

CHARACTERIZATION OF ERYTHROCYTES FROM HEALTHY INDIVIDUALS AND FROM PATIENTS WITH THE SICKLE CELL TRAIT

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INTRODUCTION

Red blood cells (RBCs, erythrocytes) are a major component of the blood. Their function is to transport oxygen to all parts of the body since they contain hemoglobin, a protein able to carry oxygen. RBCs contain approximately 250 million Hb molecules and have a characteristic biconcave shape to increase the surface area and to facilitate O₂ and CO₂ diffusion into and out of the cell [1]. Abnormalities in Hb result in serious complications, the most serious of the diseases being sickle cell disease (SCD). Polymerization of deoxygenated Hb and the formation of long stiff rodlike fibers [2] forces RBCs to assume a sickle shape in SCD.

Sickle cell trait (SCT, sicklelemlia) describes the way a person inherits both the abnormal HbS gene and normal hemoglobin (HbA) gene. People with SCT do not have the symptoms of true SCD and thus SCT is usually considered benign [3]. In extreme conditions including dehydration, hyperosmolarity, or elevated erythrocyte 2,3-DPG, individuals with SCT can develop a syndrome resembling SCD with vaso-occlusive sequelae resulting from rigid erythrocytes [3]. Approximately three million people in the United States have the SCT genotype, 40 to 50 times the number of individuals with SCD [3].

IMMOBILIZATION OF ERYTHROCYTES

Normal RBCs and RBCs from patients with the genotype for sickle cell trait were purchased from Research Blood Components, LLC (Brighton, MA). Glass petri dishes (Willco Wells, Amsterdam, The Netherlands) were coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) to increase cell adherence. Fixation of RBCs was performed by a 3-min treatment of the cells with 0.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in PBS buffer. Fixed RBCs were maintained in PBS solution at room temperature during experiments.

ATOMIC FORCE MICROSCOPY

Atomic force microscopy (AFM) allows for high-resolution topography studies of biological cells and measurement of their mechanical properties in physiological conditions. Experiments were carried out using an Asylum MFP 3D-BIO (Asylum Research, Santa Barbara, CA) AFM equipped with a "liquid cell" setup.

Erythrocytes were imaged in tapping mode with a scan rate of 0.2 Hz, and the minimal force necessary to obtain good image

contrast was determined by gradually increasing the force applied to the sample from zero force (noncontact) to the necessary minimal force. Silicon nitride probes with a nominal spring constant of 0.01 N/m (Veeco Probes, Camarillo, CA) were employed for imaging.

Stiffness measurements were carried out in contact mode using silicon nitride cantilevers with a nominal spring constant of 0.03 N/m (Veeco Probes, Camarillo, CA). Exact values for the cantilever spring constants were obtained via a thermal noise based method implemented by the manufacturer and were used in all calculations. Probes had nominal tip radii of 20 nm, as provided by the manufacturer.

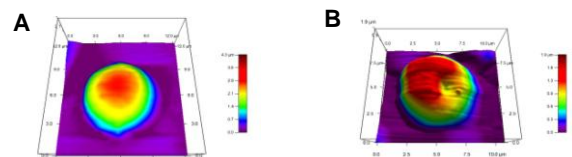


FIG. 1. THREE-DIMENSIONAL TOPOGRAPHICAL IMAGE OF A (A) NORMAL RBC AND AN (B) RBC FROM A PATIENT WITH THE SCT GENOTYPE MEASURED VIA AFM

CELL STIFFNESS DETERMINATION

Force curves were collected primarily from the central part of the erythrocyte (Fig. 1), the point of maximum indentation. For each measurement around 450-500 force curves were recorded.

The Young's modulus was calculated using the Hertz model describing the elastic deformation of the two bodies in contact under load. This theory was extended by Sneddon assuming an appropriate shape of indenter deforming elastic half space [4]. It characterizes the relationship between the applied force and the indentation depth [5]. When the shape of the AFM tip may be approximated by a paraboloid, the force as a function of indentation is described by the following equation:

$$F = \frac{4}{3} \sqrt{RE'} \Delta z^{\frac{3}{2}} \quad (1)$$

where Δz is the indentation depth, R is the tip radius of curvature,

E' is the reduced Young's modulus of the tip-sample system defined as:

$$\frac{1}{E'} = \frac{1 - \mu_{tip}^2}{E_{tip}} + \frac{1 - \mu_{sample}^2}{E_{sample}} \quad (2)$$

where E_{tip} , E_{sample} are Young's moduli of the tip and the sample, μ_{tip} , μ_{sample} are Poisson ratios. If $E_{sample} \leq E_{tip}$ (in the case of Si_3N_4 tips, the Young's modulus is 150 GPa [6]), E' can be simplified as:

$$E' = \frac{E_{sample}}{1 - \mu_{sample}^2} \quad (3)$$

For soft tissues, the Poisson ratio ranges from 0.490 to 0.499. The Poisson ratio used in the present study was assumed to be 0.5.

