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Fullerenol C$_{60}$(OH)$_{36}$ protects human erythrocyte membrane against high-energy electrons

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Abstract

Fullerenols (polyhydroxylated fullerene C$_{60}$) are nanomaterial with potentially broad applicability in biomedical sciences with high antioxidant ability, thus, we investigated the radioprotecting potential of fullerenol C$_{60}$(OH)$_{36}$ on human erythrocytes irradiated by high-energy electrons of 6 MeV. The results demonstrate that C$_{60}$(OH)$_{36}$ at concentration of 150 μg/mL protects the erythrocytes against the radiation-induced hemolysis (comparing to non-protected cells, we observed 30% and 39% protection for 0.65 and 1.3 kGy irradiation doses, respectively). The protecting effect was confirmed by 32% decreased release of potassium cations comparing to the cells irradiated without C$_{60}$(OH)$_{36}$. Measurements of the amount of lactate dehydrogenase (LDH) released from the irradiated erythrocytes showed that the size of the pores formed by irradiation was not sufficient to release LDH across the erythrocyte membranes. We also report a significant decrease of the affinity of acetylcholinesterase (AChE) for the substrate in the presence of fullerenol, indicating the relatively strong adsorption of C$_{60}$(OH)$_{36}$ to components of plasma membrane. Changes in membrane fluidity detected by fluorescence spectroscopy and conformational changes in membrane proteins detected by spin labeling suggest the dose-dependent formation of disulfide groups as an effect of oxidation and this process was inhibited by C$_{60}$(OH)$_{36}$. We suppose that scavenging

AChE, erythrocyte acetylcholinesterase; IDSL, bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-yl)disulfide; LDH, lactate dehydrogenase; LET, Linear Energy Transfer; MSL, 2,2,6,6-tetramethyl-4-maleimidopiperidine-N-oxyl; RNS, reactive nitrogen species; ROS, reactive oxygen species; TMA-DPH, 3-methylammonio-1,6-diphenyl-1,3,5-hexatriene.

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the ROS as well as adsorption of fullerenol to membrane proteins and steric protection of -SH groups against oxidation are responsible for the observed effects.

**Keywords**

fullerenol; membrane fluidity; membrane damage; thiol groups; hemolysis; radioprotection

1. **Introduction**

One of the key anticancer therapy approaches is modern radiotherapy (Bert and Durante, 2011; Specht et al., 2014). Therapeutic radiology utilizes mainly external-beam radiation treatment with photons, electrons, protons, carbon ions or neutrons (Xu et al., 2008). The latest achievements in the radiation treatment allow to adjust the dose distribution to the shape of the irradiated area. Unfortunately, despite the novel irradiation techniques the healthy tissues located in the irradiated area also undergo a radiative destruction and such undesirable side effects are still inevitable. This damage is a consequence of free radicals generated by radiation (Newhauser et al., 2011, Islam, 2017), therefore, the vast majority of commonly used radioprotectors includes substances that effectively scavenge free radicals, indirectly preventing damage to cellular structures. Broadly used radioprotectors predominantly belong to thiol compounds. Currently approved popular products for reducing the oxidative damage in irradiated healthy tissues are mesna and amifostine (WR 2721). Mesna has a relatively narrow spectrum of protective effect against damage of healthy tissue (heart and epithelium of the urinary tract respectively), whereas the spectrum of action of amifostine is wider and includes more tissues (Hensley et al., 2009). Unfortunately, both mentioned compounds may cause numerous side effects (Ścieszka et al., 2001). Moreover, numerous cases of the inhibitory effect on radiotherapy efficacy have been reported during the treatment with amifostine (Buentzel et al., 2006; Gu et al., 2014). Due to these drawbacks, new radioprotecting compounds that minimize the radiation-induced oxidative stress with no side effects, are strongly desirable. Recently, great hope is attributed to carbon nanocompounds: fullerenes, carbon nanotubes, graphene (Wang et al., 2015). Fullerenes and their water-soluble derivatives like fullerensols (polyhydroxylated fullerene C_{60}) are nanomaterials with potentially broad applicability in biomedical sciences (Djordjevic et al., 2015; Krokosz et al., 2016). Described by a general formula C_{60}(OH)_{n} where n=2-36, fullerensols are characterized by high polarity of the molecule, the presence of conjugated


double bonds, high electron affinity, excellent activity as scavengers of free radicals, and reactivity with nucleophilic substituents. All these features perfectly match the requirements for a good radioprotector, and make fullerenols the promising candidates (Grebowski et al., 2014): the results of several studies indicate that some of the water-soluble fullerenols applied in the doses exerting protective effects against oxidative damage, exhibit no cytotoxic effects (Partha and Conyers, 2009; Grebowski et al., 2013a). In addition, there are numerous evidences suggesting that water-soluble fullerenes are able to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) both in vitro and in vivo (Mirkov et al., 2004; Yin et al., 2009; Jacevic V et al., 2017).

