Sickle cell trait human erythrocytes show a significant increase in their stiffness compared to erythrocytes from healthy individuals

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Text: 2,961
Abstract: 109 words
Figures: 3
Tables: 1
References: 67
Abstract

Atomic force microscopy (AFM) allows for high-resolution topography studies of biological cells and measurement of their mechanical properties in physiological conditions. In this work, AFM was employed to measure the stiffness of abnormal human red blood cells (RBCs) from patients with the genotype for sickle cell trait. The determined Young’s modulus was compared with that obtained from measurements of erythrocytes from healthy subjects. The results showed that the Young’s modulus of pathological erythrocytes was approximately three times higher than in normal cells. Observed differences indicate the effect of the polymerization of hemoglobin S as well as possible changes in the organization of the cell cytoskeleton associated with the sickle cell trait.

Keywords: AFM; stiffness; erythrocyte; sickle cell disease
Introduction

Sickle cell disease (SCD) is an inherited blood disorder caused by a single point mutation in one of the genes encoding hemoglobin. Four polypeptide chains, two of type $\alpha$ and two of type $\beta$, form the globular protein Hb. In sickle cell hemoglobin (HbS), the normal sequence of $Val-His-Leu-Thr-Pro-Glu-Glu-Lys$ is changed to $Val-His-Leu-Thr-Pro-Val-Glu-Lys$, with the amino acid valine substituting for the glutamic acid in the $\beta 6$ site. The replacement of two charged groups by two hydrophobic ones leads to polymerization of deoxygenated Hb and to formation of long stiff rodlike fibers (Ferrone, 2004, Noguchi and Schechter, 1985, Turner, et al., 2002) which force RBCs to assume a sickle shape. Other factors such as lowered pH, RBC dehydration and hyperthermia are also known to prompt sickling (Connes, et al., 2008). Healthy RBCs are very deformable and easily pass through narrow capillaries during their approximately three-month life span. The characteristic biconcave shape of RBCs does not only increase the surface area and facilitate $O_2$ and $CO_2$ diffusion into and out of the cell (Alberts, et al., 1983, Mohandas and Gallagher, 2008) but it has been shown that it is compatible with the increased compliance of the erythrocytes (Li, et al., 2005). The abnormal morphology and rheology of the sickle cells triggers the obstruction of the microvasculature which results to the development of hypoxia, vaso-occlusive crisis and organ damage.

SCT has a very significant social impact because approximately three million people in the United States have this genotype, making it 40 to 50 times more prevalent than SCD (John Kark, 2000). People with sickle cell trait do not have the symptoms of SCD (Austin, et al., 2007, NIH) and thus SCT is not usually regarded as a disease state because it has complications that are either uncommon or mild (John Kark, 2000). Although SCT is considered to be benign, in
extreme conditions including hypoxia, acidosis, dehydration, hyperosmolarity, or elevated erythrocyte 2,3-diphosphoglycerate (DPG), individuals with SCT can develop a syndrome resembling SCD with vaso-occlusive sequelae resulting from rigid erythrocytes (Acharya, et al., 2009, John Kark, 2000, Jones SR, 1970, Kark JA, 1987). Other complications associated with SCT include increased urinary tract infection in women, gross hematuria, complications of hyphema, splenic infarction with altitude hypoxia or exercise, and life-threatening complications of exercise, exertional heat illness (exertional rhabdomyolysis, heat stroke, or renal failure) or idiopathic sudden death (John Kark, 2000, Kark and Ward, 1994, Sears, 1978).