INDENTATION DEPTH DETERMINATION

The quantitative determination of the elastic properties of a particular material can be obtained from the relationship between the applied force F and the indentation depth Δz using Eq. (1). Prior to taking force measurements on the RBC surface, the force is measured on the poly-L-lysine coated glass substrate. This curve is used as a calibration curve since no permanent sample deformation is observed [7]. The indentation produced by the AFM tip was determined by subtracting the calibration curve from the curve recorded for the erythrocyte using the Igor Pro 6.04 (Wavemetrics, Portland, OR) software program. Typical force v. indentation curves obtained from erythrocytes from patients with SCT (A) and normal erythrocytes (B) are presented in Figure 2. The force v. indentation curves obtained for normal erythrocytes (B) indicate a greater compliance than sickle trait RBCs (A).

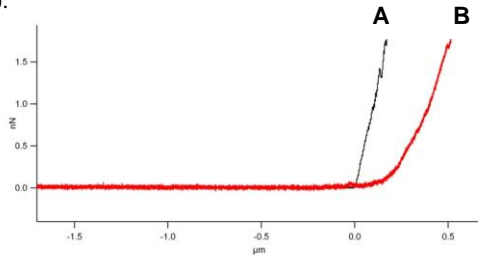


FIG. 2. TYPICAL AFM FORCE CURVES OBTAINED ON (A) SCT ERYTHROCYTE AND (B) NORMAL ERYTHROCYTE.

The presence of the internal structure of erythrocytes suggests that the calculated Young's modulus can vary revealing its distinct value as a function of the indentation depth. At greater indentation depths, there is known to be a linear dependence between the applied load and the produced deformation [7]. To determine the Young's modulus of the normal and sickle cell trait erythrocytes, we chose an indentation depth value of 200 nm to avoid the expected influence of the hard substrate on which cells were immobilized and the range of alterations in the structure of the erythrocytes.

YOUNG'S MODULUS DETERMINATION

The average values of Young's modulus for normal erythrocytes and SCT erythrocytes were obtained by fitting the Gaussian distribution to the histogram (Fig. 3A-B). The bin size of each histogram was 10 kPa and errors were estimated as the half width

of the Gaussian peak. The obtained Young's modulus of erythrocytes from patients with SCT was more than three times larger than that obtained for normal erythrocytes (32 ± 13 kPa and 100 ± 28 kPa, respectively).

It has been reported in many publications that the Young's modulus determined using AFM reflects alterations in the cell cytoskeleton [8]. Therefore, in order to explain the obtained results the erythrocytes cytoskeleton was taken into account. During indentation of erythrocytes, the AFM tip monitors the response from the cytoskeleton and hemoglobin fibers within the cytoskeleton. Any alterations occurring in these structures can influence the overall stiffness of erythrocytes.

We conjecture that the erythrocyte cytoskeleton is altered by changes in the spectrin network and membrane loss associated with polymerized HbS. These structural changes are due to a gene mutation. Hemoglobin S is responsible for creating rigid, "sickled" cells in patients with SS-SCD. Because normal erythrocytes are very compliant they assume the shape of a spherical cap (Fig. 1A) when strongly adhered to a poly-L-lysine coated glass surface. Due to an increased stiffness, the sickle trait RBC maintains (Fig. 1B) some of its characteristic biconcave shape when treated with glutaraldehyde and immobilized on a poly-L-lysine coated glass surface.

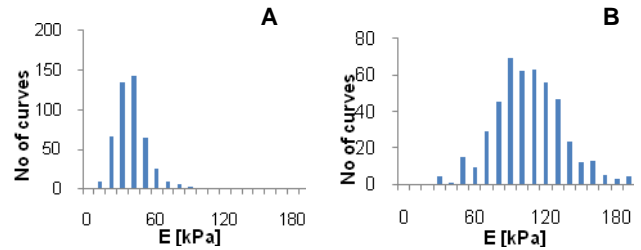


FIG. 3. HISTOGRAMS OF THE YOUNG'S MODULUS DETERMINED FOR RBCs FROM (A) NORMAL AND (B) SICKLE CELL TRAIT BLOOD SAMPLES.

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