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Research Article

The Effect of Highly Hydroxylated Fullerenol $C_{60}(OH)_{36}$ on Human Erythrocyte Membrane Organization

Jacek Grebowski and Anita Krokosz

Department of Molecular Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, 141/143 Pomorska Street, 90-236 Lodz, Poland

Correspondence should be addressed to Anita Krokosz; krokosz@biol.uni.lodz.pl

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The mechanism of the interaction of highly hydroxylated fullerenol $C_{60}(OH)_{36}$ with erythrocyte membranes was studied by electron spin resonance spectroscopy (ESR) of stearic acid derivatives labeled with a nitroxyl radical at C-12 or C-16 and with a nitroxyl derivative of maleimide covalently attached to sulfhydryl groups of membrane proteins. A significant increase in membrane fluidity in the hydrophobic region of the lipid bilayer was observed for 12-doxylstearic acid at fullerenol concentrations of 100 mg/L or 150 mg/L, while for 16-doxylstearic acid significant increase in fluidity was only observed at 150 mg/L. Fullerenol at 100 mg/L or 150 mg/L caused conformational changes in membrane proteins, expressed as an increase in the h_w/h_s parameter, when fullerenol was added before the maleimide spin label (MSL) to the membrane suspension. The increase of the h_w/h_s parameter may be caused by changes in lipid-protein or protein-protein interactions which increase the mobility of the MSL label and as a result increase the membrane fluidity. Incubation of the membranes with the MSL before the addition of fullerenol blocked the available membrane protein –SH groups and minimized the interaction of fullerenol with them. This confirms that fullerenol interacts with erythrocyte membrane proteins via available protein –SH groups.

1. Introduction

Fullerenes and their derivatives can penetrate the cells of living organisms. However, the mechanism of penetration into cells and the possible effects on the plasma membrane are still unclear [1].

A water soluble derivative of fullerene $C_{60}(CO_2H)_2$ has been shown to penetrate the cell membrane and immunofluorescence microscopy confirmed the presence of the fullerene derivative inside the cell. Differential centrifugation showed the presence of radiolabeled nanoparticles in the cytoplasm, plasma membrane, mitochondria, and microsomes [2]. The probable mechanism of penetration into the cell is related to the structural similarity of the carbon cage of fullerene derivatives to clathrin. Clathrin is a component of the coat of endocytic vesicles [2]. Additionally, nanoparticles of fullerenol $C_{60}(OH)_{24}$ can be taken up by cells via endocytosis resulting in their intracellular localization [3].

Fullerenol can interact with polar groups of phosphatidylglycerol via hydrogen bonds. This leads to disruption of the structural organization of the lipid bilayer which, in turn, causes a change in membrane fluidity [4].

Moreover, the hydrophilic molecules of $C_{60}(OH)_x$ (Figure 1) can adsorb to membrane phospholipid heads and may also interact with membrane proteins, affecting their structure and function [1]. Changes in the activities of ATPases caused by fullerenol could be the result of direct and/or indirect (via membrane fluidity changes) interaction with the enzymes. The amount of fullerenol associated with the membranes was proportional to its concentration in the incubation medium and inversely proportional to the concentration of the membrane proteins. This is probably connected to the fact that at higher concentrations of membrane proteins, the increased viscosity of the suspension can affect the diffusion of fullerenol inside the membrane [5]. Our previous work has shown that fullerenol $C_{60}(OH)_{36}$, by associating with band 3 protein, not only prevents its degradation, but can also influence the binding sites of spectrin; bands 4.1 and 4.2 proteins; and actin,

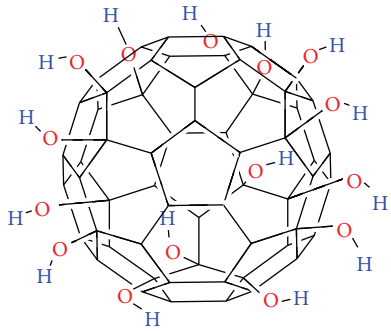

Figure 1: The structure of fullerenol.

leading to changes in the cytoskeleton that affect erythrocyte morphology [6].

