Research Article

Change in the Microviscosity of Erythrocyte Membranes and Proteins in Blood Plasma after Graphene Oxide Addition: The ESR Spectroscopy Study

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Received 7 September 2018; Accepted 19 December 2018; Published 1 April 2019

Academic Editor: Rizwan Hasan Khan

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The change in the microviscosity of erythrocyte membranes and the proteins in blood plasma after graphene oxide addition is studied by the ESR spectroscopy exploiting two spin probes with different lipophilic components in the structures. Experiments with charged spin probe 2 embedded into the erythrocyte membrane showed that the introduction of graphene oxide in small concentrations (∼70 μg/ml) into a suspension of erythrocytes did not lead to significant changes in the microviscosity of their membranes. Correlation times of hydrophobic spin probe 1 adsorbed to hydrophobic pockets of plasma proteins demonstrate a gradual slowdown at the graphene oxide injection into blood plasma that indicates a small deformation of the hydrophobic cavity of protein at the adsorption. However, this protein binding with graphene oxide does not cause the displacement of the spin probe from their hydrophobic cavities, which is evidence about small changes in the protein secondary structure. The obtained results indicate the insignificant cytotoxicity effect of small concentrations of graphene oxide for erythrocytes and blood plasma.

1. Introduction

In recent years, manufactured graphene nanomaterials have received increasing attention owing to their unique physical and chemical properties, such as high electronic conductivity, good thermal and chemical stability, and excellent mechanical strength. In addition to the above indicated properties, an oxidized form of graphene (graphene oxide (GO)) has a good water dispersibility, a facile surface functionalization, and attractive optical features. All these characteristics make this nanomaterial very attractive in many biomedical applications including biosensing, drug delivery, photothermal and photodynamic therapy, tissue engineering, and imaging of different biological processes in vitro and in vivo [1–9].

Biomolecules can interact with GO through π−π stacking interaction with the sp2-conjugated domains of the basal plane and by means of the hydrogen-bond formation between the oxygenated groups of GO and different chemical groups localized on the molecule shell. Existence of flat sp2-hybridized sections surrounded with different oxygen-containing groups resulting in mixed sp2/sp3 hybridized carbon domains facilitates efficient interaction of GO with biomolecules of different types, including small biologically active molecules and large structures like proteins and membranes.

Area of GO applications in biology and medicine is permanently extended including tissue engineering and studying of different biological processes in cells [4–6] and animal [7]. In recent years, several studies have devoted to
the elucidation of a question on biocompatibility and toxicity of graphene oxide in vitro and in vivo, but contradictory outcomes were reported. The discrepancy in conclusions on GO toxicity can be explained by the fact that this toxicity depends on various physiochemical properties of GO such as shape, size, oxidative state, and quantity of functional groups in the GO structure. In several reviews that have been published recently [3, 4, 8, 9], the numerous studies devoted to the nanotoxicology and biocompatibility of graphene-related materials were summarized. However, there are many questions that still need to be addressed, and additional experiments with a broad spectrum of cells are required.

The purpose of this work is to study the effect of graphene oxide on the microviscosity of the erythrocyte membranes and on the blood plasma proteins (primarily, on serum albumin (SA) as the prevailing protein in blood plasma) using the spin labeling method. Microviscosity is an important parameter of cell membranes that can characterize the enzymatic activity of membrane-bound enzymes, a structure and functioning of ion channels in membranes, a membrane permeability, and bioavailability of drugs. Information on structure transformation of the blood plasma proteins at adsorption on graphene oxide is very useful to evaluate the biocompatibility or toxicity of this nanomaterial. In our study, we exploit the spin probe method, which is successfully used in molecular biology, pharmacology, and biomedical researches [10, 11]. In this method, the transformation of electron spin (paramagnetic) resonance (ESR) spectrum of stable nitroxyl radical (probe or labeling) [12, 13] after binding with a biological object is controlled. In our experiments from ESR spectra of probes with lipophilic organic components, the correlation time $\tau_c$ of Brownian rotational diffusion of the probes in cell membranes and in hydrophobic cavities of plasma proteins can be evaluated before and after adsorption on graphene oxide. The obtained results give the possibility to evaluate the changes in the structure of membranes of cells and plasma proteins upon binding with graphene oxide.

