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Fullerenol $C_{60}(OH)_{36}$ could associate to band 3 protein of human erythrocyte membranes

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ABSTRACT

The present study was aimed at investigating the effect of fullerenol $C_{60}(OH)_{36}$ on chosen parameters of the human erythrocyte membrane and the preliminary estimation of the properties of fullerenol as a potential linking agent transferring the compounds (e.g., anticancer drugs) into the membrane of erythrocytes. The results obtained in this study confirm the impact of fullerenol on erythrocyte cytoskeletal transmembrane proteins, particularly on the band 3 protein. The presence of fullerenol in each of the concentrations used prevented degradation of the band 3 protein. The results show that changes in the morphology of red blood cells caused by high concentrations of fullerenol (up to 150 mg/L) did not lead to increased red blood cell hemolysis or the leakage of potassium. Moreover, fullerenol slightly prevented hemolysis and potassium efflux. The protective effect of fullerenol at the concentration of 150 mg/L was 20.3%, and similar results were obtained for the efflux of potassium. The study shows that fullerenol slightly changed the morphology of the cells and, therefore, altered the intracellular organization of erythrocytes through the association with cytoskeletal proteins.

1. Introduction

Nanotechnology was recognized by the National Cancer Institute as a discipline that can significantly contribute to a breakthrough in cancer treatment and diagnostics [1]. Many conventional therapies for cancer are limited by the side effects and properties of drugs, such as rapid removal from the organism or lack of specificity, which results in unavoidable damage of nontarget cells [2,3]. The vast majority of anti-cancer drugs after administration in an unaltered form show high toxicity towards normal cells as well, damaging primarily hepatocytes and heart muscle [4–7]. One of the solutions to limit the side effects of anti-cancer therapies is the utilization of drug carriers, that allow active transport of the medication directly to the disease-altered tissue [8]. Liposomes, linear polymers and polymer micelles represent the most popular carriers for anti-cancer drugs.

Hopes are also high for the use of new nanocomponents, such as dendrimers, nanotubes and fullerenes as drug carriers [8–12]. Drug carriers should not present toxicity or immunogenicity and should be able to deliver the agent to the targeted organ. Recent research indicates that the toxicity of initially promising compounds is too high for medical use [13–15]. Among other carriers, erythrocytes represent a potentially attractive and unique carrier for drug delivery [16]. As the natural components of the organism, they are highly biocompatible, biodegradable (easily degradable with no toxic intermediates), and they have long half-life in the circulation system [17]. They are also easy to obtain in order to load the cargo. The drug is entrapped into the erythrocytes or attached to the surface of the cells by using various physical and chemical methods. Surface modification of erythrocytes with different compounds, such as glutaraldehyde, antibodies, carbohydrates or biotin is possible to improve their target specificity and to increase their circulation half-life. [17–19]. Some of them, such as antibodies and biotin, show some substrate specificity; others, like glutaraldehyde, which is a crosslinking agent, can interact with a variety of erythrocyte membrane proteins [20,21]. Altogether, these facts indicate the superiority of red blood cells over synthetic compounds. However, erythrocytes have some drawbacks that limit their use as drug carriers. In vivo, the most common problem of using erythrocytes containing cargo is their increased elimination by the reticuloendothelial system (RES). This process severely limits the use of these cells, as every interaction with the RES increases their specificity for off-target organs, namely the spleen, liver and blood marrow [18,19]. Moreover, the use of crosslinking agents may be detrimental to the maintenance of erythrocyte integrity and could lead to accelerated damage of erythrocytes [20–22]. The shortened time of storage of the loaded erythrocytes is an additional problem [19,21].

Therefore, it is crucial to find new and efficient compounds that would serve as the linkers between erythrocytes and drugs and could be used to diminish the side effects of chemotherapy. As these molecules serve an anchoring function, they have to possess functional groups reacting both with the drug and the membrane proteins of the erythrocytes. Fullerenols $C_{60}(OH)_n$, water-soluble hydroxyl derivatives of C_{60},

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seem to be a promising group for this purpose, because they can interact and bind both erythrocytes and drugs via the hydroxyl groups present on the surface of their carbon cage. Fullerenols receive a lot of attention in the field of biomedical nanotechnology-related research, primarily due to their relatively low toxicity in comparison to other nanocompounds [23,24]. The abundance of hydroxyl groups on the surface provides opportunities for creating multiple hydrogen bonds between one fullerenol molecule and various biological compounds as the protein domains of the plasma membrane or hydrophilic lipid heads. In effect, fullerenols, by adsorption to components of plasma membranes influence the conformation and function of membranes [14,25]. Our previous work shows 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 [26].

The aim of this work was to assess the influence of fullerenol $C_{60}(OH)_{36}$ on selected parameters of human erythrocyte membranes and preliminary verification of fullerenol as an anchor to erythrocyte membranes linking drug and plasma membrane.