The red cell membrane derives its resilience and resistance to mechanical stresses from the membrane skeleton, a hexagonal lattice network composed of spectrin tetramers. These are formed by the side-by-side alignment of pairs of heterodimers attached at their ends to complexes consisted mainly of actin and of several other proteins (Alberts, et al., 1983, Li, et al., 2007, Mohandas and Gallagher, 2008). Horizontal connections between the spectrin filaments are formed at the actin junctions with proteins such as protein 4.1R, tropomyosin, tropomodulin, and adding. Vertical connections between the spectrin network and the lipid bilayer are formed at the ankyrin linkers which connect the \( \beta \)-spectrin filaments to the band 3 protein along with protein 4.2 (pallidin). A second type of vertical connection is formed at the actin junctions where, protein 4.1 binds the spectrin network to the lipid bilayer via the integral protein, glycophorin C (Cartron, et al., 1998, Iolascon, et al., 2003, Mohandas and Gallagher, 2008). Mutations in membrane proteins related to vertical connections cause hereditary spherocytosis related to membrane loss because of reduced support to lipid bilayer. Mutations in proteins associated to horizontal connections cause hereditary elliptocytosis (Tse and Lux, 1999).
Normal erythrocytes in adults contain approximately 250 million adult hemoglobin (HbA) molecules, totaling 25-30% of the cell (Ferrone, 2004). Sickle cell trait (SCT, sickleemia), in contrast, is marked by the presence of both HbS and HbA. Sickle hemoglobin comprises approximately 20-46% of the total hemoglobin content in persons with sickle cell trait (Neel, et al., 1951), with most individuals having 40-42% HbS (Wells and Itano, 1951). In the case of SCD, it has been hypothesized that increased association of HbS with the membrane proteins contributes in the change of the mechanical behavior of Sickle cells (Mohandas and Evans, 1994). HbS could alter the mechanical properties of RBCs not only by extending the spectrin filaments, and by the interaction between HbS filaments and the lipid bilayer but also by altering the functionality of the membrane proteins (Statius van Eps, 1999). There is considerable evidence that HbS is associated with the inner membrane of RBCs and in particular with the cytoplasmic tail of the band 3 protein (Ferrone, 2004).

Many case studies have shown that translation of cellular mechanics findings is crucial in many diseases, including Alzheimer’s disease, Parkinson’s disease, type II diabetes, malaria, sickle cell disease, and most recently cancer (Buehler and Yung, 2009, Cross, et al., 2007). Understanding the relationship between proteins and cellular material properties will allow for detection of diseases and new approaches to addressing medical disorders (Buehler and Yung, 2009). Recently, atomic force microscopy methods have shown that the stiffness of metastatic cancer cells is more than 70% softer than their benign counterparts (Cross, et al., 2007). Because these findings correlate well with the commonly employed immunohistochemical testing, it is reasonable to argue that AFM methods can enhance cancer detection methods. SCD is a
characteristic case where molecular changes in a protein alter their structural properties causing the initiation of a disease state. Additional reason for the SCD is the increased adherence of sickle cells on the endothelial cells and on leucocytes which can generate an inflammation and chronic vasculopathy (Hebbel, et al., 2004). AFM can return information not only on the stiffness of the cells but also on the specific interaction between proteins expressed on the cellular erythrocyte membrane and proteins expressed on leucocytes, on endothelial cells and/or the extracellular matrix (Hebbel, et al., 2004). Because of this characteristic, AFM could decisively enhance our knowledge on the biology and consequently on the therapy of SCD.

Characterization of erythrocyte mechanical properties has been conducted via micropipette aspiration (Evans, 1973, Glenister, et al., 2002), cell poking (Daily, et al., 1984), atomic force microscopy (Dimitriadis, et al., 2002, Dulinska, et al., 2006, Hategan, et al., 2003), optical tweezers (Lenormand, et al., 2001, Suresh, 2006), optical stretching (Dao, et al., 2006, Gu, et al., 2007), and magnetic tweezers (Puig-De-Morales-Marinkovic, et al., 2007), and magnetic twisting cytometry (Marinkovic, et al., 2009). However, values of mechanical moduli derived from experimental data tend to vary largely based on which of the aforementioned techniques is used (Musielak, 2009). In the present student, we quantified the stiffness of erythrocytes from patients with the genotype for sickle cell trait using atomic force microscopy, a tool for imaging and characterization of materials at the nanometer scale (Binnig G, 1986). The high force sensitivity of AFM and its ability to measure local and overall properties of individual cells under physiological conditions make this technique particularly appropriate for measuring the mechanical properties of living and fixed cells (Dimitriadis, et al., 2002, Haga, et al., 2000, Hategan, et al., 2003, Nowakowski, et al., 2001, Radmacher, 1997). We established that the
stiffness of cells from patients with the genotype for SCT was approximately three times greater than normal RBCs.

