

RHEOLOGICAL AND ELECTRICAL BEHAVIOR OF ERYTHROCYTES IN PATIENTS WITH DIABETES MELLITUS

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Abstract. Diabetes mellitus is a disease in which the body does not produce enough, or properly respond to, insulin, a hormone produced in the pancreas. The rheological and electrical properties of red blood cells in the patients with diabetes have been studied in order to assess the complications of the disease. There was an increase in the viscosity and the yield stress of diabetic erythrocyte comparing to the normal. These may lead to the increase in the aggregation and decrease in deformability of red blood cells due to reducing the negative surface electric charge. The rheological disorders could lead to microcirculation problems. On the other hand, the relative permittivity, dielectric loss, and AC conductivity of diabetic erythrocytes increased significantly compared to the control. This is due to the toxic effects of glucose on erythrocytes which lead to restructuring of erythrocytes membranes. The high glucose value leads to imbalance of electrolytes in red blood cell membrane and the dielectric spectroscopy (DS) is very sensitive to such slight changes.

Key words: diabetes, viscosity, conductivity, dielectric properties.

INTRODUCTION

Diabetes mellitus is a complex, chronic disease and an increasingly significant health problem as the incidence increases worldwide. In 2030 the World Health Organization (WHO) predicts 366 million people with diabetes (5% of the world's population) [1]. Diabetes mellitus is the most important cause of blindness in adults [13]; in addition, the risk of coronary heart disease is two to four times higher in diabetes patients. The risk of stroke or peripheral vascular disease also increases strongly.

Diabetes produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation [16], which is responsible for increased incidence of atherosclerosis [9], a major complication of diabetes mellitus [29]. An enhanced oxidative stress has been observed in these patients as indicated by increased free radical production [2], lipid peroxidation and diminished antioxidant status [11].

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Oxidative stress in cells and tissues plays an important role in the pathogenesis of diabetes mellitus (DM). Oxidative stress manifests by increased levels of free radicals and LPO; it suppresses glycolysis, protein and nucleic acid production, and enzyme activities and promotes oxidation-phosphorylation uncoupling. The rate of free radical formation during oxidative stress surpasses the rate of their neutralization by the antioxidant system. Oxidative stress is regarded not only as the main mechanism of delayed complications of DM, but also as a factor underlying the development of DM.

Toxic effects of glucose on erythrocytes manifest in restructuring of the erythrocyte membranes, disorders in hemoglobin oxygen-binding activity, modification of mechanical characteristics of the membrane and cell in general [28]. Oxidative stress caused by peroxynitrite treatment of erythrocytes leads to modification of their membrane cytoskeleton structure [27]. Changes in the cytoskeleton structure and lipid bilayer composition caused by oxidative processes lead to erythrocyte damage.

MAJOR DETERMINANTS OF RBC RHEOLOGY

Aggregation

Erythrocytes (i.e., red blood cells, RBCs) are an important determinant of the rheological properties of blood because of their large number density ($\sim 5 \times 10^6/\text{mm}^3$), particular mechanical properties, and aggregation tendency. Typically, a human RBC has a biconcave shape of $\sim 8 \mu\text{m}$ in diameter and $\sim 2 \mu\text{m}$ in thickness, and is highly deformable [24]

The RBC membrane is composed of proteins (52% in weight), lipids (40%), and carbohydrates (8%). Membrane elasticity depends on the structural interactions between the outer plasma membrane and the underlying protein skeleton. The proteins of the RBC membrane are divided into two groups: integral and peripheral (Fig. 2). Integral proteins (glycophorin and Band 3 proteins) are tightly bound to the membrane through hydrophobic interactions lipids in the bilayer [15, 19, 20]. A filamentous network of proteins is anchored to the bilayer by the integral proteins. This network has three principal components: spectrin, actin, and protein 4.1. The peripheral membrane proteins are located on the cytoplasmic surface of the lipid bilayer and can be readily released from the membrane by simple manipulation of the ionic strength of the milieu or variation in the concentrations of other proteins [19].