2. Materials and methods

2.1. Chemicals

Fullerene (99.5%) was purchased from SES Research (Houston, TX, USA) and Amberlite MB20, phenylmethanesulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), dithiothreitol (DTT) and bromophenol blue were purchased from Sigma-Aldrich (Poznan, Poland). Sodium chloride, sodium hydroxide, hydrogen peroxide, potassium phosphate monobasic and Folin–Ciocalteu reagent were purchased from POCh (Gliwice, 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) $C_{60}(OH)_{36}$ was synthesized as described earlier [26]. The structure of the obtained 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 spectroscopy MALDI-TOF (PerSeptive Biosystems Inc. Voyager Elite).

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 $\times g$ for 8 min. All procedures were carried out at 4 °C. The buffy coat was removed, erythrocytes were washed with phosphate buffered saline (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. [27] 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. [28]. The erythrocyte ghosts were kept frozen at −20 °C and used within a week of preparation.

2.5. The interaction of fullerenol with erythrocytes or erythrocyte plasma membranes

Two percent erythrocyte suspensions in PBS, pH 7.4 or erythrocyte plasma membranes (1 mg of membrane protein per mL) were incubated with fullerenol at the final concentration of 50–150 mg/L in the dark for 3 and 48 h at 37 °C.

2.6. Measurement of hemolysis

The hemolysis of erythrocytes was determined spectrophotometrically at 523 nm based on the ratio of hemoglobin (Hb) released from cells to the total cellular Hb content after hemolysis with distilled water. The ratio of hemolysis was calculated from the equation:

$$H(\%) = \frac{A_1}{A_2} \cdot 100\%$$

where H (%) is the per cent of hemolysis of the erythrocytes, A_1 is the absorbance of the supernatants of the samples of the erythrocytes incubated with or without fullerenol and A_2 is the absorbance of the supernatant of the samples of the erythrocytes incubated with or without fullerenol after complete hemolysis with distilled water (100%).

2.7. Potassium efflux

The erythrocyte suspensions were centrifuged at 650 $\times g$ for 10 min. The resulting supernatant was assayed for its potassium content. The concentration of potassium was measured by flame atomic emission spectrometry using a SpectrAA-300 apparatus (Varian, Australia).

2.8. Flow cytometry

Red blood cell suspensions after incubation were diluted to suspensions containing approximately 10^6 cells/mL and analyzed using a Becton–Dickinson, LSR II flow cytometer, with simultaneous separate detection at low angle (FSC) and right angle (SSC). The light scattered near the forward direction (low angle) is expected to be proportional to the size (volume) of the particle and is independent of the cell refractive index and shape, whereas scattering at the right angle depends on the cell shape and internal properties of the scattering particles [29]. FSC/SSC is a dual parameter contour plot histogram proportional to total cell diversity. Each measurement was done for 30,000 cells. Data were analyzed with FlowJo software 7.2.2. (Tree Star Inc., USA).

2.9. Phase contrast microscopy

Erythrocytes incubated with or without fullerenol were observed using a phase contrast microscope (Eclipse E600W Nikon, Japan) at a magnification of 1000×.

2.10. Polyacrylamide gel electrophoresis (SDS-PAGE)

Erythrocyte membrane proteins were analyzed by SDS-PAGE as described earlier [30]. Erythrocyte membranes were solubilized by addition of a solution containing: 10% SDS and 0.05% bromophenol blue in 0.25 M Tris–HCl buffer (pH 6.8) with or without 0.5 M dithiothreitol (DTT). After incubation at 90 °C for 5 min, the samples containing 1 mg/mL of protein were subjected to one-dimensional SDS-PAGE. Electrophoresis was carried out with 4% and 7.5% gels for condensation and separation, respectively, in Tris–HCl buffer pH 8.8 containing 0.1% SDS. Electrophoresis was carried out in a Bio-Rad system with a 20 mA current. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The gels were digitalized and analyzed with the software GelScan (KTE, Poland). The relative quantities of proteins in selected bands were expressed as the percentage of the total protein quantity.

2.11. Statistical analysis

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

3. Results and discussion

3.1. The influence of fullerenol on the level of hemolysis and the leakage of potassium ions from erythrocytes

In order to estimate the influence of fullerenol on erythrocyte membrane integrity, the cells were incubated with the compound in the concentration range of 50–150 mg/L at 37 °C for 3 and 48 h. Subsequently, the level of hemolysis and potassium ion leakage was investigated. The samples incubated with fullerenol for 3 h did not show an increase in hemolysis or in release of potassium ions (Figs. 1 and 2). Prolonged, 48-hour incubation caused significant autohemolysis and potassium ion leakage (Figs. 1 and 2). Fullerenol present in the medium caused a slight, concentration-dependent decrease in hemolysis, which for the final concentration of 150 mg/L proved to be statistically significant. The protective effect of fullerenol in this case was estimated at 20.3%. Similar results were obtained for the potassium ion leakage: for the final concentration of 150 mg/L, the amount of potassium released from the erythrocytes decreased by 21.6% when compared to that from the control sample. This protective effect can be attributed to interactions between the hydroxyl groups of fullerenols and the plasma membranes, e.g., creating hydrogen bonds between the –OH groups of the nanocompound and plasma membrane proteins or polar heads of membrane lipids [14,26,31]. In addition to hydrogen bonding, hydrophobic and electrostatic interactions may also contribute to adsorption of fullerenol to the plasma membrane [11]. Fullerenol has more than 30 –OH groups on the surface, which are responsible for the dipolar negative charge of this molecule and is similar to the resultant charge of the erythrocyte membranes. It can adsorb, however, to the protein domains of the membrane by van der Waals or dipolar interactions [31]. These assumptions are in agreement with data obtained via molecular modeling [25,32] as well as experimental data [26,33,34].

