THERMAL DIELECTROSCOPY STUDY OF THE BINDING OF OSO_4 TO ERYTHROCYTE MEMBRANE

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ABSTACT

Osmium tetroxide (OsO_4) is used as fixative of membrane lipids and heavy metal stain in optical and electron microscopy. The interaction of OsO_4 with membrane proteins is rather obscure. PURPOSE. To study the effect of OsO_4 on whole erythrocytes and isolated erythrocyte membranes (EMs), resealed with 75 mM NaCl, suspended in isotonic osmotically non-equilibrated media. METHOD. We used thermal dielectroscopy sensing the dielectric response of EMs due to spectrin denaturation at 49.5°C, and the osmotic response of EMs at 61°C, involving a pre-denaturation alteration in the band 3 integral protein. RESULTS. Upon heating, the erythrocyte suspension capacitance, C_s, and conductance, G_s, changed abruptly at the spectrin denaturation temperature, T_s (49.5 °C). These threshold changes, ΔC_s and ΔG_s, were regarded as measures for the intrinsic dielectric polarization of spectrin network. The Cole-Cole plot for the frequency dependence of $\Delta C_s/C_s$ and $\Delta G_s/G_s$ displayed semicircle indicating frequency relaxation of the intrinsic polarization of spectrin network with critical frequency at 2.5 MHz. With OsO_4 - pretreated erythrocytes the radius of the semicircle depended on OsO_4 concentration; at 0 – 0.2 mM it increased twofold and at 0.3 – 0.4 mM it strongly decreased to near zero. At 0.15 mM OsO_4 treatment eliminated the dielectric response of isolated EMs at 49.5°C while at the same time it retained the osmotic response at 61°C. At concentrations equal and greater than 0.3 mM OsO_4 eliminated the osmotic response of EMs at 61°C. Overall, in treating whole erythrocytes with OsO_4 the cytosolic hemoglobin appeared as primary target followed by spectrin network, while integral proteins and membrane lipids were eventually concerned by the fixative.

***Key words**: spectrin-actin cytoskeleton, OsO_4 fixation, erythrocyte membrane, thermal dielectroscopy.*

Introduction. There are two distinctive stages in the fixation of cellular and tissue proteins in the so called positive staining: cross-link formation and denaturation (Eltoum et al., 2001). While denaturation is due to the replacement of water by various organic solvents the fixation is achieved by using non-coagulant, fixing agents which chemically react with proteins and other cell and tissue components, binding to them by addition and forming inter-molecular and intra-molecular cross-links. By binding to a variety of chemical groups in cells and tissues, these agents often affect the charges at the site of attachment. This can alter the conformation and solubility of protein leading to their removal (Luftig et al., 1977).

Several cross-linking fixatives including osmium tetroxide (OsO_4, Flemming's fluid), glutaraldehyde, formaldehyde, acrolein, glyoxal, and carbodiimides are commonly used preparatory to optical (Di Scipio et al., 2008) and transmission electron microscopy (Hayat MA, 2000) because they preserve the ultrastructure of cells and tissues. Of these OsO_4 is a good cross-linking agent for unsaturated lipids and phospholipids and excellent heavy metal stain for biomembranes embeding directly into phospholipid head regions. Several stages of its fixation process remains unclear. A spin label study of the effects of glutaraldehyde and OsO_4 on erythrocyte membrane (EM) (Komorowska et al., 1982) reported that OsO_4 reacts predominantly with lipids and glutaraldehyde with membrane proteins. In the staining of cells OsO_4 and glutaraldehyde produce different effects on plasma membranes as shown by dielectric technique (Carstensen at al., 1969; E. L. Carstensen et

al., 1971). Membrane resistance, considered as accurate criterion for determining membrane condition, is lowered by the OsO_4 and glutaraldehyde-fixation of cells, nevertheless, a major portion of the fixed membrane still acts as an insulation barrier. Membrane fluidity is mostly unaffected by glutaraldehyde, which is unable to fix membrane lipids, while OsO_4 cross-links unsaturated lipids reducing membrane fluidity to zero.

The interaction of OsO_4 with membrane proteins, especially at low concentrations of the fixative is obscure and frequently disputed. Fixation of erythrocytes by OsO_4, in contrast to the fixation with potassium permanganate, did not change the electrophoretic behaviour of erythrocytes (Glaeser and Mel, 1964). OsO_4 reacts primarily with the sulfhydryl and amino groups of proteins producing denaturation without cross-linking of proteins (McMillan and Luftig, 1973). At concentrations as low as 0.25 mM OsO_4 did not affect the erythrocyte intramembrane particles while at greater concentrations (1 – 4 mM) OsO_4 turns integral proteins soluble and removes them from the membranes (Luftig et al., 1977).

