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Original article

THERMAL DIELECTROSCOPY STUDY OF SUBMEMBRANE SPECTRIN NETWORK IN ANIMAL ERYTHROCYTES

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Summary

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The shape and mechanical properties of mammalian erythrocytes depend on the conformational flexibility of their submembrane spectrin network. By contrast, avian erythrocytes are ovoid and non-deformable even though they also have such spectrin network. Using thermal dielectroscopy we isolated and studied erythrocytes from domestic mammals (cow, horse, dog and goat) and birds (chicken, turkey, pigeon and duck). Both erythrocyte suspension resistance and capacitance changed abruptly at the spectrin denaturation temperature, T_d, determined with standard deviation of ± 0.2 °C. The frequency dependence of these changes occurring at T_d, revealed the dielectric relaxation of dipoles, associated with the spectrin network. The critical frequency, f_c, of this relaxation reflected the segmental mobility of spectrin while the T_d corresponded to the thermal stability of spectrin. The spectrin network was more flexible and thermally labile in mammalian erythrocytes (f_c and T_d were 2–2.5 MHz and 50±1°C, respectively, compared to 0.6–1 MHz and 54±1 °C in avian erythrocytes). The species-related differences in T_d and f_c between mammals and birds were attributed to the cytoskeleton which existed in avian but was absent in mammalian erythrocytes.

Key words: dielectric spectroscopy, dipole polarisation, erythrocyte membrane, spectrin denaturation, undermembrane network

INTRODUCTION

Mammalian erythrocytes (red blood cells) are an important determinant of the rheological properties of blood because of their quantity, extreme deformability and elasticity, and aggregation tendency.

A mature mammalian red blood cell has three main components: lipid membrane, undermembrane network of peripheral proteins, and fluid cytoplasm. Major determinants of erythrocyte deformability are the rheological traits of the plasma membrane and intracellular fluid as well as the surface to volume ratio (Shin *et al.*, 2007). Among them the primary factor is

the deformability of erythrocyte membrane which depends on the viscoelastic properties of the submembraneous protein network and its association to the bilayer (Picart *et al.*, 2000).

A major constituent of the submembrane protein meshwork is the filamentous protein spectrin that comprises about 30% (w/w) of membrane protein content (Liu *et al.*, 1990). The meshwork is attached to the lipid membrane through transmembrane proteins (Marchesi, 2008). The interaction of the spectrin-based protein network with the cytoplasmic surface of the membrane controls the elasticity of the bilayer membrane and erythrocyte shape. Spectrin self-associates into tetramers that are in turn coupled to the short filaments of actin. The spectrin and actin attachment is stabilised by the protein band 4.1 (Ungewickell *et al.*, 1979). The association between submembrane protein network and the bilayer is mediated by band 4.1, which connects spectrin to glycophorin (Mueller & Morrison, 1981), and by ankyrin which connects spectrin to the anion exchanger (Bennett & Stenbuck, 1979). Upon direct stretching, spectrin filaments behave like stiff, non-linear springs (Rief *et al.*, 1999). In addition, the submembrane spectrin network possesses high conformational flexibility and segmental mobility that strongly contribute to the unique biconcave shape and mechanical properties of mammalian erythrocytes. Any disturbance in the structural constituents and conformational flexibility of submembrane network leads to the alteration of shape and impairment of mechanical function of the erythrocyte membrane.

Avian elliptocytic erythrocytes also have sub-membrane protein network, analogous to that in mammalian erythrocytes, the main component of which is the fibrillar protein spectrin (Granger & Lazarides, 1982). Alhough mammalian and avian erythrocytes have almost identical membrane structure and submembrane spectrin meshwork, they have different shapes and mechanical behaviour. In contrast to the enucleated, flat, biconcave-shaped mammalian 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, in addition to their undermembrane spectrin network, the avian erythrocytes possess cytoplasmic cytoskeleton tightly connecting their plasma membrane to the nucleus. Based on above reasons, the conformational flexibility and thermal stability of spectrin network is much better studied in human and mammalian than in bird erythrocytes (Chakrabarti *et al.*, 2006).

