Research Article

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The impact of orally administered gadolinium orthovanadate $GdVO_4:Eu^{3+}$ nanoparticles on the state of phospholipid bilayer of erythrocytes

ORAL VERİLEN GADOLİNYUM ORTOVANADAT $GdVO_4:Eu^{3+}$ NANOPARTİKÜLLERİNİN ERİTROSİT FOSFOLİPID ÇİFT TABAKASI ÜZERİNE ETKİSİ

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Abstract

Objectives: To assess the state of phospholipid bilayer of red blood cells (RBCs) in rats orally exposed to gadolinium orthovanadate $GdVO_4:Eu^{3+}$ nanoparticles (VNPs) during two weeks using fluorescent probes – ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole.

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Methods: Steady-state fluorescence spectroscopy: a study by the environment-sensitive fluorescent probes – 2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole (probe O10) and 2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole (probe PH7).

Results: No significant changes are detected in the spectra of the fluorescent probes bound to the RBCs from the rats orally exposed to nanoparticles in comparison with the corresponding spectra of the probes bound to the cells from the control group of animals. This indicates that, in case of the rats orally exposed to nanoparticles, no noticeable changes in physico-chemical properties (i.e., in the polarity and the *proton-donor ability*) are observed in the lipid membranes of RBCs in the region, where the probes locate.

Conclusions: No changes in the physical and chemical properties of the erythrocyte membranes are detected in the region from glycerol backbones of phospholipids to the center of the phospholipid bilayer in the rats orally exposed to VNPs during 2 weeks.

Keywords: cell membrane; erythrocyte; fluorescent probes; nanoparticles.

ÖZ

Amaç: Bu çalışmanın amacı iki hafta boyunca 2,5 diaril-1,3-oksazol orto-hidroksi türevleri floresan problemleri kullanılarak Gadolinium ortovanada $GdVO_4$ 'e oral yolla maruz kalan sıçanlarda RBC'lerin fosfolipid çift tabakasının durumunu değerlendirmektir.

Yöntemler: Floresans spektroskopisi: çevreye duyarlı floresan problemleri—2-(2'-OH-fenil)-5-fenil-1,3-oksazol (prob O10) ve 2-(2'-OH-fenil)-fenantro [9,10]-1,3-oksazol (PH7 probu).

Sonuçlar: Kontrol grubundaki hayvanların hücrelerine bağlı problemlerin karşılık gelen spektrumlarına kıyasla, nanoparçacıklara oral olarak maruz kalan sıçanların RBC'lerine bağlı floresan problemlerin spektrumlarında önemli bir değişiklik tespit edilmemiştir. Bu, nanopartiküllere oral yolla maruz kalan sıçanlarda, problemlerin bulunduğu bölgedeki RBC'lerin lipid membranlarında fiziko-kimyasal özelliklerde (yani polarite ve proton-verici kabiliyetinde) belirgin bir değişiklik gözlenmediğini göstermektedir.

Tartışma: İki hafta boyunca oral olarak VNP'lere maruz kalan sıçanlarda eritrosit membranlarının fiziksel ve kimyasal özelliklerinde, fosfolipitlerin gliserol omurgalarından fosfolipid çift tabakasının merkezine kadar hiçbir değişiklik tespit edilmemiştir.

Anahtar kelimeler: hücre zarı; eritrosit; floresan problemler; nanopartiküller.

Introduction

Nanotechnology has revolutionized the field of medicine during the last decade. Nanoparticles (NPs) have found numerous applications in various biomedical fields. It has been reported that NPs can be used as targeted drug delivery systems, biosensors, for gene delivery, bioimaging and magnetic hyperthermia [1–4].

