (range 0 ~ 200 μg/mL) of silver nanoparticle or silver ion have significant toxic effects on organism (Maqusood et al. 2010), i.e., cells (Bastos et al. 2016), tissues (Samberg et al. 2010), organs (Piao et al. 2011), and function systems (Yang et al. 2013).

Blood, which transports the foreign substances over the human body, is the most important medium to the study on toxicity of silver nanoparticles. For biomedical or other applications, injection (Xue et al. 2012), inhalation, and ingestion (Smock et al. 2014) of silver nanoparticles are the main source of intake of silver nanoparticles into the bloodstream. The interactions between silver nanoparticles and blood components, particularly red blood cells, are thus very important to the toxicity of the hazardous material. Some studies have reported that the interactions between silver nanoparticles and biomolecules rely on their surface properties (Comer et al. 2015), stability of the silver nanoparticles, and components of the biomolecules (Poblete et al. 2016). Generally, the toxicity of any nanoparticles relies on their structures, components, material properties, binding affinities to biological sites, cellular intakes, and excretions (Schrand et al. 2010; Castranova 2011). Small silver nanoparticles (diameter < 100 nm) with more released silver ions have been observed to have higher toxicity than large silver nano/microparticles to red blood cells, which can result in the oxidative stress, membrane injury, and hemolysis (Choi et al. 2011; Chen et al. 2015). Since there is a reversible conversion between silver nanoparticles and silver ions, both silver nanoparticles and silver ions may

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✉ Zhenxing Chi
zhenxingchi@gmail.com

[1] Department of Environmental Engineering, Harbin Institute of Technology, Weihai, 2# Wenhua West Road, Weihai 264209, People's Republic of China

[2] Guangzhou Key Laboratory of Environmental Exposure and Health, School of Environment, Jinan University, Guangzhou 510632, People's Republic of China

account for the toxic effects (Kawata et al. 2009). The toxicity of silver nanoparticles to hepatoma cells is reported due to the oxidative stress and independence of silver ions (Kim et al. 2009). However, high fractions of silver nanoparticles to silver ions are reported to be more toxic to lung cells (Beer et al. 2012).

Yet, it is unclear if small silver nanoparticles are more toxic than silver ions to the blood oxygen-carrying function of human body. Red blood cells (or hemoglobins) are used to carry and deliver oxygen in human blood. In this work, the effect of small silver nanoparticles (20 nm) or silver ions on the structure and function of red blood cells are studied in vitro. The toxicity of the silver nanoparticles and silver ions is assessed regarding the red blood cells in cellular level and hemoglobins in molecular level. This work may provide a theoretical guidance for risk assessment of silver nanoparticles in biomedical applications or in the environment.

Materials and methods

Reagents and apparatus

EDTA-K_2 (Tianjin Kermel Chemical Reagent Co., Ltd.)-stabilized blood samples were obtained from the Weihai Blood Centre in China (ethics statement, the study was approved by the Ethics Committee of Weihai Blood Centre). Silver nanoparticles (NanoAg) with an average diameter of 20 nm was purchased from Nanjing XFNANO Materials Tech Co., Ltd. $AgNO_3$, hemoglobin (Hb, bovine), phosphate saline buffer (PBS) with a pH of 7.4, and ethanol were purchased from Sigma-Aldrich Co., Ltd. The silver solutions were preserved in the dark and diluted to different Ag concentrations as required. Trisaminomethane (Tris, AR, 0.05 M) was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Other chemicals were analytical grade and were purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China).

