NEAR INFRARED LIGHT INDUCES POSTTRANSLATIONAL MODIFICATIONS OF HUMAN RED BLOOD CELLS PROTEINS

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There is a growing body of evidences that near infrared (NIR) light exerts beneficial effects on cells. Its usefulness in the treatment of cancer, acute brain injuries, strokes and neurodegenerative disorders have been proposed. The mechanism of NIR action is probably of photochemical nature, however it has not been fully understood. Here, using relatively simple biological model, human red blood cells (RBCs), and polychromatic non-polarized light source, we investigate the impact of NIR radiation on the oxygen carrier, hemoglobin (Hb) and anion exchanger (AE_1, Band 3). Exposition of intact RBCs on NIR causes quaternary transitions of Hb, dehydration of proteins and decreases the amount of physiologically inactive methemoglobin, as detected by Raman spectroscopy. These effects are accompanied by lowering of intracellular pH_i and changes of the cell membrane topography, documented by atomic force microscopy (AFM). All those changes are in the line with our previous studies where alterations of membrane fluidity and membrane potential were attributed to NIR action on the RBCs. The rate of the above listed changes depends strictly on the dose of NIR the cells receive, nonetheless it should not be considered as a thermal effect.

Introduction

The electromagnetic radiation ranging from 780 nm to 3000 nm (according to ISO 20473) is called near-infrared (NIR) light and from year to year it finds more and more potential applications in biology and medicine. In photobiostimulation NIR holds special place. It has been demonstrated that energy production by mitochondria is improved, the oxidative stress diminishes, cell proliferation increases and apoptosis decreases in cells treated by NIR light.[1] Although NIR photobiostimulation has been applied in various areas of clinical practice, from wound healing to neurodegenerative disorders, the basic mode of its action is still not fully understood.

The impact of NIR or visible light on red blood cells (RBCs) aminoacids, proteins and water has been intensively studied by us[2–10] and others.[11–19] So far it has been documented that RBCs exposition to NIR results in change of membrane electrokinetic potential and mechanical resistance.[4,11] The NIR radiation influences activity of enzymes: *e.g.* ATPase and acetylcholinesterase (AChE).[12,13,15] Moreover, it was shown that photochemical dissociation of hemoglobin (Hb)-ligand complexes (*e.g.* O_2, CO, NO) can be also initiated. Because of the differences in experimental setups, especially in used spectral range of NIR radiation and exposure time, it is difficult to distinguish between primary target of NIR within the cell and secondary effects which lead to modifications of cellular properties. However, taking into account the absorption spectra of water and Hb,[20,21] these seems to be the best candidates to mediate the NIR effect in erythrocytes. Especially water appears as the most rational choice.[4–10,22–25] It is omnipresent in cells: surrounds amino acids and is bounded within biological structures. Absorption of NIR radiation can significantly disturb the energy of hydrogen bonds, which in turn can lead to their disruption and increased dissociation of water molecules.[3–10,22–25] Following dehydration several secondary effects can occur like changes in the structure of water bound to the cell surface with possible alternations of membrane proteins conformations. Hb, the most abundant protein in RBCs, is in contact with plasma membrane through anion exchanger (AE_1) protein, the second most abundant protein in erythrocytes.[26] Previously it was reported[4,19] that the concentrations of deoxyhemoglobin (deoxy-Hb) and oxyhemoglobin (oxy-Hb) change in erythrocytes exposed to NIR. However, it is not clear whether modification of hydrogen bonds leads to quaternary changes of Hb or the absorption of the light energy by this metalloprotein initiates the above described changes. To our knowledge there is no evidence of beneficial effect of NIR radiation on physiologically inactive methemoglobin (met-Hb).

In spite of dilemma about primary target of NIR in cells, all researchers point that the action of radiation on processes taking place in cells, and in RBCs in particular, is strictly dependent on the

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dose of received/absorbed light. The source of light seems not to be important.[19]

By this report we add a little piece to the knowledge about NIR interaction with human erythrocytes. The exposition of human RBCs to the polychromatic and non-coherent light in the range of NIR triggers the reduction of functionally inactive met-Hb concentration, the effect which accompanies previously described transformation of oxy-Hb to deoxy-Hb. In turn these changes are accompanied by intracellular pH_i alternation and modifications of Band 3 proteins. The most important factor in these processes is the dose of the NIR radiation the cells receive.

