Assessment of Silver Nanoparticles Toxicity in Human Red Blood Cells Using ELISA and Immunofluorescence Microscopy Techniques

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Abstract—Colloidal silver and silver nanocompounds have been used for decades to diagnose and treat human diseases. The present study aims at assessing the molecular impact of silver nanoparticles on red blood cells (RBCs) following acute exposure.

Immunofluorescence, contrast phase microscopy and ELISA were used to assess the apoptotic rate as well as 2,3 DPG and ATP levels in human red blood cells. Although proved to be dose dependent, changes in terms of apoptotic rates, 2,3 DPG and ATP release as well as architectural structure in red blood cells following AgNps exposure were not significant up to a level of 20µg/ml.

Our findings could be of significance for the specific use of these silver nanoparticles in treatment of human diseases.

Keywords—silver nanoparticles, cytotoxicity, red blood cells, apoptosis, ATP production

I. INTRODUCTION

Biomedical nanoscience has a huge potential to bring benefits in detection, diagnosis and treatment of various diseases, with fewer side effects and better quality of life for the patients.[1-3] Silver nanoparticles have interesting optical properties, such as highly photothermal effect with surface plasmon resonance and strong optical absorption. Their unique size-dependent properties make these materials suitable for the attachment of biomolecules like proteins/antibodies, drugs and DNA. So far, silver nanoparticles have proven several promising effects, such as antitumor[4] gene therapy vector[5] anti-inflammatory[6] antiviral[7] and antibacterial effects[8, 9]. Even though most studies dedicated to silver nanoparticles have limited their experiments to in vitro toxicological assessments[10-12], recent data brings evidences on their in vivo toxicity. Various animal models, including zebrafish[12], Sprague-Dawley rats[13, 14] mice[15] demonstrate their toxic effects. Regardless of the administration pathway, the transit of silver nanoparticles through the blood flow is noticeable.[16] Since most of the future applications of silver nanoparticles are based on systemic administration, experiments on their interaction with human blood components are highly needed. In this context, the knowledge of the degree to which damage on red blood cells following exposure to silver nanoparticles contributes to the overall toxicity of AgNps is still limited.[17, 18] The present study aims at assessing the cellular and molecular impact of silver nanoparticles on red blood cells (RBCs) following acute exposure.

II. MATERIALS AND METHODS
A. Reagents

Commercially available colloidal silver nanoparticles (Purest Colloids Inc, Westamptom, USA) have been selected for our experiment. The initial solution was of 20 ppm concentration, 99.9% purity, 0.65 nm particle diameter as characterized by the manufacturer. Solutions of 10 µg/ml and 5 µg/ml were also prepared using appropriate dilutions. Annexin V-Cy3 Apoptosis Detection Kit was purchased from Enzo Life Sciences, ATP Colorimetric/Fluorometric Assay Kit was purchased from Biovision Inc. (San Francisco, USA) and 2,3-DPG ELISA Kit was purchased from Sunred Biological Technology Co., Ltd (Shanghai, China). All chemicals were acquired from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany)

Erythrocytes from healthy human donors were provided by the Blood Transfusion Centre of the 3rd Surgical Department, Cluj-Napoca, Romania. Blood was collected in
BD Vacutainer® Venous Blood Collection Tubes already prepared with 10-30 USP units of heparin/mL of blood in order to prevent clotting.

**B. Exposure to nanomaterials**

RBC exposure to AgNPs was performed in separate test tubes, in duplicates. For each concentration of AgNPs solution, 200 µl were mixed with 100 µl blood, followed by incubation (1 hour, 37°C).

**C. Characterization of silver nanoparticles**

Absorption spectra of the AgNPs used in the experiment were measured on a Shimadzu (UVmini-1240) spectrophotometer.

![Absorption spectra graph](image)

Fig. 1 Uv-Vis characterization of silver nanoparticles used in study.

**D. Annexin V apoptosis assay**

Annexin V, a protein that has a particularly high-affinity for phosphatidylserine, exposed on the surface of apoptotic cells, was used to assess the toxicity in RBCs following exposure to AgNPs. Detection was made in accordance with the manufacturer’s recommendations. Shortly, following incubation with NPs, 5x10^5 RBCs (as measured by flow cytometry-data not shown) were collected by centrifugation. Next, RBCs were resuspended in 500µl binding buffer and then stained with 5µl Annexin V (5 minutes, room temperature, in the dark). RBCs were further examined by fluorescence microscopy.

