Proceedings of the National Conference
On Undergraduate Research (NCUR) 2013
University of Wisconsin, La Crosse, WI
April 11-13, 2013

Physical and Mechanical Properties of the Human Red Blood Cells with Different Hemoglobin Types

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Abstract

Sickle cell anemia (SCA) is identified as a genetic disease in which a point mutation in the β-globin gene resulted from the replacement of adenine with thymine. This single nucleotide substitution results in β-globin chain producing the sickle hemoglobin (HbS). This genetic mutation produces two distinct genotypes; heterozygotes (AS) resulting in sickle cell trait (SCT) and homozygotes (SS) resulting in SCA. SCA do not appear until after six months of age when the β-globin gene expression has primarily switched to β^S-globin gene expression resulting in the conversion from predominantly fetal hemoglobin (HbF) to HbS instead of to normal adult hemoglobin (HbA). In SCA, the red blood cells (RBCs) fail to change its shape and deformability normally under deoxygenating conditions resulting in occluding the blood vessels in the microcirculation. To better understand how the physical and mechanical properties of RBCs are affected by Sickle Cell Anemia, a laser tweezer (LT) is used to manipulate the cells. By manipulating the RBCs using LT studies have been made on the mechanical properties differences of RBCs with different types of Hemoglobin [SCT (HbSA), SCA (HbSS), normal adult (HbAA), and normal infant (HbFF)].

Keywords: Laser trapping, chromatography, hemoglobin

1. Introduction

Red blood cells (RBCs) are donut-shaped cells devoid of a nucleus found abundantly in the blood. An RBC is made predominantly from two hemoglobin chains called α-globin and β-globin chains. Each of these chains carries Heme-group (iron) that binds with oxygen in the lung and release the oxygen to different parts of body tissue as the RBCs squeeze through narrow blood capillaries. Certain abnormalities in the β-globin chain can change the chemical composition of the normal hemoglobin that causes a drastic change in the physical and mechanical properties of the RBCs and results in a disease called sickle cell anemia (SCA). Since its discovery in the year 1910[1], SCA has been identified as a genetic disease in which a point mutation in the β-globin gene located on chromosome 11 has one original nucleotide, adenine, replaced with thymine resulting in the sickle hemoglobin (Hb S)[2, 3]. This genetic mutation produces two distinct genotypes; heterozygotes (AS) resulting in sickle cell trait (SCT) and homozygotes (SS) resulting in SCA. In SCA, the normally donut-shaped RBCs, change their shape and deformability under deoxygenating conditions which results in a sudden blockage of a blood vessel (vascular occlusion) in the microcirculation[4]. Vascular occlusion results in a restriction in blood supply to tissues, causing a shortage of oxygen and glucose needed for cellular metabolism required to keep tissues alive. As a result, patients with SCA suffer with acute, painful disorder of blood vessels that could lead to a central nervous system breakdown that causes impaired intellectual development and, in some patients, devastating strokes that could lead to disability and early death[5, 6]. These symptoms do not appear until after six months of age when the γ-globin gene expression has primarily switched to β^S-globin gene expression resulting in the conversion from predominantly fetal hemoglobin (Hb F) to sickle hemoglobin (Hb S) instead of to normal adult hemoglobin (Hb A). Currently there are no effective curative

therapies of SCA and patients are treated by a lifelong regular intravenous transfusion of normal RBCs and by use of hydroxyurea (HU), the only FDA approved oral medication drug, which boosts the production of HbF[7].

In this study the physical and mechanical properties of the RBCs for blood samples obtained from normal individuals, individuals with SCT, and patients with SCA who were treated by blood transfusion and HU and untreated SCA patients. For each of these samples a hemoglobin quantitation assessment was carried out by use of a High Performance Liquid Chromatography (HPLC)[8]. The mechanical properties of the RBCs was studied by use of a laser tweezer (LT)[9]. LT is a novel optical device capable of conveniently trapping and manipulating living[10-13] or non-living[14] dielectric particles whose dimensions range from tens of nanometers to tens of microns. The trap is formed by focusing a well collimated laser beam using a high numerical aperture microscope objective lens.

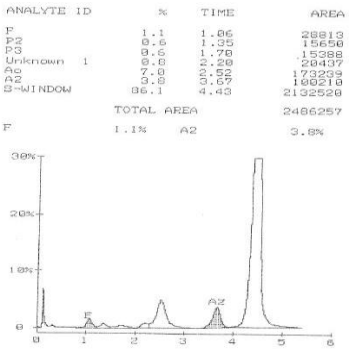
2. Materials and Experimental Method

2.1 Blood Samples & Hemoglobin Quantitation

De-identified blood samples from individuals with SCT, SCA (treated and untreated), and normal individuals were obtained from the Meharry Sickle Cell Center (MSCC) in an EthyleneDiamineteTraacetic Acid (EDTA) tube which had anticoagulant properties to prevent blood clotting. MSCC is a major testing center for genetically defected chains in the hemoglobin molecule (Hemoglobinopathy) which is located at Meharry Medical College (MMC) in Nashville, Tennessee. These samples were obtained from the individuals by either vein puncture or finger stick. Unless fresh, all samples used in the study were stored at 2°C at the MSCC. A biological material transfer agreement was processed between both institutions involved, MTSU and MMC. Blood samples from a group of unidentified volunteers in the research team with normal hemoglobin types were also included in this study. For these normal blood samples and the blood samples from SCT and the SCA patients who are treated/untreated a hemoglobin type quantitation was done by Ultra2-HPLC from Trinity Bio-Tech (Kansas City, Kansas) at the MSCC.