Material and methods

The human erythrocytes from red blood cells concentrate (RBCC) collected into a plastic pack containing citrate-phosphate-dextrose-adenine (CPDA1) anticoagulant, not older than 10 days taken from 6 donors were used for Raman spectroscopy experiments. Erythrocytes isolated from human whole blood were taken from 10 donors using citrate as an anticoagulant. Material was obtained in compliance with GMP standards certified by The Main Pharmaceutical Inspectorate (Certificate no. GIF-IW-N-4022/102/10). Blood samples and RBC concentrates were taken from the Regional Centre of Blood Donation and Blood Therapy in Wrocław. The donors were healthy volunteers informed on the scientific aim of the research. Written informed consent was obtained from the participants prior to the experiment. The experiments were approved by the Bioethics Committee in Wrocław with the resolutions numbers 146/2008 and 8/2014.

Red blood cells exposition to NIR

After removal of plasma and buffy coat the packed red blood cells were washed three times (5 min, 2000 × g, 4 °C) with Ringer's solution (RS) containing: 150 mM NaCl, 5 mM KCl, 10 mM glucose, pH = 7.4. After the last centrifugation the hematocrit was adjusted to 20%. 15 ml of RBCs suspension was then exposed to NIR radiation. The source of NIR radiation was a system comprising a halogen lamp and broadband (750–2000 nm) filter. The dose of NIR (*exposure time x irradiance*) the RBCs received depended on the experimental purpose and are indicated separately in each experimental design section. Irradiance values were determined based on radiometer measurements (RAD_10_USB, Spectra-Laser). To ensure uniform dose of NIR the cells receive, the gentle stirring (250 rpm) with teflon-coated magnetic bar were applied. None of the NIR doses induced sample temperature changes. The temperature was maintained throughout the experiments at the constant level and in none of the settings was higher than 37 °C. Its variation level was 0.1 °C.

Control samples were protected from light and treated in the same way as the exposed, except for the exposure to NIR radiation.

Acquisition of Raman spectra

In this sets of experiments RBCs were exposed to 0.69 $mW \cdot cm^{-2}$ for 10, 15, 30 and 45 minutes. The control was kept in the dark. Following the NIR exposure the suspension was centrifuged for 30 minutes at 20000 x g, at 8 °C. After supernatant removal the resulting precipitate was a hemolysate with an extremely high concentration of hemoglobin.

The spectra of hemolysates were measured on a T64000 Raman spectrometer (Jobin Yvon). Excitation wavelength was 514 nm (argon-krypton laser). The spectrometer operational parameters were: aperture d = 0.3 mm, exposure time 3 x 30 seconds and 3 mW of laser power. The spectra were analyzed as previously described[27–33] and Fig. 1 presents the representative spectrum of hemolysate recorded at this setup.

Six spectral positions were chosen: 4 describe changes of hemoglobin form and 2 define the protein secondary structures. They are summarized in Table 1.

Based on the intensity of the bands at selected spectral positions, three ratios were defined:

$$R_1 = \frac{I_{1374}}{I_{1402}} = \frac{deoxyHb}{oxyHb} \quad (1)$$

$$R_2 = \frac{I_{1590}}{I_{1610}} = \frac{deoxyHb}{metHb} \quad (2)$$

$$R_3 = \frac{I_{1645}}{I_{1685}} = \frac{\alpha\ structure}{\beta\ structure} \quad (3)$$

The parameter R_1 describes relation ratio between deoxy- and oxy-Hb; R_2 deoxy- and met-Hb; whereas R_3 α to β secondary structure of the proteins.

The final results are presented as an relative percentage change and were calculated using the equation (4),

$$R_i[\%] = \frac{R_i - R_{i0}}{R_{i0}} \cdot 100\% \quad (4)$$

where R_i is the value for NIR-exposed sample and R_{i0} is a value of its control.