Methods

AFM allows for high-resolution topography studies of biological cells and measurement of their mechanical properties in physiological conditions (Ikai A, 1997). Experiments were carried out using an Asylum MFP 3D-BIO (Asylum Research, Santa Barbara, CA) AFM equipped with a “liquid cell” setup. Normal RBCs and RBCs from patients with the sickle cell trait were purchased from Research Blood Components, LLC (Brighton, MA). RBCs were maintained in PBS at room temperature during experiments.

Erythrocyte immobilization

Cells were immobilized on AFM grade mica (Novascan Technologies, Inc., Ames, IA) coated with poly-L-lysine (PLL) (Sigma-Aldrich, St. Louis, MO) to increase cell adherence. 150 µl of 1 mg/ml PLL solution was allowed to adsorb for 5 min to an unmodified mica surface, and excess solution was drained away. RBCs of 0.5% concentration in PBS were allowed to adhere to each PLL-coated mica surface for 10 min in the incubator. Unattached cells were removed by gentle rinsing of the slide with PBS solution at 25°C. For imaging, fixation was performed by a 1-min treatment of the cells with 0.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in PBS buffer. The sample was again rinsed several times with PBS and a volume of PBS was added for experiments. Glutaraldehyde was not employed for stiffness measurements.
Erythrocyte imaging

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. While the characteristic biconcave shape was observed for the fixed normal erythrocyte (Fig. 1A) the SCT erythrocyte appeared to have an irregular biconcave shape accompanied by a decrease in size which is most likely due to dehydration associated with the polymerization of HbS (Fig. 1B).

Cell stiffness determination

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 and nominal angle of 20°, as provided by the manufacturer. The force curves were generated at a loading rate of 10,000 pN/s, and for each cell type between 500-600 measurements were collected.
**Figure 1.** Three-dimensional topographical images of a (A) normal RBC and an (B) RBC from a patient with the SCT genotype measured by AFM. While the normal RBC has the characteristic biconcave shape, the SCT erythrocyte has an irregular biconcave morphology. In addition, the SCT erythrocyte is smaller than the normal erythrocyte, most likely due to dehydration associated with HbS polymerization.

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 (Giannakopoulos, 2006, Sneddon, 1965). It characterizes the relationship between the applied force and the indentation depth (Lekka, et al., 1999, Radmacher, 1997). When the shape of the AFM tip is a four-sided pyramidal indenter, the force as a function of indentation is described by the following equation:

\[
F = \frac{3E \tan \theta}{4(1-\nu^2)} \delta^2
\]  

where \( E \) and \( \nu \) are the Young’s modulus and the Poisson’s ratio of the cell, respectively (Alcaraz, et al., 2003, Bilodeau, 1992, Rico, et al., 2005). The Poisson ratio used in the present study was assumed to be 0.5 (Dulinska, et al., 2006).
Results

Determination of the indentation depth

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 $\delta$ using Eq. (1).

When force is measured on a hard substrate, the cantilever deflection is proportional to the relative sample position resulting in a linear slope for the portion of the curve where the tip and the sample are in contact. When soft samples like erythrocytes are investigated, the recorded cantilever deflection as a function of the relative sample position is not linear due to the deformable structure of the RBC. Prior to taking force measurements on the RBC surface, the force is measured on the PLL-coated mica substrate. This curve is used as calibration curve since no permanent sample deformation is observed.