### 2. Experimental Details

#### 2.1. Materials

Graphene oxide (powder, 15–20 sheets) was purchased from Sigma-Aldrich (Europe) and used as received (Product Number: 796034). A water suspension of GO powder was ultrasonicated in the water bath for 60 minutes and then centrifuged (3000 g, 20 minutes) to remove the large fragments. An initial concentration of GO in water suspension was 300 $\mu$g/ml. GO concentration in aqueous suspension in erythrocytes or plasma solutions was 70 $\mu$g/ml. Prior to the experiment, GO suspension was ultrasonicated for 30 minutes by Ultrasonic Cleaner.

For ESR experiments as stable spin probes, we selected two nitroxyl radicals, whose structures are shown in Figure 1. These spin probes are prepared from palmitic acid and were provided by Prof. Igor A. Kirilyuk. Injection of probes 1 and 2 (Figure 1) to the suspension of erythrocytes or blood plasma was occurred by means of adding of concentrated probe solution of dimethylsulfoxide (DMSO). To escape a toxic effect of DMSO, its final concentration in suspension was not above 1%.

Erythrocytes and blood plasma used in the research were obtained from blood of male rats according to the technique described earlier [13]. Erythrocytes were obtained from the blood by means of triple washing with physiological solution (0.9% of NaCl) prepared on the sodium phosphate buffer (5 mM, pH 7.2), with further centrifugation during 20 min with the speed of 3000 rpm. Supernatant obtained after the first centrifugation was used as blood plasma. For our experiments, the obtained erythrocytes and plasma of the rat blood after centrifugation was diluted 2-fold with physiological solution. Our estimation showed that the number of erythrocytes in 1 mm$^3$ was about 9 $\times$ 10$^6$ and the SA concentration in the diluted blood plasma was about 34 mg/ml.

For samples preparation, the blood of white outbred male rats with a weight of 180–210 g was used. Blood was drawn during daylight hours from every six animals. All experiments on animals were conducted in compliance with European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and “General ethical principles of experimentation on animals” (Ukraine, 2001).

#### 2.2. Methods

##### 2.2.1. Electron Spin Resonance

ESR spectra were observed using ESR Spectrometer CMS8400 (Adani, Europe). ESR spectra of prepared samples were recorded at 25°C. An evaluation of the membranes microviscosity was controlled on the basis of analysis of the intensity and the width of lines assigned to the electron transitions in the triplet state of two stable nitroxyl radicals (spin probes) observed in ESR spectra. These spin probes interacted with external environment; in our case, it was a lipid bilayer of erythrocyte membranes and hydrophobic cavities of proteins of blood plasma (mainly with SA as the major protein of blood plasma) or water. For calculation of the probe correlation time ($\tau$), the following spectral characteristics were used: a width of the central component (at half of height) ($\Delta H_c$), an intensity of ESR spectrum components ($h_{\lambda}$, $h_0$, $h_{\mathbf{h} \Lambda}$) with the nucleus magnetic quantum number of $^{14}\text{N}$ ($M = 0, +1, -1$), an isotropic splitting constant ($\mathbb{A}_{0\mathbf{h}}$), determined as the distance (in the gauss units) between the low-field ($h_{\mathbf{h} \Lambda}$) and central ($h_0$) components of the ESR spectrum. The basic equation for evaluation of the viscosity of any medium is the modified Stokes–Einstein equation:

$$\tau = \frac{4\pi a^3 \cdot \eta}{3kT} \quad (1)$$

where $\eta$ is the medium viscosity and $a$ is the effective radius of the spin probe defined by ESR spectra [10, 11]. Probe correlation time ($\tau$), proportional to the membrane viscosity ($\eta$), is determined from the following empirical correlations [10, 11]:
of GO are usually attributed to hydroxyl and carboxyl groups [16] (Figure 2). For more detailed analysis of content of oxygen-containing groups in the GO structure, which may influence the ESR spectra, the FTIR spectroscopy was exploited. Up to now, the main characteristic absorption bands, which allow unique identification of GO, are known [16, 17]. In accordance with this assignment, the GO absorption bands of the IR spectrum in the range of 1750–1600 cm\(^{-1}\) are attributed to stretch vibrations of ν(C=O) mainly of the carbonyl and carboxylic groups (Figure 3), and the bands in the range of 1600–1500 cm\(^{-1}\) are assigned to stretch vibrations of ν(C=C) involving fragments of the sp\(^2\)-hybridized graphene domains. Near 1250 cm\(^{-1}\), the bands are attributed to the vibrations of (C–O–C) epoxy groups, and near 1100 cm\(^{-1}\) to stretch vibrations of hydroxyl and carboxylic groups (ν(C–OH)) [16, 17]. In GO film spectra used in our experiments, the most intensive absorption band is located at 1586 cm\(^{-1}\) (Figure 3), which is assigned to stretch ν(C=C) vibrations of the graphene domain. Absorption at 1364 cm\(^{-1}\) is the most likely related to the in-plane deformation vibrations of graphene hexagons and of hydroxyl groups. The wide absorption band with a maximum at 3440 cm\(^{-1}\) may be due to vibrations of ν(OH) of hydroxyl and carboxylic groups and partially to the absorption of intercalated water molecules (Figure 3). Note that the IR spectrum in the area of 1250–800 cm\(^{-1}\) has a weak absorption (Figure 3). Also, very weak bands at 1709 cm\(^{-1}\) and the small shoulder at 1620 cm\(^{-1}\) of the intensive band at 1586 cm\(^{-1}\) can be assigned to vibrations of the carbonyl and carboxylic groups, respectively (Figure 3). As the band at 1586 cm\(^{-1}\) assigned to ν(C=C) vibrations of the graphene domain has a prevailing intensity in the “finger print” region over other bands that attribute to oxygen-containing groups, we may be conclude that studied samples of GO contain a small amount of hydroxyl and carboxylic groups. Note that these groups are placed on the edge of sheets while the bands that characterize the basal surface (carbonyl and epoxy groups) are not observed practically. Schematic representation of GO used in our experiments is shown in Figure 3. Note that the ultrasound action used in these experiments for preparation of GO aqueous suspensions does not lead to the quantity increase of oxygen-containing groups. Our previous analysis of the size of the GO flakes in water suspension prepared by similar method [17] showed that we obtained mainly single layers or bilayers of GO with a lateral size ranging from 0.5 to 2 μm (the average flake size is about 1 μm).

\[ \frac{1}{\tau_{c+1}} = \frac{2 \cdot 10^8}{(h_0/h_1)^{1/2} - 1} \Delta H_0 (c e k^{-1}), \quad (2) \]

\[ \frac{1}{\tau_{c-1}} = \frac{3.6 \cdot 10^9}{(h_0/h_1)^{1/2} - 1} \Delta H_0 (c e k^{-1}), \quad (3) \]

\[ \frac{1}{\tau_{c+1/-1}} = \frac{1.5 \cdot 10^9}{(h_0/h_1)^{1/2} - 1} \Delta H_0 (c e k). \quad (4) \]

It is known that the spin probes based on the palmitic acid-type (probes 1 and 2) used in our experiments have a poor structural ordering in the membranes of cells [10]. The shape of the ESR spectra indicates predominantly isotropic and slowed Brownian rotational diffusion of these probes in lipids of the erythrocyte membranes. However, to obtain more objective information about possible anisotropic rotation of probes in the membrane, we determined correlation times of rotational diffusion of probes in erythrocyte membranes according to (2)–(4). It should be noted that, in our research, we do not aim for the determination of the absolute value of the medium microviscosity, and the main purpose of our work is an observation of the relative changes in the parameters \( \tau_c \) to ascertain the possible effect of GO on the erythrocyte membrane or on proteins of blood plasma.