Simulation results indicate that although a hydrophobic fullerene C_{60} molecule can readily "jump" into the lipid bilayer and translocate into the membrane within a few milliseconds, the $C_{60}(OH)_{20}$ molecule can barely penetrate the bilayer. The mean translocation time via diffusion for the $C_{60}(OH)_{20}$ molecule is several orders of magnitude longer than for the C_{60} molecule [7]. Therefore, the $C_{60}(OH)_{20}$ appears to "pinch" the cell membrane. This "pinch" is caused by strong interactions between the OH groups of $C_{60}(OH)_{20}$ and the lipid head groups. The strong interactions can be inferred from the close proximity of lipid head group to the $C_{60}(OH)_{20}$ adsorbed onto the lipid bilayer. Micropore formation is suppressed by the adsorption of $C_{60}(OH)_{20}$ onto the lipid membrane. This may be one of the reasons why no membrane leakage was reported for cells in contact with $C_{60}(OH)_{20}$ solution [7].

Studies which take into account the impact of fullerenol nanoparticles on the plasma membrane proteins and lipids are necessary, because these components are an integral part of the cell and are necessary for the proper cellular function.

The biological effects of the fullerenols depend on the number of hydroxyl groups on $C_{60}(OH)_x$ [8]. Our previous studies have shown that fullerenol $C_{60}(OH)_{36}$ can interact with erythrocyte membranes. Information about the impact of highly hydroxylated fullerenols on plasma membranes is vital for the assessment of their suitability in biomedical applications.

In this work we examined the mechanism of this interaction in detail by electron spin resonance spectroscopy (ESR). We measured the influence of fullerenol on the fluidity of erythrocyte membrane using two spin probes: 12-doxylstearic and 16-doxylstearic acids. In addition we studied the conformational changes in membrane proteins using the spin label 2,2,6,6-tetramethyl-4 maleimidopiperidine-N-oxyl.

2. Materials and Methods

2.1. Chemicals. Fullerene (99.5%) was purchased from SES Research (Houston, TX, USA), 5- and 16-doxylstearic acids, 2,2,6,6-tetramethyl-4 maleimidopiperidine-N-oxyl (MSL), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetracetic acid (EGTA), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Co. LLC. Other chemicals were obtained from POCh (Gliwice, Poland) (all of analytical grade). All solutions were made with ultrapure water purified by the Milli-Q system.

2.2. Synthesis of Fullerenol. Polihydroxy fullerene (fullerenol) $C_{60}(OH)_{36}$ was synthesized as described earlier [5]. The structure of the hydroxyl derivative of fullerene C_{60} was confirmed by elementary analysis, IR spectrophotometry (NEXUS FT-IR spectrometer), ^{1}H NMR (Varian Gemini 200 MHz), ^{13}C NMR (Bruker Avance III 600 MHz), and mass spectrometry MALDI-TOF (PerSeptive Biosystems Inc. Voyager Elite).

2.3. Preparation of Erythrocyte Suspensions. Blood samples from healthy adult donors were provided by the Central Blood Bank in Lodz. Erythrocytes were separated from plasma and leucocytes by centrifugation at 2000 g for 5 min at 4°C and washed three times with sodium phosphate buffered saline (PBS, 10 mM phosphate in 145 mM NaCl, pH 7.4). The buffy coat was removed each time. Erythrocytes were resuspended in PBS to obtain a hematocrit of 2%. Erythrocyte suspensions were incubated with fullerenol $C_{60}(OH)_{36}$ (50–150 mg/L) for 3 h at 37°C. After incubation, suspensions were centrifuged and supernatants were removed in order to obtain a hematocrit of 50%.

