ERYTHROCYTE MEMBRANOPATHY REVEALED BY THERMAL DIELECROSCOPY. A CASE STUDY

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ABSTRACT

Dielectic spectroscopy of heated suspensions, containing either human erythrocytes or resealed erythrocyte ghost membranes, assays the state of sub-membrane spectrin-actin skeleton and its attachment to the lipid bilayer (Ivanov and Paarvanova, 2016). Using this method across a set of frequencies we measured the changes in the complex electric impedance and capacitance of tested suspensions at the spectrin denaturation temperature, 49.5 °C. In the impedance ($-\Delta Z_{im}$ vs ΔZ_{re}) plot these changes depicted two semicircles expressing two dielectric relaxations, while the capacitance plot (ΔC_{im} vs ΔC_{re}) expressed a single semicircle, corresponding to the second relaxation.

DNAse I is an enzyme that disintegrates actin polymers. Relatedly, the impedance plot of erythrocyte ghost membranes, resealed with DNAse I, demonstrated strong reduction of the radius of first semicircle compared to the impedance plot of control membranes.

In this study we compared the impedance plot obtained with erythrocytes of a tested patient and the plot of isolated erythrocyte membranes which contained DNAse I. The two plots were identical indicating that the patient could have a kind of erythrocyte membranopathy related to reduced polymerization of actin or impaired spectrrin-actin association.

Keywords: *Hemolytic anemia, thermal dielectroscopy, erythrocyte membrane, spectrin-actin network, membranopathy.*

INTRODUCTION

The shape, deformability and elasticity of human erythrocytes depend on their plasma membrane. The latter includes a lipid bilayer with intercalated integral proteins and an under-membrane network of proteins, mainly spectrin and actin. The spectrin-actin network is attached to the lipid bilayer with numerous protein nodes of two types. The first node contains ankyrin, spectrin and the tetramers of band 3 integral protein. The second node includes band 4.1 protein bound to the spectrin-actin complex and glycophorin C integral protein. The structural alterations of above mentioned membrane proteins results in hemolytic pathologies (membranopathies), like hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and others.

Dielectric spectroscopy is a helpful method for studying dielectric polarization and dynamics of polar macromolecules (Pethig and Kell, 1987; Kuang and Nelson, 1998; Klösgen et al., 2011). We used this method to investigate molecular dynamics and dielectric polarization of erythrocyte under-membrane spectrin skeleton, based on following considerations. The complex impedance and capacitance of suspensions, containing either native erythrocytes or their resealed ghost membranes, depends on the dielectric properties of suspension medium and suspended erythrocytes. During heating, the dielectric properties of medium change in a well predictive manner, while these of erythrocytes sustain threshold changes related to the thermal denaturations of major membrane proteins, spectrin and band 3.

Upon heating spectrin denatures at 49.5 °C (T_A) (Brandts et al., 1977). Recently this denaturation at T_A was registered as a sigmoid, threshold changes in the complex impedance (ΔZ_{re} and ΔZ_{im}) and capacitance (ΔC_{re} and ΔC_{im}) of heated suspensions, containing either native erythrocytes or resealed ghost membranes (Ivanov et al., 2012; Ivanov, 2010).

As the dielectric activity of denatured spectrin is null, the sigmoid changes in dielectric parameters of tested suspensions at T_A (ΔZ_{re}, ΔZ_{im}, ΔC_{re} and ΔC_{im}) were assumed to express the dielectric properties of native spectrin network. Indeed, the frequency dependences of these changes were found to reflect the dielectric polarization of intact spectrin network and its attachment to the lipid bilayer (Ivanov and Paarvanova, 2016). The aim of this study was to use thermal dielectroscopy for evaluating some alterations in erythrocyte membrane related to the anemia of hereditary type.

MATERIALS AND METHODS

Materials. DNAase I (deoxyribonuclease I), $MgCl_2$, NaCl, mannitol and phosphate buffer were purchased from Sigma Chemicals Co, St. Louis, MO, USA.