Intracellular pH_i measurement

The RBCs (hematocrit 20%) were exposed to NIR radiation of 0.69 $mW \cdot cm^{-2}$ for 15 minutes (measurements were performed only for one dose of radiation previously determined as the most effective). The control samples were kept in dark. Next the RBCs suspensions (hematocrit 1%) was loaded with fluorescent intracellular pH_i indicator BCECF (2',7'-bis-(Carboxyethyl)-5(6)-Carboxyfluorescein) to 10 μM of the cell-permeable acetoxymetyl ester in physiological solution, at 37 °C for 30 min. The cells were washed three times with the physiological NaCl solution pH = 7,4 (20 sec at 12000 × g) and resuspended in the same medium at final hematocrit 10%. Fluorescence was recorded using SLM 8000 fluorometer (SLM Instruments). As previously described,[34,35] the calibration of the dye was done based on two excitation wavelengths: 503 nm (maximum absorption, strongly dependent on pH_i) and 430 nm (independent on samples pH_i). Emission was recorded at 530 nm wavelength. The calibration curve of chosen pH points (pH = 6.8, 7.0, 7.2, 7.4 and 7.6) was linear and is described with the following equation $pH_i = 11.443 \cdot I_{503}/I_{430} - 73.513$. To convert the fluorescence ratio of BCEF-loaded erythrocytes into intracellular pH_i values, a calibration was carried out by equalizing pH and pH_i using the K^+/H^+ ionophore nigericin (5 μM).

Atomic Force Microscopy (AFM)

The erythrocytes suspension was divided into three 3 ml samples, two of which were exposed to NIR radiation, and the third served as a control (CTR). Two NIR radiation doses were used: 1.5 mW·cm^{-2}; (NIR1.5) and 4.5 mW·cm^{-2}; (NIR4.5). Exposure time of both samples was 15 minutes.

Immediately after exposure the cells were treated with 2% glutaraldehyde (Sigma-Aldrich) in RS at 4 °C. The suspension was shaken for 2 min. After washing three times (5 min, 2000 × g, 4 °C) the erythrocytes were suspended in RS with hematocrit 0.2%. Thirty µl of the suspension were spread on a defatted microscopic slide using a silicone tip. The sample was dried for 30÷45 min at 37 °C in a laboratory incubator. Pre-dried slides were gently rinsed twice with double-distilled water (2×1 ml) to flush the remaining crystallizing salt. Then the slides were again placed in the laboratory incubator for 30 min. The samples were initially evaluated under an optical microscope and then under AFM.

Erythrocyte topography imaging was performed using a standard atomic force microscope MultiMode NanoScope III (Veeco Instruments, Santa Barbara, USA) with a maximum scanning range of 12 µm for x and y axes and 2.5 µm for z axis. The study was conducted in the tapping mode at room temperature and relative air humidity of 60%. The measurements were made using NT-MDT probe, model Etalon HA_NC-B (High Accuracy noncontact etalon probe). The probe was characterized by a cone angle of $\phi \leq 22°$ and the curvature radius of 10 nm.

Selection of the erythrocytes for the first scan was based on the control image from a CCD camera installed over the sample. After capturing the image of an entire cell two scans at a higher resolution within 1×1 µm area were made. While capturing the 1×1 µm image a similar region of an erythrocyte was selected – marked with letter "a" in Fig. 2 – in order to avoid substantial slope variability of the scanned surface, resulting from a characteristic biconcave shape of the erythrocytes. Selection of the most horizontal section facilitated subsequent image analysis within 0.25×0.25 µm range.

The input data were the images of erythrocyte surface 1×1 µm in size. Each image was subjected to the flattening procedure. Each image processed in the above-described manner was then divided into 16 smaller areas, 250×250 nm in dimension. The analysis involved characteristic ring-shaped structures. These structures were present in all analyzed images, and thanks to their specific shape they were relatively easy to find. Representative objects of interest are shown in Figs. 3A and 3B.

Determination of object dimensions was based on their surface profile. The Section Analysis tool was used to perform a cross-section of a selected structure. Fig. 4 presents a geometric analysis carried out for the shown object.

A phase characteristic of the structures located on the basis of topographic analysis was examined, based on the images taken in phase contrast. Representative analysis is shown in Fig. 5.