The leakage of potassium ions from the cells is related to disruption of active membrane transport and a result of the decrease in ATP levels, which can occur during prolonged incubation of erythrocytes [35]. As shown in our previous work, fullerenol inhibits the activity of ion-dependent ATPases as a result of both direct interaction with the enzyme and the influence of fullerenol on membrane fluidity [26].

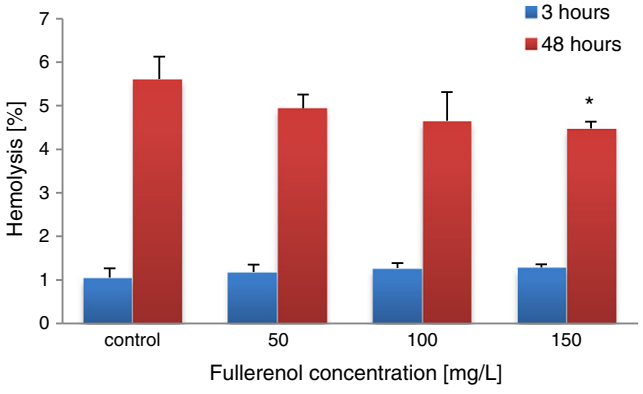

Fig. 1. The autohemolysis of erythrocytes incubated with fullerenol $C_{60}(OH)_{36}$, at a concentration range of 0–150 mg/L for 3 and 48 h at 37 °C. Asterisks are used to mark values that are statistically different in comparison with control values $^*p < 0.05$.

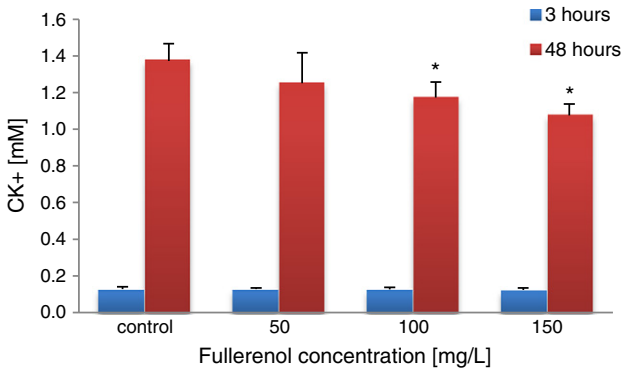

Fig. 2. The potassium leakage from erythrocytes incubated with fullerenol $C_{60}(OH)_{36}$, at a concentration range of 0–150 mg/L for 3 and 48 h at 37 °C. Asterisks are used to mark values that are statistically different in comparison with control values $^*p < 0.05$.

Inhibition of Na^+,K^+-ATPase in erythrocytes causes distortion of the ion balance and leads to osmotic hemolysis [36,37].

Despite the inhibition of Na^+,K^+-ATPase by fullerenol, an increase in hemolysis and potassium ion leakage was not observed. Blocking the leakage can result from physical "blockages" of potassium channels by fullerenol molecules. A similar effect was observed for blocking potassium ion channels by fullerene and nanotubes, where the blockage effect was dependent on the size and shape of the nanocompounds [38]. The ion channels can be flanked by the nanocompounds as the result of electrostatic interaction or plain adsorption, which influences ion exchange and, therefore, cellular metabolism.

3.2. The influence of fullerenol on the content of cell membrane proteins

In order to investigate the influence of fullerenol on the membrane proteins, erythrocyte ghosts incubated with fullerenol for 3 and 48 h at 37 °C were separated by SDS-PAGE electrophoresis in reductive and non-reductive conditions. As presented in Fig. 3, fractions identified after staining with Coomassie Brilliant Blue contained spectrins α and β, ankyrin, band 3 (AE1), 4.1 (EPB41), 4.2 (EPB42) and 4.9 (EPB49) proteins, actin, low molecular weight proteins and hemoglobin. The relative contribution of each fraction expressed as the percentage of the total amount of protein is shown in Table 1. There were no statistically significant differences between control samples and samples containing membranes incubated with fullerenol up to 100 mg/L for 3 h. Interestingly, for the samples containing 150 mg/L of fullerenol incubated in the same conditions, there was a significant decrease in the band identified as actin with a simultaneous increase in the band 3 protein fraction.

