DIELECTRIC RELAXATIONS ON THE MEMBRANE SKELETON OF HUMAN AND HEN ERYTHROCYTES

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Summary. Plenty of serious diseases are associated with worsening of blood rheological parameters, which largely depend on erythrocytes and their plasma membrane. In this regard the molecular mobility of spectrin-based under-membrane skeleton (MS) of erythrocytes plays a major role. Important data on this mobility can be obtained studying dielectric relaxations (DR) on MS.

Aim. To compare DRs on MS of nucleated hen and enucleated human erythrocytes.

Materials and Methods. Human and hen erythrocytes, suspended in isotonic low salt mannitol media, were pre-cooled (4°C, 10 hours) and heated. At the spectrin denaturation temperature (49.5 °C with human and 54.5 °C with hen erythrocytes), the complex impedance of suspension sustained change, $\Delta Z^* = \Delta Z_{re} + j\Delta Z_{im}$, which was assumed a measure for the intrinsic dielectric polarization of MS. The ΔZ_{re} and ΔZ_{im} were determined at 16 frequencies and corrected for their temperature dependence of conductivity. For human erythrocytes the complex plane (Nyquist) plot of $-\Delta Z_{im}$ against ΔZ_{re} reveals two DRs on MS, one with low and another one with high critical frequency (Ivanov and Paarvanova, Bioelectrochemistry, 2016 (110), 59-68).

Results. The pre-cooling of hen erythrocyte and not of human erythrocytes was obligatory in order to register the low and high frequency DRs on MS. While the high frequency DR was the same with human and hen erythrocytes, strong species differences were found in the low frequency DR which depends on the attachment of MS to the lipid bilayer.

Conclusion. Thermal dielectroscopy is useful method for investigation of MS and its attachment.

Keywords: *erythrocyte membrane, membrane skeleton, attachment, dielectric relaxation.*

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1. Introduction. Eventhough mammal and avian erythrocytes have almost identical plasma membrane and submembrane skeleton (MS) of peripheral proteins they both have different shapes and mechanical behaviour. In contrast to the enucleated, biconcave mammal erythrocytes, the nucleated bird erythrocytes look like flattened ovoids. Moreover, while the former have extreme deformability and elasticity the latter are rigid and practically non-deformable. These differences originate from the fact that avian erythrocytes possess cytoplasmic cytoskeleton connecting their plasma membrane to the nucleus (Fig. 1).

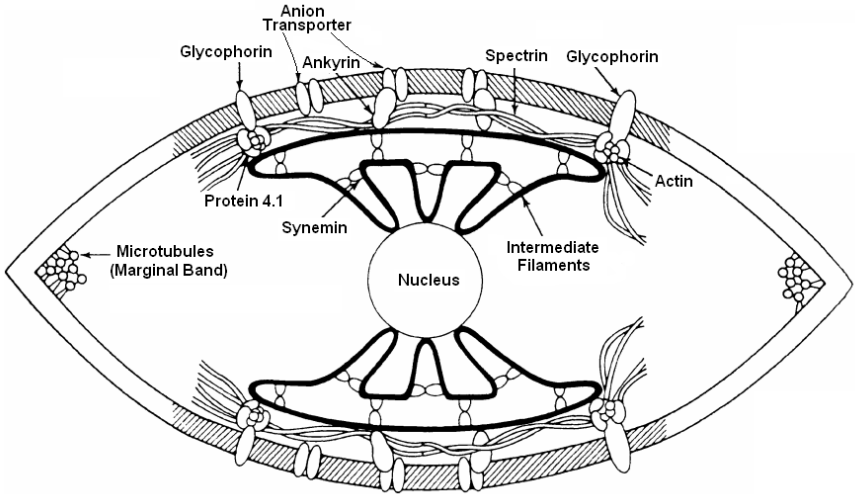

Fig. 1. Schematic drawing of the arrangement of proteins forming the cytoskeleton of a chicken red blood cell. From ref. [1].