Statistical analysis

The relative percentage change R_i (defined by eq. 4) calculated from ratios obtained from Raman spectra (defined by eq. 1–3) were analyzed by a bootstrap method as described in.[36] Briefly, 2000 bootstrap samples were generated by random sampling with replacement from original dataset. Then, the mean for each bootstrap sample was calculated and the sampling distribution of mean using bootstrap estimates was obtained. Finally, the mean and standard error of mean were computed and the lower and upper decision lines for the comparison of each of the R_i were given by plot the mean values of R_i from original dataset against the decision lines. If any one of the points plotted lied outside the respective decision lines, hypothesis H_0: the means are homogenous, was rejected at α = 0,05 level and conclude that the means are not homogenous.

The structures observed by AFM were analysed using the statistical package Statistica (StatSoft). ANOVA analysis was used to assess the significance between groups. The use of this analysis is limited by set assumptions: the variables are measurable, have normal distribution and homogeneous variance. Kolmogorov–Smirnov and Shapiro–Wilk tests allowed for the rejection of the normal distribution hypothesis for each of the parameters, and this prevented the performance of a standard analysis of variance. For this reason, the Kruskal–Wallis test, which is a nonparametric equivalent of the ANOVA, was performed to check whether samples originated from the same distribution. A post hoc analysis was used to identify the pairs of variables revealing statistically significant differences.

Results and discussion

Raman Spectroscopy

The representative final spectrum is presented in Fig. 1. The relative percentage changes of earlier defined markers (Equations 1 - 4) in relation to the exposition time of RBC to NIR are shown in Fig. 6. The positive values of R_1[%], R_2[%], and R_3[%] mean that the ratio of deoxy- to oxy-Hb, deoxy- to met-Hb and α to β secondary structure is higher than for corresponding controls. The negative values of mentioned parameters indicate that the ratio of deoxy- to oxy-Hb and α to β secondary structure is lower than for control samples. Changes in the designated parameters strongly depend on the exposure time. 10 min exposure time induces large changes in the secondary structure of proteins; the R_3[%] parameter strongly increases of 106% compared to the non-irradiated sample ($p < 0.05$). Exposure for 10 min. results also in 40% increase in the parameter R_2[%] ($p < 0.05$). Since the R_1[%] parameter (ratio of deoxy/oxy) is unchanged in comparison with the control sample increased R_2[%] parameter (deoxy/met) should be considered as a decrease of a concentration of met-Hb form. 15 min exposure causes 74% increase of the R_1[%] parameter ($p < 0.05$ vs non-irradiated sample). Simultaneously, the R_2[%] parameter increases from 40% to 78% ($p < 0.05$ vs non-irradiated sample) while the R_3[%] returns to the value of control sample. This means that the amount of deoxy-Hb increases (what was earlier observed) while the amount of met-Hb is constant or continuously decreases. The amount of met-Hb following 10 and 15 minutes of NIR irradiation decreases in comparison with intact RBCs. The measured increase of met-Hb concentration after 30 and 45 min irradiation (negative value of R_2[%]) ($p < 0.05$ vs non-irradiated, 10, 15 min) follows Hb

oxygenation (the R_1[%] decreases and after 45 minutes exposition to NIR it reaches the value characteristic for control samples). The R_3[%] parameter increases from 34% to near 95% (both $p < 0.05$ vs non-irradiated) after 30 min and 45 min exposition respectively. Since the Raman spectra were collected from hemolysate, it should be kept in mind that for all exposure times the R_3[%] parameter do not describe solely modification of Hb structure. Hb and AE_1 are the most abundant proteins of erythrocytes thus we concluded that most of the energy was absorbed by the membrane and rise of the R_3[%] value is assigned to band 3 modifications or even progressive denaturation as the secondary thermal effect. The transitions from tens (T, deoxy-Hb) into relax (R, oxy-Hb) form of hemoglobin and back is a constant and dynamic process which is equally based on hydrogen bonds and steric interactions.[37] The action of NIR could be by weakening of the hydrogen bonds thus facilitating allosteric T to R transitions of Hb.[4, 19, 22–25]

Measurements of intracellular pH_i

Red blood cells play a crucial role in regulating the acid-base equilibrium. The two components of cells: Hb and anion-exchange transporter AE_1 (band 3 protein) are fundamental for that phenomenon. Haemoglobin is the main H^+ buffer. Band 3 protein mediates transmembrane exchange of Cl^- for HCO_3^-. The results showed in paper[38] provide the first direct correlation between pH_i value during RBC volume regulation and emphasize a key role for AE_1. The spectroscopic data presented there indicate that NIR irradiation triggers the transformations between different forms of Hb. It should be reflected in the intracellular pH_i modifications. In the present study we used the method of pH_i measurement described by Kummerow *et al*.[35] We are aware of the all disadvantages of used procedure: the pH_i value is measured in average in RBCs population, and the dye inhibited an activity of Ca^{2+} -ATPase. The resting pH_i values obtained by this method are markedly higher than reported with the use of another fluorescent probe SNARF-1 (carboxy-seminaphthorhodafluor-1), see discussion in the paper.[38] However, comparison of the change in average pH_i for whole cells population of cells upon exposure to NIR seemed to be the most important in our studies.