Blood is a non-Newtonian fluid and its viscosity is therefore variable at any given temperature, depending on the shear rate. At low shear rate, RBCs can aggregate and form one-dimensional stacks-of-coins-like rouleaux or three-dimensional (3D) aggregates [24]. This is because the electrostatic repulsion of RBC is overcome by the presence of macromolecules which aggregate the cells. The process is reversible and particularly important in the microcirculation, since such rouleaux or aggregates can dramatically increase effective blood viscosity. RBCs

may also exhibit reduced deformability and stronger aggregation in many pathological situations, such as heart disease, hypertension, diabetes, malaria, and sickle cell anemia [24].

Deformability

‘Cellular deformability’ is the term generally used to characterize the RBC’s ability to undergo deformation during flow [19]. The deformation response of a RBC to fluid forces is a complex phenomenon that depends on a number of different cell characteristics including membrane material properties [15], cell geometry, and cytoplasmic viscosity [20].

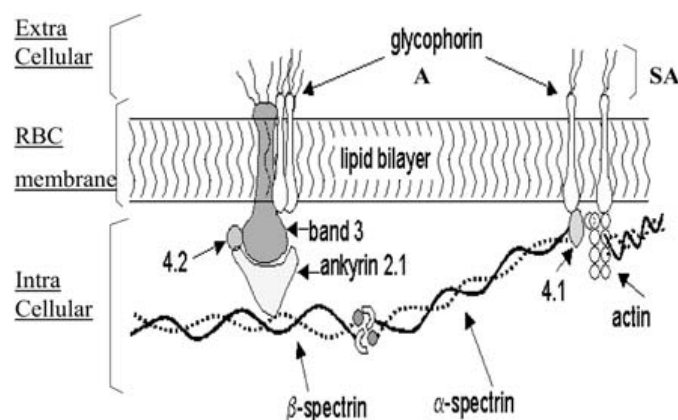


Fig. 1. RBC membrane. Schematic representation of protein orientations in the human RBC.

To undertake oxygen delivery, the RBC must be able to undergo considerable cellular deformation since its diameter (8 μm in humans) far exceeds that of the capillaries (2–3 μm) through which it must pass [19]. The proteins of the RBC membrane are divided into two groups: integral and peripheral (Fig. 2). Integral proteins (glycophorin and Band 3 proteins) are tightly bound to the membrane through hydrophobic interactions lipids in the bilayer [15, 19, 20]. A filamentous network of proteins is anchored to the bilayer by the integral proteins. This network has three principal components: spectrin, actin, and protein 4.1. The peripheral membrane proteins are located on the cytoplasmic surface of the lipid bilayer and can be readily released from the membrane by simple manipulation of the ionic strength of the milieu or variation in the concentrations of other proteins [19].

Principles of dielectric spectroscopy

In essence, dielectric studies are based on quantifying the response of a material to an electric field applied to it. The response is typically described by the material’s conductivity and permittivity. Conductivity (σ), measured in S/m,

quantifies the ability of the material to conduct the electrical charge. Permittivity (ϵ), measured in F/m, is the amount of charge that is stored by the material due to the polarization of its components. Permittivity of the material is often expressed as relative to the permittivity of vacuum ($\epsilon_0 = 8.854 \times 10^{-12}$ F/m), giving the dimensionless relative permittivity (also called dielectric constant), $\epsilon_r = \epsilon/\epsilon_0$. By dividing conductivity and permittivity by the probe constant (d/A in m^{-1} , the ratio of the distance between the electrodes and the electrodes' area), one obtains the corresponding conductance (G in S) and capacitance (C in F) of the material, respectively. The permittivity of a material tends to fall (and its conductivity to rise) in a series of step-like shifts as the frequency of the electrical field rises. These step changes, called dispersions, are due to losses of certain characteristic polarization abilities of the substance.