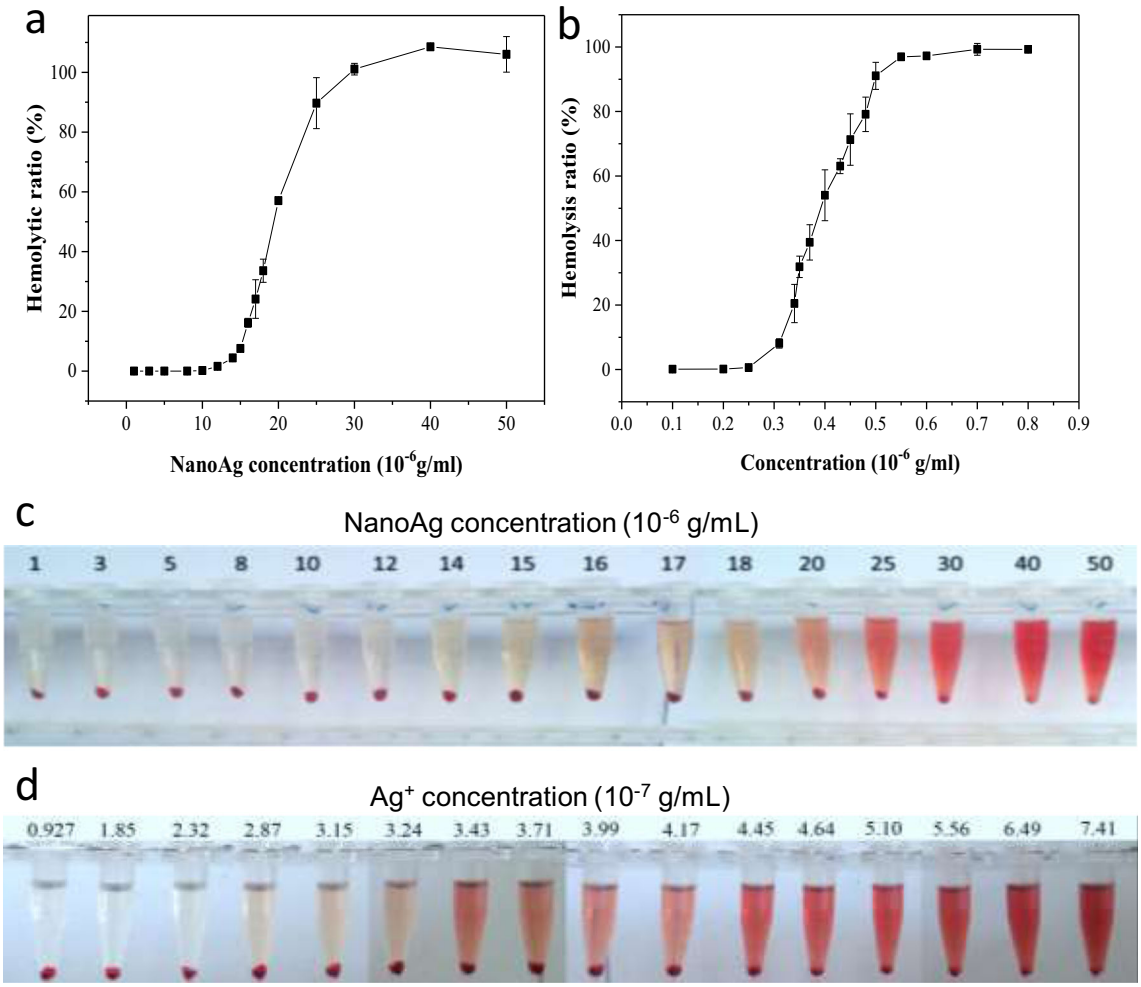

Fig. 1 Hemolytic ratios of the RBCs samples treated by nanoAg solutions (**a**, **c**) and Ag^+ solutions (**b**, **d**) with different Ag/Ag^+ concentrations. Concentration of RBCs is 8×10^7 cells/mL

