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Membrane fluidity and activity of membrane ATPases in human erythrocytes under the influence of polyhydroxylated fullerene

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ABSTRACT

The influence of fullerenol on the activities of human erythrocyte membrane ATPases and the fluidity of the plasma membrane as well as the possibility of fullerenol incorporation into the plasma membrane were investigated. Fullerenol at concentrations up to 150 μg/mL induced statistically significant decreases in the anisotropy of 1-anilino-8-naphthalene sulfonate (ANS) (14%), N,N,N-trimethyl-4-(6-phenyl-1,3,5,-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH) (7.5%) and 1,6-diphenyl-1,3,5-hexatriene (DPH) (9.5%) after a 1-hour incubation at 37 °C. The effect disappeared for ANS and TMA-DPH, but not for DPH, after washing out the fullerenol. Incubation of erythrocyte membranes with fullerenol led to decreases in the activities of Na^+, K^+-ATPase (to 23% of the control value), Ca^{2+}-ATPase (to 16% of control) and Mg^{2+}-ATPase (to 22% of control). Washing out the fullerenol lessened the inhibition of the Na^+,K^+-ATPase (37% of control) and Ca^{2+}-ATPase (23.5% of control); however, it did not influence Mg^{2+}-ATPase activity. Furthermore, fullerenol could associate with erythrocyte plasma membranes.

Our results suggest that fullerenol associates primarily with the surface of the plasma membrane; however, it can also migrate deeper inside the membrane. Moreover, fullerenol influences membrane ATPases so that it may modulate ion transport across membranes.

1. Introduction

In recent years a great deal of attention has been paid to the unique physicochemical properties of nanocompounds and their possible use in biology and medicine [1–4]. A very promising group among these is the fullerenes. Fullerenes are molecules built of rings, each consisting of 5 to 6 carbon atoms, bound by coupled π bonds. Results from many studies point to the great dependence of fullerenes' activity upon the quality, quantity and geometry of substituents in fullerene derivatives. Water-soluble fullerenes, such as hydroxylated C_{60} derivatives (fullerenols), have become a major point of interest in biomedical nanotechnology, thanks to their low toxicity compared to nano-C_{60} suspensions.

Sayes et al. [5] have investigated the toxicity of four fullerene derivatives on human skin fibroblasts and human liver cancer cells. They have shown that the largest toxic effect was demonstrated by water suspensions of C_{60}. They demonstrated that cytotoxicity depends on the C_{60} cage derivatization, and decreases along with increasing water solubility of the specimen. Differentiation of the cytotoxic effect is, in their opinion, caused by different interactions of fullerene derivatives with macromolecules in the plasma membrane.

A great deal of research has been done in order to explain the higher toxicity of C_{60} compared to $C_{60}(OH)_n$. These studies concerned the interactions of fullerenes with the lipid bilayer and the possibility of fullerene crossing the plasma membranes. It was observed that hydrophobic molecules of C_{60} were localized within the inner part of the membrane, whereas hydrophilic $C_{60}(OH)_n$ molecules were adsorbed on the heads of membrane phospholipids [6]. Due to the presence of hydroxyl groups on the surface of fullerenol molecules, it is possible for them to take part in many interactions, e.g. creating hydrogen bonds with biomolecules. Hydrogen bonds are relatively weak interactions compared to covalent bonds; nevertheless, they play an important role due to their frequent occurrence in biological systems and their roles in biochemistry and cell maintenance [7,8].

The many hydroxyl groups on the surface of fullerenol molecules make it possible for them to form hydrogen bonds between different biomolecules. In effect, fullerenol can adsorb on the heads of membrane phospholipids and interact with membrane proteins, thus influencing their functions. Membrane proteins play crucial roles in maintaining plasma membrane function. They are responsible for selective transport, the shape and architecture of the cell, and signal transduction [9].

Moreover, the high aqueous solubility and neutral pH of highly hydroxylated fullerenes and their accessibility to further modification

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make them promising agents for drug delivery to particular locations in the cell, as well as potential anti-cancer agents to kill tumor cells [10–12]. The hydroxyl groups increase hydrophilicity, which can be useful for delivering sparingly water-soluble or hydrophobic cytotoxic agents. Furthermore, fullerenol $C_{60}(OH)_{24}$ exhibits excellent cardio- and hepatoprotective effects in rats treated with doxorubicin (DOX) [13]. Fullerenols have the ability to quench various free radicals, behaving as "free radical sponges".

Membrane proteins with ATPase activity, which are responsible for enabling asymmetric concentrations of cations across the membrane at the expense of ATP hydrolysis, play a very important role in cell functioning. Among the transport ATPases worth mentioning are Na^+/K^+-ATPase, which transports Na^+ outside and K^+ ions inside the cell [14] and Ca^{2+}-ATPase, which is responsible for the transport of Ca^{2+} to the extracellular space [15]. Both enzymes require Mg^{2+} ions for the active transport of cations [16].

Impaired Na^+/K^+-ATPase function can be linked to very severe effects. It has been shown that a decrease in the activity of Na^+, K^+-ATPase may result in either apoptotic or "mixed" cell death. Depleting intracellular K^+ triggers an increase in intracellular Ca^{2+}, an event perceived as a trigger for excitotoxicity leading to necrotic cell death [17].