Some attempts have been made to use fullerenes and their derivatives as potential protectors against ionizing radiation (Zhao et al., 2005; Trajkovic et al., 2007; Bogdanowic et al., 2008; Nowak et al., 2014), however, available literature does not include exhaustive and unequivocal data on the effects of fullerenes and fullerenols on cells exposed to high energy electrons, especially their radioprotective effects on the cell membranes. For this reason, it is essential to know and to understand the mechanisms of interaction of these nanoparticles with biological systems, particularly with cellular membrane as the main barrier protecting the cell from physical and chemical agents (Johnston et al., 2010). The aim of the present study was to investigate the direct effect of fullerenol C_{60}(OH)_{36} on human erythrocyte membranes irradiated by high energy electrons of 6 MeV. We chose erythrocytes as one of the commonly used cell type for testing the effects of radioprotectors. Mammalian erythrocytes do not contain nuclei, or other organelles, however, they feature numerous antioxidant systems, making them highly radioresistant and a convenient model for the research aimed at searching of a new generation of radioprotectors. In this model system the radiolytically induced damage of cellular structure can be monitored on the basis of such biomarkers as the degree of hemolysis and the level of potassium efflux. Moreover, in order to get the deeper insights into the nature of the damage, we decided to do some additional measurements: the amount of lactate dehydrogenase released from the erythrocytes, the activity of acetylcholinesterase, the changes in the membrane fluidity and the conformational changes in membrane proteins.

2. Materials and Methods

2.1. Synthesis of fullerol

Fullerenol (polihydroxyfullerene) C_{60}(OH)_{36} was synthesized according to the modified method of Wang et al., 2005, which was described by Grebowski et al., 2013c.
The structure of the obtained hydroxyl derivative of fullerene C\textsubscript{60} was confirmed by IR spectrophotometry (NEXUS FT-IR spectrometer), \textsuperscript{1}H-NMR (Bruker Avance III 600 MHz spectrometer), \textsuperscript{13}C-NMR (Varian Gemini 200 MHz spectrometer), MALDI-TOF/TOF mass spectrometry (Axima Performance Mass Spectrometer - Shimadzu Biotech) and elemental analysis. The IR spectrum of the fullerenol was observed for the product as shown in Fig. S1 (Supplementary material), with a broad hydroxyl absorption around 3390 cm\textsuperscript{-1}, a C-O stretching absorption at 1080 cm\textsuperscript{-1} and a C=C absorption at 1618 cm\textsuperscript{-1}. The peak centered at 1370 cm\textsuperscript{-1} can be assigned to bending vibrations of hydroxyl groups. IR spectrum also showed the appearance of a C=O stretching peak at 1720 cm\textsuperscript{-1}. Acid-catalyzed hemiacetal tautomerization or pinacol coupling of fullerenol have been reported to produce such C=O groups (Kokubo et al., 2011). The spectrum of \textsuperscript{1}H-NMR is a single line on a transfer of δ = 4.71 ppm originating from the protons of hydroxyl groups (Fig. S2, Supplementary material). C\textsubscript{60} alone does not give a signal in this regard. In \textsuperscript{13}C-NMR spectrum of fullerenol weak signals were recorded at δ = 185 ppm, which suggests the presence of ketonic moieties. The signal at δ = 169.2 ppm indicates carboxylate groups. The multiplet signal at about 140 ppm is associated with the carbon atoms of the cage, while the absorption centered at δ = 77.5 ppm is indicative of a tertiary alcoholic function (Fig. S3, Supplementary material). These lines indicate the presence of aromatic systems in the molecule of fullerenol and the chemical diversity of carbon atoms in the molecule C\textsubscript{60}. Mass spectrometry confirmed the presence of hydroxyl groups connected to the C\textsubscript{60} molecule. The mass spectrum showed peaks with a high intensity at m/z = 720, which demonstrates that the cage of fullerenol has not been damaged during the synthesis. Detection of a molecular ion at m/z 1332 was indicative for the composition of fullerenol as containing 36 hydroxyl groups per C\textsubscript{60} cage (Fig. S4, Supplementary material). The elemental analysis of fullerenol (found: C, 44.27%; H, 4.38%; calc.: C, 44.45%; H, 4.23%) showed an average composition of C\textsubscript{60}(OH)\textsubscript{36}. A stock solution of fullerenol in deionized water at a concentration of 5 mg/mL was pipetted to the samples to the final concentration of 150 μg/mL in the sample. The stock solution was sonicated before adding for 10 min. Dynamic light scattering (DLS) and atomic force microscopy (AFM) were used to determine the particle size of fullerenol. Fig. S9 presents nanoparticle’s size distribution by number, with a peak at approx. 40 nm in agreement with the results obtained for other fullerenols (Husebo et al.2004, and Brant et al., 2007). However, the AFM measurements (see Fig. S10) showed that fullerenol particles dried on mica (without solvation layer) are smaller, with the average diameter of 2-10 nm. The zeta potential of fullerenol was negative with the mean value of -33.08 mV.
2.2. Preparation of erythrocytes
Blood samples from adult donors were provided by the Central Blood Bank in Łódź, Poland. Erythrocytes were separated from the blood plasma and leucocytes by centrifugation at 825×g for 5 min (4°C), washed three times with sodium phosphate buffered saline (145 mM NaCl in 10 mM sodium phosphate, pH 7.4; PBS) and the buffy coat was aspirated each time. Washed erythrocytes were resuspended in the fresh portion of the buffer to obtain a hematocrit of 2% and incubated with fullerenol for 1 h at 37°C before irradiation.

