Interaction of nanosilver particles with human lymphocyte cells

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Abstract
The damaging effects of nanoparticles were hypothesized to be the oxidative stress caused by the formation of reactive oxygen species and initiation of inflammatory reactions. In this context a study on the effects of nanosilver particles on the formation of reactive oxygen species in human lymphocyte culture was carried out. The obtained results showed that fluorescence intensity considerably increased after cells had interacted with nanosilver particles of varying concentrations, indicating the formation of reactive oxygen species and their accumulation in lymphocyte cells. Morphological study of the lymphocyte cells under the effects of nanosilver particles showed that the change in morphology depends on the concentration and size of nanosilver particles: for a size $\leq 20$ nm the lymphocyte cell significantly shrank with pronounced differences in the morphological structure of the cell membrane, but for a size $\geq 200$ nm no change was observed.

Keywords: nanosilver particle, lymphocyte, toxicity, oxidative stress
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1. Introduction
Nanotechnology is a rapidly growing tool of fabricating and utilizing nano-sized materials, among which silver nanoparticles are playing a major role in the field of nanotechnology and nanomedicine. Due to their unique physical, chemical and biological properties, nanosilver particles are frequently used in producing various engineered nanomaterial, such as antibacterial textiles, polymer films for food packaging, paints and pigments, filters for water or air treatment etc [1, 2]. Silver nanoparticles also have attractive biomedical applications due to their plasmonic and metallic properties as a diagnostic (e.g. biosensors, in vivo biomarkers) and therapeutic tool (e.g. photothermal tumor treatment) [3]. At least 20% of more than 800 available nanotechnology products contain nanosilver as an active ingredient, which causes an international group of researchers to consider nanosilver released into the water environment as one of 15 areas of concern that can threaten biological diversity [4].

The toxicity of silver nanoparticles (Ag-NPs) has been investigated for various cell types, including BRL3A rat liver cells [5], neuroendocrine cells [6], human hepatoma HepG2 cells [7], human alveolar epithelial cells [8] and germline stem cells [9]. Silver nanoparticles can be bound to different tissues and have potential effects such as induction of reactive oxygen species (ROS) and inflammation, which can be damaging to tissues [10, 11]. Silver nanoparticles and silver ions have been shown to accumulate mainly in the liver, kidney, lung and spleen and are harmful to human health. According to the experimental data [12], nanomaterials can
pass through cell membranes easily and cause severe toxic effects on human health. At higher concentrations (>44 mg L\(^{-1}\)) Ag-NPs are necrotic to cells, leading to cell membrane rupture.

Borm and Kreyling [13] showed that nanoparticles can also pass through the blood-brain barrier in mouse models. Seung-heon et al [14] demonstrated the toxic effect of Ag-NPs on the proliferation and cytokine expression by peripheral blood mononuclear cells (PBMCs). At a concentration of \(\geq 15\) mg L\(^{-1}\), nanosilver was found to have a significant cytotoxic effect on PBMCs, and phytohaemagglutinin-induced cytokine production was considerably inhibited by Ag-NPs.

Ahamed et al [15] showed that nanosilver has a potential to induce genes associated with cell cycle progression, DNA damage and apoptosis in human cells even at non-cytotoxic doses. The transmission electron microscopy (TEM) analysis [16] indicated the presence of Ag-NPs inside the mitochondria and nucleus, implicating their direct involvement in the mitochondrial toxicity and DNA damage. A possible mechanism of toxicity was proposed which involves disruption of the mitochondrial respiratory chain by Ag-NPs leading to formation of ROS and interruption of ATP synthesis, which in turn cause DNA damage.

Studying the effects of Ag-NPs on the biological functions (proliferation, cytokine release, and chemotaxis) of human mesenchymal stem cells (hMSCs) Greulich et al [17] showed a concentration-dependent activation of hMSCs at nanosilver levels of 2.5 \(\mu\)g ml\(^{-1}\) (particle diameter about 100 nm) and cytotoxic cell reactions occurred at Ag-NPs concentrations above 5 \(\mu\)g ml\(^{-1}\), while cell proliferation and chemotaxis of hMSC both decreased with increasing Ag-NPs concentrations. The release of cytokine IL-8 from hMSCs was significantly increased at high but non-cytotoxic concentrations of Ag-NPs (2.5 \(\mu\)g ml\(^{-1}\)). In contrast, the levels of IL-6 and VEGF were concomitantly decreased compared to the control group.

These experimental results clearly indicated the cytotoxic effects of Ag-NPs on human health. However, according to Sur et al [18], the toxicity of silver nanoparticles can be reduced by chemical modification, such as using lactose-modified silver nanoparticles for selective targeting of eukaryotic cells.