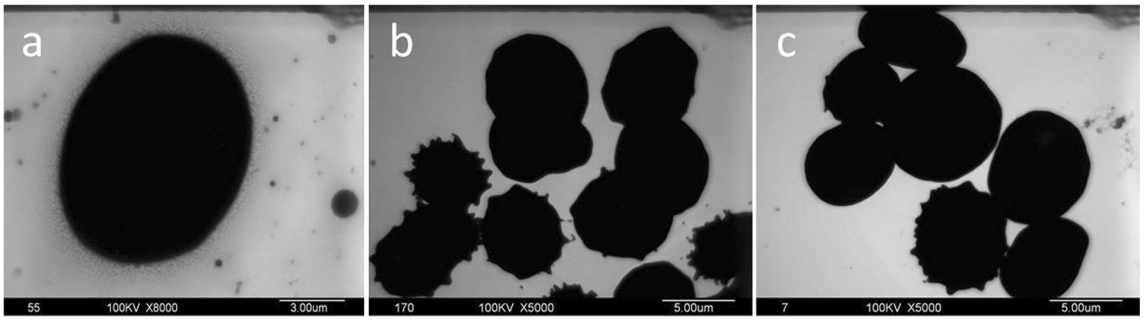

Fig. 2 TEM images of normal RBCs (**a**) and the RBCs starting hemolysis (hemolytic ratio of 10%) treated by nanoAg (**b**, 1.5×10^{-5} g/mL) and Ag^+ (**c**, 2.9×10^{-7} g/mL) solutions

UV-visible absorption spectra were measured by using a U-2900 spectrophotometer (Hitachi, Japan). Centrifugation of samples was performed by using a LDZ4-2 automatic centrifuge (Jiangsu Jintan Medical Instrument Factory). Digital dry bath incubator (HB-100, Hangzhou Bioer Technology Co., Ltd) was used for the control of temperature. The morphological changes of RBCs were observed by using a HT7700 transmission electron microscope (TEM, Hitachi, Japan) and the samples were coated by using a sputtering coater (Eiko IB5). Fluorescence measurements were performed using a F4600 fluorescence spectrometer (Hitachi, Japan). Circular dichroism (CD) spectra were measured by a Jasco J-810 CD spectrometer.

Cytotoxicity of nanoAg and Ag^+ to red blood cells

One milliliter of fresh blood sample was washed by 2 mL PBS solution for three times. The red blood cells (RBCs) sample was obtained by dispersing the centrifuged sediment in 20 mL PBS solution. 0.2 mL RBCs samples were incubated with 0.8 mL nanoAg or $AgNO_3$ solutions of different Ag concentration at 37 °C for 3 h. Each sample of 1 mL contained 8×10^7 cells. After centrifugation for 5 min at 2000 rpm, the supernatants of the RBCs samples were used to measure their hemolytic degree by spectrophotometer (hemolytic ratio = (A_{540nm} (test) − A_{540nm} (negative control))/(A_{540nm} (positive control) − A_{540nm}(negative control)) × 100%) and the sediments were treated with pure water to achieve hemolysis for the activity measurement of ATPase activity in RBCs. Three independent experiments have been done with data reported as means ± SD (standard deviation). TEM was used to measure the morphological change of RBCs. In preparation of the TEM sample, the RBCs samples were mixed with 2.5% glutaraldehyde and incubated at 37 °C for 12 h. The samples were washed with PBS solution and treated with osmium tetroxide solution (1%) for 5 h. After being washed with pure water for three times, the samples were gradually dehydrated by ethanol with increasing concentrations from 50, 70, 80, 90 to 100%. The samples were then replaced in isoamyl acetate solvent for 15 min and dried by critical point drying. The RBCs samples were coated by platinum by using the sputtering coater and measured by TEM.

Effect of nanoAg and Ag^+ on Hb

The molecular spatial structure of BHb and human hemoglobin (HHb) are almost identical. The amino acid sequences of bovine and human hemoglobins have up to 85% similarity (Chi et al. 2016). Therefore, BHb can be used instead of HHb to study the interaction between Ag^+ or nanoAg and Hb (Chi et al. 2016). With HHb substituted by BHb, the interactions between nanoAg or Ag^+ and Hb are assessed by fluorescence measurements. For the sample preparation, 1 mL Hb solution (3.0×10^{-5} M) was mixed with 1 mL Tris-HCl (5.0×10^{-3} M) and 8 mL silver solution (nanoAg or Ag^+) to obtain a final Ag concentration of 0–20×10^{-6} g/mL. The Tris-HCl solution was obtained by adjusting the pH of Tris solution to 7.4 with HCl. The excitation wavelength (λ_{ex}) was set at 280 nm and the emission wavelength (λ_{em}) range set at 280–500 nm. The slit width was 5 nm and the voltage was 400 V. The results were plotted using the Stern-Volmer equation ($F_0/F = 1 + K_{SV}[Q]$) (Geethanjali et al. 2015), where F_0 is