The obtained results are: pH_i for control samples amounted to 7.36 ± 0.03 and after 15-minute exposition to NIR-radiation intracellular pH_i decreased to 7.24 ± 0.05 (mean of two measurements).

AFM imaging of the erythrocyte surface

A 15-minute dose of NIR evokes quaternary structural changes of Hb, confirmed here by Raman spectroscopy and intracellular pH_i measurements. Here we investigated using AFM technique how topography of human erythrocyte changes after exposition to NIR. Exposure to NIR radiation with irradiance of 1.5 $mW \cdot cm^{-2}$ resulted in increased value and range of the phase shift distribution, while heightening the dose caused a significant reduction in the investigated parameter and its distribution range. Results of a statistical test for phase variable for 4 and 5 subunits indicated a statistically significant differences between NIR1.5 and NIR4.5 samples at the level of the *p* parameter equal 0.021 and 0.005 respectively, (Fig. 7).

Analysis of outside and inside diameter of the structures made up of four subunits revealed that the size of the investigated structures was reduced as a result of NIR exposure (Fig. 8). Although a marked decrease in the outer diameter in the NIR1.5 sample was not statistically significant ($p = 0.06$), it was only slightly different from this adopted for the tests ($p < 0.05$). The outer diameter in the NIR4.5 sample was smaller as compared to the control ($p < 0.05$). Samples exposure returned no statistically significant differences for a variable inside diameter (Fig. 8B). Appointed value of Kruskal–Wallis test ranks was at $p = 0.11$, which was well above the level assumed ($p < 0.05$).

Observations of erythrocyte structures built of five subunits supported the hypothesis claiming an impact of near-infrared light on the geometric features of erythrocyte membrane structures. The analysis of structures geometry that was composed of five subunits showed statistically significant differences between the control and NIR exposed samples. Level to reject the equality distribution functions hypothesis is $p = 0.0008$ for the outside diameter (Fig. 9A), and $p = 0.0003$ for the inside diameter (Fig. 9B).The *post hoc* tests for both geometric measures (OD and ID) also showed huge differences between the control cells population (CTR), and NIR1.5 and NIR4.5 exposed erythrocytes (Fig. 9A,B). Radiation dose seemed to be an extremely important factor, as for both variables the level of the null hypothesis rejection for CTR and NIR1.5 was at least an order of magnitude lower when compared to CTR and NIR4.5 populations. It must be emphasized that the values of the performed tests left no doubt as to the effect of near infrared radiation on the geometry of the structures built of five subunits.

While studying the geometrical parameters of the analyzed structures it was clearly visible that erythrocyte response to NIR was associated with reduced values of analyzed parameters. Moreover, the intensity of the NIR effect was more pronounced with increasing radiation dose. This effect was observed for outside and inside diameter of both types of structures – "fours" and "fives". Studied structures are likely to be the band 3 proteins (Anion Exchanger - AE_1), usually present in the cellular membrane in an oligomeric form.[39] The most common forms are dimeric and tetrameric structures, but other conformations are also possible. Band 3 proteins form hydrophilic channels across the membrane. Geometry of the observed structures matched the oligomeric form AE_1. Identification of the analyzed objects as aggregates of band 3 proteins was also supported by the fact that this is the most common erythrocyte membrane protein, occurring in about one million copies within a single cell.[39]

High resolution of AFM microscopy[26,40] made possible to estimate the volume and surface occupied by the protein on the outer surface of erythrocyte cells. Changes in the volume and surface area of the exposed part of the protein depend on the resting shapes of erythrocytes.[26] The described parameters are significantly decreased in stomatocytes in comparison to discocytes and echinocytes. The values obtained for the protein surface area of stomatocytes, discocytes and echinocytes were respectively: 69,5 $\pm$ 3,6 nm^2, 77.6 $\pm$ 8,5 nm^2, 83.3 $\pm$ 1,5 nm^2. The calculated values for the protein surface area on the extracellular surface of erythrocytes with different shapes show that the external leaflet of the membrane decreased around 10% during cells transformation from discocytes to stomatocytes. The results obtained by the Betz *et al*.[26]

suggest that approximately 90% of the protein volume is embedded in the membrane and in the cytosolic part of cells.