In this study we investigated the interaction of Ag-NPs with human lymphocytes, which are among the most important components of the human immune system. The adverse effect of nanoparticles on mammals was thought to be oxidative stress as a result of the generation of ROS and initiation of the inflammation signaling pathways. Cell membranes as a natural barrier would be the first to be impacted by the stress factors. One of the possible fast membrane reactions to stress is activation of lipid peroxidation (LPO) accompanied by a number of structural and functional disorders of the biological membranes.

The objective of the study is to investigate the effects of Ag-NPs on the induction of ROS in the cell culture of human lymphocytes, and cell viability, as well as morphology of the cell membranes.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were reagent grade or higher and were obtained from Sigma-Aldrich unless otherwise specified, including the following substances: NaCl, KCl, MgCl\(_2\), Na\(_2\)H\(_2\)PO\(_4\), HCl, H\(_3\)PO\(_4\), H\(_2\)O\(_2\), NaOH; phycoll, urographin, RPMI 1640, L-glutamine, HEPES, bovine serum, tanol, EDTA, DMSO, dichlorofluorescin-diacetate (DCFH-DA), penicillin and streptomycin. Lymphocyte samples were taken from donor’s peripheral blood from the Belarusian Haematology Research and Application Centre. Nanosilver solution with a concentration of 500 mg l\(^{-1}\) and an average particle size of 15 nm was prepared in Institute of Environmental Technology-VAST by aqueous molecular solution method [19], using sodium borohydride as a reducing agent and chitosan as a stabilizer. Then the solution was deionized by using cation exchange resin.

2.2. Methods of study

2.2.1. Preparation of lymphocytes from peripheral blood. 10 ml of buffer solution TBS (0.14 M NaCl, 5 mM KCl, 25 mM tris-HCl, pH 7.4) was added to 10 ml of a donor’s blood solution and the mixture was stirred and left at ambient temperature for layers separation. After 10 min centrifugation (1500 rpm) the lymphocyte layer (\(\rho = 1.077\)) was separated and diluted with TBS buffer to a larger volume. After a second centrifugation (7 min) the remaining erythrocytes were destroyed by being thoroughly suspended in 1 ml of cold water, then 1 ml of 0.3 M NaCl solution and some quantity of TBS buffer were added to the aqueous suspension. The mixture was centrifuged for 7 min and the lymphocyte fraction was suspended again in TBS buffer and counted in a Goryayev’s chamber.

Separation and purification of the donor’s blood lymphocytes were carried out by gradient phicoll-urographin method and lysis of residual erythrocytes was followed by centrifugation. So far as the separation of lymphocyte cells was carried out in a TBS buffer, which was not suitable for a longterm cells cultivation, it was replaced by a new medium RPMI 1640 to preserve cell viability for a longer incubation time. Simultaneously, for maintaining sterility a defined quantity of penicillin and streptomycin were added in order to prevent the bacterial growth during cell cultivation.

2.2.2. Lymphocyte cell treatment. After centrifugation, lymphocytes were transferred to a nutrient medium RPMI 1640 containing 0.3 mg ml\(^{-1}\) L-glutamin, 5 mM HEPES-buffer, 10% inactivated bovine serum, penicillin (100 U ml\(^{-1}\)), streptomycin (100 U ml\(^{-1}\)). The samples were placed in a CO\(_2\)—incubator at 37 °C. To study the interaction of Ag-NPs with lymphocyte cells, lymphocyte suspensions in TBS buffer containing 1.5% glutaraldehyde were incubated for varying periods in a dioxide carbon medium at 37 °C in the presence of nanosilver solution of different concentrations and particles sizes.
2.2.3. Determination of the ROS activity

Activity of the reactive oxygen species was determined by using fluorescent dye dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a substance which accumulates in the cells, and under the action of a specific esterase transforms into nonfluorescent-active dichlorofluorescin (DCFH). But in the presence of ROS, DCFH is oxidized to dichlorofluorescein (DCF) and emits fluorescence [20], as shown in figure 1.