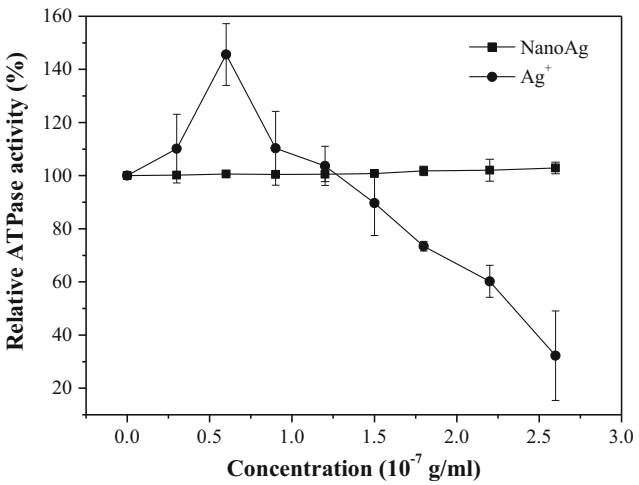

Fig. 3 The change of relative activities of ATPase with increasing the concentrations of nanoAg or Ag^+

the fluorescence intensity of Hb without quencher, F is the fluorescence intensity of Hb quenched by Ag/Ag^+, K_{SV} is the Stern-Volmer constant, and $[Q]$ is the concentration of the Ag/Ag^+ quencher, to compare the effects of nanoAg and Ag^+ on the fluorescence of Hb. The synchronous fluorescence spectra of Hb samples with the Ag/Ag^+ concentrations of ($\times 10^{-6}$ g/mL) 0, 0.2, 0.5, 1, 5, 10, and 20 were measured at $\Delta\lambda$ of 60 nm (Tan et al. 2018) to compare the effects of nanoAg and Ag^+ on the Tyr residues of Hb molecules. The resonance light scattering spectra of Hb-nanoAg, Hb-Ag^+, Hb, nanoAg, and Ag^+ solution were measured to identify if the Ag/Ag^+ and Hb molecules were well-mixed in solution (Chi et al. 2009). The CD spectra of Hb with the Ag/Ag^+ concentrations of ($\times 10^{-6}$ g/mL) 0, 4, and 8 were measured at room temperature (293 K) with the scan range of 200–250 nm, the scanning speed at 200 nm min^{-1}, and the quartz cell of 1-cm path length to study the change in α-helix structure (205–225 nm) (Tan et al. 2018) of the Hb biomolecules. The content of α-helix structure (%) can be calculated by using the equations (Cheng and Zhang 2008): MRE = observed CD/10Cnl and α-helix (%) = $[(-\mathrm{MRE}_{208} - 4000)/(33{,}000 - 4000)] \times 100$, where C is the molar concentration of Hb, n is the number of amino acid residues, l is the path length, and MRE_{208} is the MRE value at 208 nm.