Nanoparticles that contain rare Earth elements form one of the types of nanostructured materials. The future of their biomedical application seems to be promising due to their stability and low toxicity [5]. In particular, it has been reported that scintillating gadolinium orthovanadate NPs doped with europium ions $\text{GdVO}_4:\text{Eu}^{3+}$ (VNPs) can be involved in free radical scavenging *in vitro* [6]. There is some evidence that VNPs have beneficial health-related effects in experimental models. Administration of VNPs normalized sexual behavior and restored fertility in rats with neonatal reproductive dysfunction [7]. However, their practical implementation for therapeutic purposes raises questions on their safety, and the entire emerging field of nanomedicine requires developing safety assessment tests that can be used to predict the toxicity of nanomaterials. One of the mechanisms by which nanoparticles can exert toxicity at the cellular level is disruption of cell and organelle membranes, especially the lysosomal ones [8]. Cell membrane damage affects its permeability and decreases the viability of cells [9]. The loss of lysosomal

membrane integrity results in the leakage of the intralysosomal content rich in proteolytic and other hydrolytic enzymes with the significant damage to cells [10]. Furthermore, the toxic effects of NPs can be observed due to their ability to generate reactive oxygen species (ROS) [8, 11]. Reactive oxygen species are involved in free radical oxidation of polyunsaturated fatty acids (PUFAs) abundant in phospholipid bilayer of cell membranes causing lipid peroxidation. Lipid peroxidation, in its turn, increases the membrane permeability making the cells more vulnerable [12]. Thus, the evaluation of cell membrane integrity can be used to assess the toxicity of VNPs. Erythrocytes can be used as target cells for evaluating the toxicity of VNPs *in vivo*, since they are directly exposed to VNPs released into the bloodstream after their absorption in the intestine. Lipid peroxidation increases the membrane viscosity and, therefore, decreases the membrane fluidity in cells, including red blood cells (RBCs) [13, 14], which can be detected using fluorescent probes.

In this study, the state of phospholipid bilayer of RBCs was estimated by using fluorescent probes O10 (2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole). The mentioned fluorescent probes were chosen because their fluorescent characteristics depend on the physico-chemical properties of their microenvironment: the proton-donor ability, the polarity and viscosity of the microenvironment [15–19].

It is well known that the excited state proton transfer (ESIPT) reaction takes place when the ortho-hydroxy 2,5-diaryl-1,3-oxazole is in the excited state [15–19]: hydroxyl group in the ortho-position of the lateral benzene ring acts as proton donor, whereas the nitrogen atom of oxazole ring acts as proton acceptor (Figure 1). Phototautomer form (T^*) is formed in result of the ESIPT-reaction. The phototautomer is fluorescent in significantly longer wavelengths compared with the initial (or so-called “normal”) form (N^*) [15–19].

The presence of two-band fluorescence enables us to perform ratiometric measurement, i.e., to use the ratio of the phototautomer form and the initial form fluorescence intensities (I_{T^*}/I_{N^*}) as a parameter for assessment of the physical and chemical properties of the microenvironment.

Usage of ratiometric fluorescent probes allows eliminating not only the measurement error caused by the deviation of the fluorescent probe concentration (e.g., uneven content of fluorescent probe in various membranes), but also the measurement errors due to deviation in configuration and adjustment of equipment for measurements of fluorescence (e.g., deviation in the intensity of the source of excitation light, changes in the sensitivity of the photodetector, changes in focusing, etc.) [20–23].

Figure 1: Scheme of excited state intramolecular proton transfer (ESIPT) in 2-(2'-hydroxyphenyl)-5-phenyl-1,3-oxazole (probe O10) [24]. The upwards arrow represents the electronic excitation and the downwards arrow shows the emission of light (fluorescence). Corresponding maximum of absorption and the ranges of emission are shown in nanometers.