2.3. Preparation of erythrocyte membranes
Erythrocyte membranes were prepared using modified method of Dodge et al. 1963. The erythrocytes were hemolyzed with 20 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA and 0.5 mM PMSF, and the resulting 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. The protein content of the membrane preparations was determined via the method of Lowry et al. 1951. Erythrocyte membranes (1 mg_prot./mL) in PBS were incubated with fullerenol for 1 h before the irradiation.

2.4. Irradiation conditions
Human erythrocyte suspensions (Ht = 2%) or erythrocyte membranes (1 mg_prot./mL) in PBS (pH 7.4) were exposed under air to high-energy electrons from the 6 MeV ELU-6 linear accelerator. Pulse radiolysis was performed with 17 ns electron pulses which delivered doses of 65 Gy each. The total doses absorbed were 0.65 kGy or 1.3 kGy as evaluated using a Rogowski coil. The measurements were performed after 2 and 19 hrs after irradiation.

2.5. Hemolysis measurements
The percentage of hemolysis of erythrocytes, H(%), was determined spectrophotometrically based on the ratio of hemoglobin (Hb) released from cells to the total cellular Hb content after hemolysis with distilled water. H(%) was calculated on the basis of absorbance changes at 630 nm after oxidation of heme iron with K3[Fe(CN)6], according to the following equation:

\[
H(\%) = \frac{A_{630\text{sup.} \cdot \text{MetHb}}}{A_{630\text{sup.} \cdot \text{MetHb}} + \gamma \cdot A_{630\text{pelle} \cdot \text{MetHb}}} \times 100\% \tag{1}
\]
where $A_{630 \text{sup.} \text{MetHb}}$ is the absorbance of the supernatants of the samples, $A_{630 \text{pellet MetHb}}$ is the absorbance of the supernatant of the samples after complete hemolysis of the pellet with distilled water, and $r$ is the dilution factor.

2.6. LDH Release Measurement
Extracellular lactate dehydrogenase (LDH) activity was assessed by monitoring for 1 min a decrease in the absorbance at $\lambda = 340$ nm resulting from the NADH oxidation (Wroblewski and Ladue, 1955). Extracellular LDH activity was determined in supernatants after centrifugation of 2% erythrocyte suspensions (825×g, 5 min, 4°C). LDH release from erythrocytes was expressed as a concentration of NAD$^+$ in µM per packed erythrocytes.

2.7. Potassium efflux
The erythrocyte suspensions were centrifuged at 825×g for 10 min and the resulting supernatant was assayed for its potassium content, measured by flame atomic emission spectrometry using a SpectrAA-300 apparatus (Varian, Australia).

2.8. Measurement of acetylcholinesterase (AChE) activity
The activity of AChE was determined using the spectrophotometric method of Ellman et al., 1961. The rate of acetylthiocholine iodide hydrolysis was calculated as follows:

$$ V = \frac{\Delta A_{412(1 \text{min})} \cdot F}{1000 \cdot e} \cdot \text{mol}_{\text{ATCh}} \cdot \text{min} \cdot \text{mL}_{\text{packedER}} $$

(2)

where: $\Delta A_{412(1 \text{min})}$ is the increase in absorbance in min$^{-1}$ at 22°C; $F$ is the dilution factor; and $e$ = 13600 M$^{-1}$·cm$^{-1}$ is the extinction coefficient for 5-thio-2-benzoic acid (TNB).

2.9. Measurements of erythrocyte membrane fluidity
Membrane fluidity was determined by fluorescence spectroscopy with 1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) as fluorescent probe. Erythrocyte suspension was diluted with PBS (50-fold dilution to the hematocrit level of 0.04%), and TMA-DPH in tetrahydrofuran was added to a final concentration of 2 µM. The final concentration of THF was 0.1% and did not affect the results. The samples were incubated at 37°C for 15 min in the dark. Fluorescence anisotropy ($r$) was assessed at wavelengths typical for TMA-DPH probe: 365 nm for excitation and 425 nm for emission (Marczak, 2009). The widths of the excitation and emission monochromator slits were set to 10 nm. During measurement on a Cary Eclipse (Varian,
Australia) fluorescence spectrophotometer equipped with an automated polarizer accessory, the samples were thermostated at 37°C with a Peltier device and stirred with a magnetic bar. Fluorescence anisotropy (r) was calculated by the software provided with the instrument from the equation: 

\[ r = \frac{I_{vv} - I_{vh}G}{I_{vv} + 2I_{vh}G} \]

where \( I_{vv} \) and \( I_{vh} \) are the intensities of the vertically and horizontally polarized components of the fluorescent light, respectively, after excitation with vertically polarized light. \( G = I_{hv}/I_{hh} \) is a grating correction factor for the optical system (the ratio of the vertically to the horizontally polarized emission components).