The decline of the actin band when the highest concentration of fullerenol was used can suggest that the nanocompound causes association of this protein. The enrichment in the band 3 protein fraction, which has a molecular weight comparable to two molecules of actin connected by a molecule of fullerenol, suggests a possibility of interactions between fullerenol and erythrocyte cytoskeletal proteins. A prolonged, 48-h incubation resulted in the disappearance of the band 3 fraction and enrichment in the broad band of low molecular mass proteins (smearing) in control cells. This is a result of substantial fragmentation of band 3 protein, which could originate either from proteolytic or ROS-induced cleavage [39]. The presence of fullerenol in all used concentrations prevented the degradation of band 3 protein. The presented data indicate that fullerenol preferentially binds to band 3 protein and prevents its degradation. A large number of hydroxyl groups on the surface of the fullerenol carbon cage provide possibilities for interaction and attachment of other molecules, e.g., drugs [40]. At the same time, functional –OH groups can adsorb to cytoskeletal erythrocyte proteins. These properties of fullerenol can be used to improve utilization of erythrocytes as drug carriers. Prolonging the time of residence of a substance (drug) in the circulation system is possible by attaching it to residues

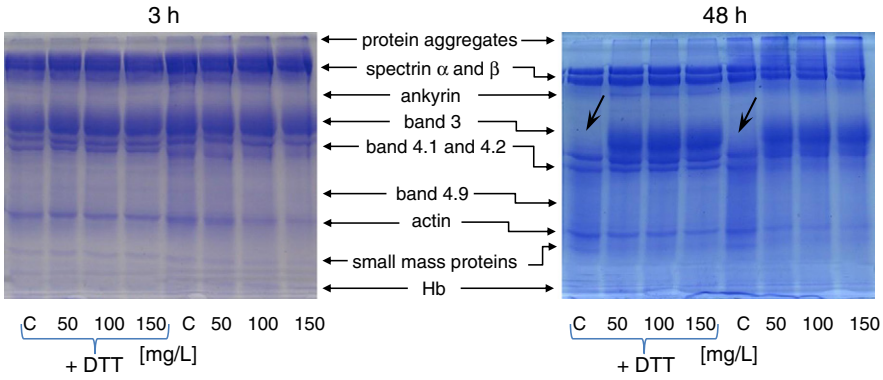

Fig. 3. SDS-PAGE of proteins of erythrocyte membranes incubated with fullerenol for 3 and 48 h at 37 °C under non-reductive or reductive conditions (DTT at 0.25 mM). Arrows indicate a complete disappearance of the band 3 protein.

of the erythrocyte membrane, such as band 3 protein or glycophorin. This method has been described by Krantz [41], and it is based on the use of "anchors" — molecules with functional groups with strong affinity for erythrocyte cytoskeletal proteins. As fullerenol is covered with polar –OH groups, it is well suited to function as an "anchor".

The band 3 protein is an ion exchanger with a fundamental role in maintaining the oxygen transport function of red blood cells and acts as an anchor for a series of key glycolytic enzymes and spectrin. The membrane alterations related to prolonged incubation cause the echinocyte or spheroechinocyte phenotypes. Finally, the cell activates a process of vesiculation, in order to eliminate proteins and lipids that have been altered by oxidative stress to protect the cell from a further chain reaction of stress and consequent removal from the circulation. Changes in band 3 protein trigger signals at the membrane to "remove" the cell, through IgG- or complement-mediated phagocytosis by the recipients' Kupffer cells [42].

In whole erythrocytes, fullerenol, by adsorption to band 3 protein can indirectly interact with cytoplasmic proteins. The results obtained with the use of isolated erythrocyte ghosts are consistent with the influence of fullerenol on peripheral and transmembrane proteins, such as ATPases [26] and band 3 protein. The influence of fullerenol on transmembrane proteins is clearly reflected by the changes in activity of Na^+/K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase, which can originate from both direct interaction with the proteins and indirect influence on the fluidity of the membrane [26]. Band 3 protein plays a central role in cytoskeleton formation, therefore, conformational changes induced by various factors in this molecule result in echinocyte formation [43]. The morphological changes of erythrocytes induced by fullerenol were investigated with flow cytometry and phase contrast microscopy and are described in Section 3.3.

It has to be noted that a 48-h incubation of erythrocytes led to aggregation of the membrane proteins. The use of reducing agents prevents these events, indicating that aggregation is promoted by the creation of –S–S– bridges [30]. Fullerenol, when used in concentrations up to 100 mg/L did not influence aggregate formation. An increase in concentration to 150 mg/L, however, promoted protein aggregation. Aggregates formed in the presence of fullerenol in these conditions could not be reduced by DDT, which confirms fullerenol–protein association.

The influence of fullerenol on the proteins is not limited to association or networking. This nanocompound can induce deformation of the protein in the binding residues. Low concentrations of $C_{60}(OH)_{20}$ (15–30 mg/L) inhibit microtubule polymerization by binding to tubulin in the ratio 9:1 [44]. Fullerenol $C_{60}(OH)_{36}$, by association with band 3

Table 1
Relative content of membrane proteins after SDS-PAGE of erythrocyte membranes incubated with fullerenol at the concentration range of 50–150 mg/L for 3 and 48 h at 37 °C under non-reductive or reductive conditions (DTT at 0.25 mM).