The cytoskeleton of avian erythrocytes includes three distinct components; the MS, marginal band, and intermediate filaments (Fig. 1). The marginal band is a hooplike bundle of continuous subplasmalemal microtubules that encircle the cell like a belt in a single plane of the flat surface of the cell [2]. It is confined to the periphery of the cell and contains essentially the same number of microtubule profiles in each individual cell. Tubulin is its major molecular component. The MS of avian erythrocytes is an interwoven network of spectrin with foci composed of actin olygomers and band protein 4.1. It is bound to the cytoplasmic surface of the plasmalemma by interaction of protein 4.1 and the intrinsic red cell membrane protein glycophorin, and interaction of spectrin-bound ankyrin and band 3 protein. Spectrin forms a lattice of tetramers composed of two heterodimers. The MS lines and interfaces with the inner surface of the entire plasmalemma. It completely encloses and is in close contact with the marginal band.

The spectrin-based MS of human erythrocytes is structurally very similar to that of avian erythrocytes. Plenty of serious diseases in human are associated with the worsening of elasticity and deformability of human erythrocytes [3], which largely depend on erythrocyte MS and its attachment to the lipid bilayer. Based on above reasons the conformational flexibility and intramolecular segmental mobility of spectrin network is much better studied in human and mammal erythrocytes than in bird

erythrocytes [4].

With this in mind we compared the dielectric relaxations (frequency dependence of intrinsic dielectric polarization) on the MS of hen and human erythrocytes. The intrinsic polarization of MS was determined as a difference between the complex impedances of erythrocytes prior to and after the denaturation of spectrin. The basic method of study was differential dielectroscopy, combined with thermal denaturation of spectrin. Two dielectric relaxations on the MS of human erythrocytes were registered which sensed the segmental mobility of native MS and its attachment to the lipid membrane [5]. This study reports that compared to human erythrocytes, the spectrin of hen erythrocytes had substantially lower segmental mobility and different, presumably milder, attachment to the lipid membrane.

2. Materials and methods.

2.1. Materials. NaCl, mannitol and phosphate buffer were purchased from Sigma Chemicals Co, St. Louis, MO, USA.

2.2. Isolation of erythrocytes. Human and hen erythrocytes were isolated by centrifugation (250 x g, 5 min) from freshly drawn heparinized blood. The upper layer of white blood cells was discarded. Erythrocytes were next washed two times in excess cold isotonic solution of 280 mM mannitol containing 5 mM NaCl and 5 mM phosphate buffer, pH 7.4. The tested suspensions were prepared suspending washed erythrocytes in the indicated washing medium, hematocrit 0.45. Prior to usage the suspensions were incubated in a refrigerator at 4°C for 10 hours.

2.3. Thermal analysis of the complex impedance, Z, of sample suspensions* [5]. The tested suspension, volume 0.07 μl, was brought out of the refrigerator and immediately heated with high heating rate of 3.0 °C /min in order to denature spectrin. At this heating rate the membrane impermeability to ions was maintained in the temperature interval of 37-56°C [6]. During the heating of the suspension its complex impedance, $Z^* = Z_{re} + j.\ Z_{im}$, was continuously measured and separated into its real (Z_{re}) and imaginary (Z_{im}) parts. 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 reversibly stored in the sample is represented by the reactance, Z_{im}.

As shown in Fig. 2, both dielectric parameters, Z_{re} and Z_{im}, exhibit sharp sigmoid changes at the temperature of spectrin denaturation, T_A [7]. The magnitude of the detected change in Z_{re} at T_A was initially defined as $\Delta Z_{re} = (Z_{re})_{\mathrm{native}} - (Z_{re})_{\mathrm{denatured}}$, where $(Z_{re})_{\mathrm{native}}$ and $(Z_{re})_{\mathrm{denatured}}$ are the real part of suspension impedance at the native state of spectrin (at a temperature 3°C less than T_A) and at the denatured state of spectrin (at a temperature 3°C greater than T_A), respectively. This change, however, levels off within a substantial temperature interval of about 6°C where it is superimposed on the continuous, thermally induced variation of electrolyte conductivity. To compensate for the temperature effect on conductivity, which had no relation to the temperature-induced denaturation at T_A, the change in Z_{re}, taking place over an equal temperature interval prior the denaturation at T_{A}, was similarly calculated and subtracted from the initial ΔZ_{re}. The change in Z_{im} at T_{A}, i.e., ΔZ_{im}, was likewise determined and corrected.