2.10. Spin labeling measurements of the –SH groups concentration in erythrocyte membranes

To the erythrocyte membranes at a concentration of membrane protein of 1 mg/mL, bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il)disulfide (IDSL) spin label was added to a final concentration of 80 μM. The samples were incubated at room temperature for 3 min. The ESR spectra were recorded on a Bruker 300E ESR spectrometer with frequency 9.74GHz, microwave power 10 mW, center field 3473 G (Fig. S7, Supplementary material). The \( P \) parameter was calculated from the equation:

\[ P = \frac{I-I_0}{I_0} \]

where \( I \) is the high-field peak height of spin label in the analyzed sample, and \( I_0 \) is the high-field peak height of spin label in PBS (Weiner, 2006). The GSH concentration was estimated by a standard curve that was made in the range of GSH concentrations from 0 to 60 μM.

2.11. Spin labeling of erythrocytes membranes with MSL

Changes in protein conformation were estimated according to Grebowski and Krokosz (2015) by spin labeling of the erythrocyte membranes with 2,2,6,6-tetramethyl-4-maleimidopiperidine-N-oxyl (MSL). MSL was added to the erythrocyte membranes to a final concentration of 0.2 mM and samples were incubated at 4°C for 1 h. Then the excess of the label was removed by washing with PBS several times. All ESR spectra of MSL bound to the membrane proteins were measured at ambient temperature using a Bruker 300 E ESR spectrometer. The spectra were analyzed by measuring the ratio of the peak heights of weakly \( (h_w) \) to strongly \( (h_s) \) immobilized components in the low field peak, thus, the value \( h_w/h_s \) expresses the ratio of population of weakly to strongly immobilized residues (Fig. S5, Supplementary material).
2.12. Statistical analysis
All experiments were run 3-8 times. Values were expressed as the mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, all using the GraphPad 4.0 Software (La Jolla, CA).

3. Results
3.1. Hemolysis of erythrocytes in the presence of fullerenol under ionizing radiation
Figure 1 shows the hemolysis of erythrocytes incubated with fullerenol at 150 μg/mL followed by irradiation with 0.65 kGy or 1.3 kGy. The hemolysis was measured after 2 h and 19 h after the irradiation. Addition of fullerenol at 150 μg/mL prior to irradiation resulted in 30%-40% decreases in erythrocyte hemolysis compared to erythrocytes irradiated with 0.65 kGy or 1.3 kGy without fullerenol, irrespectively of the time after irradiation. The degree of hemolysis of irradiated erythrocytes in the samples containing fullerenol is similar as for the control samples (without irradiation) for 2-hour post-radiation incubation. However, the efficacy of fullerenol in protection against hemolysis diminished when the time after irradiation was prolonged to 19 hours. Above results demonstrate that fullerenol is able to effectively protect the erythrocytes against the radiation-induced hemolysis but is less effective in protection against post-radiation damage. The unirradiated samples incubated with fullerenol for 3 h did not show any influence on hemolysis as it was shown by Grebowski et al., 2013b.
Fig. 1. The effect of fullerenol (150 µg/mL) on the hemolysis of irradiated erythrocytes. Fullerenol was added 1 hour before irradiation with a dose of 0.65 or 1.3 kGy. The results were obtained 2 and 19 h after the end of irradiation. The bars represent mean ± SD for 4-8 independent measurements.

3.2. The influence of ionizing radiation on the release of LDH in the presence of fullerenol

Activity of lactate dehydrogenase released from erythrocytes incubated with fullerenol (150 µg/mL) followed by irradiation with 0.65 and 1.3 kGy dose is shown in Table 1. The results indicate no substantial change in LDH activity after 2 hours from irradiation irrespective of incubation with or without fullerenol.

Table 1. Influence of irradiation in the presence of 150 µg/mL fullerenol on the release of LDH from erythrocytes measured as enzymatically catalyzed decrease of NADH oxidation. Fullerenol was added 1 hour before the irradiation with a dose of 0.65 or 1.3 kGy. The results were obtained after 2 hours after the end of irradiation. a

<table>
<thead>
<tr>
<th>LDH</th>
<th>C_{NAD+} (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.122 ± 0.013</td>
</tr>
<tr>
<td>Control with FulOH</td>
<td>0.122 ± 0.019</td>
</tr>
<tr>
<td>0.65 kGy</td>
<td>0.123 ± 0.018</td>
</tr>
<tr>
<td>0.65 kGy+ FulOH</td>
<td>0.121 ± 0.019</td>
</tr>
<tr>
<td>1.3 kGy</td>
<td>0.137 ± 0.028</td>
</tr>
<tr>
<td>1.3 kGy+ FulOH</td>
<td>0.118 ± 0.015</td>
</tr>
</tbody>
</table>

aThe results are mean values ± SD of 7-12 independent measurements. b Control samples were not irradiated.