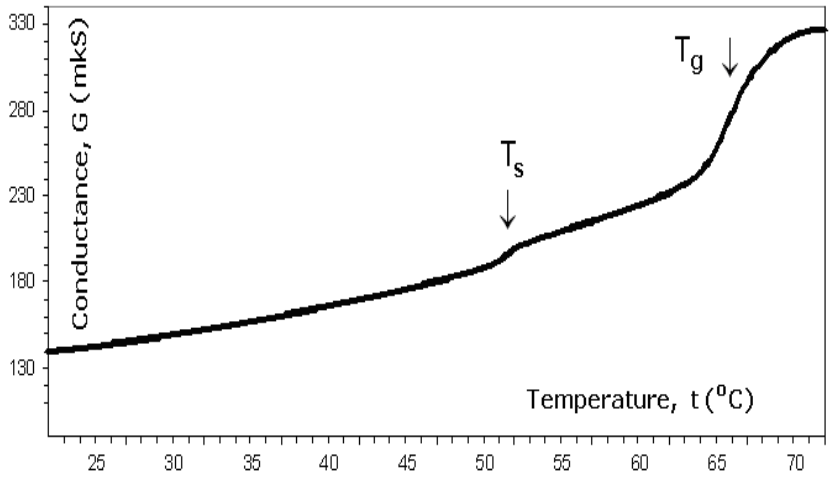

Fig. 1. Temperature profile of suspension conductance, G. The suspensions contained either erythrocytes or resealed EMs suspended in isotonic 10 mM NaCl and sucrose. Hematocrit, frequency and heating rate were 0.65, 0.1 MHz and 3°C/min, respectively. Shown are the temperature, T_s, of spectrin denaturation and the inducing temperature, T_g, for mid-point leakage of cytosolic ions.

With above in mind we studied the interaction of OsO_4 with human erythrocytes and isolated erythrocyte membranes (EMs) at minute, pre-fixation concentrations. We used thermal dielectroscopy on heated suspensions of erythrocytes and EMs, pre-treated by OsO_4. The method allows differentiate the effects of OsO_4 on spectrin and band 3, the major peripheral and integral proteins of EM, respectively, each one comprising about 30 % (w/w) of membrane protein content (Liu et al., 1990).

Materials and Methods. Osmium tetroxide (OsO_4), sucrose and NaCl were purchased from Sigma Chemical Co, St. Louis, MO, USA. About 1 ml heparinized blood samples were obtained from healthy donors. The erythrocytes were immediately isolated (3000 x g, 5 min) and washed twice with 10 ml working isotonic solution containing 10 mM NaCl and sucrose. To treat erythrocytes by OsO_4 100 µl cells were suspended in 2.5 ml working medium containing the indicated concentration of the fixative. After 20 min incubation at 20°C the cells were isolated and once washed in excess working medium. Erythrocyte membranes (EMs) were hypotonically isolated and resealed with 75 mM NaCl as described previously (Ivanov, 2010). They were treated with OsO_4 and studied as described for the whole erythrocytes.

For thermal dielectroscopy study 50 µl of washed erythrocytes or EMs were suspended in the working solution, hematocrit 0.65, and heated from 20 to 70°C at 3.0°C/min heating rate in a sample cuvette as previously described (Ivanov, 2010). At this hematocrit the suspension capacitance, *C*, is dominated by the capacitance of erythrocyte membranes. Data for the complex admittance, *Y**, and capacitance, *C**, of the heated suspension were collected in order to derive the suspension conductance, *G*, and capacitance, *C*, as a function of temperature and frequency. The core instrument was a Solartron 1260 Impedance Analyzer (Schlumberger Instruments, Hampshire, England) interfaced to Toshiba PC using the Miniscan software. *Y** and *C** were measured at the

indicated frequencies between 0.05 and 13 MHz, scanned serially with integration time of 1 s. The duration of each scan was less than 10 s.

In order to decrease electrode polarization platinum electrodes, low suspension conductance (about 40 μS), high hematocrits, low electrode voltage (50 mV) and frequencies above 50 kHz were used. Considering the amplitude of the electric voltage, U, between electrodes constant, the power, W, that the field dissipated into heat (the energy loss) was $W = G U^2 \sim G$.

Results and discussion. Temperature-induced variations in the conductance, *G*, of heated suspensions of erythrocytes or resealed EMs are shown in Fig. 1. Superimposed over the continuous Boltzmann type temperature dependence of *G* two sharp, threshold changes were registered with mid-point temperatures T_s and T_g, respectively. The temperatures, T_s and T_g, slightly depended on the heating rate and at heating rates extrapolated to zero their values were 49.5°C and 61.0°C, respectively. During repeated measurements of samples, taken from a same blood, the T_s and T_g temperatures displayed variations by ± 0.2 °C from respective mean values.