Isolation of human erythrocytes. Human erythrocytes were isolated by centrifugation (1000 x g, 5 min) of freshly collected heparinized blood from the clinical laboratory of Thracian University, Medical Faculty, Stara Zagora, Bulgaria. After precipitation of erythrocytes, the plasma and upper layer of white blood cells were removed. Prior to use, erythrocytes were washed three times in a large volume of cold isotonic solution of 10 mM NaCl and mannitol.

Preparation of erythrocyte ghost membranes. Cold (1 °C) suspension of washed erythrocytes, hematocrit 0.80, was vigorously diluted in 15 volumes of 1 °C-cold hypotonic solution, containing 2 mM $MgCl_2$ and 5 mM phosphate buffer, pH 7.8 and left at cold for 5 min. A proper volume of cold 0.75 M NaCl was added to the hemolysate in order to obtain the final concentration of 70 mM NaCl in medium. The membranes were resealed (37 °C, 20 min) and isolated (4000 x g, 12 min). Prior to usage the resealed membranes were once washed in excess (1:20) volume of 5 mM NaCl and 150 mOsm mannit.

Deoxyribonuclease I (DNAse I) is actin-specific enzyme causing depolymerization of actin polymers (Sheetz, 1979; Nakashima and Beutler, 1979). To allow DNAse I to depolimerize actin and dissociate the spectrin-actin link the enzyme was incorporated in the erythrocyte ghost membranes during the hemolysation step. Two types of resealed erythrocyte ghost membranes were prepared, one with and one without DNAse I, and the results were compared.

Thermal and frequency analysis of the complex impedance, Z* and complex capacitance, C* of suspensions (Ivanov, 2010). The sample suspensions contained either washed erythrocytes or resealed erythrocyte ghost membranes, suspended in the last washing medium at hematocrit 45 %. Using a syringe the sample suspension (volume 70 μl) was injected into a conductometric cuvette. The latter was made up of a glass tube (length 120 mm, outside diameter 4 mm, wall thickness 0.5 mm) containing two platinum electrodes spaced at about 4 mm. The cuvette was tightly inserted in a hole drilled in an aluminium block which was heated at a constant rate (2.0 °C/min). At this heating rate the membranes retain its impermeability to ions in the temperature range of 37 to 56 ° C (Muravlyova et al., 2013).

During the heating, an alternating voltage of 150 mV was applied between the electrodes. The complex impedance, $Z^* = Z_{re} + j.\ Z_{im}$, and capacitance, $C^* = C_{re} - j.\ C_{im}$, of tested sample were continuously measured and separated into their real (Z_{re}, C_{re}) and imaginary (Z_{im}, C_{im}) parts using Solartron 1260 Impedance Frequency Analyzer (Schlumberger Instruments, Hampshire, England) controlled by a computer equipped with Miniscan software. The Z* and C* values were measured at 16 frequencies between 0.01 and 12 MHz scanned sequentially with an integration time of 0.5 s. Here, j is the imaginary unit, $j^2 = -1$. The resistance, Z_{re}, irreversibly dissipates the energy of electric field as heat in the sample, while the electric energy stored in the sample is represented by the reactance, Z_{im}. Capacitance, C_{re}, represents the ability of sample to store charges, while the imaginary capacitance, C_{im}, is proportional to the power with which the field dissipates energy to

move the free ions (conductive loss) and rotate the dipoles (dielectric loss).

In the low salt suspensions of cells, alternating electric field aligns the intrinsic and induced dipoles of all types producing dielectric polarization. In general, the real and imaginary parts of Z^* and C^* are frequency-dependent, the frequency-dependence of which may be used to describe the inertia of dipoles involved. With increasing the frequency, f, each dielectric polarization is abolished (relaxed) above a specific critical frequency, f_{cr}, which is inversely related to the relaxation time constant of dipoles. In the case of dipole relaxation with a single critical frequency (relaxation of Debye type), the implicit $-Z_{im}$ vs Z_{re} dependence (Nyquist plot) has the typical form of a semicircle the top point of which corresponds to the critical frequency of relaxation, f_{cr} (for example Fig. 2). Due to the same reason the C_{im} vs C_{re} dependence (Cole-Cole plot) represents a semicircle with top point corresponding to the same f_{cr} (for example Fig. 3).