3.3. Effect of fullerenol and ionizing radiation on the potassium efflux

The potassium efflux from erythrocytes irradiated alone or irradiated after preincubation with fullerenol, are shown in Fig. 2. It was found that the presence of 150 µg/mL fullerenol in the samples irradiated with a dose of 1.3 kGy caused a decreased release of K⁺ from erythrocytes by about 32% comparing to the samples without fullerenol. However, prolongation of post-radiation incubation declined the protective effect of fullerenol to about 10%. The unirradiated samples incubated with fullerenol for 3 h did not show an increase in release of potassium ions (Grebowski et al., 2013b).
Fig. 2. Potassium efflux from irradiated erythrocytes. Fullerol (150 μg/mL) was added to samples 1 h prior to the irradiation with a dose of 0.65 or 1.3 kGy. The results were obtained 2 and 19 h after the end of the irradiation. The bars represent mean ± SD values for 5-11 independent measurements.

3.4. The influence of ionizing radiation on AChE activity in the presence of fullerol

We calculated the kinetic constants $V_{\text{max}}$ and $K_m$, from the Lineweaver-Burk curves derived from the Michaelis-Menten kinetics. The results listed in Table 2 show that fullerol itself does not affect the activity of the enzyme (control samples exhibit the same activity as without FulOH) while the irradiation of the erythrocytes in the presence of fullerol at a concentration of 150 μg/mL significantly reduced the enzyme affinity for the substrate.

Table 2. Kinetics parameters obtained from Lineweaver-Burk curves for acetylcholinesterase activity of human erythrocytes incubated with fullerol (150 μg/mL) followed by irradiation, and measured 2 hours after the irradiation.\(^a\)

<table>
<thead>
<tr>
<th>AChE</th>
<th>$V_{\text{max}}$ (μmol/mill/min packed ER)</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^a)</td>
<td>13.78 ± 0.56</td>
<td>62.29 ± 8.84</td>
</tr>
<tr>
<td>Control(^b) with FulOH</td>
<td>13.80 ± 0.58</td>
<td>56.20 ± 4.65</td>
</tr>
<tr>
<td>0.65 kGy</td>
<td>13.43 ± 0.86</td>
<td>83.85 ± 7.98</td>
</tr>
<tr>
<td>0.65 kGy + FulOH</td>
<td>14.53 ± 1.23</td>
<td>108.88 ± 10.18</td>
</tr>
<tr>
<td>1.3 kGy</td>
<td>13.99 ± 0.86</td>
<td>100.65 ± 12.42</td>
</tr>
<tr>
<td>1.3 kGy + FulOH</td>
<td>14.06 ± 1.51</td>
<td>110.78 ± 4.65</td>
</tr>
</tbody>
</table>

\(^{a}\) The results are mean values ± SD of 3-5 independent measurements. \(^{b}\) Control samples were not irradiated. \(^{c}\) $p<0.05$ vs. irradiated sample (0.65 kGy).

3.5. The influence of ionizing radiation on the fluidity of erythrocyte membranes in the presence of fullerol
Figure 3 presents the anisotropy coefficients \((r)\) of TMA-DPH labeled erythrocyte membranes and one can see that the \((r)\) values were reduced in a dose-dependent manner after 2 h from irradiation of erythrocyte membranes with a dose of 0.65 kGy or 1.3 kGy, compared to non-irradiated ones. When the samples containing 150 μg/mL of fullerenol were exposed to 0.65 kGy irradiation, a decrease of \((r)\) was still observed, however, during irradiation with 1.3 kGy dose the presence of fullerenol prevented the decrease of the anisotropy coefficient (see Fig. 3). One can mention that fullerenol at a concentration of 150 μg/mL induced statistically significant decrease in the anisotropy of TMA-DPH by 7.5% after a 1-hour incubation at 37 °C as it was shown by Grebowski et al., 2013c.

![Graph showing the effect of fullerenol on anisotropy coefficient (r) for TMA-DPH labeled erythrocyte membranes and irradiated with a dose of 0.65 or 1.3 kGy. The results were obtained after 2 hours after the end of the irradiation. Samples indicated as FulOH contained fullerenol at concentration 150 μg/mL added 1 h prior to the irradiation. The bars represent mean ± SD values for 4 independent measurements.](image)

**Fig. 3.** The effect of fullerenol on anisotropy coefficient \((r)\) for TMA-DPH labeled erythrocyte membranes and irradiated with a dose of 0.65 or 1.3 kGy. The results were obtained after 2 hours after the end of the irradiation. Samples indicated as FulOH contained fullerenol at concentration 150 μg/mL added 1 h prior to the irradiation. The bars represent mean ± SD values for 4 independent measurements.

### 3.6 Concentration of the \(-\text{SH}\) groups in erythrocyte membranes

For the samples irradiated with 0.65 or 1.3 kGy, a dose-related decrease in the concentration of \(-\text{SH}\) groups in erythrocyte membranes is observed, as shown in Fig. 4. The presence of fullerenol during irradiation resulted in the protection of \(-\text{SH}\) groups against oxidation, by about 25% for a 0.65 kGy dose and about 42% for a 1.3 kGy dose versus non-irradiated control. The unirradiated samples incubated with fullerenol for 3 h did not show any influence on the concentration of \(-\text{SH}\) groups (data not shown).
3.7. Conformational changes of membrane proteins determined with MSL