2.4. Dielectric relaxations on spectrin-based membrane skeleton [5]. As spectrin network is dielectrically inactive at its denatured state, the ΔZ_{re} and ΔZ_{im} changes are assumed to represent the intrinsic dielectric polarization of native MS, i.e., the contribution of intact spectrin network to the dielectric properties, resistance and reactance, of native erythrocyte membrane [5, 8]. This contribution

is studied analyzing the $-\Delta Z_{re}$ vs ΔZ_{im} dependence (Nyquist plot) according to the methods of dielectric spectroscopy [9].

For human erythrocytes the complex plane (Nyquist) plot of $-\Delta Z_{im}$ vs ΔZ_{re} exhibits two semicircle arcs, one above the abscissa axis and another one below this axis (Fig. 3). Each semicircle arc corresponds to a dielectric relaxation of Debye type (ie, having a single critical frequency, f_{cr}) on erythrocyte plasma membrane. The first dielectric relaxation is detected at low frequencies (0.05 - 1.0 MHz) which, at their initial interval, do not allow penetration of the field into cytosole. Its amplitude is strongly subdued after severance of the two attachment sites between spectrin skeleton and integral proteins. This relaxation is assumed to involve a direct piezoeffect on flexible spectrin filaments. The piezoeffect is driven by a mechanical force which originates from the frequency-dependent charging (Maxwell-Vagner effect) of lipid membrane and is applied on spectrin through the attachment sites and direct cohesion between spectrin and lipid bilayer. The second dielectric relaxation is detected at frequencies (1.0 - 10 MHz) which allowed the field to penetrate through the membrane and interact directly with spectrin dipoles. Its critical frequency has the value of 2.5 MHz.

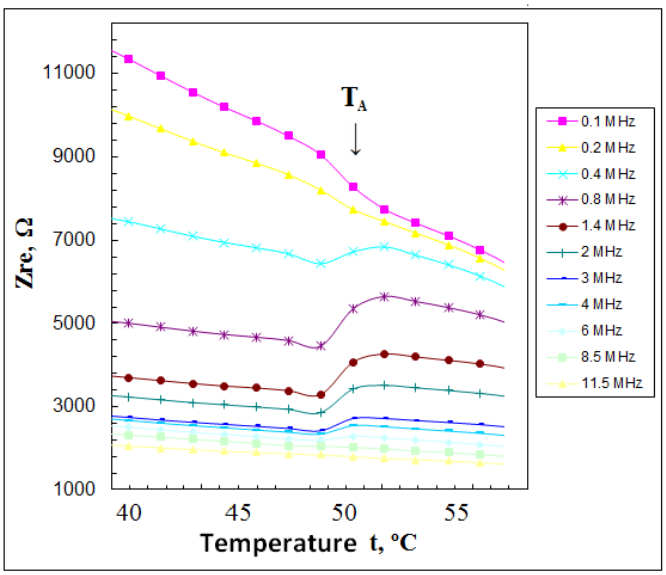

Fig. 2. Effect of frequency on the temperature profile of the real impedance, Z_{re}, of human erythrocyte suspension. Arrow indicates the denaturation temperature, T_A, of spectrin. The hematocrit and heating rate were 0.50 and 3°C/min, respectively. The washing and suspension media were isotonic solution of 280 mM mannitol containing 5 mM NaCl and 5 mM phosphate buffer, pH 7.4.