Statistical processing of the obtained data was performed with variation statistics methods by means of standard software packages Statistica 7 for Windows.

2.2.2. Fourier-Transform Infrared (FTIR) Spectroscopy of GO.

Infrared absorption spectra of GO were obtained by employing the FTIR spectrometer adapted to isolation of biomolecules in low-temperature matrices and also modified for registration of FTIR spectra of inhomogeneous films with high scattering [14, 15]. FTIR spectra of GO films were observed in the range of 4000–600 cm\(^{-1}\) with apodised resolution of about 3.0 cm\(^{-1}\). GO films were obtained on the ZnSe substrate by the dried-droplet method from aqueous suspension at the temperature of 30–40°C.

3. Results and Discussion

3.1. GO Characterization by FTIR Spectroscopy.

In accordance with the product specification, the graphene oxide sheets used in our experiment contained up to 10% edge-oxidized groups in the structure. The edge-oxidized groups

![Figure 1: Structure of spin probes used in the experiment.](image-url)
3.2. ESR Spectroscopy Study

3.2.1. Characterization of the Erythrocyte Suspension after Graphene Oxide Doping. Hydrophobic spin probe 1 is practically insoluble in water, while it has a high solubility in polar and nonpolar organic solvents. Figure 4(a) represents the ESR spectrum of probe 1 in solution that was prepared by dilution of the concentrated solution of this probe in DMSO by physiological solution; as a result, the DMSO concentration was about 0.5% by volume. A broad singlet band that can be observed in the spectrum is characteristic of the spectra of individual lipophilic nitroxyl radicals in liquid. The singlet band is result of the rapid exchange interaction between the nitroxyl fragments of spin probe 1 [10]. However, probe 2 with an alkyl tail and a positive charge at the quaternary nitrogen atom in the structure shows a narrow triplet in the ESR spectrum indicating fast rotational mobility of probe in micelles which are created by molecules of probe 2 in water (Figure 4(b)) [13].

Figures 5 and 6 represent the ESR spectra of probes 2 and 1 in erythrocyte solutions, respectively, in the absence of GO. Note that probe 2 with a positive charge and an alkyl tail in the molecule structure is similar to the structure of phosphatidylcholine that is a major component of biological membranes. So, spin probe 2 can be easily incorporated into a viscous medium of the lipidic bilayer of the erythrocyte membrane. At detectable concentrations of probes in the erythrocyte solution (2·10^{-3} M) for probe 2 in the ESR spectrum, a triplet is observed that gives a possibility to calculate a correlation time of spin probe 2 localized in this lipid membrane. We observed a slowdown of Brownian rotational diffusion of spin probe 2 after injection in erythrocyte solution (Figure 5, Table 1) that indicates this probe embedding in erythrocyte membranes [6]. At the same time, for uncharged lipophilic probe 1 in the erythrocyte solution, a broad singlet is observed in the ESR spectrum (Figure 6) with a faint hint on the triplet. Thus, quantity information about correlation time of probe 1 in the erythrocyte solution cannot be derived from this spectrum as well as from the spectrum of this probe in the erythrocyte suspension after adding GO (Figure 7).

It is known that the ESR signal from GO is well observed at room temperature, but the ESR signal of reduced GO can be observed only below 100 K [18]. Since our samples of GO are reduced (contain a small amount of hydroxyl and carboxylic groups), this guarantees the absence of additional lines from our GO in the ESR spectra.

Figure 8 shows the ESR spectrum of spin probes 2 in erythrocytes solution after adding GO (in the result of dilution, the GO concentration in the solution was about 70 μg/ml). Correlation times determined from ESR spectra of spin probes 2 in the erythrocyte membranes obtained immediately after injection as well as after 1 and 4 hours of the incubation are presented in Table 1. As we do not observe any transformation in the ESR signal after 1 and 4 hours of erythrocyte incubation with GO, we limited our measurements with 4 hours. Basic reason of such time limitation is also caused by possible erythrocytes aging as these cells were kept at 25°C.