2.4. Preparation of Erythrocyte Membranes. Erythrocyte membranes were prepared according to the method of Dodge et al. [9] with some modifications. Hemolysis was carried out at 4°C with 20 volumes of 20 mM sodium phosphate buffer (pH 7.4), containing 1 mM EDTA, 1 mM EGTA, and 0.5 mM PMSF as protease inhibitors. The membrane ghosts were washed successively with 20, 10, and 5 mM ice-cold phosphate buffer (pH 7.4) until the ghosts were free of residual hemoglobin. Protein concentration in the membrane preparations was determined by the method of Lowry et al. [10]. Plasma membranes (1 $mg_{protein}$/mL) were incubated with $C_{60}(OH)_{36}$ (50–150 mg/L) for 1 h at 37°C.

2.5. Spin Labeling of Erythrocytes and Erythrocyte Membranes. The electron spin resonance (ESR) spectra of erythrocytes labeled with 12-doxylstearic acid (12-DS) and 16-doxylstearic acid (16-DS) were used to monitor the fluidity of membrane lipids [11, 12]. Ethanol solution of spin label (10 mM) was added to erythrocyte suspension at a hematocrit of 50% (1 : 200) and incubated for 30 min at room temperature.

From the ESR spectra the ratio of the low-field peak height to the mid-field peak height, h_{+1}/h_0, was calculated, characterizing the mobility of the spin label in the microenvironment of fatty acids. The spectra of 12-DS and 16-DS are shown in Figures 2 and 3.

Changes in the membrane protein conformation of erythrocytes were estimated according to Gwoździński et al. [13] by spin labeling of the erythrocyte membranes with 1 mg of MSL per 25 mg of protein and incubation at 4°C for 1 h.

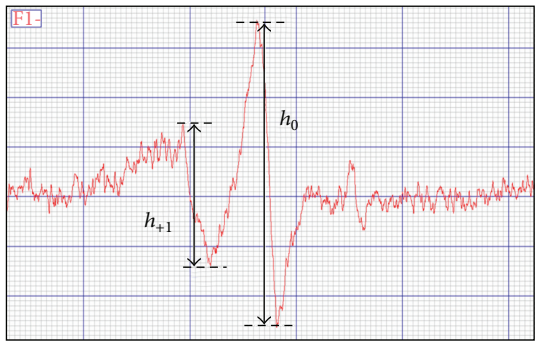

Figure 2: ESR spectrum of erythrocytes labeled with 12-doxylstearic acid. Measurement parameters: center field: 3473 G, frequency: 9.74 GHz, microwave power: 10 mW, and sweep width: 100 G.

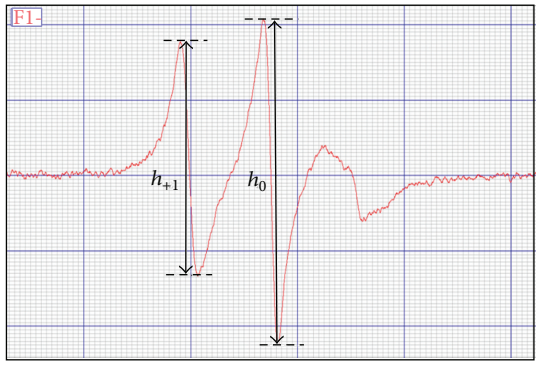

Figure 3: ESR spectrum of erythrocytes labeled with 16-doxylstearic acid. Measurement parameters: center field: 3473 G, frequency: 9.74 GHz, microwave power: 10 mW, and sweep width: 100 G.

Excess label was removed by washing with 10 mM phosphate buffer, pH 7.4. The spectra of MSL attached to the membrane proteins were analyzed by measuring the ratio of weakly (h_w) and strongly (h_s) immobilized components in the low-field peak (h_w/h_s). The spectrum of MSL is shown in Figure 4.

ESR spectra were obtained on a Bruker ESP 300E spectrometer, measurement parameters: frequency 9.74 GHz, microwave power 10 mW, center field 3473 G, sweep width 100 G, modulation amplitude 1.013 G, and time constant 5.12 ms.

2.6. Statistical Analysis. All experiments were performed at least three times. Values were expressed as the mean ± standard deviation (SD) of at least three independent experiments. For each experiment, blood samples (control and those incubated with chemical compounds) were taken from the same individual.

Data were analyzed by one-way ANOVA, followed by Tukey's post hoc test, all using the GraphPad 4.0 software.