Bearing this in mind, it is reasonable to investigate the interactions of fullerenols not only with the lipid bilayer of the plasma membrane, but also with the proteins anchored to it. It is known, for example, that the activity of ATPases can be influenced either by a direct interaction with other molecules or by changes in the fluidity of the lipid bilayer [18].

The aim of this work was to assess the influence of fullerenol $C_{60}(OH)_{\sim 30}$ on selected parameters of erythrocyte membranes. In our work, the influence of fullerenol on the activity of human erythrocyte membrane ATPases and the fluidity of the plasma membrane were investigated for the first time.

2. Materials and methods

2.1. Chemicals

Fullerene (99.5%) was purchased from SES Research. Bovine serum albumin (BSA), phenylmethanesulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane (Tris), ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), adenosine 5′-triphosphate disodium salt hydrate (ATP), ammonium heptamolybdate tetrahydrate, poly(vinyl alcohol) (PVA), trichloroacetic acid (TCA), ouabain, 1-anilino-8-naphthalene sulfonate (ANS), 1,6-diphenyl-1,3,5-hexatriene (DPH), N,N,N-trimethyl-4-(6-phenyl-1,3,5,-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH) were purchased from Sigma-Aldrich. Sodium chloride, potassium chloride, calcium chloride, magnesium chloride hexahydrate, potassium phosphate monobasic, copper(II) sulfate(VI), sodium carbonate, sodium citrate monohydrate, malachite green oxalate salt, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), Folin–Ciocalteau reagent, pyrene, hydrochloric acid, sulfuric acid, methanol and tetrahydrofuran (THF) were purchased from POCh (Poland).

Other chemicals were of the best quality commercially available. All solutions were made with water purified by the Milli-Q system.

2.2. Synthesis of fullerenol

Polihydroxyfullerene (fullerenol) $C_{60}(OH)_{\sim 30}$ was synthesized according to the method of Wang et al. [19], which was modified to increase the number of hydroxyl groups bound to C_{60}. In our work the reaction time between fullerene, sodium hydroxide and hydrogen peroxide (NaOH, H_2O_2) was prolonged up to 25 min under stirring. Next, the additional incubation in a water-bath at 60 °C was carried out for 20 min. Moreover, the time of hydrolysis with deionized water was prolonged from 10 min to 24 h. After precipitation of fullerenol by methanol and resolubilization in deionized water, traces of NaOH were removed by ion-exchange chromatography with Amberlit MB-20.

The structure of the obtained hydroxyl derivative of fullerene C_{60} was confirmed by IR spectrophotometry (NEXUS FT-IR spectrometer), ^1H NMR (Varian Gemini 200 MHz), ^{13}C NMR (Bruker Avance III 600 MHz) and mass spectroscopy MS-ESI (Varian 500MS).

2.3. Preparation of erythrocytes

Blood samples from healthy adult donors were provided by the Regional Blood Bank in Lodz. Erythrocytes were separated from blood plasma and leukocytes by centrifugation at 400 *g* for 8 min. All procedures were done at 4 °C. The buffy coat was removed, erythrocytes were washed with PBS (pH 7.4) and resuspended in the same buffer to obtain a hematocrit of 2%.

2.4. Preparation of erythrocyte membranes

Erythrocyte membranes were prepared according to the method of Dodge et al. [20] with some modifications. Hemolysis was carried out at 4 °C with 20 volumes of 20 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 0.5 mM PMSF to inhibit proteases. The membrane ghosts were washed successively with 20, 10 and 5 mM ice-cold Tris–HCl 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. [21]. The erythrocyte ghosts were kept frozen at −20 °C and used within a week from of preparation.

2.5. Measurements of membrane fluidity

Erythrocytes (hematocrit of 2%) in PBS were incubated with $C_{60}(OH)_{\sim 30}$ at concentrations of 50, 100 and 150 μg/mL at 37 °C for 1 h. Then the fullerenol was removed from some of the samples by rinsing 3 times with PBS and centrifugation (400 *g*, 5 min). The erythrocytes, with or without the washes to remove fullerenol, were then diluted 50-times to a hematocrit of 0.04% with PBS. Solutions of DPH or TMA-DPH in tetrahydrofuran and ANS in PBS were added to the erythrocyte suspensions to final concentrations of 2 μM, 2 μM, or 20 μM, respectively. The final concentration of THF in suspensions with DPH or TMA-DPH was 0.1% and did not affect the results. The samples were incubated at 37 °C for 15 min in the dark. Fluorescence anisotropy was assessed at the following excitation and emission wavelengths: 360 nm and 435 nm for DPH, 365 nm and 425 nm for TMA-DPH, 370 nm and 480 nm for ANS [22].

Fluorescence anisotropy (r) was calculated automatically by software provided with the instrument, according to $r = (I_{vv} - I_{vh}G)/(I_{vv} + 2I_{vh}G)$, 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 widths of the excitation and emission monochromator slits were set to 10 nm for all fluorescent probes. During measurement, samples were thermostated at 37 °C with a Peltier device and stirred with a magnetic bar.

Measurement of fluorescence anisotropy was performed on a Cary Eclipse (Varian, Australia) fluorescence spectrophotometer equipped with an automated polarizer accessory.

