Ortho-Hydroxy Derivatives of 2,5-Diaryl-1,3-Oxazole as Fluorescent Probes to Monitor the Changes in Human Erythrocyte Membranes under the Influence of Low Molecular Weight Cryoprotectants

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***Abstract*-It has been shown that derivatives of 2,5-diaryl-1,3-oxazole can be used as fluorescent probes to monitor the changes in physico-chemical properties of human erythrocyte membranes under the action of low molecular weight cryoprotectants such as glycerol and 1,2-propanediol. It has been found that increase in cryoprotectant concentration increases hydration of erythrocyte membrane. The minimal concentration of the cryoprotectant, which causes the perturbation of the erythrocyte membrane, has been estimated to be 0.5 % and 5.0 % for glycerol and 1,2-propanediol, correspondingly. Using a set of fluorophores with different locations in lipid bilayer enabled us to show that the increase in the membrane hydration occurs in polar regions of the membrane (in the areas of glycerol residues of phospholipids, carbonyl groups of phospholipids and in the area of methylene groups in the vicinity of carbonyl groups of phospholipids), while no changes in hydration have been observed in the most hydrophobic regions of the lipid bilayer: in the area of the methylene groups of phospholipids near the centre of lipid bilayer and in the center of lipid bilayer. The suggested fluorescent probes can be used to monitor the changes of physico-chemical properties in different regions of lipid bilayer under the action of the cryoprotectants and to determine optimal concentrations of cryoprotectants for the low-temperature storage of cells.**

Keywords- Fluorescent Probe; Human Erythrocyte Membrane; Cryoprotectants; Glycerol; 1,2-Propanediol

I. INTRODUCTION

Low molecular weight protectants such as glycerol, 1,2-propanediol and dimethylsulfoxide are widely used in biology, medicine and biotechnology for the long-term storage of the human and animal cells at low temperatures [1]. Unfortunately, at high concentrations cryoprotectants can influence membrane integrity of cell membranes even before freezing [2]: such negative influence vary between different types of cells. Thus, in order to make optimal choice of cryoprotectant concentration for the low-temperature storage of the definite type of cells one should study the influence of the cryoprotectant on the structural integrity of the cell membrane before freezing.

Due to high sensitivity and informativity, fluorescent methods are a valuable tool to study structural modifications of biomembranes [3]. In literature one can find a few examples of the fluorescent probe usage to study the influence of low molecular weight cryoprotectants (such as glycerol; 1,2-propanediol; dimethylsulfoxide) on cell membrane structural integrity: e.g. Giraud et al. [4] used fluorescence anisotropy measurements of diphenylhexatriene (DPH) to study action of glycerol on human spermatozoa membrane; Dyubko et al. [5] used 3-hydroxy-4′-(N,N-dimethylamino)flavone (FME) to study an influence of low molecular weight cryoprotectants on rat liver microsome membranes. In each of the above cases only one fluorescent probe was used to study the changes in lipid membranes: such experimental scheme did not allow to monitor the changes of physico-chemical properties in different regions of lipid bilayer.

On the other hand, to the best knowledge of the authors, there are no reports about the usage of fluorescent probes to study the influence of low molecular weight cryoprotectants on human erythrocyte membranes.

This article describes the possibility to use a few ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole as fluorescent probes to monitor the changes in different areas of human erythrocyte membrane (i.e. both in polar and apolar regions of lipid bilayer) under the influence of cryoprotectants (glycerol and 1,2-propanediol).

II. EXPERIMENTAL PROCESS

Synthesis and purification of 2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole, 2-(2′-OH-phenyl)-5-(4′-phenyl-phenyl)-1,3-oxazole and 2-(2′-OH-phenyl)-9,10-phenantr-1,3-oxazole were described elsewhere [6, 7]. Glycerol and 1,2-propanediol were from “Sigma-Aldrich Russia”.

