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Erythrocyte deformability and its variation in diabetes mellitus

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Erythrocyte deformability improves blood flow in the microvessels and in large arteries at high shear rate. The major determinants of RBC deformability include cell geometry, cell shape and internal viscosity (i.e., mean cell hemoglobin concentration and components of the erythrocyte membrane). The deformability is measured by several techniques but filtration of erythrocytes through micro-pore membranes and ektacytometry are two sensitive techniques to detect changes in erythrocytes under varied experimental and diseased conditions. Diabetes mellitus (DM) is a metabolic disorder, characterized by varying or persistent hyperglycemia, which induces several changes in the erythrocyte membrane and its cytoplasm, leading to alteration in the deformability. A decreasing trend of deformability in these patients is observed. The shape descriptor form factor, as determined by processing of erythrocyte images, increases with the increase of blood glucose levels and shows a pattern similar to filtration time of erythrocyte suspensions through cellulose membranes. Fluidity of the membrane as measured in erythrocytes of these patients is decreased. With prolonged diabetic conditions the deformability of erythrocytes is further decreased, which may complicate the flow of these cells in microvessels.

Keywords: Diabetes mellitus, Ektacytometry, Erythrocyte deformability, Fluidity, Micro-pore filtration, Shape descriptors

The human erythrocyte has long intrigued biophysicists, cell biologists and biochemists by its relatively simple geometry and its fascinating mechanical and physiological properties. There is still much to be understood about the pronounced resilience of the erythrocyte as it undergoes marked deformation in the circulatory system and rapidly recovers its resting shape once the shear stress is removed[1,2]. The complexities associated with blood flow in cardiovascular system are primarily attributed to the flow properties of blood and viscoelastic properties of blood vessels. The varying shear rates at different locations of the cardiovascular system affect the flow properties of blood. The deviation from the homogeneous flow in large vessels to heterogeneous flow in microvessels is primarily attributed to the physiological flow conditions and rearrangement of the cellular components, which is basically dominated by erythrocytes. The multi-profile flow in arterioles is reduced to single profile of erythrocytes in capillaries, the vessels which are primarily responsible for exchange of gases and metabolic products with deformability of erythrocytes as a dominant factor[3].

Erythrocyte deformability improves flow in the microvessels and in large arteries at high shear rate. The major determinants of this include cell geometry, cell shape and internal viscosity (i.e., mean cell hemoglobin concentration and components of the erythrocyte membrane)[4].There are various techniques used to measure the deformability in different experimental and diseased conditions. This brief review outlines the recent developments related to the erythrocyte deformability and its variation in diabetes mellitus.

Major determinants of erythrocyte deformability

Red cell geometry—The undeformed diameter of the cell is around 8 μm**,** and its average membrane surface area (SA) and mean cell volume (CV) are approximately 135 μm^2 and 94 μm^3, respectively. The mean surface area is considerable greater than required to enclose a sphere of volume 90 μm^3. The availability of this excess area is a major factor in allowing the erythrocyte to deform uniaxially at constant SA and CV under varied physiological conditions[2,3]. These parameters are further affected by

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the aging of erythrocytes[5] and various diseased conditions such as malaria[6], spherocytosis[7], etc.

Rheological characteristics of intracellular fluid—The flow properties of erythrocytes are affected by the concentration and physicochemical properties of hemoglobin. At the normal mean cellular hemoglobin concentration (MCHC) of 32 g/dl, the intracellular fluid viscosity is around 7 millipascal sec (mPas) and it has no elastic behavior. This parameter increases with the increase of MCHC in a non-linear fashion[2] but decreases in patients with hypochromic anemia[8].

Rheological characteristics of erythrocyte membrane—The erythrocyte membrane consists of two domains, the lipid bilayer and the cytoskeleton[9]. Phospholipids and cholesterol compose most of the lipid bilayer. The membrane phospholipids are amphipathic; each leaflet has a hydrophilic domain (on the exterior cytoplasmic and extracellular surfaces) and a hydrophobic domain (between the two leaflets of the bilayer). The phospholipids are asymmetrically dispersed in the bilayer. The outer half of the bilayer contains sphingomyelin, glycolipids and phosphatidylcholine, while the inner half (facing the cytoplasm) is composed of phosphatidylinositols, phospatidylserine and phosphatidylethanolamine[9,10]. Cholesterol is distributed evenly throughout the lipid domain, which alters flexibility and provides stability to the membrane.