Location and orientation of probes O10 (2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole) in the cell membrane is shown in Figure 2. Probe O10 is located in the area of glycerol backbones of phospholipids (closer to the center of the lipid bilayer), in the area of carbonyl groups of

Figure 2: Location and orientation of fluorescent probes O10 (2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole) in phospholipid membranes [15, 24]. Two molecules of phosphatidylcholine from the outer leaflet are shown to denote the location of the probe.

phospholipids and in the area of hydrocarbon chains of phospholipids (near the carbonyl groups of phospholipids). Probe PH7 is located in the area of hydrocarbon chains of phospholipids (closer to the center of the bilayer).

The location and orientation of probes O10 and PH7 in the cell membrane are proposed on the basis of their fluorescent properties in lipid membranes [15, 24], calculations of their location using a method of molecular dynamics [24] and their structural similarity to the fluorescent probes with a known location and orientation in lipid membranes [25].

The aim of our research was to assess the state of cell membranes in RBCs of rats consuming VNP solution and obtaining a dose of 20 µg/kg of weight during 2 weeks by means of the fluorescent probes O10 (2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole).

Materials and methods

Study design and animals

A total of 20 female adult Wistar Albino Glaxo (WAG) rats whose weight ranged from 160 to 190 g were provided by the vivarium of Kharkiv National Medical University. The rats were divided into two equal groups in a random order. Group A included the animals orally administered a 1% VNP water solution on a daily basis except on Sundays ($n=10$). A dose of 20 µg/kg of weight was given. Group B consisted of intact animals served as controls ($n=10$). The rats were housed in cages in standard laboratory conditions of room temperature ($24 \pm 2^\circ\text{C}$) and relative humidity of 50–60%. Access to water and food was provided *ad libitum*. Cervical dislocation technique was used to sacrifice the animals used in this study. Blood was collected using sterile sodium citrate vacutainer tubes. The blood samples were used to prepare erythrocyte suspensions.

Experimental procedures carried out in this study were performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes, based on the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

Characteristics of nanoparticles

Water colloidal solution of $\text{GdVO}_4:\text{Eu}^{3+}$ nanoparticles were obtained using the method reported earlier [26]. Eight milliliter of sodium ethylenediaminetetraacetate (EDTA 2 Na) solution (0.01 mol/L) was added to water solution of rare-earth chlorides (10 mL, 0.01 mol/L). It was followed by the addition of Na_3VO_4 (8 mL, 0.01 mol/L) drop by drop ($\text{pH}=13$). The mixture was intensively stirred by a magnetic stirrer until the formation of yellowish transparent solution was observed. The solution was colorless and transparent and scattered light under the side illumination (Tindal cone). To remove excessive ions, it was cooled and dialyzed against water for 24 h. We used a dialysis membrane with a molecular weight cutoff of 12 kDa whose pore size reached 2.5 nm.

Fluorescence measurements

For all the fluorescence measurements, the cells were fluorescently labeled via the same procedure. Because the solubility of fluorescent probes O10 and PH7 is limited in water, we dissolved the probes in acetonitrile to the initial concentration of $\sim 2.10^{-4}$ mol/L (stock solution). An aliquot of the probe stock solution was added to the RBC suspensions to achieve a final probe concentration of $\sim 5.10^{-6}$ mol/L (such a concentration of the probe corresponds to a lipid-to-probe molar ratio of $\sim 200:1$). Then, the cell suspensions were incubated with the probes at room temperature for 1 h before fluorescence measurements took place. The fluorescence spectra were measured on a fluorescence spectrometer “Lumina” (Thermo Fisher Scientific) at room temperature. The measurements were performed in a 10×10 mm cuvette. The fluorescence spectra of the probes were determined in the range of 340–630 nm, with an increment of 0.1 nm. Data were collected with 0.02 s interval. The slits on the excitation and emission monochromators were 5 nm. The excitation wavelength was 330 nm. The accuracy of the fluorescence intensity measurements of the samples was 8.3 and 21.5% for probes PH7 and O10, respectively.