3. Results

3.1. Plasma Membrane Fluidity. Figure 5 shows the changes in the ratio of the h_{+1}/h_0 parameter obtained from the ESR spectra of erythrocytes labeled with 12-DS and 16-DS after

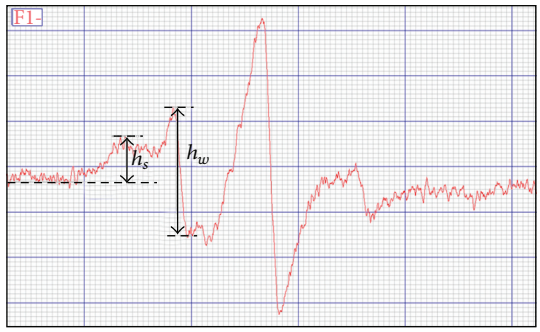

Figure 4: ESR spectrum of erythrocyte membrane proteins labeled with MSL. Measurement parameters: center field: 3473 G, frequency: 9.74 GHz, microwave power: 10 mW, and sweep width: 100 G.

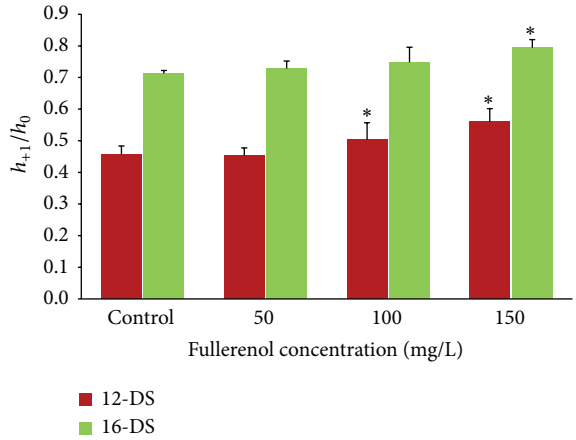

Figure 5: The influence of fullerenol on the membrane fluidity of erythrocyte membrane labeled with 12-DS and 16-DS. Erythrocytes were incubated with fullerenol at 50, 100, and 150 mg/L for 3 h at 37°C. Control: erythrocytes incubated without fullerenol. Bars are mean ± SD for five independent samples. * values statistically different in comparison with control values, $P < 0.05$.

incubation with $C_{60}(OH)_{36}$ in the concentration range 50–150 mg/L for 3 h at 37°C.

The ratio of h_{+1}/h_0 is a semiquantitative parameter determining the mobility of the acyl chains of fatty acids in the erythrocyte membrane. An increase in the value of the h_{+1}/h_0 parameter was observed indicating an increase in plasma membrane fluidity under the influence of fullerenol at 150 mg/L for both spin labels. For the 12-DS label an increase in membrane fluidity was observed even at the lower fullerenol concentration of 100 mg/L.

3.2. Membrane Protein Conformation. In order to evaluate the effect of $C_{60}(OH)_{36}$ on the conformation of membrane proteins two experimental protocols were used. First, membrane suspensions (3 $mg_{protein}$/mL) were incubated for 1 h at 4°C with the MSL and then diluted to a protein concentration of 1 mg/mL and incubated for 1 h at 37°C with fullerenol using a concentration range of 50–150 mg/L.