The major integral proteins of bilayer are the band 3 and glycophorin. The spectrin protein of the membrane skeleton self-associates to form tetramers that are in turn coupled to short filaments of actin to form a network on the inner surface of the bilayer[11]. The spectrin and actin attachment is stabilized by the protein band 4.1 (ref. 12). The attachment between the skeleton and the bilayer is mediated by ankyrin, which connects spectrin to band 3 (ref. 13), and by band 4.1 to glycophorin[14]. Direct stretching of individual spectrin chains under the action of an atomic force microscopy (AFM) tip reveals that spectrin behaves as a highly non linear spring; the extending force increases disproportionately with relative chain extension. The calculated value of spring constant as ~ 0.01 pN/nm suggests a relatively stiff chain[15]. Recent laser tweezer-imposed deformations of the erythrocyte that yield, from simple analyses, a shear modulus (μ » 0.002 to 0.003 pN/nm). This value is two- to three-old lower than that deduced from micropipette aspiration[16]. In the latter efforts, assumptions about actin protofilaments being tangent to the membrane and rotationally mobile prove consistent, to a degree, with fluorescence polarization experiments[17]. In combination with spectrin the formation of structure-function relationship as a quasi-two-dimensional meshwork of spectrin-actin, supported by proteins band 3-ankyrin, protein 4.1, glycophorin C, etc., imparts a resilence to the overlying plasma membrane, and its ability to withstand the stresses of circulation[18]. Any variation in the structural constituents leads to the impairment of functional capability of the membrane.

Measurement of erythrocyte deformability

Erythrocyte deformability is the measure of the ability of the cells to deform under applied shear stress. In cardiovascular system the erythrocytes deform in large as well as small vessels. But the deformability is of prime importance in the capillaries, the vessels where the exchange of metabolic products takes place. As there are difficulties in measuring deformability under *in vivo* conditions, this is generally measured under *in vitro* conditions by various techniques which are described elsewhere[2,3]. Some of the recent techniques, based on different principles, are discussed here.

Measurement of erythrocyte deformability by shear flow: ektacytometry—The alteration in diffraction pattern of erythrocytes depends on the shape of the erythrocytes while flowing through a channel or being sheared between cylinder in cylinder or cone and plate geometry. During flow the erythrocytes are elongated and their diffraction pattern changes from circular to elliptic form. While maintaining the constant shear stress the elongation index is directly related to the deformability of erythrocytes[19]. Based on this principle two rheoscopic instruments, Laser-assisted optical rotational cell analyzer (LORCA: AMC, Amsterdam, Netherlands)[20] and a Shear stress diffractometer (RHEODYN SSD, Myrenne, Germany)[21] are developed. These are based on shearing of the blood samples at a known shear rate. During applied shearing the diffraction pattern of erythrocytes is recorded by a CCD camera and analyzed by a computer. The elongation index (EI), calculated from the lengths of major and minor axes of the erythrocyte shape, forms the measure of the deformability. Both these techniques perform

satisfactorily in the measurement of erythrocyte deformability[22] but require frequent cleaning while carrying out measurement of large number of samples.

Based on flow of suspension through a slit the deformability of erythrocytes is also measured by similar principle, by RheoScan-D (Sewon Meditech, Korea)[23]. Figure 1 shows the schematic of this system which is consisting of laser, a CCD video camera, screen, vacuum generating mechanism and pressure driven slit rheometry, For measurement, the vacuum generating mechanism is connected with the slit element, which allows the fluid to flow through the slit and to be collected in the waste sample chamber, as driven by the differential pressure and stops flowing after the equilibrium pressure is reached.

For deformability measurement in the RheoSCAN-D, the erythrocyte suspension is prepared by suspending the whole blood in a solution of 0.14 mM polyvinylpyrrolidone (PVP, Molecular weight 360,000) at 0.5% hematocrit. During flow of suspension in the disposable rectangular slit at a known shear stress the images of erythrocytes are recorded by a CCD camera which is linked to a frame grabber integrated with a computer. The diffraction pattern at a known shear stress in elliptic form is processed. The elongation index (EI) as a measure of the RBC deformability is determined from an iso-intensity curve in the diffraction pattern using an ellipse-fitting program by (A-B)/(A+B), where A and B are major and minor axes of ellipse[23].

Measurement of erythrocyte deformability by passage of suspensions through membrane micropores—The filtration techniques basically deal with the flow of erythrocytes suspension in different types of filter membranes. The erythrocyte deformability is quantified in terms of passage time required for a known volume of suspension to flow through the membrane. Based on type of membrane and pressure applied, several filtration techniques are developed[24-30]. For measurements the applied pressure should be comparable to that as in microcirculation, below 10 Pa. However 15-25 Pa would be a good limit[3]. As erythrocytes flowing under low pressure may block the pores, a low hematocrit (less than 10%) is preferred[31].