The major protein of the human erythrocyte membrane, spectrin, unfolds at 49.5 °C (T_d) (Brandts *et al.*, 1977) producing concomitant discocyte-spherocyte transformation and radical deterioration of the mechanical properties of erythrocytes (Kucera *et al.*, 1986). Heating suspensions of human erythrocytes and isolated erythrocyte membranes, a threshold decrease in the electric capacitance and resistance has been detected at a temperature that tends to the same T_d value at heating rates projected to zero (Ivanov, 2010; Ivanov *et al.*, 2012). Several lines of evidence implicated the thermal denaturation of spectrin in these changes (Ivanov, 1997) and at the same time rejected the possible explanation of these changes based on the accompanying shape transformation (Ivanov *et al.*, 2012). Using several chemical agents which specifically cross-link spectrin net-

work of human erythrocyte membrane these changes were recently ascribed to the collapse of the dielectric polarisation of spectrin which takes place almost instantaneously during the spectrin denaturation (Ivanov *et al.*, 2012).

In this study, applying the same method of thermal dielectroscopy, we compared the thermal stability and conformation flexibility of erythrocyte spectrin network in mammals and birds. With this in mind we studied how the dielectric erythrocyte membrane polarisation depends on temperature and frequency. To subtract the dielectric polarisation of spectrin network from that of the whole membrane we denatured spectrin at T_d and determined the difference. The thermal stability of spectrin network was represented by the T_d and the conformational flexibility was assessed by the critical frequency, f_c, for relaxation of the dielectric polarisation of spectrin network.

MATERIALS AND METHODS

Mammalian and bird erythrocytes were isolated from fresh blood of mammals (2 cows, 1 horse, 3 dogs, 1 goat, 2 sheeps, 1 pig and 2 rabbits) and birds (3 chickens, 2 turkeys, 1 pigeon and 1 duck) housed in the experimental farms of the Trakia University, Stara Zagora, Bulgaria. About 0.5 mL heparinised blood was obtained from each animal and washed with 3 mL isotonic solution containing 10 mM NaCl, sucrose and 5 mM glucose. The erythrocytes were immediately isolated by centrifugation (3000×*g*, 5 min) after carefully removing the upper layer of white blood cells. Mammalian red blood cells were isolated likewise except that more extensive washings were provided. The isolated cells were immediately used. At least two samples of erythrocytes from each animal were tested.

About 50 μL of washed erythrocytes were resuspended in the washing solution, haematocrit 0.50. The suspension was heated from 20 to 60 °C at 3.0° C/min heating rate in a sample cuvette as previously described (Ivanov, 2010). During heating the complex admittance (Y*) and capacitance (C*) of the suspension were measured and separated into their real and imaginary parts allowing determination of the suspension resistance (R) and capacitance (C). The core instrument was a Solartron 1260 Impedance Analyzer (Schlumberger Instruments, Hampshire, England) interfaced to Toshiba PC using the Miniscan software. The device removes the noise and harmonic distortions and has 0.1% accuracy and 0.001 dB resolution. Y* and C* were measured at the indicated frequencies between 0.05 and 13 MHz, scanned serially with integration time of 0.5 s. At these frequencies the suspension method secured that C depended mainly on the capacitance of erythrocyte membranes including the capacitance of submembrane spectrin network (Pethig & Schmueser, 2012). The duration of each scan was less than 10 s. The amplitude of the generator output of the analyzer was set at 100 mV.

To eliminate the strong frequency dependence, due to the Maxwell-Wagner-Sillars interfacial polarisation of erythrocyte membranes (Pethig & Kell, 1987), the change in C detected at T_d was expressed as $\Delta C/C_o$. Here $\Delta C=C_o–C_d$, where C_o is the suspension capacitance at the native state of spectrin (at a temperature 3 ^oC less than T_d) and C_d is the suspension capacitance at the denatured state of spectrin (at a temperature 3 °C greater than T_d). The change in the suspension resistance, R, is expressed likewise. Thus, the $\Delta C/C_o$ and $\Delta R/R_o$ exhibi-

ted the relative contribution of spectrin network to the dielectric polarisation and dielectric loss, respectively, of erythrocyte membranes considering this contribution nil at the denatured state of spectrin.