Fig. 5 presents the $h_w/h_s$ ratio measured for isolated human erythrocyte membranes irradiated in the absence and presence of fullerene. Irradiation of samples without fullerene with the dose of 1.3 kGy induced the decrease in $h_w/h_s$ ratio that was not observed for the samples containing fullerene at 150 μg/mL during the irradiation. As it was shown by Grebowski and Krokosz (2015) fullerene at 150 μg/mL caused a slight increase of the $h_w/h_s$ ratio after a 1-hour incubation at 37 °C.
Fig. 5. Conformational changes in erythrocyte membrane proteins expressed as \( h_d/h_h \) ratio for MSL spin label. Fullerol (150 μg/mL) was added to the samples of erythrocyte membranes 1 h prior to the irradiation with a dose of 0.65 or 1.3 kGy. The results were obtained 2 hours after the end of irradiation. The bars represent mean ± SD values for 3 independent measurements.

4. Discussion

The absorption of ionizing radiation energy of several mega electronvolts (MeV) is non-specific and leads to the ionization of atoms and molecules (Alpen, 1998; Beyreuther et al., 2015). High-energy electron beam radiation may also induce formation of micropores and electroporation of cell membranes (Soszyński and Bartosz, 1997; Neamtu et al., 1999). The ratio between the direct and indirect effects of radiation on cellular components depends on the Linear Energy Transfer (LET) value and damage type / biological effects correlate with a density of the ionizing radiation. For a low LET radiation (e.g. X, γ, \( \alpha \)) it is assumed that 70-90% of the damage is caused by indirect action, i.e. the toxic effect is due to the radiolysis of water (Krokosz et al., 2006). Therefore, a substantial water content in living cells (ca. 70% of \( \text{H}_2\text{O} \)) makes the radiolysis of water much more probable than the radiolysis of other molecules. Regardless the primary site of the attack, the airborne products of water radiolysis and radiolysis of biomolecules lead to the oxidative modifications of biomolecules and cellular damage (Adams et al., 2015). Some protective substances such as antioxidants can be used in order to prevent such kind of radiolytic damage made to cellular structures (Cai et al., 2010; Citrin et al., 2010; Kamran et al., 2016). The principal function of an antioxidant is to scavenge the radical and non-radical water radiolysis products and to reduce the oxidized molecules. In addition, antioxidants diminish the negative effect of ionizing radiation by modifying the enzymatic activity of antioxidant defense systems and DNA repair systems, as well as regulation of signaling pathways. For human erythrocytes, which have no nucleus, the essential ways of protection against radiation are: (i) enzymatic systems of antioxidant defense, (ii) non-enzymatic removal of the products of water radiolysis, and (iii) the reduction/repair of oxidized biomolecules.

In the present study we investigated the ability of polyhydroxylated fullerene \( \text{C}_{60}(\text{OH})_{36} \) to reduce the damage induced by high-energy electrons (6 MeV) in the human erythrocyte plasma membrane. Irradiation of the cells under aerobic conditions mainly triggers the production of hydroxyl radicals, the main species responsible for the cellular damage. Due to high electron affinity, hydroxyl radicals are able to oxidize almost every biomolecule present in the living cell with extremely high rate of reaction (Gwoździński,
1991; Weidinger and Kozlov, 2015). As a result, the oxidized forms of biomolecules disrupt the integrity of the whole cellular membrane.

Taking into account the relatively high concentration of C$_{60}$(OH)$_{36}$ used in our study, it can be concluded that the protective effect of fullerenol on radiation-induced hemolysis (Fig. 1) is to a large extent related to its ability to scavenge ROS, as was previously confirmed by us and other research groups (Kato et al., 2009; Krokosz et al., 2014; Hao et al., 2017). In our previous work we observed higher viability of human lymphocytes X-irradiated with the dose of 50 Gy in the presence of 75 mg/L of fullerenol (Nowak et al. 2014). However, the protection was higher for the concentration of fullerenol 150 mg/L, thus, in our current studies we decided to apply 150 mg/L of fullerenol. It is worth to underline, that this high concentration of fullerenol used in our work had no influence on the examined parameters of erythrocytes excluding a slight increase of the $h_6/h_5$ ratio after a 1-hour incubation at 37 °C. The great number of -OH groups in C$_{60}$(OH)$_{36}$ can facilitate formation of a stable free radical form of fullerenol C$_{60}$(OH)$_{35}$–O• (Kato et al., 2009). However, the great number of –OH groups does not prevent soluble clusters formation due to hydrophobic and π-π interactions (Zanzoni et al., 2016). In this paper we used the same conditions (phosphate buffered saline, concentration of fullerenol, temperature, sonication) in all the samples, that is why we did not focus on the influence of fullerenol nanoparticles diameters on the measured effects. As we can see in Supplementary Material (Fig. S9), fullerenol particles were of different diameters in the experimental medium as we measured by DLS with the main peak at about 40 nm. However, the AFM image (Fig. S10) showed that the fullerenol particles are smaller with the average diameter of 2-10 nm.