The initial flow method has been found to be within the specified range of applied pressure which also minimizes the influence of gravitational field. The filtration time of erythrocyte suspension (at 5% hematocrit in phosphate buffered saline containing 0.5% albumin) through cellulose membrane by initial flow method, by an online computer system, is measured[32]. The schematic of the hemorheometer is given in Fig. 2. It consists of a syringe fixed into a filter-membrane holder. The cellulose micropore filter membrane of pores of 20 ± 5 μm diameter and 400 μm thickness (Schleicher and Schull, Germany) is placed in the holder. A three-way valve controlls the flow of suspension through the tortuous paths of the membrane. A sheath of light obtained from an LED operating at 640 nm is passed through the erythrocyte suspension and the transmitted light intensity (TLI) is measured by a photodetector. The time taken by the known volume of erythrocyte suspension (0.6 ml) to pass through the membrane is inversely proportional to the deformability of erythrocytes.

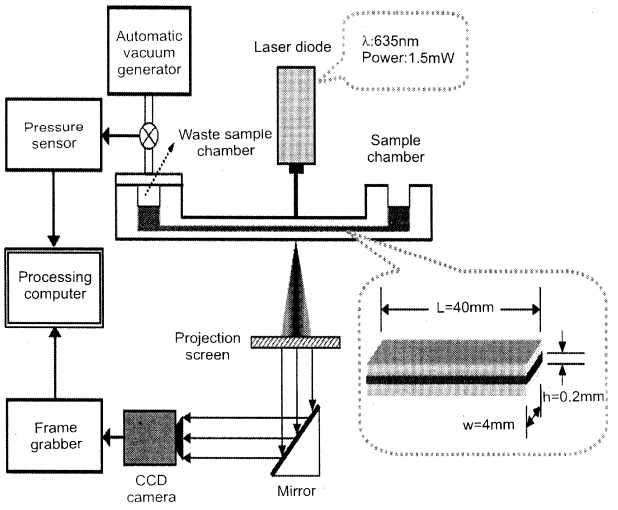

Fig. 1—Schematic diagram of slit-flow ektacytometer

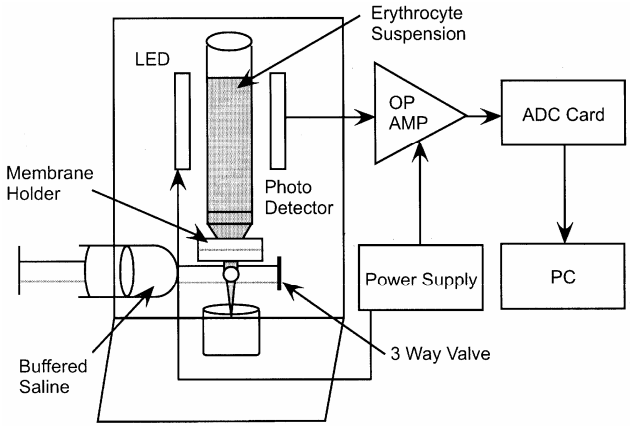

Fig. 2—Block diagram of optical hemorheometer

Measurement of membrane dynamic properties—Erythrocyte membrane lipid bilayers are fluid, and individual phospholipids diffuse rapidly throughout the two dimensional surface of the membrane. The proteins and cholesterol are integral parts of the membrane. Fluidity consists of rotational diffusion, which is related to microviscosity and hindered anisotropic rotations[33-35]. The measurement of membrane physical properties is achieved by fluorescence polarization with probe molecules inserted into the membranes[36]. Three different fluorescent probes, which enter the membrane lipid bilayer at different levels, are generally used. Both 1,6-diphenyl-1,3,5-hexatrien (DPH) and perylene when inserted into the lipid bilayer in the region of the carbon 12 to 16 fatty acid (FA) chains give indications of the degree of order owing to the difference of their shape[37]. The third probe trimethylamino-diphenylhexatrien (TMA-DPH) stays at a superficial level near the phospholipids polar heads[38].

The basic principle of measuring fluorescence polarization is that the sample is excited with vertically polarized light, and emission is measured through polarizers both parallel ($\mathbf{I}^{\parallel}$) and perpendicular ($\mathbf{I}^{\perp}$) to the excitation polarizer[39].

Fluorescence polarization is expressed as anisotropy (**r**) in which:

$$r = \frac{I\parallel - I^{\perp}}{I\parallel + 2\,I^{\perp}}$$

Using the Perrin equation[40] anisotropy is directly related to microviscosity by:

$$\frac{r_0}{r} = 1 + \frac{t}{t_r}$$

where r_0 = maximum fluorescence anisotropy; t = fluorescence lifetime: and t_r = the rotational correlation time. A parameter arising from rotational Brownian motion and describes how readily a molecule can tumble in solution.