**E. Cell microscopy**

Annexin V-stained red blood cells were placed on a glass slide and covered with a glass coverslip. Following AgNPs administration and immunostaining, RBCs were observed using an OLYMPUS FSX-100 microscope with acquisition module and red fluorescence filter for Cy3 detection. Image data were further processed using FSX-BSW software (Ver.03.02.12).

![Annexin V-Cy3 apoptosis detection](image)

Fig. 2 Annexin V-Cy3 apoptosis detection by fluorescence microscopy following erythrocytes treatment with 5 µg/ml (A), 10 µg/ml (B) or 20 µg/ml (C) AgNPs. (scale bar = 20 µm)

**F. ELISA measurement of ATP concentration**

The metabolic function of RBCs following exposure to AgNPs was assessed by measuring cellular ATP concentrations. Reagent preparation: Reaction Mix was prepared according to the producer protocol from: ATP Assay Buffer, ATP Probe (ready to use as supplied), ATP Converter (obtained by separate dissolution in 220 µl Assay Buffer) and Developer Mix (dissolved in 220 µl Assay Buffer).

Sample preparation: colloidal silver solutions (20 µg/ml, 10 µg/ml, 5 µg/ml) - for every concentration at 200µl solution, 100µl blood was prepared. After one hour incubation, 0,4 g KCl were added, followed by centrifugation (4 minutes at 4000g and 0°C).

Preparation of the plate: Standard 96 well plates were filled in duplicate with 50 µl of Reaction Mix (44 µl Assay Buffer, 2 µl ATP probe, 2 µl ATP converter, 2 µl developer mix). We used 20 µl of supernatant for each probe well. For the blank well we used 20 µl supernatant from untreated blood sample. 30 µl Reaction Mix was added in each probe and in the blank well. The plate was incubated for 30 minutes at 37°C, being protected from light. The absorbance at 470 nm was read with a MR-9602G Microplate Reader (Perlong Medical Equipment Co, Beijing, China) and concentrations were calculated using the microplate reader software provided by the manufacturer.
G. ELISA measurement of 2,3 DPG concentration

Reagent preparation: Five standard probes, were prepared in duplicate from 120 µl Original standard and 120 µl Standard Diluent for Standard no.5. For Standard no.4,3,2,1 we used 120 µl of previously prepared solution and 120 µl Standard Diluent. Sample preparation: From each colloidal silver solution (20 µg/ml, 10 µg/ml, 5 µg/ml) we extracted 200 µl solution for 100 µl blood and incubated it for one hour, then added 0.4 g KCl and centrifuged it (4 minutes at 4000g and 0ºC). Preparation of the plate: 48 well plates were used as provided by the manufacturer of the assay. Standard wells were filled with 50 µl of standard reagents. The plate was incubated for one hour at 37ºC and further read using a MR-9602G ELISA Microplate Reader.

H. Statistical data analysis

Data was expressed as mean and standard deviation. Abnormal data was analyzed using nonparametric tests (the Kolmogorov-Smirnov test). The Wilcoxon Test was used to compare the same parameters within different groups. In all tests, the Alpha error level was less than 0.05. Data was analyzed using the Microsoft Office Excel Application, as well as the SPSS Statistics platform.

III. RESULTS

A. Uv-Vis characterization of silver nanoparticles

As seen in Fig. 1A silver nanoparticles used in the experiments displayed a specific peak at 420 nm.

B. Annexin V apoptosis assay

The ability of silver nanoparticles to induce apoptosis (programmed cell death) was assessed using Cy3-Annexin V staining. This assay is used to determine membrane integrity based on phosphatidylserine migration to the extracellular surface of apoptotic cells when disruption occurs. In our study, apoptosis rate was correlated with the fluorescence intensity of the three solutions tested. (Fig. 2) Annexin V fluorescence evaluates phosphatidylserine binding exposed at the surface of apoptotic cells. In addition, the auto fluorescence levels, a strong indicator of erythrocytes health status was found similar among the tested Ag solutions. (Fig. 3 A) Next we assessed the morphology of human red blood cells contrast phase techniques following Ag treatment. As observed in Fig. 3B the vast majority of RBC displayed a healthy status independent of concentration.