2.6. Measurement of the amount of membrane-bound fullerenol

Suspensions of erythrocyte membranes with different concentrations of membrane protein (0.5, 1.0, 1.5 mg/mL) were incubated with

fullerenol (50–150 μg/mL) at 37 °C for 1 h and then washed with Tris–HCl buffer to remove unbound fullerenol. The membranes were then dissolved in a 2%-solution of dodecyl sulfate (SDS). After a 10-minute incubation at ambient temperature, UV–Vis absorption spectra were taken and the absorbance at 348 nm was read against the absorbance of dissolved membranes as a background.

The quantity of fullerenol bound to the erythrocyte membranes was taken from a calibration curve made for fullerenol solutions over a concentration range of 4–70 μg/mL in SDS solution.

2.7. Measurements of Na^+,K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activity

ATPase activities were determined by measuring the differences in the level of liberation of inorganic phosphate from ATP during a 1-hour incubation of the membrane preparations at 37 °C [23,24].

Suspensions of erythrocyte membranes at membrane protein concentrations of 1.2 mg/mL were incubated with fullerenol (50–150 μg/mL) at 37 °C for 1 h. After this time, fullerenol was removed from half the samples by washing with a Tris–HCl buffer.

Enzyme activities were determined in medium containing: 100 mM Tris–HCl buffer (pH 7.4), 85 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA and 1 mM ATP. Reaction was started by mixing the ATP containing solution (100 μL) with the solution containing the erythrocyte membranes (50 μL) after incubation with fullerenol (with or without rinses to remove fullerenol). Samples were incubated for 1 h at 37 °C. The reaction was stopped by the addition of 200 μL of ice-cold 0.6 M trichloracetic acid. Samples were centrifuged at 9200 *g* for 5 min and the supernatant was used for the determination of the amount of liberated inorganic phosphate. The activity of Na^+/K^+-ATPase was determined from the difference in the formation of inorganic phosphate in the absence or presence of 0.1 mM ouabain. The activity of Ca^{2+}-ATPase was determined from the difference in the formation of inorganic phosphate in the presence or absence of 0.2 mM $CaCl_2$. The level of ATPase in the absence of Ca^{2+} ions and the presence of ouabain was determined as the basal Mg^{2+}-ATPase activity, and calculated from the difference in the formation of inorganic phosphate in the presence and absence of Mg^{2+} and K^+ ions. The amount of liberated inorganic phosphate was determined according to the improved malachite green assay [25], adapted for use in 96-well plates.

Supernatant samples of 40 μL were placed in 96-well plates. Next, 160 μL of water was added, followed by the addition of 40 μL of malachite green solution (0.9 mM malachite green oxalate in 0.35% PVA solution). After a 10 minute incubation at room temperature, 40 μL of ammonium heptamolybdate (14 mM in 3.15 M H_2SO_4) was added. After 10 min of incubation, absorbance at 610 nm was read using a Cary 50 UV–Vis spectrophotometer, equipped with a Cary 50 MPR plate reader (Varian Scientific Instruments).

Pi concentration was taken from a calibration curve made using 0–20 μM of KH_2PO_4 as a standard.

The enzyme activity was expressed in nmol Pi mg_{prot}^{-1} h^{-1}.

2.8. Statistical analysis

All experiments were run at least 5 times. Values were expressed as the mean ± standard deviation (SD) of at least five independent experiments. Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, all using the GraphPad 4.0 software.

3. Results

3.1. The influence of fullerenol on erythrocyte membrane fluidity

Fluorescent probes were used to evaluate the fluidity of erythrocyte membranes. ANS, TMA-DPH and DPH were applied, which are known to reflect membrane fluidity at the membrane surface, in the hydrophilic layer, and in the hydrophobic core of the lipid bilayer, respectively [22,24,26]. In this study, human erythrocytes were treated with fullerenol at different concentrations and its effects on the membranes were evaluated by measuring fluorescence anisotropy (r).

Fullerenol at concentrations of 50–150 μg/mL induced a statistically significant decrease in the anisotropy of ANS after a 1-hour incubation at 37 °C (Fig. 1A). This observation reveals that the fluidity of the outer layer of the plasma membrane increased.

When fullerenol was removed from the erythrocyte suspension after a 1-hour incubation, the fluorescence anisotropy of ANS was the same as for samples incubated without fullerenol (Fig. 1B).

It can be concluded that fullerenol did not attach strongly to the membrane surface. Therefore, it did not influence the fluidity of the hydrophilic region of the erythrocyte membrane after its removal from the suspension by washing with PBS.

The fluorescence anisotropy of TMA-DPH decreased after a 1-hour incubation with fullerenol at 100 or 150 μg/mL (Fig. 2A), whereas the fluorescence anisotropy of TMA-DPH did not change at 50 μg/mL of fullerenol.

The removal of fullerenol from the erythrocyte suspension reduced the effect of its influence on the fluorescence anisotropy of TMA-DPH (Fig. 2B).