Results. Fig. 2 shows the temperature profile of the real impedance, Z_{re} of human erythrocytes, washed and suspended in low salt isotonic mannitol medium. As previously reported, Z_{re} sustains sharp frequency-dependent change due to the thermal denaturation of spectrin at 49.5°C (T_A). Similar set of curves was obtained for the reactance, Z_{im} (not shown). Heating pre-cooled suspension of hen erythrocytes, similar set of curves were yielded for the temperature dependence of Z_{re} and Z_{im}, obtained at the indicated frequencies (not shown). For hen erythrocytes the sharp changes in Z_{re} and Z_{im} took place at 54.5°C as previously reported [10]. As inspected under microscope, the hen erythrocytes did

not alter their flat ovoid shape during the change of complex impedance at the spectrin denaturation temperature. The omission of pre-cooling did not affect the changes in Z_{re} and Z_{im}, obtained with human erythrocytes, however, these changes were totally absent with hen erythrocytes (not shown).

Using above temperature curves (Fig. 2) as basic data, the respected changes in Z_{re} and Z_{im} at the temperature of spectrin denaturation were determined and corrected as explained in the Materials and methods part, section 2.3. The obtained complex plain (Nyquist) plots of $-\Delta Z_{im}$ vs ΔZ_{re} for hen and human erythrocytes are compared in Fig. 3.

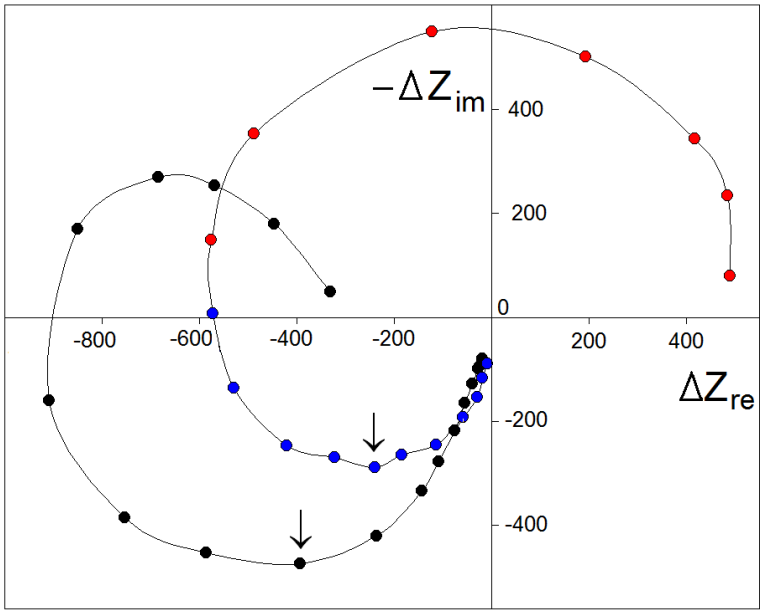

Fig. 3. Complex impedance (Nyquist) plot of erythrocyte suspension at the temperature of spectrin denaturation. Shown is the $-\Delta Z_{im}$ vs. ΔZ_{re} dependence, where ΔZ_{im} and ΔZ_{re} are the corrected changes in imaginary and real components of complex impedance, associated with the denaturation of spectrin. Suspension contained erythrocytes of human (colored circles) and hen (●). Arrow indicates the critical frequency of dielectric relaxation on spectrin segments. Other details as for Fig. 2.

The Nyquist plot for human erythrocytes had the same form as that previously reported [5]. It contains the above mentioned two arcs, the low frequency one (red circles) and the high frequency one (blue circles). Both arcs had a perfect semicircle shape as their horizontal diameters were exactly twice greater than their vertical radiuses (Fig. 3). The latter feature is characteristic for the dielectric relaxations of pure Debye type, ie, having a single critical frequency, f_{cr} (single relaxation time, $\tau = 1/(2\pi f_{cr})$).