In the case of cell suspensions, three major dispersions are identified: the α -, β - and γ -dispersions. The α -dispersion is caused predominantly by the activity of ions by the charged surfaces of cells and particles. The γ -dispersion is due mainly to the bipolar rotation of water molecules. Of particular interest in biological cells is the β -dispersion, resulting from the build-up of electrical charge at the cell membranes. Under the influence of an electric field applied to a cell suspension, the ions present in the electrolytic medium migrate towards the electrodes. The cytoplasm of the cells is also conducting but due to the presence of the non-conducting plasma membrane, the charged ions inside the cells are constrained to the cell volume. Trapped inside the membrane, the ions accumulate at the sides of the cell, and the cell becomes polarized. Clearly, only cells with undamaged membranes capable of electrical insulation contribute to the increase in capacitance. Most dead cells autolyse shortly after death and their membranes rupture, while non-cellular material cannot store electrical charge. Thus, only viable is measured. Each living cell in the suspension assumes the behavior of a tiny electrical capacitor and the overall capacitance of the suspension rises as a function of the total biovolume (i.e. the volume fraction of the suspension which is enclosed by an intact membrane). Measuring the capacitance over a predetermined range of electrical field frequencies is the basic idea of scanning dielectric spectroscopy. The typical frequency range used in bioprocesses monitoring is in the order of 0.1–10 MHz, where the β -dispersion occurs. At the lower frequencies of this range, there is enough time for electrical charge to build up at the cell membranes. However, at the high-frequency end of the spectrum, the electrical field changes direction too rapidly for the cell membrane to polarize, and the biomass no longer contributes to the measured capacitance. The net rise from the background capacitance (C_∞) at high frequencies to the increased capacitance at low frequencies is expressed as ΔC and can be attributed to the charge-storing properties of the biomass. The frequency corresponding to half of the measured DC is called the characteristic frequency (f_c) [7, 8]

MATERIALS AND METHODS

CLINICAL SAMPLES

Samples of heparinized venous blood were collected from twenty patients with diabetes mellitus. The patients were previously diagnosed and were under the supervision of medical professionals during this period. Blood from 10 healthy individuals were used as a normal control for this study.

RHEOLOGICAL PROPERTIES

In order to determine the rheological properties of blood with anomalous flow properties, it is necessary to use an instrument in which all the samples under test is exposed to a uniform shear stress and shear rate, and in which each of these effects is separately determinable. The rheometer employed in this study is the Brookfield *DV-III* Programmable Rheometer. It is a cone-plate viscometer that measures fluid parameters of shear stress and viscosity at given shear rates. The applied shear rate was 12 to 375 s⁻¹, and the measurements were carried out at temperature 25 °C. The data was collected from the rheometer by means of software program “Rheocalc for Windows”. The apparent viscosity of the blood which is a non-Newtonian fluid is not constant but rather depends on the magnitude of the shear stress or shear rate, and can be calculated as ratio of shear rate to shear stress. It decreases as the shear rate increases (Flow curve). The analysis of the flow curve was performed by applying the Bingham plastic model

$$F = F_0 + \eta D \quad (1)$$

where F is the shear stress (dyne/cm²) and D is the shear rate (s⁻¹), and the yield stress (F_0) and viscosity (η) can be calculated.

DIELECTRIC PROPERTIES

The dielectric properties of RBCs can be investigated by measuring the dielectric properties of blood suspension, which has the benefit of measuring viable cells close to its physiological state, and to avoid any induced changes in the sample during preparation or rouleaux formation during settling in the measuring tubes [26]. The dielectric measurements were carried out using *LCR* meter type HIOKI 3531, manufactured in Japan, in the frequency range 40 kHz to 5 MHz. The measuring cell is a parallel plate conductivity cell with platinum electrodes, coated with platinum black layer [12], with area 4 cm² and separating distance 2 cm. The blood samples were diluted in isotonic buffered saline (pH 7.4 and conductivity 0.627 S/m), and the hematocrit was adjusted at 3%. The samples were incubated in water bath at 37 °C during measurement.