The obtained results show the lack of influence of fullerenol, at concentrations of 100–150 μg/mL, on the fluidity of the outer monolayer of

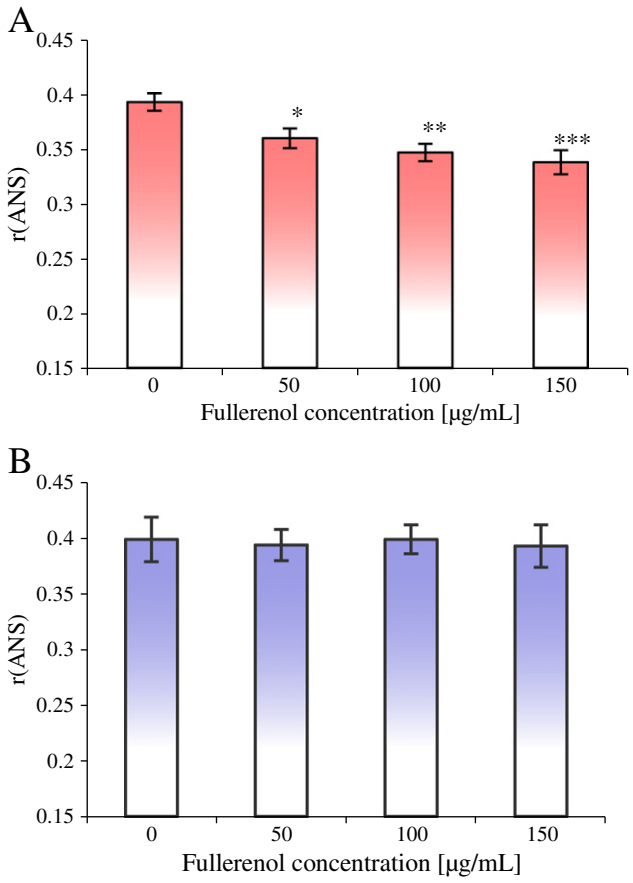

Fig. 1. Fluorescence anisotropy of ANS incorporated into the erythrocyte membranes in the presence of fullerenol; (A) red bars — fullerenol was not washed out, (B) blue bars — fullerenol was washed out. Asterisks are used to mark values that are statistically different in comparison with control values (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

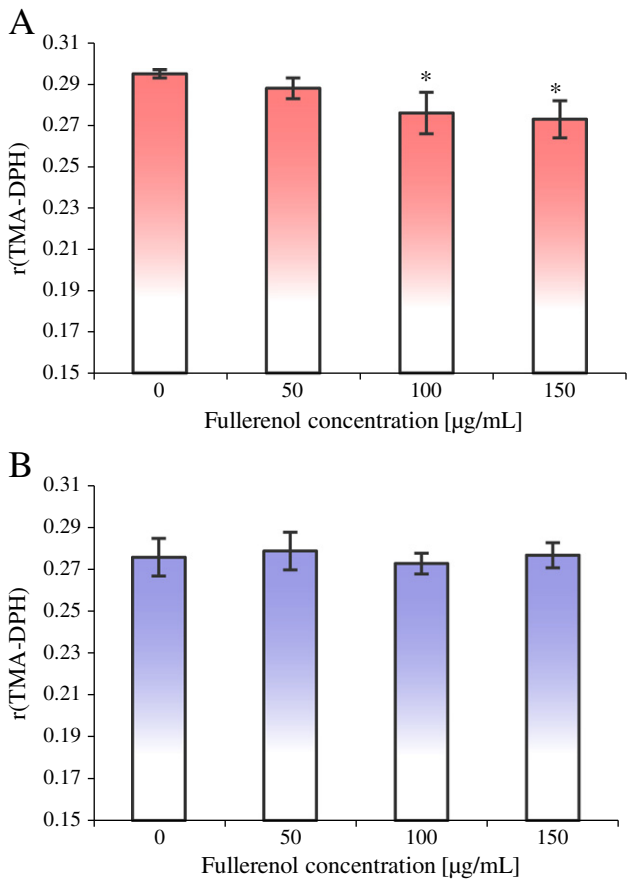

Fig. 2. Fluorescence anisotropy of TMA-DPH incorporated into the erythrocyte membranes in the presence of fullerenol; (A) red bars — fullerenol was not washed out, (B) blue bars — fullerenol was washed out. Asterisks are used to mark values that are statistically different in comparison with control values (*$p<0.01$).

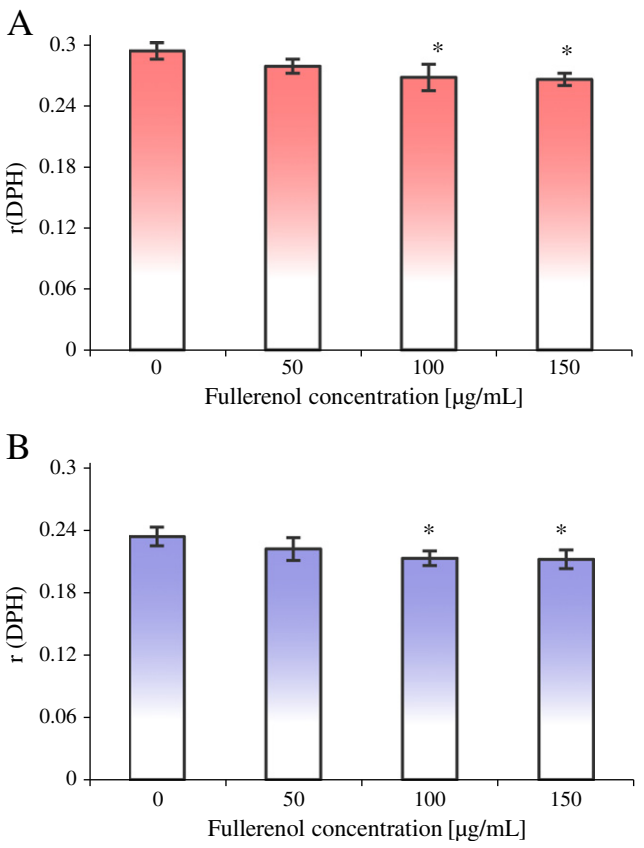

Fig. 3. Fluorescence anisotropy of DPH incorporated into the erythrocyte membranes in the presence of fullerenol; (A) red bars — fullerenol was not washed out, (B) blue bars — fullerenol was washed out. Asterisks are used to mark values that are statistically different in comparison with control values (*$p<0.01$).

the erythrocyte membrane after its removal from the suspension by washing with PBS.