We also evaluated a environment polarity close to the nitroxyl fragment of spin probe 2 in the erythrocyte membrane by measuring parameter A_{iso} which is determined as the distance (in gausses (Gs) units) between the low-field (h_{iso}) and central (h_{0}) components of ESR spectra. When the nitroxyl fragment of the spin probe is in a water environment, A_{iso} usually has a value about 17.2 Gs, while in the lipophilic environments in a cell membrane, this value is usually close to 14–15 Gs [10, 11]. In experiments with the nitroxyl fragment of the spin probe 2, we obtained that A_{iso} is equal to 15.4 Gs (Table 1). This A_{iso} value and the slowdown of Brownian rotational diffusion of spin probe 2 in the erythrocyte solution (Figure 5) indicates its embedding in the lipidic bilayer of erythrocyte membranes. In this case, the nitroxyl fragment of probe 2 is located between the phospholipid heads in the membrane, so its rotational mobility is suppressed. Thus, such spin probe is very sensitive to variation in density of phospholipid packing in cell membranes, i.e., to microviscosity of the cell membrane.
As it follows from the ESR spectra of the spin probe 2 embedded in erythrocytes (Figure 5) as well as from Table 1, the addition of GO to the erythrocyte suspension (Figure 8) does not cause an essential influence on the probe correlation time. After one hour of incubation of graphene oxide with erythrocytes, the probe correlation times demonstrate a slight tendency to slowdown that indicates a weak increase in microviscosity of erythrocyte membranes. Analysis of the ESR spectrum of spin probe 2 in suspension after 4 hours of component mixing confirms this tendency. This observation is evidence of a weak influence of weak oxidized graphene on erythrocyte membranes. We can maintain that there is no essential cytotoxic effect for weak oxidized graphene in the concentration of 70 μg/ml, in contrast to the substantial increase in the microviscosity of erythrocyte membranes induced by multiwalled carbon nanotubes observed earlier [19]. The obtained results indicate preservation of the intact structure of cell membranes at the interaction with the GO sheets in small concentration. This observation is extremely important for experiments with different living cells adsorbed onto the graphene/graphene oxide surface that opens doors for the different applications (e.g., differentiation of stem cells into neurons on the graphene surface) [1].

3.2.2. Characterization of Blood Plasma after Graphene Oxide Doping. Plasma of blood is a liquid medium of whole blood through which the blood cells are circulated. Blood plasma is a complex system with the major components such as proteins, ions, lipids, and carbohydrates. Some major proteins include serum albumin (SA), γ-globulins, and fibrinogen; among them SA is one of the most abundant proteins in blood [20]. SA performs several important biological functions within the vascular system. SA possesses the unique capacity to bind different endogenous and exogenous compounds, performing very essential transport function. It binds mainly water insoluble substances, such as lipids and also potentially toxic compounds such as bilirubin.

In contrast to the erythrocyte membranes, blood plasma as the complex system with a few basic components possesses many binding sites [21–24] for spin probes 1 and 2. However, due to prevailing of SA in this complex system (more than 50%), we concentrate our attention on the probe binding only with this protein. This protein contains in the structure three hydrophobic pockets that effectively bind the molecules of fatty acids, steroids, and other hydrophobic substances. These pockets can provide an effective binding of spin probe 1 with SA. Note that the effective adsorption of blood plasma proteins on carbon nanotubes was reported earlier [25].

Figure 9 represents the ESR spectrum of spin probe 1 which demonstrates a triplet band due to its adsorption into hydrophobic cavities of plasma proteins. The interaction of spin probe 1 with SA retards the rapid exchange interaction between the nitroxyl fragments of this probe. The spin probe 1 embedding into hydrophobic cavities is also confirmed by the $A_{iso}$ value which is 11.8 Gs.