We measured only the inner and outer diameter of selected structures on the surface of the membrane. The declining diameters may indicate a change in cell shape under the influence of NIR radiation and participation in the process of protein AE_1. Simultaneously, lowering pH_i is a factor which changes the shape of the discocytes to stomatocytes. Intracellular pH_i is thus tightly regulated in the RBC, but modulated by the cell's volume.[40]

At the same time changes in deoxy-Hb and met-Hb concentration were detected. The oxygen-linked transition of hemoglobin (T to R) could play a key role in the regulation of anion exchanger activity AE_1.[41] This protein is composed of two domains: a membrane-spanning domain (55 kDa) responsible for anion exchange across the membrane, and a cytoplasmic domain (cdb3, 43 kDa) which binds cytoskeletal proteins ankyrin and glycolytic enzymes: aldolase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, and the protein tyrosine kinase. The domain cdb3 also binds to hemoglobin.[42] This interaction is mainly electrostatic and both forms of hemoglobin are associated differently, deoxy-Hb (T-state) binds to cdb3 more tightly than oxy-Hb (R-state) thus conformational changes of Hb are transferred on band 3 and may influence the activity of the membrane spanning domain.[41–44]

Changes in activity of the AE_1 protein are associated with the change of the cell shape. This protein is present in the membrane in two conformations: outward-facing allowing the outflow of anions from the cell and inward-facing responsible for the influx of anions from the external environment.[45] The equilibrium ratio between these forms $K_i/K_0 = 15$ what means that the stream of anions into the cell is predominant. With the decrease of intracellular pH_i the concentration of Cl^- ions in the cell is growing, which enforces the outward-facing conformation of AE_1 protein. Simultaneously, the cytoplasmic part of the protein is protonated. A sudden outflow of anions primarily of Cl^- from the cell occurs when the interaction between the AE_1 protein, band 4.1 protein, and glycophorin A is lost. It increases the number of the outward-facing the AE_1 conformations which also increases the fraction of unfolded spectrin molecules and favors stomatocytosis.[45]

The phase image can be generated as a consequence of variations in properties of materials such as friction, viscoelasticity, hardness as well as a difference in electrical or magnetic properties. Another effect visible in the phase contrast images is a difference in hydrophilicity. Hydrophilic elements of the structure cause positive phase shift (light parts of the image), and hydrophobic ones cause negative phase shift (darker parts).[46,47] The data collected from the phase shift angle measurements showed that the properties of the analyzed structures were altered in response to NIR exposure. The nature of these changes was dependent on the radiation dose. In the case of a lower dose sample (NIR1.5) the AFM probe captured much larger differences in the phase lag angle within the imaged structures than for the non-radiated control sample.