The measured parameters were capacitance C and conductance G , from which the relative permittivity ϵ' and conductivity σ can be calculated as follows:

$$\epsilon' = \frac{C d}{\epsilon_0 A} \quad (2)$$

$$\sigma = G \frac{d}{A} \quad (3)$$

where A is the area of the electrode, d is the distance between the two electrodes, and ϵ_0 is the vacuum permittivity. The dissipation factor $\tan \delta$ can be given by:

$$\tan \delta = 2 \pi f \frac{C}{G} \quad (4)$$

The permittivity can be expressed in complex quantity as:

$$\epsilon^* = \epsilon' - J\epsilon'' \quad (5)$$

The real part represents the permittivity constant and is given by:

$$\epsilon' = \epsilon_\infty + \frac{\epsilon_s - \epsilon_\infty}{1 + \omega^2 \tau^2} \quad (6)$$

where ϵ_s is the limiting low frequency permittivity, and ϵ_∞ is the permittivity value at the end of the dispersion. And the imaginary part ϵ'' (the dielectric loss):

$$\epsilon'' = \frac{(\epsilon_s - \epsilon_\infty) \omega \tau}{1 + \omega^2 \tau^2} \quad (7)$$

To separate the AC conductivity component from the total conductivity measured (DC and AC) the following relationship was applied [21]:

$$\sigma_{AC} = \omega \epsilon_0 \epsilon'' \quad (8)$$

where

$$\epsilon'' = \epsilon' \tan \delta \quad (9)$$

STATISTICAL ANALYSIS

In this study, the values are expressed as mean \pm standard deviation. The significance of the difference between each value presented by various groups was evaluated by the Student t-test and values with $p < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Erythrocyte membrane proteins serve specific functions. Band 3, for instance, which is the most abundant erythrocyte transmembrane protein, is responsible for anion exchange at the level of plasma membrane. Other transmembrane proteins serve as pumps or channels for the movement of ions and the transport of glucose and other small molecules. In addition, cytoskeletal proteins are important for the maintenance of the biconcave shape and structural integrity of the erythrocyte.

Table 1

Demographic overview of patients

Subject characteristics	Control	Diabetic
Number (<i>n</i>)	10	20
Gender (Male/Female)	2/8	1/19
Age (Years)	36 ± 4.1	47 ± 2.8
Age Range	24–60	25–80
Duration of diabetes (Years)	–	5.1 ± 1
Non-Insulin/ Insulin treated	–	8/12
Glucose (mg/dL)	85 ± 7.4	242 ± 26

The analysis of the rheological properties of blood samples from diabetic patients yields two important parameters; yield stress and viscosity (Fig. 1). The yield stress is a sensitive index of blood fluidity at low shear rates. When no shear stress is applied, the red blood cells, placed in suspension, adhere face-to-face and form aggregates called rouleaux. While the low shear viscosity is a function of the aggregability of the red blood cells, the viscosity in the high shear region depends on their deformability. RBCs from diabetic patients were found to be more rigid than normal and have reduced deformability due to the interaction of hemoglobin with the membrane that contributes to the cellular rigidity and alteration of the membrane. This reduced deformability appears as increase in the yield stress and the viscosity in the high shear region. Hyperglycaemia resulting from uncontrolled glucose regulation is widely recognized as a causal link between diabetes and diabetic complications. Erythrocyte membrane glycosylation reduces the negative surface electric charge [25] due to cleavage of terminal sialic acid components of glycophorin A, leading to accelerated aging of erythrocytes. It also increases erythrocyte aggregation [18] and accumulates advanced glycation end products (AGEs), which have been associated with erythrocyte deformability defects and with microvascular complications of diabetes [5].