Introduction of GO suspension into the blood plasma labeled with spin probe 1 causes gradual rise in microviscosity of the SA shell (surrounded by water molecules) and some other plasma proteins with time employing this spin probe (Table 1). So after 4 hours of the blood plasma incubation with GO, we observed a decrease in the $A_{iso}$ value by about 9%. After such signal transformation with time, we decided to prolong the experiment up to 20 hours. We can observe a noticeable influence of GO on the correlation time of spin probe 1 (Table 1), which shows 33–40% slowdown after 20 hours of incubation. This slowdown is caused by the time-expanded adsorption of SA onto the hydrophobic surface of GO (Table 1). The interaction of SA with GO induces a small deformation of the hydrophobic cavity which increases the binding energy of spin probe 1 with SA accompanied with correlation time slowdown. Important fact is that the SA interaction with GO does not cause the displacement of lipophilic probes from the SA hydrophobic cavities, which indicates preservation of the native structure.

![Figure 4: ESR spectra of spin probes 1 (a) and 2 (b) in the physiological solution.](image)

![Figure 5: ESR spectrum of spin probe 2 in the erythrocyte suspension (control measurement).](image)

![Figure 6: ESR spectrum of probe 1 in the erythrocyte suspension (control measurement).](image)
Behavior of spin probe 2 in blood plasma (Figure 10) differs from spin probe 1 considerably. First of all, the high value of $A_{iso}$ (16.8 Gs) indicates the arrangement of spin probe 2 in water environment practically. Introduction of GO into blood plasma labeled with spin probe 2 does not lead to noticeable changes in the correlation time, especially in the first 1–4 hours. Only after 20 hours of the spin probe incubation, we can note a small decrease in the correlation time that can be caused by weak pressing of this probe to GO by proteins or by the interaction of the positively charged nitroxyl head with the oxygen group of graphene oxide. In any case, we observe a weak sensitivity of spin probe 2 to conformational changes of the protein surface.

4. Conclusions

Applications of two spin probes with different nitroxyl radicals differ in the hydrophobicity/hydrophilicity of the structure, allowing us to analyze the microviscosity of erythrocytes membranes and the proteins of blood plasma at GO doping. Experiments with hydrophilic spin probe 2 showed that the introduction of weakly oxidized GO (with an average flake size of 1 µm) into a suspension of erythrocytes did not lead to significant changes in the microviscosity of their membranes. After four hours of incubation, an insignificant increase in the microviscosity is observed, apparently due to the sorption of GO on membranes. The obtained results indicate the stability of the structure of erythrocyte membranes upon the interaction with GO, which can be considered a sufficiently cytocompatible material in small concentrations. Hydrophobic spin probe 1 bound with the hydrophobic pockets of serum albumin or other plasma proteins shows a gradual increase in the microviscosity of the water-protein phase of these proteins.
within the first 20 hours of GO doping. The interaction of proteins (mainly serum albumin) with GO induces only the small deformation of the hydrophobic cavity which is accompanied with correlation time slowdown of spin probe I. As protein-graphene oxide interaction does not cause the displacement of spin probe I from the protein hydrophobic cavities, this observation indicates a preservation of the native structure of proteins at adsorption on GO.

The obtained data indicate that GO induces the small increase in the microviscosity of erythrocyte membranes and protein shell of blood plasma. All these observations confirm an insignificant cytotoxicity of weak oxidized graphene in small concentrations (~70 μg/ml) for erythrocytes and blood plasma.

Thus, the proposed method of estimation of cytotoxicity of carbon nanomaterials by the spin probe method allowed us to clearly demonstrate and confirm the absence of expressed cytotoxicity of GO in small concentrations. This experimental observation shows additional opportunities of using GO for complex biosystems like blood cells.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors are grateful to Prof. Igor A. Kirilyuk from N. N. Vorozhtsov Novosibirsk Institute of Organic Chemistry, Novosibirsk, Russia, for synthesis of spin probes used in this work. V. A. Karachevtsev and A. Yu. Ivanov are grateful to National Academy of Sciences of Ukraine (Grant no. 15/19-H within the program “Fundamental Problems of the Creation of new Nanomaterials and Nanotechnology,” with Grant nos. 07-01-18 and 0117U002287) for financial support.

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