![Fig. 3 A) Autofluorescence image of human RBC incubated for 60 minutes with AgNps at various concentrations b)Following AgNps treatment as described above, to evaluate structural changes in shape and architecture cells were visualized by phase contrast microscopy. (X 400 magnification).](image)

C. 2,3-DPG Concentration

2,3-DPG was another parameter used in order to evaluate the toxic effect of silver nanoparticles on RBC membrane. The concentration of 2,3-DPG was correlated with the absorbance measured spectrophotometrically at 540 nm for the studied solutions. We observed that the higher the silver concentration the higher the 2,3-DPG release, (whiteout any statistical significance) emphasizing once again the dependence of cell destruction on nanomaterial dosage.

D. ATP concentration

ATP concentration was calculated using the absorbance, measured spectrophotometrically at 570 nm. The results show a
proportional growth between erythrocyte destruction and ATP release from apoptotic cells. As seen in Fig. 4B, although the increase was dose dependent, we didn’t obtained a statistical significance compared with control.

IV. DISCUSSIONS

Targeted drug delivery is an outstanding aspect regarding the use of nanoparticles in medicine, thoroughly examined at the moment.[19-23] Silver nanoparticles stimulate the deep interest of researchers because they are among the leading nanotech products used as antimicrobial agents.[2]

Several authors suggested that nano-silver particles and the Ag+ ions on their surface (in an oxidized state)[24] bind to sulfur- and phosphorus-containing biomolecules such as DNA or other biological moieties, thereby potentially causing cell toxicity.[25] Other authors showed that cytotoxicity of AgNPs is related to free radical formation that can damage cell membrane.[26] The nanoparticle chemistry, size, shape, agglomeration, surface and surface functionalization are major factors that influence the biokinetics of silver nanoparticles and consequently, their cytotoxicity. The nanoparticles used in the experiments in this study were sized between 0.5 and 0.65, with an Uv-Vis absorption peak displayed at 420 nm. (Fig. 1A)

The results presented in this study unveiled a dose-dependent cytotoxicity with low metabolic activity although this not proved to be statistically significant. These findings were also confirmed by other authors.[27]

The mechanisms of antimicrobial activity of silver ions and silver nanoparticles and their toxicity to human tissues are not fully characterized.[28] Red blood cells are the main cells carrying oxygen to tissues. Using an in vitro system and human red blood cells we evaluated the capacity of cells incubated with silver nanoparticles to release increased amounts of ATP in response to acute exposure. For the first time, our results show an increase in ATP concentration following AgNp administration, closely related to the increase in concentration. When erythrocytes were transiently exposed to AgNPs, ATP release increased. In addition, erythrocyte 2,3-DPG concentration was analyzed following AgNp treatment. 2,3-Disphosphoglyceric acid (also known as 2,3-DPG), a glycolytic precursor of 1,3-Bisphosphoglyceric acid (1,3-BPG) present in human erythrocytes at approximately 5 mmol/L, binds deoxygenated hemoglobin beta subunits by lowering their affinity to oxygen. 2,3-DPG is an allosteric effector enhancing the release of remaining oxygen molecules bound to hemoglobin, thus promoting the ability of RBCs to release oxygen in hypoxic tissues.

The concentration of 2,3-DPG was correlated with the absorbance of the standard solution measured spectrophotometrically at 540 nm. In this study, 2,3-DPG concentration was proportionally elevated after the interaction with growing nanoparticle concentrations. Physiologically, an increase in 2,3-DPG levels occurs to counteract the hypoxia associated with a stress stimulus, in this case, silver nanoparticles.

Currently, there is no consensus on whether or not toxicity mechanisms obtained in vitro may be compared with those expected to occur in vivo, as in in vitro studies one bolus dose is delivered, which is usually rather high. [20, 27, 29] When AgNps are administered in the blood stream, red blood cells are one of the first biological moieties being affected. Therefore, we tailored our research from a clinical perspective (i.e: when AgNps are administered in systemic circulation).[19] For a better understanding od the concentration range, it was compared to previous published studies.[13] Treatment doses up to 20 μg/ml used in this study proved to be well below the threshold level for cytotoxicity.

Human exposure to nanoparticles, either accidental or deliberate when its biomedical properties are exploited, is inevitable. Therefore, it is important to understand the parameters affecting nanosilver toxicity, especially in mammalian cells.[29, 30]

V. CONCLUSIONS

This study aims at assessing the degree of toxicity of silver nanoparticles on red blood cells. The results presented in this study unveiled a dose-dependent cytotoxicity with low metabolic activity although this not proved to be statistically significant.

Overall, a detailed understanding of the pharmacokinetics and toxicological properties of AgNPs and a balanced evaluation of
the risk/benefit ratio are required before recommending them for administration in humans.

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