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Morphological changes of the red blood cells treated with metal oxide nanoparticles

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ABSTRACT

The toxic effect of Al_2O_3, SiO_2 and ZrO_2 nanoparticles on red blood cells of Wistar rats was studied in vitro using the atomic force microscopy and the fluorescence analysis. Transformation of discocytes into echinocytes and spherocytes caused by the metal oxide nanoparticles was revealed. It was shown that only extremely high concentration of the nanoparticles (2 mg/ml) allows correct estimating of their effect on the cell morphology. Besides, it was found out that the microviscosity changes of red blood cell membranes treated with nanoparticles began long before morphological modifications of the cells. On the contrary, the negatively charged ZrO_2 and SiO_2 nanoparticles did not affect ghost microviscosity up to concentrations of 1 μg/ml and 0.1 mg/ml, correspondingly. In its turn, the positively charged Al_2O_3 nanoparticles induced structural changes in the lipid bilayer of the red blood cells already at a concentration of 0.05 μg/ml. A decrease in microviscosity of the erythrocyte ghosts treated with Al_2O_3 and SiO_2 nanoparticles was shown. It was detected that the interaction of ZrO_2 nanoparticles with the cells led to an increase in the membrane microviscosity and cracking of swollen erythrocytes.

1. Introduction

Unique and remarkable properties of metal oxide nanoparticles (NPs) ensure their wide applications in mining, aerospace, refinery and chemical industries, etc. (Miller et al., 2010; Li, 2013; Kim et al., 2014). Besides, NPs are very promising for biomedicine and tissue engineering (Mortensen et al., 2006; Stark, 2011; Parveen et al., 2012). Meanwhile, metal oxide NPs are well-documented to pass through different protective barriers and accumulate in the lungs, brain, liver, spleen, bones and other organs (Rothen-Rutishauser et al., 2006; Nel et al., 2009; Stark, 2011; Paula et al., 2012; Kumar et al., 2012; Lamprou et al., 2013). For this reason, it is very important to study carefully the toxicity of such NPs which is the dark side of bionanotechnology.

In spite of the huge quantity of the data concerning the toxic effect of NPs in literature, many of them are often fragmentary and contradictory. Thus, there is a common belief that SiO_2 NPs possess stable physical and chemical properties, an enormous surface-to-volume ratio, low toxicity and the ability to be functionalized with a range of molecules and polymers. Due to the remarkable properties of silica oxide NPs they are present good perspectives in biomedical applications including optical imaging, cancer therapy, and targeted drug delivery (Paula et al., 2012). Conversely, it has been reported that exposure to SiO_2 NPs results in cellular morphological modifications, mitochondrial dysfunction, and oxidative stress in red blood cells (RBCs) (Gerashchenko, 1998) and human embryonic kidney cells (Wang et al., 2009), induces both necrosis and apoptosis of endothelial cells (Duan et al., 2013). Such contradictions have arisen due to a wide variety of the synthesis protocol, the chemical composition, the size and shape of the NPs, their surface charge, capping ligands, etc. As a consequence, the result of physical, the chemical and biological interactions between NPs and the lipid membrane of the cells might be quite different.

Since the numerous experiments with NPs showed that irrespective of the method of their administration route into the living body, NPs will inevitably get into the blood flow, it is of interest to study the NP interaction with blood cells (Mocan, 2013). In this case, the simplest and the most informative method for studying the surface morphology and the structure of RBCs is the atomic force microscopy (AFM). In contrast to the scanning electron microscope, an atomic force microscope is simple to use, does not require a vacuum and can be operated with aqueous

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media. The latter allows to obtain the surface topography of biological objects with high resolution in their natural environment, or in saline solution (Dufrene, 2002; Jena and Hörber, 2002; Francis et al., 2009; Frederix et al., 2009). Moreover, AFM method can easily identify changes in structure of cells under various external influences, as well as to determine their resistance to toxic substances and other exogenous factors.

Microviscosity measurements of RBCs subjected to NPs is also of great interest, as it allows evaluating the plasticity of their membranes (Abdelhalim, 2011; Honerkamp-Smith et al., 2013; Hormel et al., 2014). Due to the plasticity, erythrocytes are able to undergo considerable deformation when passing through narrow and curved capillaries with subsequent recovery of their original shape. However NPs induced conformational changes of RBCs can alter the membrane plasticity that drastically affects the ability of erythrocytes to perform their functions, especially the respiratory one. The possible increase/decrease in the membrane resistance accompanied by the change of cell morphology after the interaction with NPs can leads to disruption of their ductility and the loss of their ability to deform.

This paper presents the results of the AFM studies and the fluorescence analysis of the toxicity of SiO_2, ZrO_2 and Al_2O_3 nanopowders with respect to RBCs.

2. Materials and methods

2.1. Nanopowder characterization

Three different commercially available metal oxide nanopowders were used in this study. The SiO_2 powder was purchased from Sigma-Aldrich Chemie Gmbh (Germany). TZ 3YS nanopowder of partially yttrium-stabilized zirconium dioxide (ZrO_2 + 3 mol%Y_2O_3) was produced by TOSOH (Japan). Highly pure Al_2O_3 powder was grade HITK (Germany).