Fullerenol concentration [mg/L]	3 h				48 h			
	Control membrane proteins	50	100	150	Control membrane proteins	50	100	150
Reductive conditions (DTT — 0.25 mM)								
Protein aggregates	1.61 ± 0.64	1.67 ± 0.47	1.71 ± 0.56	1.64 ± 0.51	2.20 ± 0.80	3.91 ± 1.29	4.38 ± 2.00	4.91 ± 1.52*
Spectrin α	18.43 ± 2.96	18.54 ± 1.73	18.63 ± 0.58	18.67 ± 1.52	11.50 ± 1.24	12.58 ± 2.07	12.72 ± 1.35	12.33 ± 1.11
Spectrin β	16.39 ± 2.61	16.23 ± 2.01	16.17 ± 2.46	16.84 ± 1.97	16.60 ± 1.63	17.17 ± 1.20	15.79 ± 3.58	15.11 ± 1.03
Ankyrin	3.06 ± 1.02	3.39 ± 1.30	3.28 ± 1.19	2.82 ± 0.98	3.97 ± 1.13	4.79 ± 1.07	4.96 ± 1.58	4.73 ± 1.05
Band 3	28.34 ± 1.70	30.59 ± 2.07	31.17 ± 1.34	32.43 ± 1.08*	3.50 ± 7.00	27.87 ± 2.09*	31.07 ± 1.56*	32.34 ± 2.91*
Band 4.1	9.48 ± 1.26	9.29 ± 0.85	8.80 ± 0.59	8.52 ± 0.45	11.78 ± 1.57	10.63 ± 2.47	8.82 ± 0.64*	8.34 ± 1.60*
Band 4.2	9.10 ± 1.05	7.58 ± 0.91	7.89 ± 0.50	7.03 ± 0.65	10.37 ± 2.25	8.74 ± 1.63	8.76 ± 1.37	9.61 ± 4.34
Band 4.9	3.22 ± 0.06	3.36 ± 0.26	3.15 ± 0.17	3.04 ± 0.25	4.50 ± 4.27	3.67 ± 3.22	3.69 ± 3.24	3.52 ± 3.06
Actin	8.89 ± 0.66	8.03 ± 0.87	8.24 ± 1.37	6.41 ± 0.50*	11.46 ± 1.28	7.54 ± 1.10*	6.14 ± 1.78*	5.22 ± 1.34*
Small mass proteins	1.95 ± 0.27	1.93 ± 0.31	1.65 ± 0.23	1.78 ± 0.42	5.22 ± 0.40	3.88 ± 0.54*	3.53 ± 0.84*	3.51 ± 0.85*
Non-reductive conditions								
Protein aggregates	2.54 ± 0.84	2.91 ± 0.80	3.21 ± 1.00	3.42 ± 0.67	3.62 ± 0.54	4.87 ± 0.88	4.81 ± 0.96	5.40 ± 1.01*
Spectrin α	19.22 ± 2.99	18.12 ± 1.64	18.73 ± 1.89	20.16 ± 1.94	13.81 ± 0.24	12.41 ± 1.10	12.79 ± 1.05	13.39 ± 3.13
Spectrin β	15.98 ± 1.57	18.35 ± 4.39	17.13 ± 3.34	16.84 ± 2.70	16.88 ± 1.53	13.91 ± 1.67*	12.35 ± 1.98*	11.36 ± 1.11*
Ankyrin	3.91 ± 0.64	3.67 ± 1.33	3.67 ± 1.18	3.47 ± 1.15	4.15 ± 0.89	4.57 ± 1.12	4.18 ± 1.14	3.79 ± 0.85
Band 3	28.39 ± 0.68	28.92 ± 0.94	29.11 ± 1.56	30.38 ± 1.06*	3.54 ± 6.12	27.43 ± 2.40*	27.28 ± 3.67*	26.49 ± 2.76*
Band 4.1	8.47 ± 1.06	9.30 ± 0.61	8.89 ± 0.65	8.87 ± 0.54	12.11 ± 2.66	7.18 ± 0.51*	6.76 ± 0.68*	6.34 ± 0.90*
Band 4.2	8.01 ± 1.30	7.13 ± 0.96	6.91 ± 0.39	6.80 ± 0.71	9.06 ± 2.11	5.53 ± 0.59*	4.85 ± 0.70*	4.06 ± 0.33*
Band 4.9	3.27 ± 0.91	1.99 ± 1.45	3.39 ± 0.04	3.38 ± 0.20	5.55 ± 1.56	4.90 ± 0.73	5.59 ± 1.03	3.81 ± 0.53
Actin	9.74 ± 0.46	7.20 ± 1.80	7.40 ± 1.16	5.84 ± 0.75*	8.09 ± 1.17	3.40 ± 0.42*	2.77 ± 0.39*	2.52 ± 0.36*
Small mass proteins	1.98 ± 0.55	2.01 ± 0.39	2.24 ± 0.55	2.23 ± 0.49	6.81 ± 1.92	2.83 ± 0.60*	2.45 ± 0.50*	2.15 ± 0.44*

Asterisks are used to mark values that are statistically different in comparison to control values $^*p < 0.05$.

protein, not only prevented its degradation, but also influenced the binding sites of spectrin, band 4.1 and 4.2 proteins or actin, leading to changes in the cytoskeleton affecting erythrocyte morphology [45–47].