The fluorescence anisotropy of DPH decreased after a 1-hour incubation with fullerenol at 100 μg/mL, and at 150 μg/mL was 90.5% of control (Fig. 3A). This meant that the fluidity of the hydrophobic region of the erythrocyte membrane increased after fullerenol treatment at higher concentrations. Fullerenol at 50 μg/mL did not influence the fluorescence anisotropy of DPH.

Washing with PBS did not influence the effect of fullerenol treatment (Fig. 3B). The maintenance of the increase in the fluidity of the hydrophobic region of the plasma membrane even after three washing steps confirms the stable incorporation of fullerenol into the hydrophobic inner region of the lipid bilayer of the erythrocyte membrane.

3.2. The amount of fullerenol incorporated into erythrocyte membranes

In order to check if fullerenol molecules can associate with the erythrocyte plasma membrane, an experiment was set up in which membrane preparations with a specific protein concentration (0.5, 1.0 or 1.5 mg/mL) were incubated for 1 h at 37 °C with $C_{60}(OH)_{\sim 30}$ (Fig. 4). It was observed that the amount of fullerenol bound to the membrane increased proportionally to the fullerenol concentration in the sample. The amount of fullerenol incorporated into the plasma membrane was 2.50 $\mu g_{C60(OH)\sim 30}/mg_{MPr}$ for 50 μg/mL of $C_{60}(OH)_{\sim 30}$ in samples in which the protein concentration was 1 mg/mL, whereas in samples in which the fullerenol concentration was 3-fold higher (150 μg/mL) the amount of fullerenol incorporated was 6.76 $\mu g_{C60(OH)\sim 30}/mg_{MPr}$ (MPr-membrane protein).

3.3. The influence of fullerenol on erythrocyte membrane ATPases

Incubation of erythrocyte membranes with fullerenol for 1 h led to a decrease in the Na+/K+-ATPase activity proportional to the

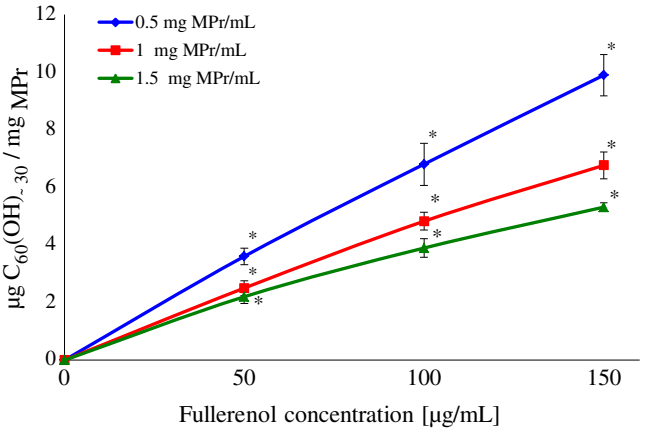

Fig. 4. The amount of fullerenol incorporated into the erythrocyte membranes. Suspensions of erythrocyte membranes with different concentrations of membrane proteins (0.5, 1.0 and 1.5 mg/mL), were incubated with fullerenol (50–150 μg/mL) for 1 h at 37 °C. Asterisks are used to mark values that are statistically different in comparison with control values (*$p<0.001$).