The change in the real part of capacitance, ΔC_{re}, in T_A, was determined by subtracting the suspension capacitance at the denatured state (3 °C after T_A) from the suspension capacitance in the native state of spectrin (3 °C prior T_A). This change was corrected, taking into account the overlapping of the thermally induced change in the conductivity of suspension in the given temperature range. The remaining changes in the real and imaginary part of the impedance and the capacitance (ΔC_{im}, ΔZ_{re} and ΔZ_{im}) at T_A were determined and corrected in the same way. These changes (ΔC_{re}, ΔC_{im}, ΔZ_{re} and ΔZ_{im}) quantify the dielectric polarization of the spectrin undermembrane network in the native state of the spectrin because in the denatured state the spectrin network does not exhibit dielectric polarization.

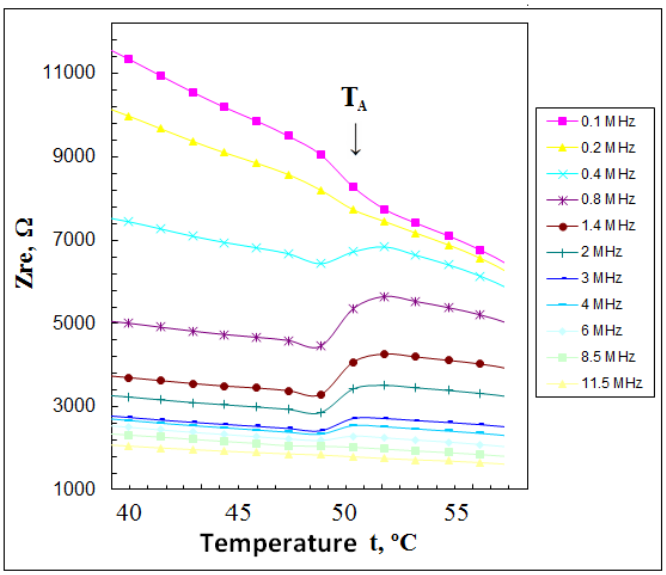

Fig. 1. *Thermal profile (thermogram) of the real part of impedance, Z_{re}, of suspension, obtained at the indicated frequencies. Shown is the effect of spectrin denaturation at T_A on these thermograms. The suspension contains either erythrocytes or resealed erythrocyte ghost membranes.*

RESULTS AND DISCUSSION

Dielectric parameters of heated suspensions sharply changed at T_A, the spectrin denaturation temperature, as exemplified by the thermograms of suspension resistance in Fig. 1. Using these thermograms as raw data, the sigmoid changes in ΔZ_{re}, ΔZ_{im}, ΔC_{re}, and ΔC_{im} at T_A were further determined, corrected and subjected to frequency analysis, as explained in the Materials and

methods section and in previous reports (Ivanov and Paarvanova, 2016).

The dependence of - ΔZ_{im} on ΔZ_{re}, known as Nyquist or impedance plot, was practically the same when tested suspension contained either erythrocytes or resealed erythrocyte ghost membranes (Fig. 2). This underlined the predominant role of plasma membrane in determining the dielectric properties of tested suspensions. The implicit frequency dependence between - ΔZ_{im} and ΔZ_{re} was expressed by two semicircles - one above the abscissa axis, and a second one below this axis. According to the theory of dielectroscopy each semicircle revealed separate dielectric relaxation involving the dipoles on spectrin-actin skeleton.

The second relaxation occurs at high frequencies (between 1.0 and 12 MHz) when the electric field penetrates into erythrocyte cytoplasm and indicates direct interaction of the field with spectrin tetramers (Ivanov and Paarvanova, 2016).