In the experiments, hydroperoxide was used as a reactive oxygen species, therefore, it was necessary to know whether H$_2$O$_2$ is able to oxidize DCFH to DCF. 1 mM H$_2$O$_2$ was added to lymphocyte suspension previously loaded with DCFH. As a result, the fluorescence intensity increased considerably, validating the use of this probe to monitor the formation of ROS in lymphocytes. According to the schema presented in figure 1, inside the blood cells 2′,7′-dichlorofluorescein-diaceotate is hydrolyzed to 2′,7′-dichlorofluorescin and then oxidized to dichlorofluorescein in the presence of H$_2$O$_2$ [20]. Therefore, for quantitative determination of reactive oxygen species DCFH was prepared as follows: 5 μl of 10 mM DCFH-DA solution in DMSO was mixed with 45 μl DMSO and 1 μl of 0.01 N NaOH. The mixture was incubated 30 min with lymphocyte suspension in TBS buffer at ambient temperature and then neutralized with 1 ml of 25 mM NaH$_2$PO$_4$ (pH 7.4) and left on the ice. 5 mM DCFH was added to the cuvette containing 1.75 ml TBS buffer. After 60 s a defined quantity of nanosilver solution with defined concentration was added to the cuvette and fluorescent intensity was measured on a spectrofluorometer CM 220.

2.2.4. Morphological characterization of the lymphocyte cells

Determination of morphological characteristics of human lymphocyte cells in the presence of nanosilver particles was carried out on AFM NT-206 in contact mode at temperature 20 ± 3 °C, relative humidity 58 ± 20% and atmospheric pressure 100 ± 3 kPa.

3. Results and discussion

3.1. Influence of nanosilver concentration on the formation of ROS in human lymphocytes

Figures 2(a), (b) illustrates the dependence of DCFH fluorescence intensity in human lymphocytes on the incubation...
time with Ag-NPs of different concentrations. The experimental data indicated that fluorescence intensity linearly increased with increasing contact time from the beginning to 60 min, even at much more prolonged incubation time (figure 3). The data also showed that the higher the nanosilver concentration the greater the increment of fluorescence intensity. This witnessed the formation of ROS and their accumulation in lymphocyte cells.

Figures 3(a)–(d) demonstrates the effects of extended incubation times on DCFH fluorescence intensity in lymphocyte cells. As observed in figure 2, at a given Ag-NPs concentration the fluorescence intensity increased with increasing incubation time. The data depicted in figure 3 also showed that fluorescence intensity partially decreased after 24 h incubation at a higher concentration of nanosilver solution (75 and 100 μg ml⁻¹, figures 3(c) and (d)). It could be explained by the fact that some lymphocytes might have died under the effect of Ag-NPs at high concentration. From these results it can be concluded that the increase in fluorescent intensity of DCFH in human lymphocytes under the action of Ag-NPs with increased concentration confirmed the ability of nanosilver particles to induce the formation of ROS in lymphocyte cells, which may adversely affect viability of human lymphocytes. These results were in conformity with the experimental data presented in other works on the oxidative effects of nanosilver particles on human organisms, e.g. hepatoma cells [7], germline stem cells [9], PBMCs and hMSCs [14, 17].

3.2. Visualization of the interaction of Ag-NPs with a human lymphocyte

Topographic images of a human lymphocyte cell treated with Ag-NPs and immobilized by glutaraldehyde on a silicon substrate are presented in figures 4(a) and (b), in which one can see an uneven surface of the lymphocyte membrane. Figure 5(a) presents a TEM micrograph of Ag-NPs produced by aqueous molecular solution method using chitosan as a stabilizer and AFM topographic images of these Ag-NPs immobilized on a silicon substrate (figure 5(b)).

The image in figure 5(a) shows that Ag-NPs in the aqueous solution have an average particle size of about 15 nm, but under the objective of AFM the apparent size of these particles greatly increased up to a hundred nanometers (figure 5(b)). This is because the chitosan layer on the nanosilver particle is transparent for TEM, but non-transparent for AFM imaging. The average particle size of the Ag-NPs was about 150–200 nm, much greater than those on the TEM micrograph.

Figure 6 illustrates the morphological changes of human lymphocytes under the action of silver nanoparticles of different sizes (concentration of the nanosilver solution was...
40 mg L\(^{-1}\)). After 6 h of interaction with Ag-NPs of size \(\leq 20\) nm the lymphocyte cell significantly shrank with pronounced differences in morphology of the cell membrane (figure 6(a)), but for a size of \(\geq 200\) nm with the same nanosilver concentration and incubation time, the cell’s geometry remained unchanged (figure 6(b)).

4. Conclusion

Fluorescence intensity of the probe DCFA-DA in the lymphocyte culture considerably increased under the affect of Ag-NPs in a dose-dependent manner, suggesting the formation of ROS and their accumulation in the cultured cells. This
result also supported the use of ROS as a means for validation of the toxicity of nanoparticles to human health. Morphological study of the lymphocyte cells in the presence of nanosilver particles was carried out using AFM method. Morphological changes depended on the concentration and size of nanosilver particles: for a size $\leq 20$ nm the lymphocyte cell significantly shrunk with pronounced differences in topography of the cell membrane, but there were no effects for a size $\geq 200$ nm with the same nanosilver concentration and incubation time.

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