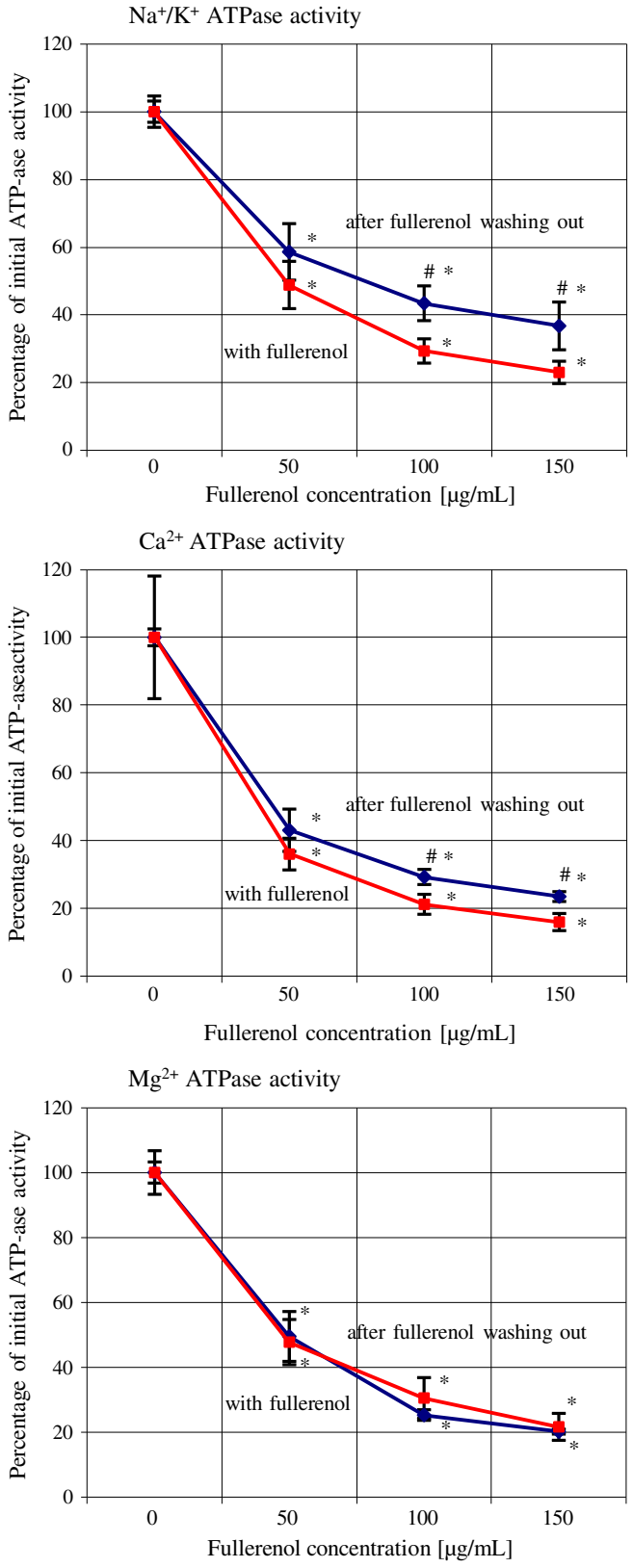

Fig. 5. Initial activities for Na^+/K^+-ATPase, Ca^{2+}-ATPase and basal Mg^{2+} dependent ATPase activity, expressed as nmol Pi/(mg protein × h), were: 143.3 ± 6.6, 226.0 ± 5.6 and 115.5 ± 7.8, respectively. Results are expressed as the percentage of the initial ATPase activity with standard deviation shown as error bars. Asterisks are used to mark values statistically different in comparison with control (* $p<0.001$); hashes — difference between samples containing fullerenol and after its removal by washing with PBS (# $p<0.05$).

fullerenol concentration (Fig. 5). Fullerenol inhibited the Na+/K+-ATPase activity by 51% at 50 μg/mL of fullerenol and by 77% at 150 μg/mL of fullerenol. The decreases in the ATPase activity were still observed when fullerenol was removed from the membrane suspensions by washing three times with PBS. In this case, the inhibition of the Na+/K+-ATPase activity was smaller, by about 10–14%, in comparison with the samples containing fullerenol during the ATPase activity assessment.

Analogous experiments were made for Mg^{2+}-ATPase and Ca^{2+} ATPase. ATPases were inhibited with increasing fullerenol concentration either in the presence of fullerenol or after its removal by washing with PBS.

The decrease in the activity of Ca^{2+}-ATPase after removing fullerenol was lower by about 7–8% compared with samples in which fullerenol was present during the Ca^{2+}-ATPase activity assessment.

Mg^{2+}-ATPase activity was inhibited by fullerenol to the same extent for samples either with fullerenol present or absent during the assessment.

4. Discussion

Numerical simulations performed with the use of molecular dynamics done by Qiao et al. [27] have shown that fullerenols are able to penetrate the plasma membrane. Hydrophobic C_{60} particles easily penetrate the lipid bilayer, as shown by both molecular modeling [27] and experimental data [28,29]. Fullerenol particles, however, because of the presence of many hydroxyl groups on their surface, have a hydrophilic character, which makes their transport across the lipid bilayer of the plasma membrane more difficult. Qiao et al. in 2007 [27] estimated the penetration time of $C_{60}(OH)_{20}$ through DPPC as 9 orders of magnitude longer compared to that of C_{60}.

In this work, fluorescent probes ANS, DPH and TMA-DPH were used to investigate the influence of fullerenol on the fluidity of the plasma membrane.

Fluidity was estimated on the basis of the anisotropy coefficients (r) of the probes, which were located in different regions of the erythrocyte membrane. According to Shinitzky and Barenholz [30], anisotropy is inversely proportional to the fluidity of the membrane. The results obtained in our work show that fullerenol increased the fluidity of the membrane at concentrations between 50 and 150 μg/mL. The largest decrease in the value of the anisotropy coefficient (r) was observed in the case of the ANS probe (Fig. 1) located on the hydrophilic surface of phospholipid heads and, therefore, providing information about the organization of the membrane at its surface (Fig. 6).

For TMA-DPH (used to monitor the fluidity in the hydrophobic–hydrophilic region of the membrane) (Fig. 2) and DPH (providing information from the hydrophobic region between the two leaflets of the membrane bilayer) (Fig. 3), significant changes were observed only at fullerenol concentrations above 100 μg/mL. Thus, it can be assumed that fullerenol binds mostly to the surface of the membrane, but can also migrate deeper inside the membrane. This is in agreement with the data of Monticelli et al. [6], which revealed that hydrophilic particles of $C_{60}(OH)_{20}$ adsorb more strongly to the membrane phospholipid heads.

The removal of fullerenol from an erythrocyte suspension caused increases in the anisotropy coefficients (r) for ANS and TMA-DPH back to the levels observed in control erythrocytes. This observation indicates that the adsorption of fullerenol is probably due to the formation of hydrogen bonds, and removing it from the incubation medium leads to the dissociation of fullerenol molecules from the membrane surface.