3.3. Changes in shape of the erythrocytes analyzed by flow cytometry and phase contrast microscopy

The changes in erythrocyte size and shape were assessed on the basis of microphotography and light dispersion parameters FSC-A and SSC-A obtained by flow cytometry. Utilization of flow cytometry provides a fast and very precise estimation of the physico-chemical and biological properties of cells [21,48,49]. Fig. 4 shows the diagrams and histograms obtained for erythrocytes incubated in the presence and absence of $C_{60}(OH)_{36}$ for 3 and 48 h in accordance to the frontal (FSC) and lateral (SSC) light dispersion.

After a 48-h incubation in all investigated samples, a fraction of cells with an increased FSC parameter was detected (Fig. 4A). This effect, however, cannot be attributed to the presence of fullerenol, as control cells displayed the same features and no concentration-dependence was observed. Prolonged incubations of erythrocytes led to exhaustion of cellular reductive components and structural and functional changes in the membranes: degradation of phosphatidylserine [50] and sialic acid, which are the main components establishing the negative charge on the outer side of the membrane [51]. All these effects can be a direct result of prolonged incubation at 37 °C and cause significant changes in morphology and size of the erythrocytes.

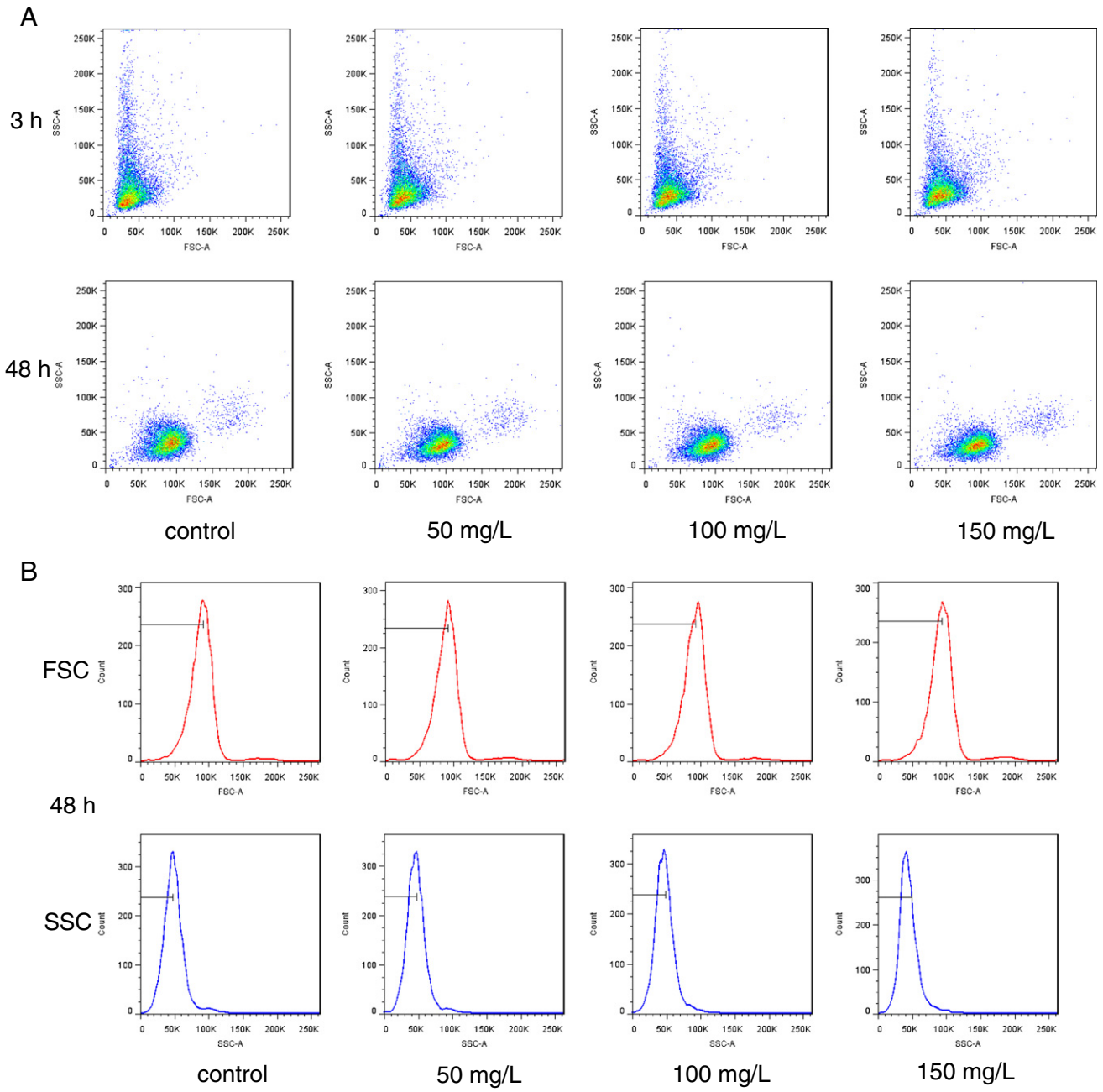

Fig. 4. Flow cytometry analysis of fullerenol-induced changes in RBC morphology. (A) Scattering diagrams of human control erythrocytes and erythrocytes incubated for 3 and 48 h with fullerenol in concentrations ranging from 50 to 150 mg/L. The FSC-A/SSC-A diagram is a dual parameter contour plot proportional to the total cell diversity. (B) The FSC-A histograms represent the light scattered near the forward direction (proportional to the volume of the particles). The SSC-A histograms represent scattering at the right angle (dependent on cell shape and internal properties).

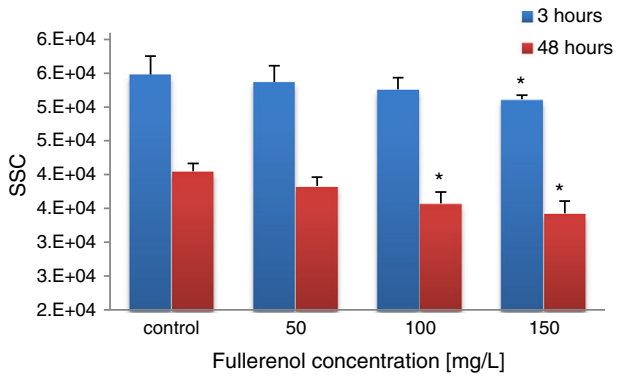

Fig. 5. Flow cytometry analysis of the shape of control erythrocytes and erythrocytes incubated with fullerenol in the concentrations ranging from 50 to 150 mg/L. (*) Significantly different from control ($p < 0.05$); one-way ANOVA and a posteriori Tukey's test.

The SSC-A histograms provide information about the shape and structure of the outer cellular membrane. After analyzing the percentage of unaffected cells it was apparent that this parameter was slightly less in the samples treated with fullerenol at the concentration of 150 mg/L for 3 h (Fig. 5). The changes become enhanced with an increased incubation time and fullerenol concentration (Fig. 4B), to reach 15% of morphologically changed cells in comparison to the control when incubated with 150 mg/L for 48 h.