Wash protocol for RBCs

To prepare RBC suspension for incubation with the fluorescent probes, RBCs were washed. Briefly, when the rats were sacrificed, their whole blood was collected into sterile sodium citrate vacutainer blood collection tubes. Then 100 μ L of blood was added to 12×75 mm capped polystyrene test tubes containing 1 mL of normal saline solution (0.9% NaCl, Yuria-Pharm, Ukraine). This was followed by centrifugation at 1000 rpm during 5 min. The supernatant was discarded. Erythrocytes were resuspended in 1% of normal saline solution and centrifuged again in the same conditions. This step was repeated to have a total of three washes. One milliliter of 0.9% NaCl and 10 μ L of RBC mass were mixed. The suspension obtained as a result was at once used for the incubation with the fluorescent probes O10 (2-(2'-OH-phenyl)-5-phenyl-1,3-oxazole) and PH7 (2-(2'-OH-phenyl)-phenanthro[9,10]-1,3-oxazole).

Statistical analysis

Non-parametric Mann–Whitney U test was selected to compare two independent groups of variables. Its choice was substantiated by the outcome of the Shapiro–Wilk normality test showing the non-Gaussian distribution. The data obtained as a result of the statistical analysis were presented in the form of medians and interquartile ranges. Differences were considered statistically significant at $p < 0.05$. Statistically analyses were performed by GraphPad Prism 5.0 (GraphPad software, USA).

Results

For this study, the composition of spindle-like $Gd_{(0.9)}Eu_{(0.1)}VO_4$ nanoparticles was synthesized. Their average size was 8×25 nm. A transmission electron microscopy (TEM) image of nanoparticles used in this research is available in Figure 3.

Figure 3: Representative transmission electron microscopy (TEM) image of orthovanadate $GdVO_4:Eu^{3+}$ nanoparticles obtained for this study in colloidal solutions. Their average size is 8×25 nm.

The results of the fluorescence measurements are presented in Figure 4 and Table 1.

No noticeable statistically significant ($p > 0.05$) changes are observed in the spectrum of fluorescent probe O10 bound to the RBCs from the rats orally exposed to VNPs during two weeks in comparison with the corresponding spectrum of the probe bound to the cells from the control group of animals (Figure 4). The ratios of the fluorescence intensities of the phototautomer and normal forms (I_{T^*}/I_{N^*}) of probe O10 calculated for the rats orally exposed to nanoparticles and for the control group coincided within the accuracy of analysis (Table 1). This suggests that, in case of the rats orally exposed to nanoparticles, no noticeable changes in physico-chemical properties (i.e., in the polarity and the *proton-donor ability*) are observed in the lipid membranes of RBCs in the regions, where probe O10 locates: in the area of glycerol backbones of phospholipids (closer to the center of the lipid bilayer), in the area of carbonyl groups of phospholipids and in the area of hydrocarbon chains of phospholipids (near the carbonyl groups of phospholipids).

In case of probe PH7, slight difference between the spectrum of the probe bound to the RBCs from the rats orally exposed to VNPs during two weeks and the corresponding spectrum of the probe bound to the cells from the control group of animals is observed (Figure 4). However, it was statistically insignificant ($p > 0.05$). Taking into account the accuracy of the fluorescence intensity measurements of the samples (8.3%), the observed difference between the spectra is negligible. The ratio of the fluorescence intensities of the phototautomer and normal forms (I_{T^*}/I_{N^*}) of probe PH7 calculated for the rats orally exposed to

Figure 4: Fluorescence spectra of probes O10 (panel A) and PH7 (panel B) in erythrocyte suspensions: (a) the control group of rats (black solid line), (b) the animals orally exposed to gadolinium orthovanadate $\text{GdVO}_4\text{:Eu}^{3+}$ nanoparticles (VNPs) during two weeks (red dash-dot-dot line).

nanoparticles coincided within the accuracy of analysis with the corresponding ratio calculated for the control group (Table 1). This indicates that, in comparison to the control group (group B), no significant changes in physico-chemical properties (i.e. in the polarity and the *proton-donor ability*) of the lipid membranes of RBCs are observed for experimental group (group A) in the membrane area, where probe PH7 locates: i.e., in the area of hydrocarbon chains of phospholipids closer to the center of the bilayer.