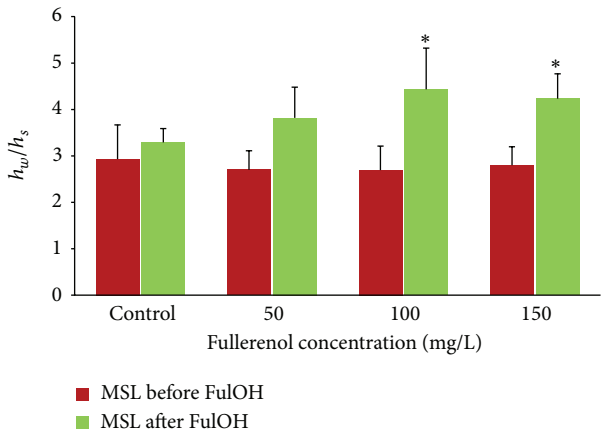

Figure 6: The influence of fullerenol on the membrane protein conformation (h_w/h_s parameter) of MSL-labeled membranes. (1) Erythrocyte membranes were incubated with MSL for 1 h at 4 °C, and then spin-labeled membranes were incubated with fullerenol at 50, 100, and 150 mg/L for 1 h at 37°C ("MSL before FulOH"). Bars are mean ± SD for three independent samples. (2) Erythrocyte membranes were incubated with fullerenol at 50, 100, and 150 mg/L for 1 h at 37°C, and then membranes were incubated with MSL for 1 h at 4°C ("MSL after FulOH"). Bars are mean ± SD for four independent samples. Controls are membranes incubated only with MSL according to the scheme detailed above. * values statistically different in comparison with control values, $P < 0.05$.

In the second set of experiments the 1 mg/mL protein concentration membrane suspensions were first incubated for 1 h at 37°C with fullerenol at 50–150 mg/L and subsequently incubated with the MSL for 1 h at 4°C.

In both cases, excess label was washed out with phosphate buffer; pH 7.4 and ESR spectra were obtained. The ESR spectrum of MSL-labeled membrane proteins recorded at ambient temperature is shown in Figure 4. The weakly (h_w) and strongly (h_s) immobilized components are visible in the low-field line. The h_w/h_s ratio was calculated being a parameter which is sensitive to conformational changes in proteins.

The influence of fullerenol on the membrane protein conformation (h_w/h_s parameter) of MSL-labeled membranes is shown in Figure 6. An increase of the h_w/h_s ratio in erythrocyte membranes incubated with high concentrations of fullerenol (above 100 mg/L) and labeled with MSL is visible. However if fullerenol was added after labeling erythrocyte membranes with MSL, no changes were observed.

4. Discussion

Fullerenes are a significant class of nanomaterials with a wide range of potential applications in the biomedical sciences. It is therefore very important to understand the mechanisms of the interaction of these nanoparticles with biological systems and in particular with the cell membrane. The cell membrane is the first barrier that protects cells against the impact of different physical and chemical factors. Computer simulations show that fullerenol, that is, hydroxylated C_{60} fullerene is able to penetrate the plasma membrane [7].

Membrane fluidity was determined using lipid spin labels: 12-doxylstearic acid and 16-doxylstearic acid, analogs of fatty acids. The nitroxide group with an unpaired electron in different positions in the carbon chain of stearic acid gives information about changes in the indicated region of the lipid bilayer [13].

Fluidity is a physicochemical feature of cell membranes which plays an important role in regulating the basic functions of the membranes such as permeability, ion transport, membrane enzyme activity, and osmotic resistance. Changes in this parameter depend mainly on lipid-lipid or protein-lipid interactions and the content of cholesterol in the outer monolayer of the membrane [14–17].

A significant increase in membrane fluidity was observed for 12-DS at fullerenol concentrations of 100 mg/L and 150 mg/L, while for 16-DS a significant increase was observed only at the higher concentration, 150 mg/L. Both spin labels are located in the hydrophobic core of the lipid bilayer. The results of research conducted by several groups [4–7] have shown that fullerenol is mainly adsorbed at the membrane surface to the polar head group of phospholipids, membrane proteins, and the –OH groups of cholesterol by hydrogen bonds. However, the results obtained in this work show that fullerenol can penetrate into the membrane interior to the depth of the twelfth carbon atom, where hydrophobic and electrostatic interactions between the fullerenol core and acyl chains of fatty acids occur. An increase in the fluidity of plasma membrane was also observed at the depth of the sixteenth carbon atom but only at the highest fullerenol concentration of 150 mg/L.

On the basis of their studies on model membranes, Brisebois et al. [4] suggest that the presence of fullerenol nanoparticles in the polar region of phospholipids induces lipid segregation in the lipid bilayer. Moreover, fullerenol can disturb the acyl chain packing and increase the membrane fluidity.