There is an inverse relationship between the two parameters; the higher the anisotropy the lower the fluidity. Fluidity is the opposite of microviscosity. Erythrocyte membrane fluidity is determined by fluorescence polarization, performed on a spectrofluorometer equipped with a polarization accessory[36].

Morphological parameters and deformability of erythrocytes—The morphological alterations in erythrocytes during the disease process reflect the changes which are taking place in the cardiovascular system. Turchetti *et al*.[41] carried out morphological analysis of erythrocyte and showed that bowls are the most deformable red cells while the discocytes having a stiffer form. The morphological changes in erythrocytes are further analyzed by their shape descriptors which are obtained by processing of their microscopic images. These descriptors are projected area and perimeter of erythrocytes. Based on these the form factor (FF), given as the ratio of projected square perimeter to area of erythrocyte, is calculated, which is correlated with the deformability of erythrocytes [32]. In case of closely resembling images the precise shape analysis could further be carried out by wavelet transforms[42].

Alteration of erythrocyte deformability in diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by varying or persistent hyperglycemia (elevated blood glucose), attributed to the decreased production of insulin or improper utilization of glucose[43]. Diabetes is the most common cause of polyneuropathy, with approximately 50% diabetics affected within 25 years of diagnosis[44] and is responsible for over 50% non-traumatic amputations[45], nephropathy[46,47] and retinopathy in adults[48]. Diabetics have a higher incidence and prevalence of large vessel disease[49,50] and occurrence of non-enzymatic glucosylation of hemoglobin[51].

Diabetes induced changes in erythrocytes

Diabetes mellitus, being a metabolic disorder, affects the functioning of the erythrocytes through interaction with its membrane and intracellular constituents. Some changes are associated with the impairment of glucose utilization process, whereas, others are induced by the dysfunctional mechanisms due to disease process, thus affecting the erythrocytes properties[52]. Some of these changes which directly or indirectly affect the functional characteristics of erythrocytes are given below:

Oxidative stress—Diabetes erythrocytes have increased malondialdehyde (MDA) (an indicator of lipid peroxidation)[53,54], and decreased glutathione (GSH) and membrane -SH group[55] compared with normal erythrocytes[55,56]. Oxidative stress and increased insulin production contribute to endoplasmic reticulum stress, protein misfolding and induction of the unfolded protein response, leading to

pathological protein[57,58]. The oxidative stress may further induce erythrocytes shape changes as observed under *in vitro* conditions by incubation of erythrocytes with H_2O_2 or ascorbate/Fe^{2+}, which transform discocytes to echinocytes due to involvement of membrane proteins[57].

Lipids—The electronic spin resonance, using spin-labeled fatty acids, has shown structural changes at a depth of 0.6-0.8 nm from the membrane surface in the lipid bilayer of diabetic erythrocytes[59]. The membrane cholesterol is increased but there is four fold increase in phospholipids concentration in the membrane leading to a highly significant decrease in the ratio of cholesterol to phospholipids[59,60].

Skeletal proteins—Spectrin and actin are the two main structural proteins that together form a sub-membraneous cytoskeletal meshwork that is responsible for the viscoelastic properties of the erythrocyte membrane[61]. The spectrin-actin network combined with protein 4.1, which provides erythrocyte membrane the ability to withstand the stresses of circulation, has its origins in various levels of structural organization[52,61,62]. The labeling of erythrocyte membranes with [3H]-borohydride, which labels glucose residues bound to proteins, revealed that several proteins are heavily glycosylated compared with non diabetic membrane. In particular, the proteins beta-spectrin, ankyrin, and protein 4.2 are the most glycosylated while the spectrin is oxidatively damaged[63].

Enzymes and ionic balance—The alteration of activity of (Na^+/K^+) ATPase, which plays a central role in the regulation of intra- and extra-cellular homeostasis, is thought to be linked to several complications in diabetes mellitus[64]. In diabetic patients serum and intra-erythrocyte sodium and serum potassium levels are increased significantly as compared to control subjects. The (Na^+/K^+)ATPase levels are significantly decreased which may cause disturbance of intracellular ionic balance and thus acceleration of cellular ageing[65]. Magnesium in the cell is largely associated with ATP, as the complex Mg-ATP. ATP is less stable when it is not complexed with magnesium, so the loss of magnesium makes the cell more susceptible to stress, leading to an increased uptake of Ca^{2+} (ref. 66, 67), and diminished Ca^{2+}-ATPase activity in comparison to healthy individuals[68].