Red blood cells (erythrocytes) were obtained from human blood by centrifugation at 3000 rpm for 3 minutes (the blood was centrifuged three times). The obtained erythrocytes were resuspended in physiological solution (0.15 M) at room temperature (20 °C). After addition of cryoprotectants the suspensions of erythrocytes were equilibrated at 20 °C for 40 minutes.

The suspension of erythrocytes, used for the fluorescence measurements, had absorption 0.02 at 545 nm. For all the fluorescence measurements, the cells were fluorescently labeled using the same procedure. Since the solubility of the probes is limited in water, the 0.2 mM stock solution of the probes in acetonitrile was used. An aliquot of 0.2 mM stock solution of the probe in acetonitrile was added to physiological solution with erythrocytes (i.e. acetonitrile final concentration was ≤ 0.5%) to achieve final probe concentration of 1 μM. The cell suspension was then incubated with the probes in the dark at room temperature for 1 hour before fluorescence measurements.

Fluorescence spectra were recorded on a Hitachi F850 steady-state fluorescence spectrometer at room temperature. The slits on excitation and emission monochromators were 5 nm. The mesurements were made in a 10 mm×10 mm cuvette. An excitatation wavelemgth was 330 nm. Emission was recorded in the range of 340 – 620 nm, with an increment of 1 nm. Data were collected with a 1 s interval.

III. RESULTS AND DISCUSSION

Ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole have been chosen for the study because of their high sensitivity to the parameters of the environment, such as polarity, viscosity and hydrogen-bonding ability [6-12]. In the excited state the ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole are capable of isomerization via the excited state proton transfer (ESIPT) reaction (Fig. 1): hydroxyl group in the ortho-position of the lateral benzene ring acts as protonodonor and the nitrogen atom of oxazole ring acts as proton acceptor [6-12]. The result of the ESIPT reaction is the formation of phototautomeric form (T*), fluorescent in significantly longer wavelengths in comparison with the initial form (N*) [6-12].

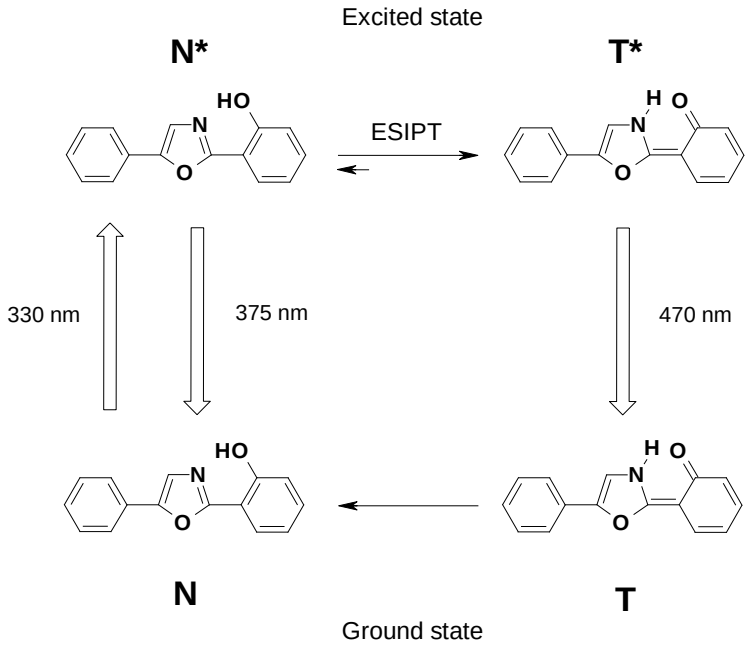

Fig. 1 Scheme of excited state proton transfer (ESIPT) reaction in 2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole. Upwards arrow shows the electronic excitation and downwards arrow represents the light emission (fluorescence). Corresponding maxima of absorption and fluorescence are measured in nanometers

The position and intensity of the fluorescence of each form depend not only on the chemical structure, but also on the parameters of the molecule microenvironment [6-12].