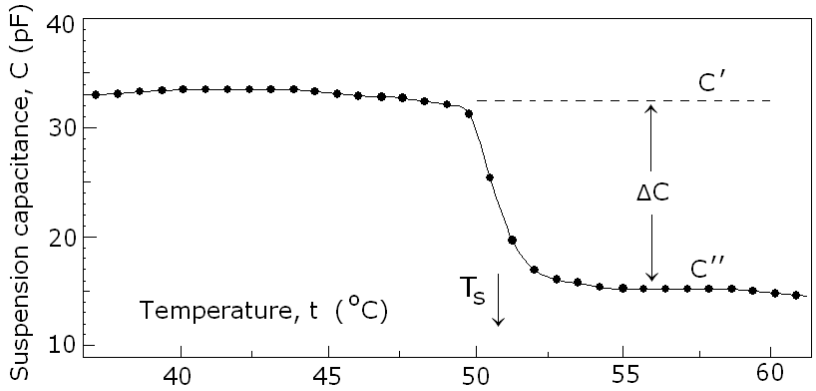

Fig. 2. Temperature profile of the suspension capacitance, C. The suspensions contained either erythrocytes or resealed EMs. Shown is the change in C, ΔC = C′ – C″, at the spectrin denaturation temperature, T_s. All other details are as indicated for Fig. 1.

The change in the suspension conductance at T_g is explained by the out leakage of cytosolic ions due to thermally-induced transition in EMs involving a pre-denaturational change in band 3 (Ivanov et al., 2011).

In addition to suspension conductance, *G*, the suspension capacitance, *C*, also changed abruptly at T_s (Fig. 2). According to microcalorimetry studies the heat denaturation of spectrin takes place at 49.5°C (Brandts et al., 1977) coinciding with T_s. Applying a number of specific chemical modifications of spectrin network the changes in *C* and *G* at T_s were directly ascribed to the collapse of the dielectric polarization of spectrin network related to the concomitant denaturation of spectrin (Ivanov et al., 2012).

The detected change in *C* at T_s was defined as $\Delta C = C' - C''$, where C' is the suspension (membrane) capacitance at the native state of spectrin (at a temperature 3°C less than T_s) and C'' is the suspension (membrane) capacitance at the denatured state of spectrin (at a temperature 3 °C greater than T_s) (see Fig. 1). The change in the suspension conductance, *G*, was defined likewise. With increasing the hematocrit of suspensions the *C* and ΔC both increased almost linearly (not shown). To eliminate this dependence on hematocrit the detected change in *C* at T_s was further expressed in normalized form as $100\Delta C/C'$ (in %). The change in the suspension conductance was expressed in similar normalized form, $100\Delta G/G'$ (in %). The $\Delta C/C'$ and $\Delta G/G'$ are assumed both to quantify the intrinsic dielectric polarization of spectrin network at its native state considering this polarization nil at denatured state of spectrin.

The normalized representations, $100\Delta C/C'$ and $100\Delta G/G'$, of the intrinsic polarization of spectrin network displayed strong dependence on frequency. The Cole-Cole plot between these variables (Fig. 3) expresses this frequency dependence as a semicircle. Theoretically this indicates frequency relaxation of a polarization mechanism of Debye type, i.e., a polarization mechanism with single relaxation time. Based on the data in Fig. 3 the critical frequency of the intrinsic polarization mechanism of spectrin network was determined about 2.5 MHz. At the same time the

critical frequency of the accompanying interfacial polarization of erythrocytes, due to the Maxwell-Wagner effect of charge accumulation on both sides of plasma membranes, was determined about 0.30 MHz at 46°C (not shown). These considerably different values, 2.5 MHz compared to 0.30 MHz, for the indicated critical frequencies demonstrate that the mechanism, specific for dielectric polarization of spectrin network, was different to that of the interfacial polarization of plasma membranes. According to previous study (Ivanov et al., 2012) this mechanism could involve dipoles associated to the highly flexible segments of spectrin fibrillar molecule.

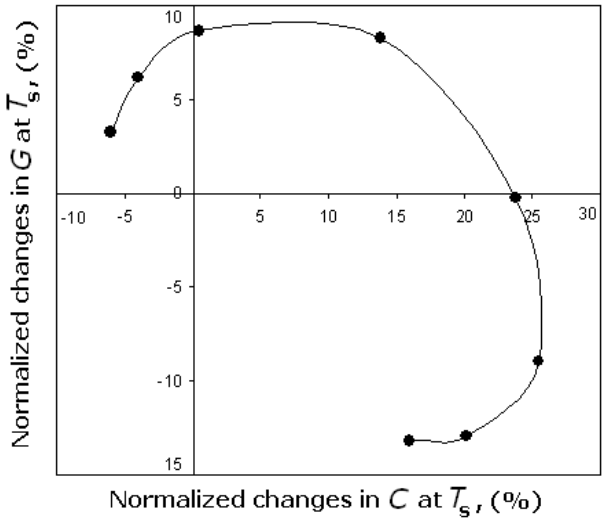

Fig. 3. Cole-Cole plot between the normalized changes in suspension capacitance, C, and conductance, G, at the spectrin denaturation temperature, T_s. The suspensions contained either whole erythrocytes or resealed EMs. All other details are as indicated for Fig. 2.