If NIR action is related to changes in the hemoglobin structure, then absorption of NIR radiation by water may facilitate the transition of oxy-Hb into deoxy-Hb. A transition of Relax form into Tense form is associated with dehydration.[48–50] Additionally, Colombo *et al.*[48] concluded that deoxy-Hb coexists in two allosteric states: i) one state of low affinity to O_2, and to anions with low hydration for deoxy-Hb, ii) the second with increasing in O_2^- affinity and increasing in protein hydration which is accompanied by the anion dissociation from deoxy-Hb. An increased efflux of Cl^- as a result of the changing pH_i may further reduce the amount of Hb complexes with anions. The resulting deoxy form (T) may be transformed into oxy-Hb or met-Hb. Met-Hb may exhibit oxy-like or deoxy-like structure. In the first case Fe (III) coordinates 6 ligands (the 6th ligand is a water molecule) or 5 ligands (water is removed). Pentacoordinate met-Hb molecule can be much more easily reduced to deoxy-Hb.[51,52] This process takes place during the 15 minutes of NIR exposure. It was correlated with higher intensity of amide band, corresponding to α-helix structures, and was accompanied by variability of membrane fluidity[2] and minimum value of zeta potential.[4] Increased intensity of amide I band may indicate the initial dehydration. Dose increasing triggered the second phase of changes: elevated amount of met-Hb and deoxy-Hb, but also much smaller boost of R_3[%] marker. Hydrophobicity of the erythrocyte surface (NIR4.5) returned to its initial level. However, this was a different state of the cell than before the exposure. Longer exposure times may trigger structural changes in proteins, including hemoglobin. Dehydration may lead to protein degradation, aggregation, and the changes in the secondary structure affect the value of R_3[%] parameter. This would be an irreversible process,[4] accompanied by elevated concentration of deoxy-Hb and met-Hb. Hemoglobin modifications should induce both cell volume and intracellular pH_i changes, which in turn must affect the structure of band 3 protein, as indicated by AFM data.[41–44] This work gives the evidence that exposition of human erythrocytes to NIR light is able to decrease the amount of physiologically inactive hemoglobin. This transformation is accompanied by decrease of intracellular pH and modifications of the AE_1 proteins.

While described effects were consistent with the experiments, the very mechanism of NIR action can be proposed as a hypothesis.

Absorption of NIR radiation by OH groups excite overtone stretching vibration resulting in weakening hydrogen bonds formed by them and modification of bonded water structure.[23–25] The results obtained from the analysis of AFM images reaffirms our suggestion.[3–10] The consequence of this process is a transformation of oxy-Hb into deoxy-Hb. Met-Hb in these conditions undergoes a similar process changing conformation from oxy-like structure to the deoxy-like. When the hemoglobin oxy-Hb is auto-oxidized to met-Hb, the met-Hb is recycled back to deoxy-Hb by antioxidative enzymes facilitated by the formation its deoxy-like structure. Regardless of other antioxidant mechanisms in RBCs, lowering the pH_i induces formation of active forms of very potential antioxidative enzyme peroxiredoxin (Prxs) from inactive dimeric to active decameric form. Decameric form is located into hydrophobic area of RBCs membrane from the inside of the cell. Structural deformations inside hydrophobic part of membrane were presented in our previous study.[2] The high abundance of Prxs (the third most abundant protein in cytozol of erythrocytes with concentration 5.6 mg·ml^{-1} or 14 million copies per cell) proves that these proteins are important players in peroxide detoxification in RBCs.[53–55] Studies of the properties of Prxs revealed that in erythrocyte hemolysates in a dose-dependent manner it

significantly protects RBCs from ROS by binding to hemoglobin, thus preventing both induced and spontaneous oxidation to met-Hb and membrane lipid peroxidation.[55] The formation of Prxs decamers entails the radical changes in proteins secondary structure.[54] The R_3 parameter is a measure of the changes taking place in both Hb and Prxs during exposition to NIR. The smallest concentration of met-Hb after 15 min of exposure may indicate the highest antioxidant activity of all antioxidant enzymes including Prxs effectively protecting RBCs against oxidative stress which has been confirmed by our earlier experiments.[5]

Conclusions

NIR radiation is a potent modulator of cellular components. In human RBCs it probably interacts with both Hb and the cellular membrane. As a consequence it facilitates reduction of met-Hb to deoxy-Hb and oxy-Hb forms. But it must be underlined that the action of NIR is strongly dependent on the dose of radiation. Moreover, contrary to widely studied benefits of exposition of tissues to red and near red light emitted by lasers we have showed that the polychromatic light 750–2000 nm range cut from non-polarized affects also the proteins. We believe that the NIR light has a potential to replace currently using high energy sources in photobiostimulation practices because it is relatively safe in application and cheaper thus can be wider available.

It is also essential to preserve the precautions in the use of NIR radiation as a research method.

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Table 1 The spectroscopic bands of different forms of haemoglobin (oxy-Hb, deoxy-Hb and met-Hb) and protein secondary structure (α and β).

Band Position [cm^{-1}]	Assignment
1374	deoxyHb[27]
1402	oxyHb[27]
1590	deoxyHb[29]
1610	met-Hb[30]
1645 – 1655	amid I (structure α)[27]
1685 – 1687	amid I (structure β)[32]