The presence of two-band fluorescence allows to perform ratiometric measurement, i.e. to use the ratio of phototautomeric form and the initial form fluorescence intensities (I_{T*}/I_{N*}) as a parameter for evaluation of the physical and chemical properties of the microenvironment. The use of ratiometric fluorescent probes allows to exclude the measurement error associated with the deviation of the fluorescent probe concentration (e.g., uneven distribution of the fluorescent probe in various membranes) and the measurement error associated with a deviation of fluorescence equipment settings (deviation of the intensity of the exciting source, a change in focus, changes in the sensitivity of the photodetector, etc.) [13].

A few ortho-hydroxy derivatives of 2,5-diaryl-1,3-oxazole that differ in their lipophilicity [8-11] were selected for the present study (Fig. 2): 2-(2′-OH-phenyl)-5-phenyl-1,3-oxazole (probe **1**), 2-(2′-OH-phenyl)-5-(4′-phenyl-phenyl)-phenyl-1,3-oxazole (probe **2**) and 2-(2′-OH-phenyl)-9,10-phenantr-1,3-oxazole (probe **3**). It is expected that the regions of location of selected probes in the membrane are different and correspond to the lipophilicity of the probes (Fig. 2) [8-11]. Expected

location and orientation of probes **1**, **2** and **3** in lipid membranes is based on their fluorescence properties in lipid membranes [8-11] and on the basis of their structural similarity with fluorescent probes with known location in lipid membranes [3]. The location of the probes in lipid membranes: probe **1** – in the area of glycerol residues of phospholipids and in the area of carbonyl groups of phospholipids; probe **2** – in the area of carbonyl groups of phospholipids and in the area of methylene groups of phospholipids near the polar region of the bilayer; probe **3** – in the area of methylene groups of phospholipids near the center of the bilayer and in the center of the bilayer (Fig. 2).

The fluorescence spectra of the probes **1**, **2** and **3** in human erythrocyte membranes before and after the action of glycerol are shown on Fig. 3.

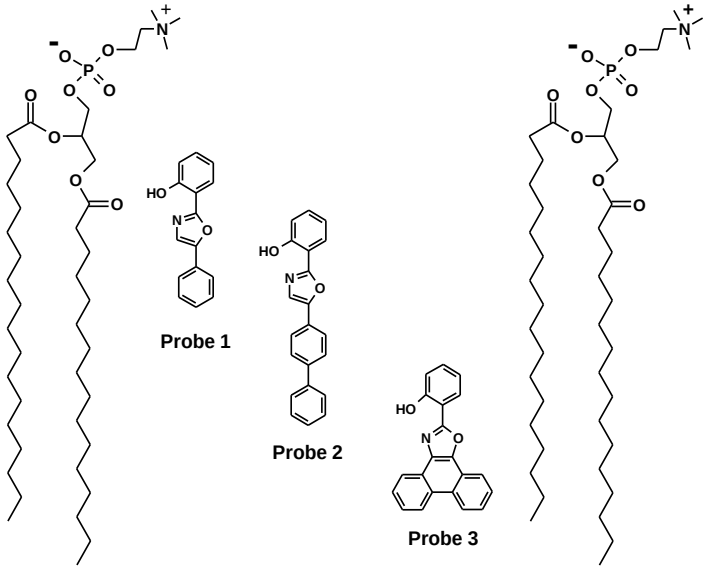

Fig. 2 Expected location and orientation of fluorescent probes 1, 2 and 3 based on their fluorescence properties in lipid membranes [8-11] and on the basis of their structural similarity with fluorescent probes with known localization in lipid membranes [3]. Two molecules of phosphatidylcholine from the outer monolayer are shown to denote the localization of the probes