The erythrocyte cell membrane integrity was also assessed by the amount of lactate dehydrogenase (LDH) released from the irradiated erythrocytes (Table 1). There was no effect of radiation nor fullerenol observed on the LDH release, but such negative result can be reasoned due to the size of LDH, being a tetramer with a molecular weight of about 140 kDa twice bigger than hemoglobin (65 kDa). The radiation doses applied in the present study did not disrupt the continuity of the membrane to form sufficiently big pores able to release such a large protein as LDH. However, the results of LDH experiments have to be interpreted with a caution because the radiation doses used (0.65 and 1.3 kGy) could inactivate some fraction of LDH, thus underestimating the enzyme activity (Kowalczyk et al., 2008).

Results presented in Fig. 2 demonstrate, as expected, that irradiation increased the amount of potassium released from the erythrocytes along with increasing radiation dose (van de Watering, 2011). Formation of the pores can be the effect of the lipid peroxidation or
denaturation of integral proteins followed by their aggregation. Additionally, the absorption of ionizing radiation may lead to electroporation of the membrane. This process of reversible formation of transient pores can be induced by short, high-intensity electric pulses (Neamtu et al., 1999). Regardless the mechanism of formation of the pores induced by radiation in the erythrocyte membrane, their size, as calculated by Soszyński and Bartosz (1997), is smaller than the size of hemoglobin molecule, but sufficient for the leakage of small inorganic ions which can easily penetrate the pores. Fig. 2 demonstrates that fullerene reduces the potassium leakage from irradiated cells. However, fullerene could irreversibly inhibit Na,K-ATPase (Grebowski et al., 2013c) influencing active ion transport. In this work we observed only slight reduction of the radiation-induced $K^+$ efflux by fullerene. There is only 10% protection 19 h after irradiation. Rokitskaya and Antonenko (2016) reported that fullerene $C_{60}(OH)_{24}$ could induce ion permeability of a planar lipid bilayer membrane via the formation of ion pores or conductive defects with a preference for cations over anions. However, incubation of intact human erythrocytes with fullerene $C_{60}(OH)_{36}$ for 48 h did not induce enhanced potassium efflux (Grebowski et al., 2013b). Reduction of the leakage of potassium ions by $C_{60}(OH)_{36}$ may result from chemical and physical action of fullerene - the first one is a protection of the membrane due to fast reaction with $^•OH$ radicals whereas the physical protecting effect origins from a “blockage” of the pores by fullerene molecules (Grebowski et al., 2013c). Moreover, Zeta potential of fullerene in buffered system at pH 7.4 was negative. Thus, the electrostatic interactions could influence the cation flux as well as membrane permeability.

The hypothesis on the adsorption of fullerene molecules to components of plasma membranes was confirmed by the results obtained for erythrocyte acetylcholinesterase (AChE). Erythrocyte AChE is biochemically identical with the enzyme that is present in the nervous tissue. It is characterized by lower interindividual differences and it is resistant to external factors. The inhibition of AChE activity in blood is correlated with its inhibition in target tissues as indicated in earlier studies (Padilla et al., 1994; Kale et al., 1999; Soreq and Seidman, 2001). The inhibition of erythrocyte AChE activity is useful biomarker of the exposure to many chemical and physical toxic factors (Colovic et al., 2013; Kwiatkowska et al., 2014). The values of $K_m$ and $V_{max}$ for AChE of irradiated erythrocytes are presented in Table 2. With increasing radiation dose a decrease in affinity of the substrate to the enzyme was observed. Irradiation of erythrocytes in the presence of fullerene resulted in a further reduction of affinity of the substrate to AChE. This indicates that the interaction of fullerene with plasma membrane promoted damage induced by high-energy electrons. In contrast, no
reduction in $V_{\text{max}}$ of the enzyme was observed in the range of radiation doses or fullerenol concentration used (see Table 2). It should be emphasized that AChE is a protein anchored in the plasma membrane by a GPI structure, which protrudes completely above the membrane surface. The active center of the enzyme is a deep, narrow gorge at the depth of 2 nm. Such a structure of the active center, which is located in the so-called "catalytic triad", limits the possibility of penetration by fullerenol. Moreover, AChE does not contain -SH groups in its active site, and the active group of catalytic triad is OH group of serine. There may be an electrostatic repulsion between the amino acids of catalytic triad (glutamic acid, serine and histidine) and fullerenol. However, Asp 74 in peripheral binding site is able to form hydrogen bond between its carboxylate moiety and fullerenol (Johnson and Moore, 2006) and such interaction could modify the turnover of AChE by physical blocking of the gorge entrance, or an allosteric interaction between the active and peripheral sites involving conformational changes in the protein molecule.

Microviscosity of plasma membrane (Fig. 3) indicates the increase in membrane fluidity of irradiated erythrocytes, and this effect may be associated with oxidation of lipids and membrane proteins (Strugala et al., 2016). Under irradiation with high-energy electrons lipid peroxidation is not the dominant process (Todoriki and Hayashi, 1994). Therefore, the increased membrane fluidity is due to oxidation of thiol groups and/or conformational changes in membrane proteins. The appearance of new lipid-protein interactions may cause local changes in microviscosity without affecting the arrangement of the lipids (Gwoździński, 1991; Terry et al., 1999).