Changes in the shape of the cells observed by using flow cytometry are in accordance with microscopic observations of erythrocytes exposed to fullerenol. Fig. 6 shows the light microscopic images of erythrocytes incubated with different concentrations of fullerenol (1000× magnified). Non-treated cells after a 3-h incubation at 37 °C have the characteristic shape of discocites. In the samples incubated in the same conditions with 150 mg/L fullerenol, slight changes were observed, including the formation of dendrites, which are typical for echinocyte forms. After a 48-h incubation, a substantial number of control cells showed signs of echinocytic transformation and aggregation. Fullerenol seemed to slightly enhance this effect at a concentration of 150 mg/L. These results are consistent with previous data obtained via flow cytometry. Formation of echinocytes is caused by a number of factors, which, among other things, affect the conformation of band 3 protein, the main foundation of the cytoskeleton [43]. Moreover, it is a transmembrane anion exchanger protein responsible for chloride and bicarbonate transport, which is correlated with potassium and sodium active transport by Na^+/K^+-ATPase. Fullerenol, by inhibiting the function of Mg^{2+}-dependent membrane ATPases [26], may be able to disturb the distribution of lipids in the inner and outer membrane layers, thus, triggering the collapse of the discoidal shape of erythrocytes [52]. Further, the altered morphology of the cells can be explained by the formation of hydrogen bonds between the nanoparticles and the lipid head groups. The presence of each nanoparticle engaged a number of lipids to reduce their areas per lipid molecule. It is not impossible that a synergistic effect of fullerenol on membrane ATPases, band 3 protein and lipids is responsible for part of the echinocytic transformation.

Our data show that fullerenol can slightly alter the morphology and, therefore, the inner-organization of the cells through association with cytoskeletal proteins. These results confirm that fullerenol is able to interact with transmembrane cytoskeletal proteins, in particular band 3 protein and ATPases.

4. Conclusion

The presented data are promising in the context of utilizing fullerenol as a drug carrier. The interaction between band 3 protein and hydroxyl groups of the compound creates a possibility to use fullerenol as an "anchor" between erythrocyte and an anti-cancer drug.