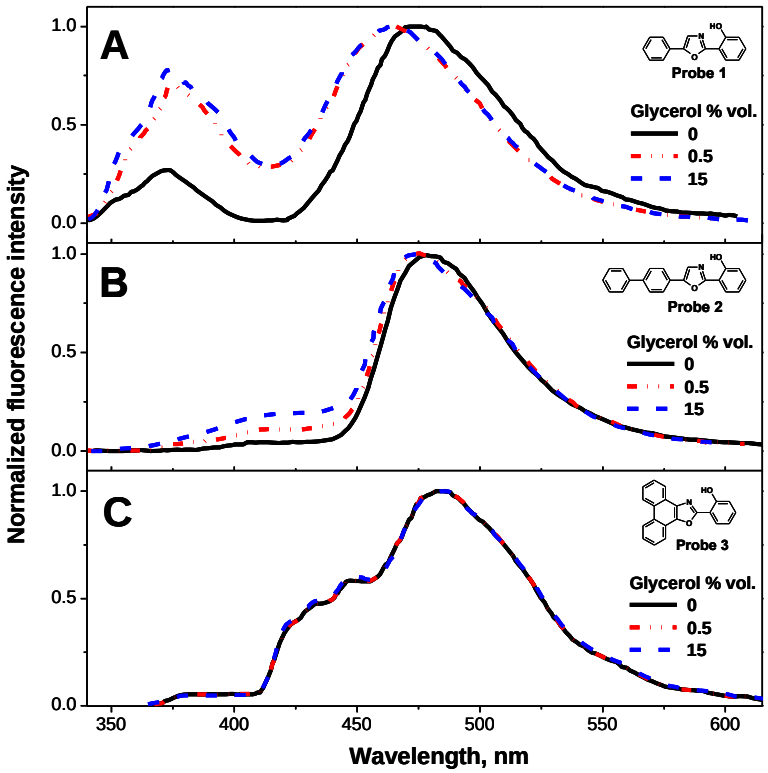

Fig. 3 Normalized fluorescence spectra of probes 1 (A), 2 (B) and 3 (C) in human erythrocyte membranes in the presence of different concentrations (% vol.) of glycerol: (a) 0 (solid black line), (b) 0.5 (dash-dot-dot red line), (b) 15 (dashed blue line)

Two fluorescence bands are observed in each fluorescence spectrum presented in Fig. 3: short-wavelength band is the fluorescence of the initial form (N*, see Fig. 1) and long-wavelength band is the fluorescence of the phototautomer form (T*, see Fig. 1).

According to Fig. 3, the action of glycerol on erythrocyte membranes resulted in the increase of the fluorescence intensity of the normal form (I_{N*}), in the decrease of the ratio I_{T*}/I_{N*} and in hypsochromic (i.e. short-wavelength) shift of the phototautomer fluorescence of the probes **1** and **2**, at the same time, no noticeable changes were observed for the probe **3**. Similar results were observed in case of action of high concentrations of 1,2-propanediol on the erythrocyte membranes (not shown).

The observed hypsochromic shift of phototautomer fluorecscence bond and decrease of the ratio I_{T*}/I_{N*} for probes **1** and **2** in erythrocyte membranes after the action of the cryoprotectants are indicative of an increase in polarity and hydrogen-bonding ability in the lipid membrane regions, where probes **1** and **2** are located. Such changes point to the increase in hydration of the lipid membrane [14-16] in the regions, where probes **1** and **2** are located: in the area of glycerol residues of phospholipids, in the area of carbonyl groups of phospholipids and in the area of methylene groups of phospholipids near the polar region of the bilayer. The increase in hydration may be caused by the perturbation of the membrane lipid packing [15]. Moreover, taking into account that glycerol and 1,2-propanediol are cryoprotectants penetrating into membrane [5, 17-20] (i.e. so-called "membrane-permeable cryoprotectants" [20]), one can suggest that the abovementioned increase in the hydration of the erythrocyte membrane may be due to the accumulation of hydrated cryoprotectant molecules in the membrane [5].

The influence of cryoprotectant concentration growth on the ratio of phototautomeric form and the initial form fluorescence intensities (I_{T*}/I_{N*}) of probes **1**, **2** and **3** is shown on Fig. 4.