Alterations in erythrocyte deformability

Diabetes mellitus, as mentioned above, produces a series of changes in the various constituents of the erythrocyte membrane and its interior. Each one of these constituents affects the functional characteristics of erythrocytes through impairment of the deformability.

Based on measurement of erythrocytes deformability by different methods several investigators have found that the deformability decreases with the increase of severity of the disease[69-72]. Early impairment in red blood cell deformability appears in patients with normal renal function, and progressive impairment in red blood cell deformability is associated with renal function loss in all patients regardless of the presence or absence of diabetes[70]. The aging process of the erythrocytes also affects the deformability as the erythrocyte deformation index is decreased with its aging process in a nonlinear fashion, and increasingly greater changes were observed in the later part of the erythrocyte life span[5]. The ektacytometry procedure has been found to be sensitive to detect membrane bound specific changes in diabetes[63].

The slit ektacytometry[23] has been an effective technique not only to measure the deformability of erythrocytes but also to differentiate the influence of the severity of the disease. Figure 3 shows the comparison of the erythrocytes elongation index (EI) in diabetic and healthy subjects. The EI is reduced significantly indicating the loss of deformability in diabetic erythrocytes.

For further analysis of the effect of increasing level of glucose the patients with different glucose

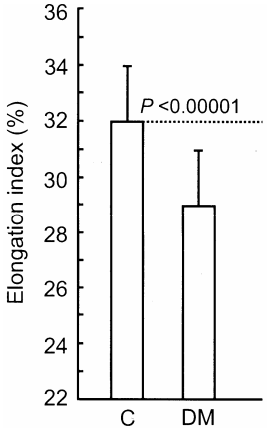

Fig. 3—Comparison of erythrocyte elongation index of healthy controls (C) and diabetes mellitus (DM) subjects at shear stress 3 Pa

concentrations were selected. The filtration time of each category of samples was determined and their blood smears were prepared for analysis of shape descriptors. Table 1 shows the comparison of the form factor (FF) with filtration time of erythrocytes suspensions. With the increase of glucose level in blood the filtration time of erythrocytes is increased, indicating the loss in deformability. The increase in the FF corresponds to the deviation from discoidal shape of normal erythrocytes[32].

Further analysis of the microviscosity of erythrocyte membranes provides unambiguous proof of the structural deterioration of erythrocyte membranes in diabetes[73]. Alterations in membrane lipid-protein interactions together with the increased glycosylation-derived internal viscosity may consequently imply altered viscoelastic properties of erythrocyte membranes. These alterations underlie the impaired deformability of red blood cells in the diabetic state[74].

Conclusion and Future Perspectives

The increasing number of diabetes patients internationally emphasizes the need of rapid and precise detection of changes in blood cells. The present emphasis has been on the measurement of glucose level in blood but not on the consequence of this increase which is clearly shown by the decrease in erythrocyte deformability. For angiopathic changes both parameters, duration of hyperglycemia and changes in deformability, are important. The portable glucose meter, after proper calibration, could help in determining the blood glucose level. Similarly the deformability needs to be measured by techniques which are less costly and easily available. Ektacytometry and micro-pore filtration could be suitable for rapid analysis of erythrocyte deformability of large number of samples; in clinical environment the ektacytometer with its disposable fluidic chip may be more appropriate. More research is required to develop another technique or parameter which could provide data on the consequences of hyperglycemia. Fluidity measurement provides information on the changes in the bilayer only; hence this is not suitable to detect overall changes in erythrocytes. Morphologic analysis, similar to malaria detection[75], could be a preferred procedure but that requires more research prior to its implementation as a regular diagnostic procedure. Such an approach will also be suitable for monitoring the effect of regular exercise, diet and hypoglycemic drugs on routine basis.

There are several direct and indirect mechanisms associated with the membrane for maintenance of normal deformability. Some of these mechanisms are impaired during diabetes. Based on collection of hemorheological, biochemical and clinical data from large number of patients, similar to malaria[76], the artificial neural network procedure could be developed. This could be utilized for severity analysis of diabetes based on the available parameters.

Table 1—Comparison between filtration time and form factor at various glucose levels (based on data in ref. 32). [Values are mean ± SD]

S. No.	Glucose (mg%)	Filtration time (s)	Form factor
1	< 120	1.56 ± 0.15	0.98 ± 0.07
2	120-160	1.65 ± 0.12	1.13 ± 0.14
3	161-200	2.06 ± 0.15	1.16 ± 0.19
4	201-240	2.49 ± 0.29	1.21 ± 0.15
5	. > 241	3.47 ± 0.39	1.31 ± 0.21

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