Previous studies showed that C$_{60}$(OH)$_{36}$ applied in the range of 50-150 μg/mL increased fluidity of the erythrocyte membrane (Grebowski et al., 2013c), but under the stress generated by high-energy electrons, fullerenol exhibit a protective effect that may be related to scavenging of ROS and alteration of the interaction of fullerenol with membrane structures damaged by radiation. The thiol groups play an important role in maintaining the structure and function of the membrane proteins. Changes in the content of available -SH groups may provide information on both their oxidation as well as changes in conformation and interactions between protein subunits. The number of available -SH groups was determined with the IDSL spin label (Weiner, 2006): it is a hydrophobic biradical joined by a disulfide bond that can be easily exchanged with -SH groups present in the studied systems. Figure 4 indicates that the decrease in the amount of -SH groups is proportional to the radiation dose. Reduction of the content of available thiol groups may result from oxidation or formation of conjugates. Fullerenol exhibit a statistically significant protective effect for -SH groups which
may be related to two non-exclusive processes, the first one is the scavenging of ROS, especially 'OH radicals, and another one is a significant steric hindrance caused by fullerene adsorbed to erythrocyte membranes, that may be a physical barrier to the oxidation of the -SH groups (Krokosz et al., 2014).

The -SH groups are readily oxidized by ionizing radiation under aerobic conditions, resulting in the formation of disulphide bridges or derivatives of sulfenic and sulfinic acids (Chauvin and Pratt, 2017). These reactions lead to conformational changes in proteins that can be monitored by \( h_{\omega}/h_{s} \) parameter changing as a result of the rotation of the 4-maleimido-TEMPO spin label (MSL) joined to the available -SH groups by covalent bond. 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 may also bind to –SH groups of membrane Na,K-ATPase (Grebowski and Krokosz 2015, Yasuoka-Yabe and Kawakita 1983). Irradiation of erythrocytes with a dose of 1.3 kGy resulted in a slight decrease in the \( h_{\omega}/h_{s} \) parameter (Fig. 6) which is consistent with our previous results for gamma radiation (Szweda-Lewandowska et al., 2003). Fullerene present during irradiation of erythrocyte membranes maintained the \( h_{\omega}/h_{s} \) parameter at the control level.

Protein unfolding is expected to increase the population of weakly immobilized residues (\( h_{\omega} \)) in relation to the population of strongly immobilized residues (\( h_{s} \)). The previous work of Gwoździński et al. (2003) suggested the observed \( h_{\omega}/h_{s} \) changes are caused by the dissociation of the spectin-actin complex as well as by the decrease of interactions of this complex with other proteins. In our previous studies we also noticed that fullerene changed the conformation of the erythrocyte membrane proteins and adsorbed to the band 3 protein, thereby indirectly affecting cytoskeletal proteins: spectrin, actin, proteins 4.1 and 4.2 (Grebowski et al., 2013b; Grebowski and Krokosz, 2015), which were the binding site for the MSL. Zanzoni et al. (2015) revealed that fullerene C\(_{60}(\text{OH})_{24} \) clusters adsorbed reversibly to monomeric ubiquitin and bound specifically to dimeric ubiquitin.

Our findings support the view that fullerene can induce functional perturbations of proteins and can interact with membrane lipids.

5. Conclusions

In the present study we investigated the ability of fullerene C\(_{60}(\text{OH})_{36} \) to reduce damage induced by high-energy electrons (6 MeV) in human erythrocyte plasma membranes. We observed that fullerene lowered the post-radiation hemolysis, potassium efflux and oxidation of thiol groups as well as prevented the radiation-induced increase in membrane
fluidity and protein conformation changes. Nevertheless, we also noticed that fullerenol enhanced toxic effects of high-energy electrons toward membrane acetylcholinesterase.

Taking into account the relatively high concentration of fullerenol used in our study, it can be concluded that the protective effect of fullerenol on radiation-induced damage to erythrocyte membrane is, to a large extent, related to its ability to scavenge ROS. However, physical “blockages” of the radiation-induced pores by fullerenol molecules cannot be excluded. Adsorption of fullerenol molecules to components of plasma membranes was also confirmed by the results obtained for erythrocyte acetylcholinesterase. These results indicated that the interaction of fullerenol with plasma membrane promoted the damage induced by high-energy electrons.

Under irradiation with high-energy electrons lipid peroxidation is not the dominant process. Therefore, increased membrane fluidity is supposed to be due to oxidation of thiol groups and/or conformational changes in membrane proteins. The protective effect of fullerenol observed in this study may be related to scavenging of ROS by fullerenol and enhancement of the interaction of membrane structures damaged by ionizing radiation with fullerenol.

Fullerenol C$_{60}$(OH)$_{36}$ protects human erythrocytes against high-energy electrons induced damage, however, can enhance radiation-induced functional perturbations of membrane proteins.

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**References**


Graphical abstract
Highlights

- fullerol $C_{60}(\text{OH})_{36}$ protects human erythrocytes against high-energy electrons induced damage
- protective effect of fullerol is, to a large extent, related to its ability to scavenge ROS
- fullerol can enhance radiation-induced functional perturbations of membrane proteins