The removal of fullerenol from the incubation medium had no influence on the fluidity of the hydrophobic region (Fig. 3). Molecules of fullerenol that penetrated the lipid bilayer of the plasma membrane

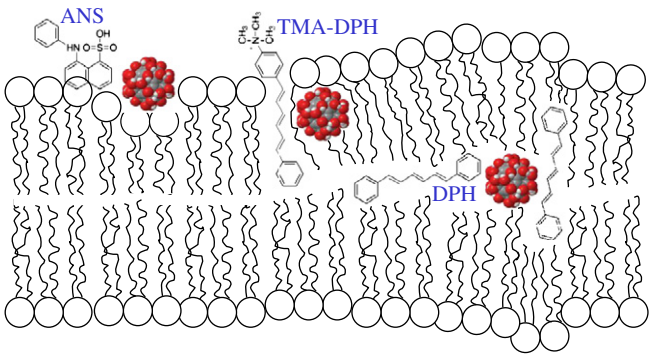

Fig. 6. The location of the fluorescent probes ANS, TMA-DPH, DPH and fullerenol in the lipid bilayer.

could not be removed by washing with PBS. Thus, fullerenol influenced the fluidity of the inner part of the lipid bilayer even after washing. Moreover, these results confirm that fullerenol could penetrate the plasma membrane.

The possibility of fullerenol associating with erythrocyte membranes was confirmed in the next experiment, in which erythrocyte ghosts were incubated with fullerenol for 1 h at 37 °C. The amount of fullerenol associated with the membranes was relatively proportional to its concentration in the incubation medium, and inversely proportional to the concentration of the membrane proteins (Fig. 4). This is probably connected to the fact that at higher concentrations of membrane proteins, the higher viscosity of the suspension can affect the diffusion of fullerenol inside the membrane.

Xiao et al. [31] suggested a possible mechanism for fullerene penetration through membranes via association with proteins. This model assumed that the transport of PVP-C_{60}-fullerene across the membrane was controlled by certain proteins: protein I (8 kDa; present in the cell membrane) and protein II (53 kDa; present in the membrane and cytosol). Penetration through the membrane was possible only for conjugates of C_{60}-protein II while PVP remained outside the cell. Other authors suggested endocytosis as a mechanism of fullerenol transport through the plasma membrane [32,33, reviewed in Ref. 34]. The next step was to investigate the activities of membrane ATPases under the influence of fullerenol. Significant decreases in Na^+/K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were observed after fullerenol treatment (Fig. 5). Data obtained by other research groups suggested that fullerenol was able to inhibit the activity of mitochondrial Mg^{2+}-ATPase. In this way it disabled the phosphorylation of ADP and the regeneration of ATP [35]. Not only fullerenol, but also C_{60} aggregates, could promote changes in the ATPase activity. Prylutska et al. [36] have shown that the lipophilicity of C_{60} aggregates and their affinity for the plasma membranes of thymocytes were high enough to promote the transport of its particles inside the membrane, even if C_{60} was in aggregates. In conclusion, C_{60} could accumulate in the lipid bilayer and suppress the activity of Ca^{2+}-dependent, membrane associated ecto-ATPases.

Our results indicated that fullerenol might inhibit the activity of the membrane ATPase system despite the presence of –OH groups on the surface of the carbon cage, which gave it a hydrophilic character and, as a result, limited the penetration of the bilayer by the nanoparticles.

Functional changes in Na^+/K^+-ATPase under the influence of fullenerol could be associated with two effects. The first is the interaction of fullerenol molecules with subunits of the enzyme. The Na^+/K^+-ATPase is a tetramer composed of two identical catalytic subunits α and two glycosylated β subunits [37]. The latter are crucial for the appropriate location of the enzyme in the membrane and for its proper functioning, as they regulate the activity of the α subunits [38]. Molecules of fullerenol penetrating the membrane could presumably interact with β subunits and through changes in their structure influence the action of the whole enzyme.

These postulates are supported by the results of Calvaresi and Zerbetto in 2010 [39], who proved using computational methods the possibility of blocking the catalytic subunits of ATPases via interaction with fullerenols. As models they used rat Na^+/K^+-ATPase (1MO8 in PDB), which through ATP hydrolysis maintains an appropriate gradient of Na^+ and K^+ ions on both sides of the membrane, and bovine mitochondrial F1-ATPase (1EFR), participating in oxidative phosphorylation providing ATP synthesis. In both models the interaction of C_{60} with the enzymes can be described as the inhibition of the catalytic mechanism. Within Na^+/K^+-ATPase, C_{60} binds to the very mobile α1 subunit region delimited by Gln396-Ala416 [40,41]. On the other hand, in F1-ATPase, C_{60} interacts with both the α and β subunits [41], hindering the rotational movement necessary for the functioning of the protein.

Another explanation for the attenuation of Na^+/K^+-ATPase activity could be the influence of fullerenol molecules on the lipids of the plasma membrane (Fig. 7) and, in consequence, changes in its fluidity. Such changes have an impact on the ATPase activity [18]. For example, cholesterol is a compound playing a major role in the regulation of the structure and dynamics of the lipid bilayer. It can moderate the activities of various membrane transporters such as Ca^{2+} channels, Na^+/K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase in different cells, including erythrocytes and endothelial cells [42].