Fig. 3 shows that both semicircle arcs, respectively both dielectric relaxations are present in the Nyquist plots of hen and human erythrocytes. The high frequency dielectric relaxation of human erythrocytes involves segmental motions of spectrin filaments elicited by the direct interaction of electric field with MS. The critical frequency of this relaxation was 2.5 MHz with human erythrocytes and 1.5 MHz with hen erythrocytes (Fig. 3).

The low frequency dielectric relaxation of human erythrocytes involves spectrin filaments and their attachment sites to the lipid membrane. Substantial differences between its semicircle arc in human and hen erythrocytes could be indicated. With hen erythrocytes this semicircle arc occupied the second quadrant only. In addition, it had strongly reduced amplitude indicating significantly lower changes in membrane resistance and reactance (Fig. 3). Exactly the same depressed shape and reduced magnitude of the low frequency arc have been reported with human erythrocytes in case their spectrin-actin-glycophorin binding site was severed through specific depolymerization of actin by DNA-ase I [5]. Thus, it could be assumed that in respect to human erythrocytes the attachment of MS of hen erythrocytes to the lipid membrane has different, possibly milder nature.

3. Discussion. Upon the denaturation of spectrin, the resistance (Z_{re}) and reactance (Z_{im}) of erythrocyte plasma membrane both change. Based on these changes and using the methods of dielectroscopy two dielectric relaxations on the spectrin-based MS of human erythrocytes have been recently reported [5]. The first relaxation engaged the region between 10 kHz and 1.0 MHz and had low critical frequency. The second one had higher critical frequency. This study reports two similar dielectric relaxations on the MS of pre-cooled hen erythrocytes. The pre-cooling of hen erythrocytes was obligatory in order to yield changes in Z_{re} and Z_{im} at the spectrin denaturation temperature, while it was not needed for human erythrocytes. This difference could be possibly associated with the disintegrating effect of low temperatures (4°C) on the microtubules of marginal band of bird erythrocytes [2, 11].

The high frequency dielectric relaxation of pre-cooled hen erythrocytes was quite similar to that in human erythrocytes. It had a critical frequency of 1.5 MHz, compared to 2.5 MHz with human erythrocytes. Similar value, based on the Cole-Cole plot of the relative changes in complex capacitance at the spectrin denaturation temperature, was recently reported for pre-cooled hen erythrocytes [10]. These finding indicate substantially reduced segmental mobility of spectrin filaments in hen MS, compared to that in human erythrocytes.

The results indicate substantial species differences concerning the low frequency relaxation on MS which involves the attachment of MS to the lipid membrane. Hence, the dielectric changes at the spectrin denaturation temperature evidence how the attachment of native MS to the lipid membrane affects the barrier properties (Z_{re}) and the reversible energy storage part (Z_{im}) of erythrocyte membrane. For human and hen erythrocytes the denaturation of spectrin reduced the reversible energy storage part (Z_{im}) possibly due to the elimination of piezoelectric effect on MS. This reduction of Z_{im} was much greater with human erythrocytes compared to the hen erythrocytes (Fig. 3). Hence, we conclude the energy storage part and the attachment strength were substantially lower in the membrane of hen erythrocytes, compared to human erythrocytes.

For human erythrocytes, when the electric field remained outside cells ($f < 0.3$ MHz) the denaturation of spectrin reduced Z_{re} indicating positive effect of the native MS on the barrier properties of erythrocytes. Surprisingly, at such conditions the Z_{re} of hen erythrocytes was increased indicating negative impact of native MS on the barrier properties. The latter outcome could be due to the weaker attachment of MS in hen erythrocyte membrane.

4. Conclussion. The obtained results indicate that there are species differences in the segmental flexibility and in the attachment of spectrin-based membrane skeleton of human and avian erythrocytes.

In combination with specific biochemical methods and appropriate models the experimental approach used allows future studies to get insight into the molecular mechanisms underlying these differences.

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