The Cole-Cole plot of $100\Delta C/C'$ and $100\Delta G/G'$ for OsO_4 - pretreated erythrocytes is exhibited in Fig. 4. Binding of OsO_4 to erythrocytes produced bimodal, concentration – dependent effect on the intrinsic dielectric polarization of spectrin network. At concentrations 0 - 0.2 mM OsO_4 progressively augmented this polarization to twofold while at concentrations of 0.3 – 0.4 mM OsO_4 attenuated it to near zero. Based on the report of others (Luftig et al., 1977) this attenuations of the spectrin polarization could be possibly related to the concomitant solubilization and removal of peripheral membrane proteins and consequent disturbance of overall spectrin network structure.

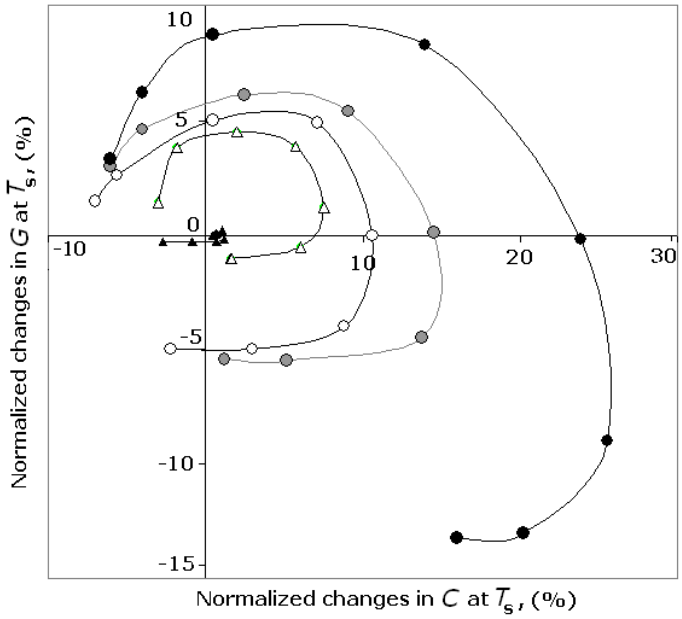

Fig. 4. Effect of OsO_4 on the Cole-Cole plot between the normalized changes in suspension capacitance, C, and conductance, G, at the spectrin denaturation temperature, T_s. The erythrocytes were treated and washed with OsO_4 at the indicated concentration: 0 mM – open circles, 0.1 mM (0.0025 %) – gray circles, 0.2 mM (0.005 %) – black circles, 0.3 mM (0.0075 %) – open triangles and 0.4 mM (0.01 %) – black triangles. All other details are as indicated for Fig. 3.

Finally we studied the impact of OsO_4 on hemoglobin – free EMs, isolated from whole erythrocytes and resealed with 75 mM NaCl. The end-points of this impact were the changes which OsO_4 produced on the dielectric response of EMs at 49.5°C, due to the spectrin denaturation, and on the osmotic response of EMs at 61°C, involving a pre-denaturation alteration in the band 3 integral protein (Ivanov et al.,

2011). At low concentrations of 0 – 0.04 mM OsO_4 did not produce any effect on the normal dielectric response of EMs at the spectrin denaturation temperature (Fig. 3), while at greater concentrations (0.08-0.15 mM) OsO_4 produced strong inhibitory effect similar to that shown in Fig. 4. At 0.15 mM OsO_4 fully eliminated the dielectric response at 49.5°C while at the same time it retained the osmotic response at 61°C indicating strongly disturbed composition and structure of the spectrin network and spared structure of band 3. At concentrations equal and greater than 0.3 mM OsO_4 eliminated the osmotic response of EMs at 61°C indicating that at this concentration of OsO_4 the EMs became fixed and able to preserve their volume and ion content in osmotically non-equilibrated media (not shown).

The augmentation of erythrocyte spectrin network polarization at low OsO_4 concentrations could be possibly ascribed to the stripping of spectrin network by the associated cytosolic proteins induced by OsO_4.

Conclussion. Comparing the results obtained with whole erythrocytes and isolated EMs it could be deduced that in treating whole erythrocytes with OsO_4 the greater part of the fixative was absorb by the cytosolic hemoglobin. The remaining part was bound to spectrin network, while integral proteins and membrane lipids were the last target of the fixative.

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