RESULTS

During the heating, suspension capacitance (C) did not vary until a denaturation temperature, T_d, was reached when it suddenly went down (Fig. 1). In repeated experiments with the samples of erythrocytes from a given species the T_d was determined with variability of ± 0.2 °C. Similar threshold change was registered for suspension resistance (R) at the same temperature (not shown). The temperature for the threshold changes in C and R depended on the heating rate. It was about 56.5 °C at 3.0 °C/min heating rate and about 54 °C at low heating rates (0.5 °C/min).

Chicken erythrocytes have a cytoplasmic skeleton that strongly stabilises their shape. As demonstrated on Fig. 2, chicken erythrocytes entirely preserved their shape after thermal denaturation (56 °C, 10 min) of their submembrane spectrin network. This finding supports

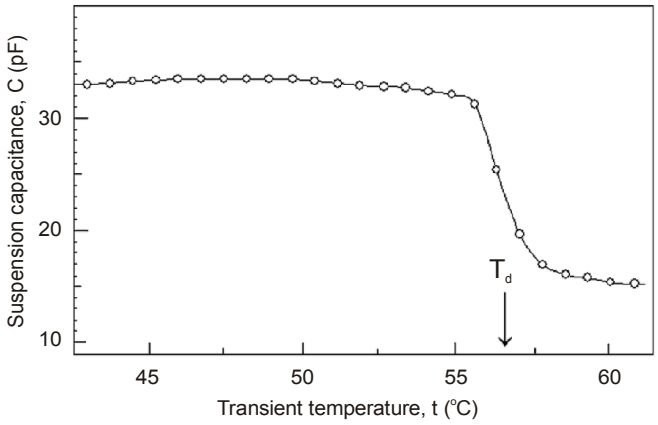

Fig. 1. Temperature profile of the capacitance (C) of suspension containing chicken erythrocytes. The suspension medium was isotonic solution of 15 mM NaCl and sucrose. The haematocrit, frequency and heating rate were 0.50, 0.1 MHz and 3°C /min, respectively. The denaturation temperature, T_d, of spectrin at this heating rate is indicated by arrow.

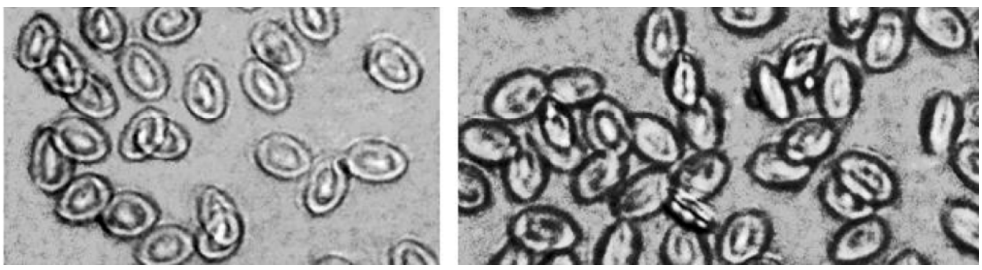

Fig. 2. Microphotographs of chicken erythrocytes at 20 °C (left panel) and after the thermal denaturation of spectrin by heating at 56 °C for 10 min (right panel).