We can assume that interactions will be limited to those with surface and transmembrane proteins, such as ATPases [26] and band 3 protein. The inhibition of potassium ion leaks and subsequent changes in membrane electrostatic charge combined with interactions with band 3 proteins can eventually lead to changes in erythrocyte epitopes. Such changes can result in increased capture by the RES and targeting to specific organs (liver, spleen or bone marrow), which can be an advantage in light of the targeted therapies. Moreover, the data presented in this study show that changes in the morphology of erythrocytes triggered by interaction with fullerenol in high concentrations (up to 150 mg/L) do not lead to an increase in hemolysis or potassium ion leakage, but even prevent them in a moderate manner.

In conclusion, fullerenol in the range of concentrations between 50 and 150 mg/L does not show significant toxic effects in erythrocytes. Fig. 7 shows a proposed mechanism of interaction between fullerenol and cytoskeletal proteins. These results reveal the need of further investigation into the interactions between biological membranes and soluble fullerene derivatives in order to verify their toxicity and possibilities of utilization as drug carriers in more detail.

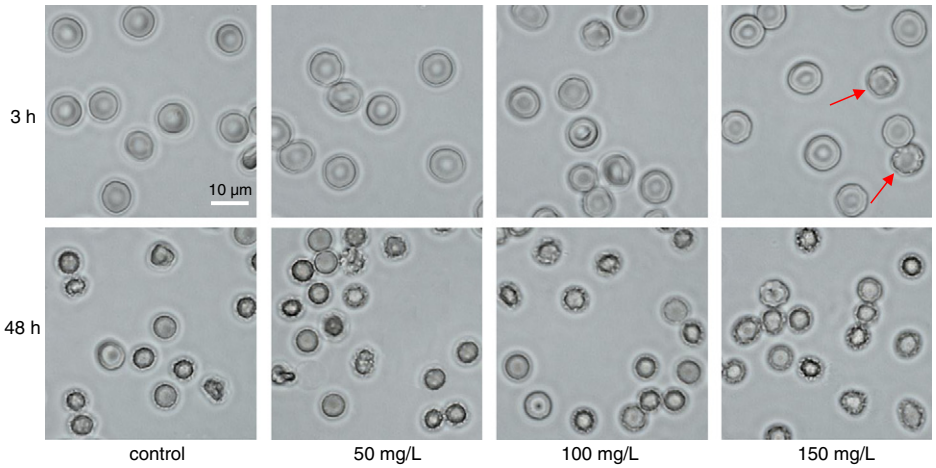

Fig. 6. Microscopic images of erythrocytes exposed to fullerenol. The cells were incubated in the presence of fullerenol at 50–150 mg/L for 3 and 48 h at 37 °C. Echinocyte formation for 150 mg/L of $C_{60}(OH)_{36}$ — indicated by arrows. All images are at the same scale (scale bar: 10 µm).

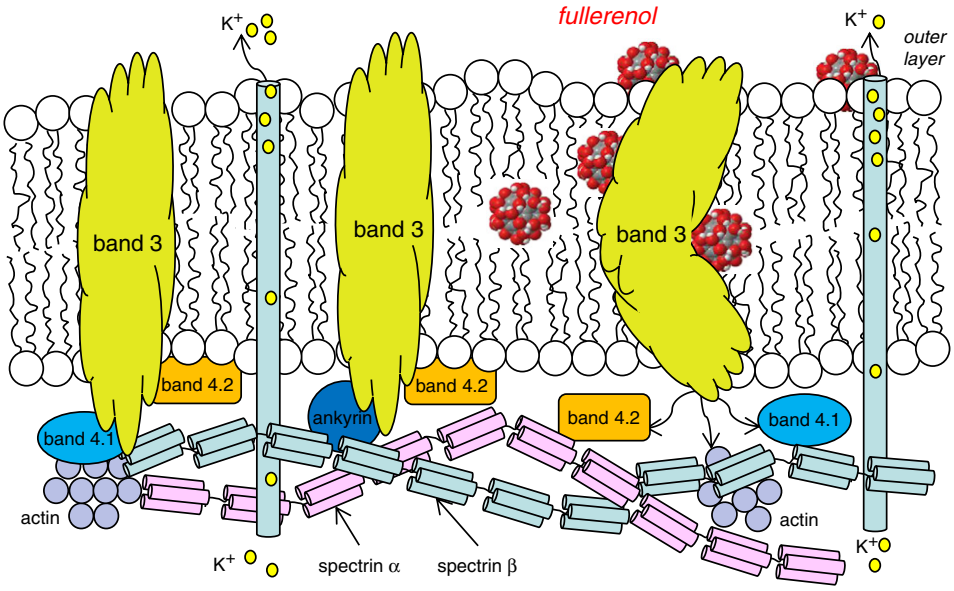

Fig. 7. Fullerenol interactions with the band 3 protein and the location of fullerenol in the lipid bilayer. Fullerenol $C_{60}(OH)_{36}$, by associating with band 3 protein, does not only prevent its degradation, but can also influence the binding sites of spectrin, band 4.1 and 4.2 proteins or actin, leading to changes in the cytoskeleton affecting erythrocyte morphology.

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