The results obtained in the present work indicate that despite the hydrophilic nature of fullerenol nanoparticles, they can penetrate deeper into the hydrophobic core of the lipid bilayer and change its fluidity. Similar results were obtained in our previous work using the fluorescent label 1,6-diphenyl-1,3,5-hexatriene (DPH) [5]. DPH provides information from the hydrophobic region between the two leaflets of the membrane bilayer. It is worth noting that the increase in fluidity in the hydrophobic core of the lipid bilayer of the plasma membrane was observed at high concentrations of fullerenol (100 mg/L or higher) using either the fluorescent label DPH or the spin label 12-doxylstearic acid.

The ability of fullerenol to interact with membrane proteins was confirmed in the experiment wherein the isolated erythrocyte membranes were labeled with a nitroxide-maleimide spin label (MSL). MSL binds to 75–90% of –SH groups of the spectrin-actin complex, band 3 protein, and bands 4.1 and 4.2 proteins at neutral pH. MSL can also bind to –SH groups of membrane Na,K-ATPase [18]. The h_w/h_s parameter, which reflects the conformational changes

of membrane proteins, was analyzed from the ESR spectra of MSL [13, 19, 20].

Protein unfolding is expected to increase the population of weakly immobilized residues (h_w) in relation to the population of strongly immobilized residues (h_s), and therefore the maleimide spin label can be used as a structural probe for protein unfolding and refolding [18].

Fullerenol at 100 mg/L and 150 mg/L causes a statistically significant increase in the h_w/h_s parameter when fullerenol is added before the MSL to the membrane suspension. The increase of the h_w/h_s parameter may be a consequence of changes in lipid-protein or protein-protein interactions thus increasing membrane fluidity and mobility of the MSL label. Mendanha et al. [21] observed an increase in rigidity and a decrease in the h_w/h_s parameter in erythrocyte membranes after treatment with H_2O_2.

On the other hand, when fullerenol is added after the labeling of the membrane proteins, no changes are observed. Incubation of the membranes with the MSL before addition of fullerenol blocks the available –SH groups and minimizes the interaction of fullerenol with the membrane proteins. This suggests that fullerenol interacts with erythrocyte membranes at the functional sites of proteins with available –SH groups.

Our previous results have shown that fullerenol nanoparticles can bind to the erythrocyte cytoskeleton and membrane proteins [5, 6]. Moreover, we have proved that fullerenol $C_{60}(OH)_{36}$ preferentially binds either to band 3 protein (a transmembrane protein responsible for anion exchange which also acts as an anchor for many cytoskeletal proteins, including spectrin) or to membrane ATPases. In this work we show that interaction between fullerenol and membrane proteins is based on the available –SH groups of membrane proteins.

According to Soszyński and Bartosz [22], a decrease of the h_w/h_s parameter reflects an increase in protein-protein interactions. Decrease in the h_w/h_s parameter is observed in cell membranes exposed to oxidative stress, which causes the formation of disulfide bonds and aggregation of proteins. This observation is confirmed by the results of Koziczak et al. and Krokosz and Szweda-Lewandowska [23, 24]. They have reported that ionizing radiation causes a decrease in the h_w/h_s parameter as a result of conformational changes of membrane proteins, for example, spectrin or by oxidation of available –SH groups by $^{\cdot}OH$ radicals.

Very recent studies on nanodiscs have indicated that the structure of membrane proteins has a significant effect on lipid bilayer structure and should be taken into account in future membrane component studies [25].

5. Conclusions

The results obtained in this work using ESR spectroscopy confirm that highly hydroxylated fullerenol can penetrate the hydrophobic core of lipid bilayers in erythrocyte membranes, especially at higher concentrations of fullerenol. Fullerenol $C_{60}(OH)_{36}$ increases the fluidity of the lipid bilayer in the hydrophobic region of the erythrocyte membrane. Conformational changes in membrane proteins are observed under the influence of $C_{60}(OH)_{36}$ which are connected to increased mobility of the MSL label. Fullerenol interacts with erythrocyte membrane proteins via the available protein –SH groups.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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