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. The force vs. indentation curves obtained for normal erythrocytes indicate a greater compliance than that of the sickle trait RBCs.
Data processing

Data was imported into MATLAB (The MathWorks, Natick, MA) and the value of $E$ was obtained by fitting the theoretical curve generated by Eq. (1) to the experimental data up to a minimum depth of 250 nm, which is approximately 10% of the total thickness of the cell. The method is in agreement with the well known fact that the effect of the substrate over which the cells are placed is negligible if the indentation depth is less than 10% of the total thickness of the specimen (Dimitriadis, et al., 2002). Figure 2 shows the MATLAB output of the pyramidal indenter model fit to the force v. indentation curve obtained via experimentation. It appears that

Figure 2. Theoretical model for a pyramidal shaped indenter fitted to the experimental data for normal and SCT erythrocytes obtained via AFM. In these curves, the Young’s modulus values for the normal and SCT cell are 1.30 and 3.30 kPa, respectively. The curves shown above are representative of the 500-600 force curves obtained for each normal and SCT erythrocytes. Minor variations can be seen in other curves obtained during experimentation, representative of the standard deviation we observe in the calculation of the Young’s modulus.
the theoretical model for a pyramidal indenter fits the SCT data up to a greater indentation depth (700 nm) than that of the normal RBC (400 nm), indicating that SCT follow the elastic model for larger indentation depth.

The average values of Young’s modulus for normal erythrocytes and SCT erythrocytes were obtained by fitting the Gaussian distribution to the generated histograms of the measured Young’s modulus values (Figure 3A-B, respectively). The measured Young’s moduli and standard deviations were: \( E_1 = 1.10 \text{kPa}, \sigma_1 = 0.40 \text{kPa} \), and \( E_2 = 3.05 \text{kPa}, \sigma_2 = 1.09 \text{kPa} \), for normal and sickle cell trait, respectively.

**Figure 3.** Histograms of the Young’s modulus determined for RBCs from (A) normal and (B) sickle cell trait blood samples. The Young’s modulus values obtained from fitting the theoretical model to the experimental data were fitted with the Gaussian distribution. Normal RBCs are more elastic and their Young’s modulus values have a lesser standard deviation (\( E_1 = 1.10 \text{kPa}, \sigma_1 = 0.40 \text{kPa} \)) while SCT erythrocytes have a stiffness approximately three times greater as well
as a larger standard deviation ($E_2 = 3.05 \text{ kPa}, \sigma_2 = 1.09 \text{ kPa}$). The observed increase in stiffness for the pathological erythrocytes is likely due to changes in the affinity of spectrin and actin filaments resulting from the presence of abnormal hemoglobin, HbS.

**Discussion**

The main goal of this work was the determination of the erythrocyte’s stiffness in blood samples taken from patients with the sickle cell trait genotype. The obtained Young’s modulus of erythrocytes from patients with SCT was approximately three times larger than that of normal erythrocytes.

Hemoglobin S is responsible for converting normal RBCs into “sickled” cells in patients with SS-SCD. At high concentrations, deoxy sickle hemoglobin results in the formation of a seven stranded polymer structure which increases RBC rigidity (Turner, et al., 2006). As expected, we observe the characteristic biconcave shape for normal erythrocytes (Figure 1a) when imaging on a PLL-coated mica substrate. In contrast, due to an increased membrane stiffness and dehydration, the sickle trait RBC digresses from its characteristic biconcave shape (as shown in Figure 1b) and also experiences a decrease in size.