The first relaxation occurs at low frequencies (between 0.05 and 1 MHz) and mirrors the charge accumulation on both sides of the lipid bilayer (Maxwell-Vagner effect). It involves the two attachment sites of spectrin network to the lipid bilayer because the specific disconnection of any of these attachment sites reduces the radius of the first semicircle (Ivanov and Paarvanova, 2016). This effect is demonstrated in Fig. 2 (left) on erythrocyte ghost membranes resealed with DNAse I. The strong reduction of the radius of first relaxation could be related to the presence of DNAse I which specifically depolymered actin destroying the attachment site which involves the protein 4.1 - spectrin - actin complex and glycophorin C.

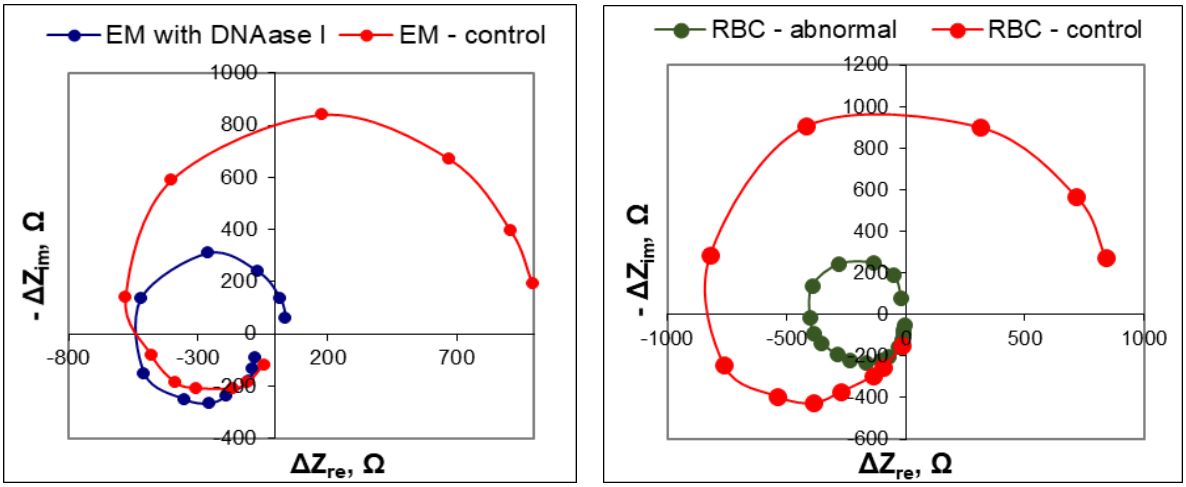

Fig. 2. *Impedance plot showing the dependence of - ΔZ_{im} on ΔZ_{re} at the denaturation temperature of spectrin. Left: the suspension contained erythrocyte ghost membranes resealed with and without DNAase I. Right:the suspension contained either erythrocytes from a healthy donors (n=30) or abnormal erythrocytes obtained from a patient with membranopathy (n=1).*

We have studied erythrocytes from more than 30 healthy blood donors and obtained approximately the same impedance plot in all cases (Fig. 2, right). There was, however, a strong exception with the erythrocytes of a single patient. Compared to control erythrocytes the impedance plot of latter erythrocytes (Fig. 2, right) differed by its strongly reduced radius of the first semicircle just like the impedance plot of healthy erythrocyte ghost membranes resealed with DNAse I (Fig. 2, left). This allows us to assume that this patient could have abnormal erythrocytes with hereditary membranopathy associated with a defect in the spectrin-actin-protein-4.1-glycophorin C node. It is reasonable to assume that such a defect should tolerate the approachment of cytoskeleton to the

lipid bilayer resulting in substantial immobilization of spectrin filaments. This assumption is supported by the large suppression of the changes in ΔZ_{im} and ΔZ_{re} at low frequencies (Fig. 2, right).