Results and discussion

Hemolysis of RBCs caused by nanoAg and Ag^+

Figure 1a, c, shows that the RBCs start hemolysis with the hemolytic ratio of 10% when the concentration of nanoAg increases to 1.5×10^{-5} g/mL. The hemolytic ratio achieves almost 100% when the concentration of nanoAg increases to 3×10^{-5} g/mL. However, the RBCs start hemolysis (Fig. 1b, d) when the concentration of Ag^+ is 2.9×10^{-7} g/mL and the hemolytic ratio achieves 100% only when the concentration of Ag^+ increases to 5.1×10^{-7} g/mL. The phenomenon clearly shows that Ag^+ at these low concentrations can induce more hemolysis of RBCs. The effect of nanoAg and Ag^+ on the morphology of RBCs was examined by TEM. Figure 2a shows the morphology of RBCs with a radius of 3 μm and smooth surface. Figure 2b, c, shows the cytoplasmic projections and deformed structures of RBCs when the cells start hemolysis, due to the treatment of nanoAg (1.5×10^{-5} g/mL) and Ag^+ (2.9×10^{-7} g/mL) solutions, respectively. Regarding the bilayer-couple hypothesis for RBCs (Sheetz and Singer 1976), the possible reason for the hemolysis phenomena is the intercalation of nanoAg or Ag^+ in the lipid monolayers of cell membrane, which can cause severe damages to the membrane receptors, molecular channels, and osmatic pressure. In literature, Ballinger et al. (1982) have observed severe hemolysis of RBCs when the Ag^+ concentration increases up to 50 μM (2.4×10^{-6} g/mL). Chen

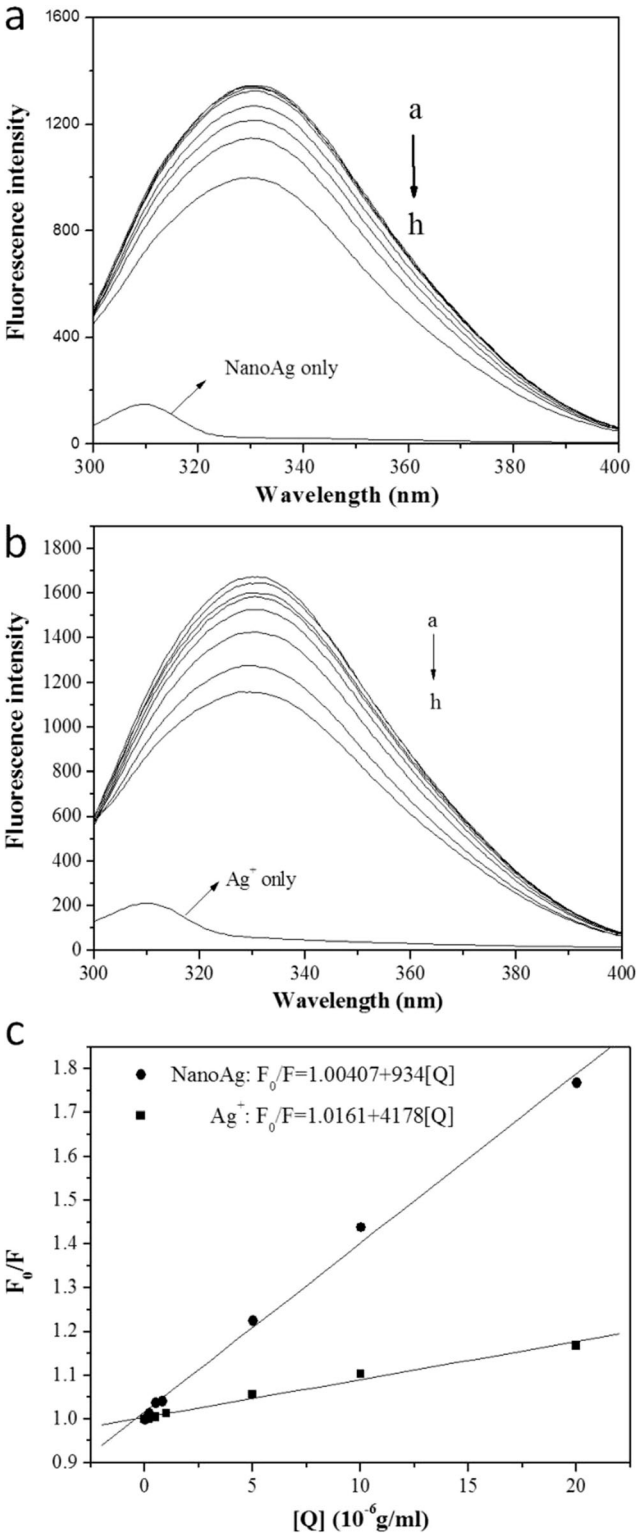

Fig. 4 Fluorescence spectra of Hb under different nanoAg (**a**) and Ag^+ (**b**) concentrations. Stern-Volmer plots (**c**) for the quenching of Hb by the nanoAg and Ag^+ under different concentrations of ($\times 10^{-6}$ g/mL) 0, 0.2, 0.5, 0.8, 1, 5, 10, and 20 (sub-a to sub-h)