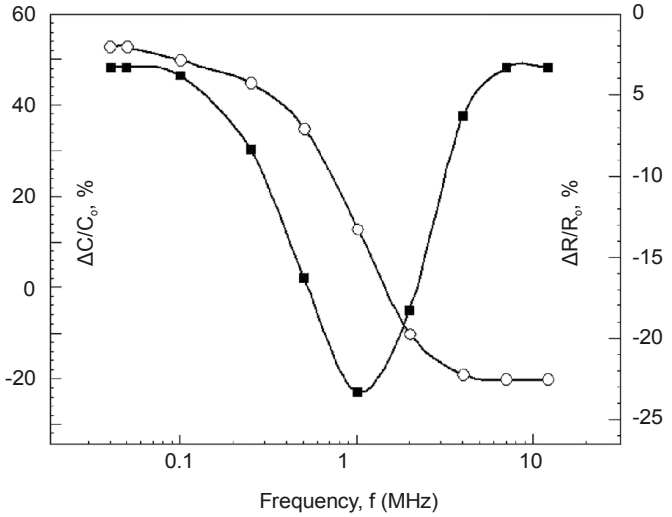

Fig. 3. Frequency dependence of the changes in hen erythrocyte suspension capacitance, $\Delta C/C_o$, (o) and resistance, $\Delta R/R_o$, (■), occurring at spectrin denaturation temperature, T_d. $\Delta C = C_o - C_d$, where C_o and C_d are the values of suspension capacitance prior to and after the denaturation of spectrin at T_d, respectively. The changes in suspension resistance at T_d are expressed likewise. Other details as for Fig. 1.

the thesis that the concomitant changes in C and R at T_d did not depend on cells' shape change, but possibly, were related to alteration in the passive electric properties of plasma membranes consequent to the denaturation of spectrin network.

In order to gain insight into this membrane alteration we obtained the frequency profiles, i.e., Bode representation, of the changes in C and R at T_d (Fig. 3). According to the data presented in Fig. 3, the changes in suspension capacitance and resistance ($\Delta C/C_o$ and $\Delta R/R_o$) were constant and positive within a broad interval of low frequencies. In this interval spectrin network increased the capacitance and decreased the dielectric loss of membranes. Above this interval the capacitive contribution went down and leveled off while the dielectric loss peaked at the same critical frequency, f_c, where the decrease in capacitive contribution was centered. This frequency behaviour is characteristic for the Debye-type dielectric relaxation processes. According to Fig. 3 the critical frequency, f_c, of this dielectric relaxation in chicken erythrocyte membrane was about 1.0 MHz.

Similar results were obtained with erythrocytes of other studied animal species: (cattle, horse, dog, goat, turkey, pigeon and duck) (data not shown). Apparently the dielectric polarisation of spectrin substantially contributed to the capacitance of plasma membranes and this contribution was eliminated upon the thermal denaturation of this protein network at a temperature, T_d, specific for the respective species. The critical frequency for the dielectric relaxation of spectrin network varied between 2.0 and 2.5 MHz with the tested mammalian erythrocytes and between 1.0 and 0.7 MHz with avian erythrocytes (data not shown). The denaturation temperature T_d

of spectrin was determined for erythrocytes of other species as well (Table 1). It

Table 1. Species variations in the critical temperature, T_d, for the threshold change in erythrocyte suspension capacitance (C), and resistance (R), during heating, compared to the mean body temperature of respective species. T_d is assumed representative for the denaturation temperature of spectrin of erythrocyte undermembrane network.

Species origin of erythrocytes	T_d of erythrocytes (°C)*	Mean body temperature of species (°C)
Man	49.5	37.0
Sheep	51.5	38.5
Cow	52.0	38.0
Pig	49.0	38.0
Goat	51.0	38.5
Dog	51.0	38.5
Horse	48.5	38.0
Rabbit	50.0	38.3
Chicken	54.5	40.5
Turkey	54.0	40.0
Pigeon	54.5	41.0
Duck	54.5	41.0

* the T_d values were obtained at various heating rates ranging from 3.0 to 0.5 °C/min for at least two samples of every species. Shown below are the values of T_d projected to zero rate of heating. For birds the body temperature corresponds to the daytime period (Laurila *et al.,* 2005).

could be seen that T_d, i.e., thermal stability of erythrocyte spectrin network was almost the same within the group of mammals (about 50 ± 1 °C) and substantially higher for birds (about 54 ± 1 °C).