The difference between the dielectric properties of spectrin-actin skeletons of healthy erythrocytes and the abnormal erythrocytes of tested patient was even stronger expressed by Cole-Cole plot (Fig. 3 left). This plot revealed only the gamma relaxation on spectrin since electrode polarization impeded the measurement of suspension capacitance at frequencies below 100 kHz. As shown in Fig. 3, the gamma relaxation on the spectrin of patient's erythrocytes was largely suppressed indicating strong immobilization of spectrin-actin skeleton.

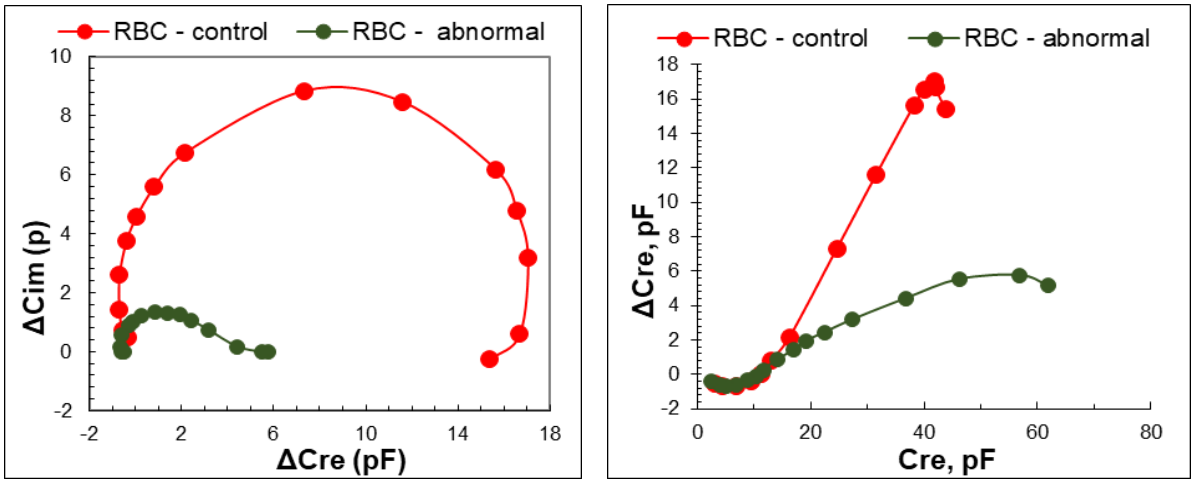

Fig. 3. *Left: capacitance plot of erythrocytes showing the dependence of ΔC_{im} on ΔC_{re} at the denaturation temperature of spectrin. Right: relationship between the capacitance of erythrocyte suspension, C_{re}, and its change, ΔC_{re}, at the denaturation temperature of the spectrin. The suspension contained either erythrocytes from a healthy patients (red circles) or abnormal erythrocytes obtained from a patient with membranopathy (green circles).*

At large hematocrit values (45 vol % in our study), the suspension capacitance, C_{re}, and its change, ΔC_{re}, at the spectrin denaturation temperature are entirely determined by the capacity of plasma membrane of suspended erythrocytes. Hence, useful information could be obtained comparing the ΔC_{re} vs C_{re} dependence of healthy erythrocytes and of patient's erythrocytes, as shown in Fig. 3 (right). We see that reducing the frequency the C_{re} increased, reaching almost equal values with the control and abnormal erythrocytes. The ΔC_{re} also increased with C_{re}, however, at much stronger rate with control erythrocytes while the increase was faint with abnormal erythrocytes. This result also indicates that, compared to control erythrocytes, substantial portion of spectrin-actin skeleton of abnormal erythrocytes was immobilized.

CONCLUSION

The presented results show that the method of thermal dielectroscopy can be used for mass screening and diagnosis of hemolytic anemias of hereditary membranopathy type. The method delivers a lot of information for the spectrin-actin skeleton and its attachment to the lipid bilayer of erythrocyte membrane whose congenital alteration represents the molecular basis of this type of anemia.

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