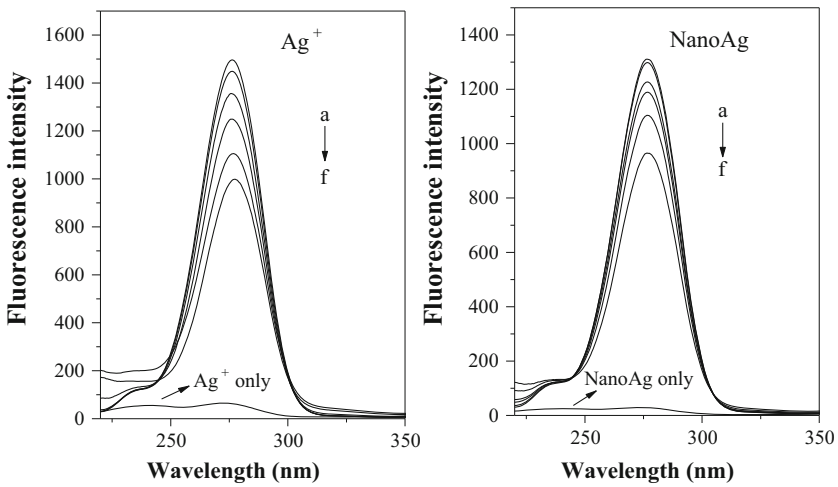

Fig. 5 Synchronous fluorescence spectra ($\Delta\lambda = 60$ nm) of Hb under different nanoAg or Ag^+ concentrations of ($\times 10^{-6}$ g/mL) 0, 0.5, 1, 5, 10, and 20 (sub-a to sub-f)

et al. (2015) have reported that a nanoAg (diameter of 15 nm) concentration of 5×10^{-6} g/mL can cause about 10% hemolytic ratio of RBCs. These works agree well with our results. According to the observations, both nanoAg and Ag^+ can cause hemolysis of RBCs, but Ag^+ is likely to be more toxic.

Effect of nanoAg and Ag^+ on the ATPase activity of RBCs

The decrease of the activity of ATPase can cause the disorder of cell energy metabolism, ion transport, and signal transduction to affect the normal function of cells. Therefore, the activity of ATPase was chosen to reflect the influence of nanoAg and Ag^+ on the function of RBCs in the study.

Figure 3 shows that the relative ATPase activity firstly increased (stimulation) and then largely decreased (toxic effects) to 30% of the initial level with the increase of Ag^+ concentration from 0 to 2.6×10^{-7} g/mL. The increase of the ATPase activity at a low Ag^+ concentration can be understood as hormesis. Such a low ATPase activity of 30% is not sufficient to maintain the bioactivity of RBCs, indicating that Ag^+ has a high cytotoxicity to RBCs. With a similar level of concentrations ($< 2.6 \times 10^{-7}$ g/mL), nanoAg does not have obvious influence to the ATPase activity.

Change in fluorescence characteristics and molecular structure of Hb

Fluorescence quenching is a widely used technique to study the effect of toxic substances on proteins (Chi et al. 2017; Zhou et al. 2017). The interaction between the toxic substances and the fluorescent amino acid residues can induce the decrease of fluorescence intensity. Figure 4a, b, shows the fluorescence spectra of Hb under different nanoAg and Ag^+ concentrations (0, 0.2, 0.5, 0.8, 1, 5, 10, and 20×10^{-6} g/mL), respectively. By adding nanoAg or Ag^+, the spectra peak (330 nm) of Hb decreases gradually. After plotting the fluorescence intensities by using Stern-Volmer equation (Fig. 4c), the F_0/F values of nanoAg quenching are higher