DISCUSSION

Mammals and birds are warm-blooded, homeothermic animals with precise thermoregulation that maintains at a roughly constant level their internal body temperature regardless of the changes in ambient temperature. Mean body temperature exhibits species variations which are substantially higher in the group of birds compared to that in mammals. The middle of the range of typical body temperatures of mammals is about 38 °C while that of birds is about 40 °C and increases to 43 °C during flight (Prinziger *et al.*, 1991). According to Table 1 the T_d, i.e., thermal stability, of erythrocyte spectrin network was almost the same within the group of mammals (about 50 ± 1 °C) and substantially higher for birds (about 54 ± 1 °C). This is in contrast to the segmental mobility of erythrocyte spectrin, as represented by f_c, being greater in mammals compared to that in birds.

In these experiments, the temperature for the threshold changes in C and R depended on the heating rate. Using chicken erythrocytes it was determined to be 56.5 °C at 3.0 °C/min heating rate and about 54 °C at low heating rates (0.5 °C/min). This threshold temperature (about 54 ^{o}C at heating rates projected to zero) was close to the spectrin denaturation temperature of human erythrocytes (49.5 °C). In addition the frequency dependences of the changes in C and R, obtained with hen erythrocytes (shown below), were the same as those obtained with human erythrocytes (Ivanov *et al.*, 2012). Based on these arguments we assumed that the changes in C and R, detected with hen erythrocytes, also revealed the denaturation of submembrane spectrin network.

Recently a dielectric relaxation process was reported in the membrane of human erythrocytes, ascribed to dipoles related to their undermembrane spectrin network (Ivanov *et al.*, 2012). In this paper we present a comparative study on similar dielectric relaxation in the plasma membrane of mammalian and bird eryth-

rocytes. For the first time this relaxation was exhibited by the Bode dependence of the changes in the real and imaginary part of erythrocyte suspension impedance that took place at the spectrin denaturation temperature (Fig. 3). The obtained frequency behaviour reveals a Debye-type dielectric relaxation that considers the frequency response of an ideal, non-interacting population of dipoles to outside alternating electric field. As it was previously documented for human erythrocytes (Ivanov *et al.*, 2012) the latter dipoles were assumed bound to the spectrin network of tested erythrocytes.

In addition we evaluated species differences in the critical frequency, f_c, for this dielectric relaxation and the denaturation temperature, T_d, of erythrocyte spectrin. T_d and f_c are important as they correspond to the thermal stability and conformational flexibility, respectively, of the spectrin network in respective erythrocytes. The f_c in mammalian erythrocyte membranes was substantially higher compared to that in avian erythrocytes. By contrast, the T_d was determined substantially higher in avian erythrocytes, compared to mammalian ones. These species differences could be possibly due to the cytoplasmic skeleton that exists in avian erythrocytes and is absent in mammalian erythrocytes. The avian cytoskeleton contains protein filaments and microtubules that project from the nucleus and interact extensively with the plasma membranes including the undermembrane spectrin network (Doherty & McMahon, 2008). This could impose conformational restrictions to the spectrin network of avian erythrocytes decreasing its segmental mobility and increasing its thermal stability.

As shown in Table 1 the higher T_d in birds corresponded to the higher mean body temperature in these species compared to mammals. This result is in line with the report that cells derived from different species varied in their heat sensitivities in a manner correlated to the normal body temperature of the animal that was the source of the particular cell line (Raaphorst *et al.*, 1979).

In conclusion, on the basis of tested mammalian and bird species, the erythrocyte spectrin network was found to be more conformationally flexible and thermally labile in mammals compared to that in birds. Using thermal dielectroscopy we demonstrated that the spectrin network of avian erythrocytes had lower intramolecular mobility and greater thermal stability compared to mammalian erythrocytes. The biological sense of these differences could be to render greater thermal resilience to avian erythrocytes facilitating the cooling capability of avian blood during flight.

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