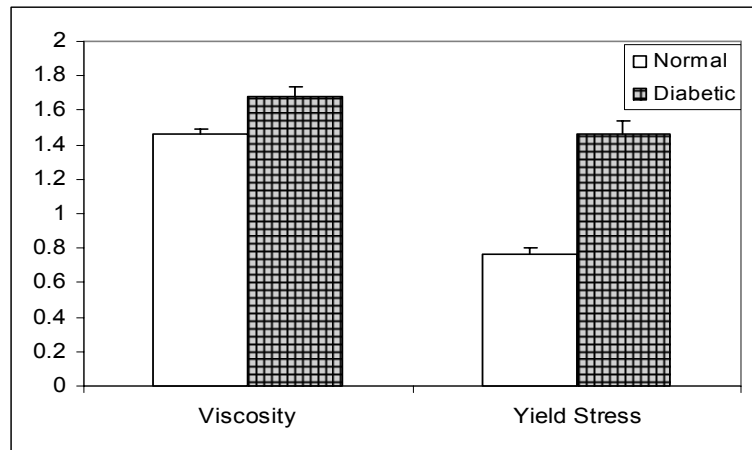


Fig. 2. Viscosity (in cp) and yield stress (in D/cm²) of normal and diabetic erythrocytes.

One of the important subjects in biophysics is the investigation of the dielectric properties of cells and of structural parts of the cell (membrane, cytoplasm, etc.). These can provide valuable knowledge about deferent cell structures, their functions and metabolic mechanisms [23].

Table 2

Electrical parameters of normal and diabetic erythrocytes

	τ (s)	$\Delta\sigma$	ϵ_{∞}	ϵ_s	$\Delta\epsilon$
Normal					
Mean	1.25E-07	3250.2	3279.3	22983	19684
\pm SE	3E-09	59	530.83	531.14	531.13
Diabetic					
Mean	1.4E-07	3820.9	3231.8	28115	24036
\pm SE	3.19E-09	88.94	71.506	879.14	859.95
P value	0.0005	0.0018	0.6175	0.0012	0

The dielectric properties of the biological membrane, under the influence of applied electric field, are characterized by several parameters: relative permittivity or dielectric constant (ϵ'), dielectric loss (ϵ''), dielectric strength ($\Delta\epsilon'$), relaxation time (τ) and AC conductivity. The permittivity is a measure of its polarizability in the electric field. It is related to the structural arrangement of the lipid bilayer and with the conformation and localization of proteins in the membrane, consequently with the spatial distribution of charge and dipolar groups at the hydrophobic interface [4]. This polarization does not occur instantaneously, and the associated time constant is called the relaxation time τ [17]. The dielectric strength (the difference between the limits of (ϵ_s and ϵ_{∞}) is the maximum field strength the

material can withstand without breakdown. It is an intrinsic property which depends on the membrane conformation. The conductivity depends on the dynamical ionic transport through the membrane. It takes into account both the structural ionic and polar group arrangements and the dynamical ionic transport processes of the membrane [3]. Fig. 3a shows the dielectric dispersion (the corresponding frequency dependence of permittivity) of normal and diabetic blood.