In general, one can make a conclusion that no changes in the physical and chemical properties of the erythrocyte membranes are detected in the region from glycerol backbones of phospholipids to the center of the phospholipid bilayer in the rats orally exposed to VNPs during 2 weeks.

Table 1: The ratio of the fluorescence intensities of the photo-tautomer and normal forms (I_T/I_N) of probes O10 and PH7 in RBC membranes of rats (Me [IQR]).

Groups of animals	Probe O10 (I_{485}/I_{372})	Probe PH7 (I_{492}/I_{382})
Control	6.8 [4.6; 9.2]	35.0 [31.4; 38.9]
Rats orally exposed to nanoparticles	6.2 [4.0; 8.3] p>0.05	33.0 [29.9;36.3] p>0.05

Differences were considered statistically significant at $p<0.05$.

Discussion

It is important to note that human RBCs are present in blood vessels for 120 days and directly contact with NPs after their absorption into the bloodstream from intestine. Furthermore, they contain oxygen-transporting protein hemoglobin and, thus, constantly experience high oxygen tension [27]. In addition, oxidative stress-induced damage to RBC membranes may affect oxygen delivery to tissues leading to hypoxia [28]. All these facts make RBC membrane a sensitive and valuable marker for evaluating VNP toxicity.

Despite several beneficial effects of VNPs reported earlier, some experiments demonstrate that at high doses (0.5 mL, concentration—0.2 g/L) in case of intramuscular injections to rats, VNPs can transiently activate lipid peroxidation, evidenced by elevation of circulating malondialdehyde and conjugated dienes [29]. However, at lower therapeutic doses (20 $\mu\text{g/kg}$ of weight) used in this study at oral exposure we did not observe changes in the RBC membrane, which are characteristic of lipid peroxidation (i.e., an increase in viscosity and a decrease in fluidity). A more ordered, rigid and viscous membrane is observed in conditions of lipid peroxidation activation due to the free radical oxidation of polyunsaturated fatty acids (PUFAs) found in phospholipid molecules. They are characterized by higher flexibility and, thus, the ability to pack more loosely compared with saturated fatty acids. Thus, when PUFAs are oxidized by ROS, the relative amount of saturated fatty acids in phospholipids increases, providing the increase in viscosity and the decrease in fluidity [13, 30, 31]. Thus, the lack of statistically significant changes in the fluorescence of two probes in RBC suspensions observed in this study is indicative of the absence of lipid peroxidation activation under the influence of VNPs. Our findings allow us to assume that fatty acid composition of phospholipids in RBC membranes is not affected and PUFAs remain unoxidized by ROS.

We believe that evaluation of the state of RBC membrane by the fluorescent probe technique can be used as a

relatively simple, cost-effective and informative method to assess the toxicity of nanoparticles.

Our research has some limitations. Firstly, we did not assess the features of fatty acid composition of erythrocyte membrane phospholipids to judge directly whether PUFAs are oxidized or not. Secondly, levels of oxidative stress markers such as TBA (thiobarbituric acid)-reactive substances, conjugated dienes, reduced glutathione, membrane SH groups, and others were not evaluated, since the use of two independent fluorescent probes is sufficient to demonstrate oxidative stress-associated changes in RBCs.

Conclusion

No changes in the physical and chemical properties of the erythrocyte membranes are detected in the region from glycerol backbones of phospholipids to the center of the phospholipid bilayer in rats orally exposed to gadolinium orthovanadate $\text{GdVO}_4\text{:Eu}^{3+}$ nanoparticles during 2 weeks, i.e., VNPs at the concentration studied don't promote ROS-mediated cell membrane damage in RBCs.

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