Therefore, it is possible to conclude that the observed decrease in ATPase activity is a consequence of changes in the microviscosity of the erythrocyte membrane, caused by the incorporation of $C_{60}(OH)_{\sim 30}$ into its interior.

Despite the reduced toxicity of fullerenol compared to nano-C_{60} suspensions [5], the interactions between fullerenol and membrane ATPases may cause abnormalities in cell functioning. Improper ATPase activity can lead to very serious events. In the case of Na^+, K^+-ATPase, a decrease in activity results in apoptosis [17]. Inhibition of Na^+,K^+-ATPase leads to ion imbalance and osmotic hemolysis in erythrocytes [43].

Furthermore, impairing the function of Ca^{2+}-ATPase also has very serious consequences. An increase in the concentration of intracellular calcium ions promotes an increase in proteolysis via Ca^{2+}-dependent proteases [44]. Inhibiting the function of other membrane ATPases (not participating in ion transport), which are dependent on Mg^{2+} ions, can cause abnormal lipid distribution between the outer and

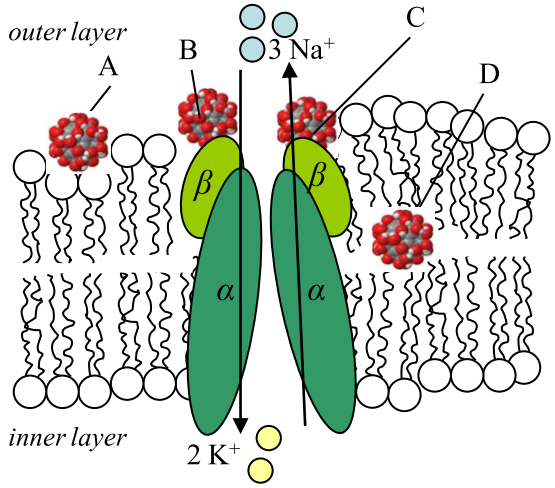

Fig. 7. Fullerenol interactions with Na^+/K^+-ATPase subunits spanning a lipid bilayer. (A) Attachment of fullerenol to the outer layer of the lipid bilayer; (B) fullerenol interactions with β subunits of Na^+/K^+-ATPase; (C) simultaneous fullerenol interactions with an enzyme and a lipid bilayer; (D) incorporation of fullerenol into the hydrophobic, inner region of the lipid bilayer of the plasma membrane.

inner layer of the membrane, thus changing the discoidal shape of the erythrocytes [45].

The concentrations of fullerenol used in our work were rather high, up to 150 mg/L so the observed effects were explicitly pronounced. Such high nanoparticle concentrations are hardly expected in real applications. However, fullerenes including fullerenol could accumulate in organisms, achieving higher concentrations compared to the concentrations applied. Moreover, nanoparticles could be released to the environment by means of waste disposal or accidentally during or after use, increasing exposure to them [46,47].

Derivatives of fullerenes are prone to activate interactions with protein molecules; however, the type of interaction is highly dependent on the functional groups attached to the carbon "fullerene cage" [48–51]. The fullerenol used in our work is rich in –OH groups on its surface, and therefore can interact with the functional groups of amino acids via hydrogen bonds.

Similar results were obtained by Ciolkowski et al. [24] during an investigation of the influence of PAMAM-OH dendrimers on the activities of erythrocyte membrane ATPases. They demonstrated that hydrogen bonds are responsible for the interaction between PAMAM-OH and the cell surface. Fullerenol has a spherical shape, and the presence of –OH groups on the surface of that molecule can have similar effects on membrane proteins.

The obtained results suggest that fullerenol could be transported by erythrocytes in the circulatory system. On the other hand, the irreversible inhibition of membrane ATPases by fullerenol points to the toxicity of the nanoparticles in the examined system, leading to ionic imbalance and the erythrocyte shape alterations. These potentially harmful effects to cells may be useful if we take into account that erythrocytes with altered shape are removed from circulation and absorbed by the liver and the spleen. These biological effects may be a useful/promising approach in targeted therapy of hepatocellular and spleen cancer.

Since a decrease in the activity of transport ATPases may result in the apoptosis of cancer cells, fullerenol could also be regarded as a new candidate for cancer chemotherapy.

In conclusion, fullerenol induced decreases in the anisotropy of plasma membranes as determined with fluorescent labels ANS, TMA-DPH and DPH after a 1-hour incubation at 37 °C. The effect disappeared after washing out the fullerenol for ANS and TMA-DPH, but not for DPH. Incubation of erythrocyte membranes with fullerenol led to decreases in Na^+,K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase activities. Washing out the fullerenol reduced the inhibition of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities; however, it did not influence Mg^{2+}-ATPase activity. Furthermore, our results show that fullerenol can associate with erythrocyte plasma membranes. Fullerenol associated mostly with the surface of the plasma membrane; however, it could also migrate deeper inside the membrane. The changes in the activities of ATPases caused by fullerenol could be the result of its direct and/or indirect (via membrane fluidity changes) interaction with the enzymes. Moreover, fullerenol inhibited membrane ATPases, so it could modulate ion homeostasis, which regulates cell death. The obtained results could help elucidate the molecular mechanism of $C_{60}(OH)_{\sim 30}$ and other fullerenols and their derivatives.

Anita Krokosz and Jacek Grebowski contributed equally to this publication.

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