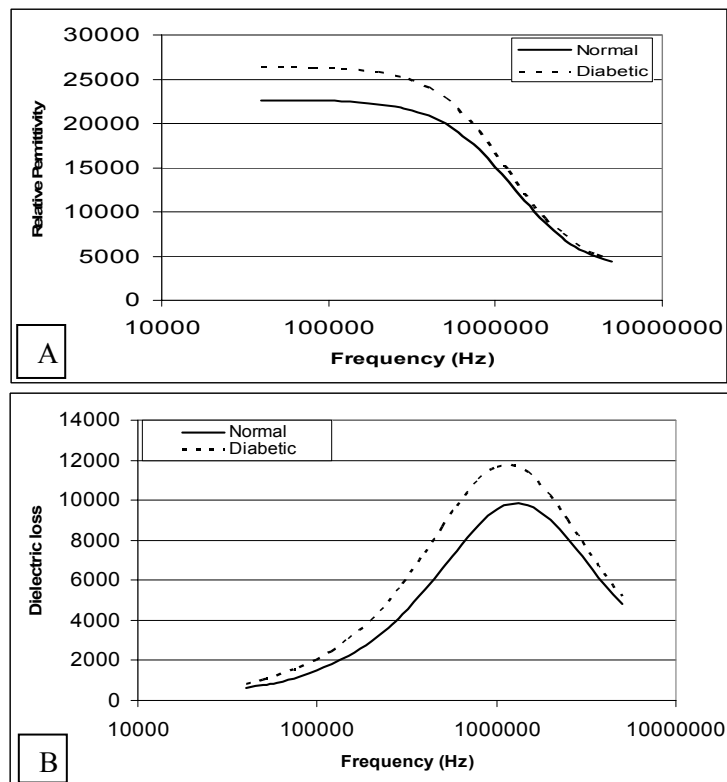


Fig. 3. Relative permittivity (A) and dielectric loss (B) for normal and diabetic erythrocytes.

The dielectric loss curve (Fig. 3b) can be evaluated by the total area under the loss curve. It is proportional to the total concentration of dipoles in the material and their dipole moment, irrespective of their distribution of relaxation times [22]. Most of the calculated dielectric parameters of diabetic RBCs show increased values compared to normal (Table 2). In 1999 Hillier *et al.* [10]., reported that hyper and hypo-glycaemic excursions lead to changes in the electrolyte balance in blood (a decrease in sodium ion concentration, and an increase in potassium ion concentration), cells and interstitial fluid in healthy subjects and in patients with diabetes. These variations cause changes in the red blood cells membrane potential,

which can be estimated by determining the permittivity and conductivity of the cell membrane through the dielectric spectrum. The same result was reported by [30] who found that glucose is able to directly affect the impedance of the blood especially below 1 MHz.

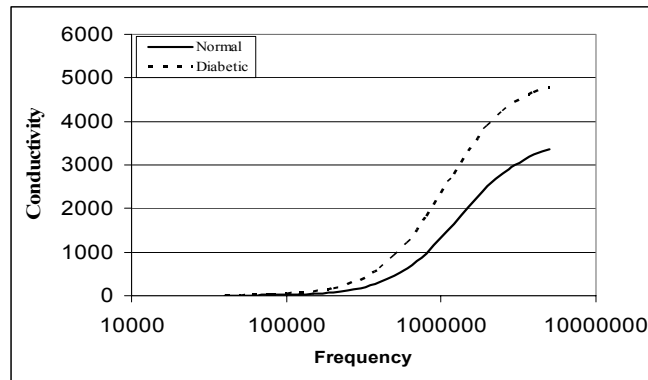


Fig. 4. AC electrical conductivity of normal and diabetic erythrocytes.

Based on the change in the dielectric measurements due to glucose concentration, many researchers try to use the dielectric spectroscopy as a non-invasive technique for blood glucose monitoring. The technique is based on impedance spectroscopy in the frequency window characterized by the β -dispersion. The clinical-experimental studies with the non-invasive technique in healthy subjects have shown that changes in blood glucose following intravenous or oral administration of glucose could be tracked [6, 14] under controlled conditions.

CONCLUSION

The obtained results show there is an increase in rheological (viscosity and yield stress) and electrical properties (dielectric constant, dielectric loss, relaxation time and ac conductivity) of diabetic erythrocytes compared to healthy individuals. The rheological disorders of diabetic erythrocytes (increased aggregation and decreased deformability) will cause microvascular complications of diabetes.

There is controversy about the effect of glucose on the electrical properties of red blood cells. Many researchers had found only a slight variation of glucose, while others said that there is a relationship between increased concentration of glucose and the increase in the electrical properties. These results reinforced the opinion, which states an increase of electrical properties with the increase of glucose. Finally, the obtained results confirm the use of these properties as a way to measure the concentration of glucose without intervention.

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