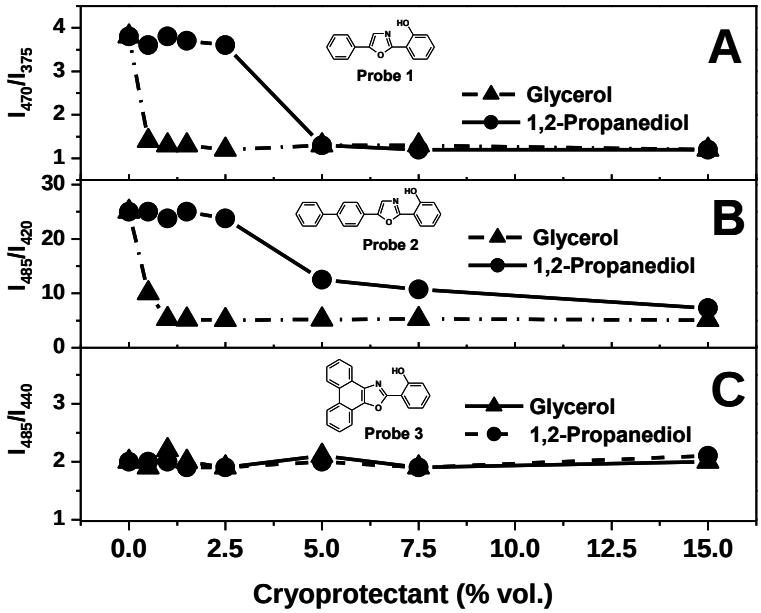

Fig. 4 The influence of cryoprotectants (glycerol (squires) and 1,2-propanediol (triangles)) on the fluorescence intensity ratio I_{T*}/I_{N*} of probes 1 (A), 2 (B) and 3 (C) in human erythrocyte membranes

It is found that initial concentration of cryoprotectant that causes perturbation of human erythrocyte membrane is 0.5 and 5 % vol. for glycerol and 1,2-propanediol, correspondingly.

Thus, the ortho-hydrohy derivatives of 2,5-diaryl-1,3-oxazole can be used to determine optimal concentrations of cryoprotectants for the low-temperature storage of cells.

According to Figs. 3 and 4, there are no changes in the fluorescence spectra (and, thus, in the ratio I_{T*}/I_{N*}) of probe **3** in human erythrocyte membranes after the action of the cryoprotectants. This result shows that the used cryoprotectants do not affect the regions, where probe **3** is located: i.e. the area of methylene groups of phospholipids near the center of the bilayer and the center of the bilayer.

Summarizing the results for probes **1-3**, one can conclude that the suggested set of fluorescent probes enables to monitor the changes of physico-chemical properties in different regions of lipid bilayer under the influence of the cryoprotectants.

IV. CONCLUSIONS

It has been shown that derivatives of 2,5-diaryl-1,3-oxazole can be used as fluorescent probes to monitor the changes in physico-chemical properties of human erythrocyte membranes under the action of low molecular weight cryoprotectants such

as glycerol and 1,2-propanediol. It has been found that increase in cryoprotectant concentration increases hydration of erythrocyte membrane. The minimal concentration of the cryoprotectant, which causes the perturbation of the erythrocyte membrane, has been estimated to be 0.5 % and 5.0 % for glycerol and 1,2-propanediol, correspondingly. Using a set of fluorophores with different locations in lipid bilayer enabled us to show that the increase in the membrane hydration occurs in polar regions of the membrane (in the areas of glycerol residues of phospholipids, carbonyl groups of phospholipids and in the area of methylene groups in the vicinity of carbonyl groups of phospholipids), while no changes in hydration have been observed in the most hydrophobic regions of the lipid bilayer: in the area of the methylene groups of phospholipids near the center of lipid bilayer and in the center of lipid bilayer. The suggested fluorescent probes can be used to monitor the changes of physico-chemical properties in different regions of lipid bilayer under the action of the cryoprotectants and to determine optimal concentrations of cryoprotectants for the low-temperature storage of cells.

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