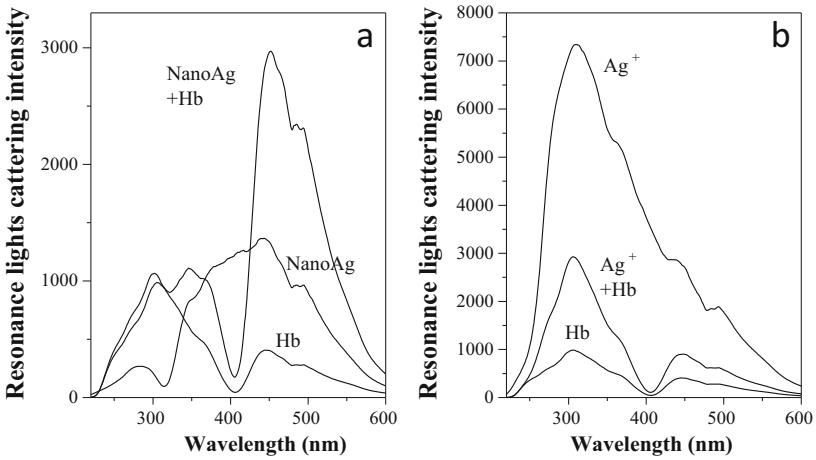

Fig. 6 Resonance light scattering intensity of Hb-nanoAg (**a**) and Hb-Ag^+ (**b**) solutions

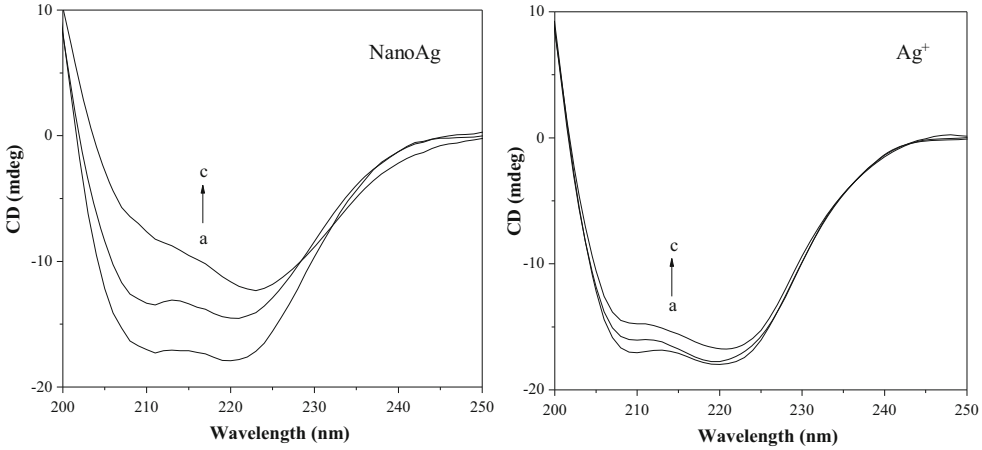

Fig. 7 Circular dichroism (CD) spectra of Hb samples treated by nanoAg or Ag^+ with the nanoAg/Ag^+ concentrations of ($\times 10^{-6}$ g/mL) 0, 4, and 8 (sub-a to sub-c)