Structural characterization of the nanopowders was made by transmission electron microscopy (TEM) using a JEM 1400 instrument (JEOL, Japan). Zeta-potential of the nanopowders in isotonic phosphate buffer solution (PBS) of pH = 7.4 was determined using a Zetasizer Nano (Malvern Instrument, UK). All nanopowder samples were prepared at a concentration of 2 mg/ml.

2.2. Preparation of RBC

RBCs were extracted from the fresh blood of Wistar rats after decapitation under light nembutal anesthesia. The animals were used with the approval of Local Ethics Committee of Siberian State Medical University (Permission No. 1923 from 28.03.2011). The blood was diluted to a tenfold volume with PBS, pH 7.4 containing 0.136 M of Na_2HPO_4 and 0.044 M of KH_2PO_4. After sedimentation of cells by centrifugation at 600*g* for 10 min, the supernatant was poured off and the washing procedure was repeated four times. During the last three washings, the volume ratio blood:isotonic buffer was 1:4. The procedure of cell preparation was described in detail elsewhere (Kozelskaya and Panin, 2014).

2.3. AFM-study of RBC

The nanopowders were diluted with the isotonic phosphate buffer and mixed with erythrocytes to obtain the final NP concentrations of 4 ng/ml–2 mg/ml. The cell count in 1 μl of the buffer was 820,000. The incubation time was about 2–5 min. After that, the resulting erythrocyte suspension volume of 20 μl was deposited onto a glass slide as a thin smear. The smear was predried for 10 min in the air at 24 °C and humidity of 40%. After evaporation of excessive surface moisture, the smear was investigated using a Solver HV (NT-MDT, Russia) atomic force microscope with silicon nitride cantilevers (Veeco, USA spring constant k = 0.01 N/m) in the tapping-mode. The measurements were performed under atmospheric conditions at room temperature.

2.4. Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared after hemolysis in the hypotonic phosphate buffer (2.75 mM of KH_2PO_4 and 8.5 mM of Na_2HPO_4, pH 7.4). After sedimentation of erythrocyte ghosts by centrifugation at 5500*g* for 40 min, the supernatant was poured off and the washing procedure was repeated four times.

2.5. Microviscosity analysis of erythrocyte membranes

The effect of the nanopowders on the membrane microviscosity was studied with a Spectrofluorophotometer RF-5301 (Shimadzu, Japan). 4 ml of the hypotonic phosphate buffer containing erythrocyte ghosts together with one of nanopowders were placed into 1 × 1 × 4 cm^3 quartz cuvette. The ghost protein concentration in the buffer determined by the Warburg-Christian methods was 0.10–0.25 mg/ml. The cuvette with the ghost suspension was inserted into a spectrofluorophotometer thermostat for 10 min. The intensity of the intrinsic fluorescence of tryptophan residues in protein membranes was measured. In all experiments the cuvette temperature was 36 °C. To measure the microviscosity of the lipid bilayer near proteins (protein-lipid interaction), we used the excitation wavelength λ = 281 nm and spectral slit width 1.5/5. The microviscosity of the lipid bilayer far from proteins (lipid-lipid interaction) was measured with the excitation wavelength λ = 337 nm and the spectral slit width 1.5/3, with the maxima of emission intensity observed at λ = 374 nm and λ = 393 nm (the vibronic emission peaks of excited pyrene monomers), and λ = 468 nm (the emission maximum of excited pyrene dimer).

To measure microviscosity, the concentration of NP (c) in phosphate buffer was calculated as follows. At first, we found the volume of one NP by measuring its diameter from the TEM images. Then, we obtained the mass of a NP and calculated the molar concentration of the NPs in phosphate buffer in the following way:

$$c = \frac{\text{NPs mass}}{\text{volume of PBS} \times \text{one NP mass} \times \text{Avogadro constant}}.$$

The value of the microviscosity of intact erythrocytes was taken as 1 arbitrary unit.

2.6. Statistical analysis

Microviscosity measurements were replicated tree times. The results were expressed as mean ± standard deviation. Differences were considered significant as $p < 0.05$.

3. Results

3.1. TEM investigations of the nanopowders

Fig. 1 shows the TEM-images of SiO_2, ZrO_2 and Al_2O_3 NPs subjected to preliminary sonication in an ultrasonic bath at room temperature for 30 min. Agglomeration of the silicon dioxide NPs is well pronounced, only a few isolated particles with an average size of about 30 nm can be observed (Fig. 1a). The zirconium dioxide nanopowder is characterized by spheroid-like particles, which are approximately 80–100 nm in diameter. The ZrO_2 clusters consisting of several primary particles are shown in Fig. 1b. Finally, disc-like Al_2O_3 particles are clearly seen in Fig. 1c. The diameter of Al_2O_3 NPs varies from 15 to 60 nm. It should be noted that the alumina NPs are less prone to agglomeration as compared with other nanopowders, but their sizes are various.