Our findings show that the Young’s modulus of normal erythrocytes is 1.10 kPa with a standard deviation of 0.40 kPa, while erythrocytes from patients with the genotype for sickle cell trait have a Young’s modulus of 3.05 kPa with a standard deviation of 1.09 kPa. Other techniques,
such as micropipette aspiration and optical tweezers, result to RBC moduli values in similar range (Dao, et al., 2003, Evans, 1973, Suresh, 2006). AFM has previously been used in the measurement of the Young’s modulus of various types of cells e.g., platelets (1-50 kPa), lymphocytes (1.24 kPa), fibroblasts (1-5 kPa), mesenchymal stem cells (1-2.5 kPa) and osteoblasts (1-200 kPa) (Cai, et al., 2010, Cai, et al., 2010, Domke, et al., 2000, Radmacher, et al., 1996, Schafer and Radmacher, 2005, Simon, et al., 2003, Takai, et al., 2005, Yim, et al., 2010).

The threefold increase in the stiffness of SCT erythrocytes is probably due to changes in the affinity of the spectrin and actin filaments that comprise the membrane skeleton. It has been proposed that SCT erythrocytes show an increased concentration in Ca$^{+2}$ which results in a higher cytoskeleton rigidity via an increased binding of Band 3 to the spectrin bound ankyrin (Liu, et al., 2005, Takakuwa, 2000). Another possible source of the measured increased stiffness is the dehydration of the SCT erythrocytes that promotes polymerization of deoxygenated HbS (Maher and Kuchel, 2003, Stocker, et al., 2003). It has been shown that activation of the Gardos channel by an increase of the intracellular free Ca$^{+2}$ concentrations results to expulsion of potassium and H$_2$O. In addition, spectrin which is the main protein responsible for the mechanical strength of the erythrocyte has been found to bind to hemoglobin via the Band 3 protein that binds almost exclusively to hemoglobin fibers (vonRuckmann, et al., 1997, Walder, et al., 1984). Thus, interaction between the HbS fibers that are connected to the membrane and the HbS fibers in the cytoplasm could also contribute to the measured increase in stiffness (Brugnara, 2001).
In summary, the present study suggests that sickle hemoglobin promotes a threefold increase in the stiffness of sickle trait erythrocytes. Our data conclude that Young’s moduli and standard deviations were $E_1 = 1.10 \text{kPa}$, $\sigma_1 = 0.40 \text{kPa}$, and $E_2 = 3.05 \text{kPa}$, $\sigma_2 = 1.09 \text{kPa}$, for normal and sickle trait erythrocytes, respectively. In contrast to normal RBCs with a characteristic biconcave shape, SCT erythrocytes deviate from this configuration with irregularities in the expected morphology and a decrease in both diameter and height.

**Conflict of Interest statement**

The authors have no conflicts of interest to declare.
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Figure Captions

Figure 1. Three-dimensional topographical images of a (A) normal RBC and an (B) RBC from a patient with the SCT genotype measured by AFM. While the normal RBC has the characteristic biconcave shape, the SCT erythrocyte has an irregular biconcave morphology. In addition, the SCT erythrocyte is also smaller than the normal erythrocyte, likely due to dehydration associated with HbS polymerization.

Figure 2. Three-dimensional topographical images of a (A) normal RBC and an (B) RBC from a patient with the SCT genotype measured by AFM. While the normal RBC has the characteristic biconcave shape, the SCT erythrocyte has an irregular biconcave shape. In addition, the SCT erythrocyte is also smaller than the normal erythrocyte, likely due to dehydration associated with HbS polymerization.

Figure 3. Histograms of the Young’s modulus determined for RBCs from (A) normal and (B) sickle cell trait blood samples. The Young’s modulus values obtained from fitting the theoretical model to the experimental data were fitted with the Gaussian distribution. Normal RBCs are more elastic and their Young’s modulus values have a lesser standard deviation ($E_1 = 1.1$ kPa, $\sigma_1 = 0.4$ kPa) while SCT erythrocytes have a stiffness approximately three times greater as well as a larger standard deviation ($E_2 = 3.05$ kPa, $\sigma_2 = 1.09$ kPa). The observed increase in stiffness for the pathological erythrocytes is likely due to changes in the affinity of spectrin and actin filaments resulting from the presence of abnormal hemoglobin, HbS.