than the F_0/F values of Ag^+ quenching, which shows that the fluorescence intensity (F) of Hb is more sensitive to Ag^+ with increasing the quencher concentrations. Tyr residue is a typical amino acid of Hb biomolecules and has fluorescence. The synchronous fluorescence spectra of Hb samples (Fig. 5) have been measured at $\Delta\lambda$ of 60 nm to compare the effects of nanoAg and Ag^+ on the Tyr residues (Hou et al. 2017). With the nanoAg or Ag^+ concentration increases from 0, 0.2, 0.5, 1, 5, 10 to 20×10^{-6} g/mL, the fluorescence intensities of the peak at 276 nm both decrease. The decrease by Ag^+ quenching ($\Delta F = 500$) is higher than that by the nanoAg quenching ($\Delta F = 340$); meanwhile, there is a slight peak shift from 276 to 278 nm observed by Ag^+ quenching. The small red shift of spectra may be related with a change of environment or slight deformation of protein skeleton (Wu et al. 2007). Since the toxicity of foreign substances relies on not only the intermolecular binding affinity but also the distribution of the molecules, resonance light scattering spectra of Hb-nanoAg, Hb-Ag^+, Hb, nanoAg, and Ag^+ solutions have been measured (Fig. 6). It can be seen from Fig. 6b that the F of the mixture of Hb-Ag^+ ($F = 3800$) is much lower than that of Ag^+ ($F = 7400$), and much higher than that of Hb ($F = 800$), which shows that there is no polymerization between Ag^+ and Hb, and the mixture of Hb-Ag^+ becomes more homogenous with Ag^+ added. However, the mixture of Hb-nanoAg ($F = 3000$) is much higher than Hb ($F = 800$) and nanoAg ($F = 1400$), which means that the polymerization occurred between Hb and nanoAg.

Table 1 The contents of α-helix structure of Hb after treatments of nanoAg or Ag^+ (calculated from the data of CD spectra)

NanoAg/Ag^+ concentration (g/mL)	0×10^{-6}	4×10^{-6}	8×10^{-6}
NanoAg treated	38.96%	24.21%	15.82%
Ag^+ treated	38.24%	35.68%	31.47%

Figure 7 shows the CD spectra of Hb samples treated with the Ag/Ag^+ concentrations of 0, 4, and 8×10^{-6} g/mL. The changes of Hb CD spectra indicate the interactions between nanoAg and Ag^+ with the amino acid residues of Hb skeleton, which can cause possible loss of functions of Hb (Melo et al. 1997). The two negative peaks (208 nm for the π–π* transition of α-helix; 222 nm for the π–π* transitions of α-helix and random coil) of CD spectra get smaller with increasing the concentration of nanoAg or Ag^+, which means a loss of α-helix content of Hb after the conjugation (Lu et al. 2007; Tan et al. 2018). From Fig. 7, nanoAg results in more loss of the content of α-helix structure than Ag^+. MRE_{208} has been calculated by using the data from CD spectra for α-helix content (%). Table 1 shows the contents of α-helix structure of Hb after treatments of nanoAg or Ag^+. After Hb being treated with nanoAg of 4 and 8×10^{-6} g/mL, its α-helix content largely decreased from 38.9 to 24.21% and 15.82%, respectively. After Hb being treated with Ag^+ of 4 and 8×10^{-6} g/mL, its α-helix content only decreased to 35.68% and 31.47%, respectively. The nanoAg is more toxic to the secondary structure of Hb than Ag^+ at the concentration level of 10^{-6} g/mL, which may result in severe loss of the biological activity (Giacomelli et al. 1997; Brandes et al. 2006). The deformation of protein structure by nanoAg is related with the formation of bioconjugate system with Hb (Bhunia et al. 2017). Therefore, in molecular level, Ag^+ is more toxic to the amino acid residues while nanoAg (20 nm) is more toxic to the secondary structure of Hb (Ag/Ag^+ concentration of 10^{-6} g/mL).

Conclusions

This work studied the toxicity of small silver nanoparticles (20 nm) and silver ions to the RBCs with the silver concentration level of 10^{-6} g/mL. RBCs started hemolysis when treated by nanoAg of 1.5×10^{-5} g/mL or Ag^+ of 2.9×10^{-7} g/mL. A low ATPase activity of 30% was observed after

RBCs being treated with Ag^+ of 2.6×10^{-7} g/mL, while the nanoAg did not obviously affect the ATPase activity. In molecular level, Ag^+ was more toxic to the amino acid residues than nanoAg according to the change of fluorescence characteristics of Hb. However, the nanoAg was more toxic to the secondary structure of Hb (loss of α-helix content).

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Compliance with ethical standards

The study was approved by the Ethics Committee of Weihai Blood Centre.

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