Ultra2-HPLC employs principles of ion exchange chromatography and spectrophotometric detection. For Hemoglobinopathy screening, the RBCs are first hemolyzed (the cell membrane is ruptured releasing the hemoglobin). The resulting hemoglobin mixture is then put under high pressure so that the mixture may be injected into a net-positively charged column. The positive charges in this column follow a gradient starting from high magnitude descending to lower. At a moderately alkaline pH, all hemoglobins carry a variable net negative charge with magnitude distinctive to that hemoglobin. The hemoglobin present in our samples were F, A S, and C. In this order F has the weakest and C is the strongest negative charge. When these differently negatively charged hemoglobin types are injected into the positively charged column of Ultra2-HPLC, the C type will bind strongly with the column at first and F bind weakly at last. Then the column is washed with appropriately blended buffers which cause elution of Hemoglobin F first and hemoglobin C at last from the column which would be detected by a spectrometer and the percentage of the different types of Hemoglobin is recorded on a computer [Fig. 1b].

(a)



(b)

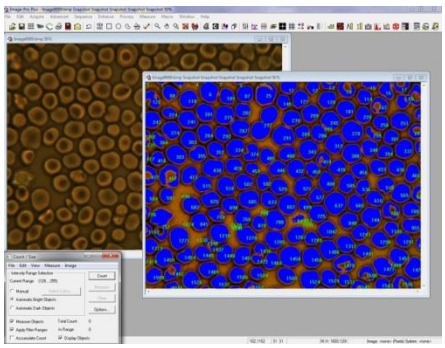


Figure 1. (a) Hemoglobin quantitation by Ultra2-HPLC for SCA patient 90 days post transfusion. The large peak represents the 86.1% of HbS found in the individual's sample. (b) Physical properties measurement for RBCs using Image-Pro Plus 6.2

2.2 Free RBC's imaging and Physical Properties

Each of the three blood samples were diluted in fetal bovine serum (FBS) in about a 1:100 dilutions. The FBS provided a suitable environment for the cells to be suspended in while the dilution provided a large concentration of cells in the viewing range of the microscope. Each sample was placed in a well slide, and several images were taken with a digital camera attached to the microscope and interfaced with a computer. In each image several properties of the RBCs were measured using image processing, enhancement, and analysis software-Image-Pro Plus 6.2 (Media Cybernetics). This was carried out by programing Image-Pro Plus 6.2 to highlight bright pixels of a certain intensity which was set to be in the range of the intensity of light transmitted through the cells. The program was then used to count all the highlighted pixels, grouping them if they shared adjacent sides. Calibrating the size of a pixel to an area in square meters for a magnification of 100X we were able to find over a dozen size characteristics of the cells including mean diameter and area [Fib. 1b].

2.3 Laser Tweezers

After the measurements studying the physical properties of the free cells were processed, similar measurements on the RBCs for SCA, SCT (adult and infant), and normal (adult and infant) were conducted using LT. The core element of a LT is a well collimated laser beam sharply focusing via a microscope objective lens as shown in Fig. 2a.

To briefly explain how laser tweezers work we will recall the Lorentz force which deals with the force of an electric field and a magnetic field on a charged particle. When these two fields are orthogonal to one another we see that a charged particle originating from rest will "bounce" up and down the axis of the electric field but it will propagate down the axis that is orthonormal to both the electric and magnetic fields, or the Poynting vector [Fig. 2b]. But when we introduce a charged particle into an electromagnetic wave [Fig. 2c] we get a path carved out by the charged particle that for all intended purposes here can be very accurately simplified to the particle following an electric field centered and propagating along the Poynting vector [Fig. 2d]. This is the reason a dielectric particle is needed for the trapping process. The opposing forces on the bounded positive and negative charges counterbalance one another trapping the dielectric particle in space. The beam is focused through the OL so that there is a high gradient in the electric field pointing towards the beam waist which, if positioned correctly, will tightly trap the particle on the focal plain of the microscope with strength proportional to the power of the laser. And since a laser follows a Gaussian distribution, the intensity of the electric field will very drastically increase close to the center of the trap so that if the dielectric in question is deformable to some extent such as a RBC then we will witness a squeezing effect on the dielectric. Note that it is this understanding that this exploration into the physical and mechanical properties of RBCs is based upon.

However, a LT that can trap on the focal plain, manipulate, and at the same time produce images of the trapped RBCs requires a well-designed experimental set-up. The design for our experimental set-up is shown in Fig. 2e. Following the design (from top left) we have a linearly polarized infrared diode laser source (LS) producing 8 watts at 1064nm with a beam size of 4mm. The power was controlled by a $\lambda/2$-wave plate (W) and polarizer (P) combination. In order to ensure the position of the trap on the focal plain of the microscope we use a series of converging and diverging lenses beginning with the beam expander (BE) and through lenses (L1-4). The mirrors (M1-4) are used to align and direct the laser beam through a port of an inverted microscope (IX 71-Olympus). A perfectly aligned beam would then be redirected for a normal incidence angle at the center of the back of an objective lens (OL) using a dichroic mirror (DM) positioned at 45° inside the microscope. The OL has a 100X magnification and a 1.25 numerical aperture (Edmund Optics). The microscope is equipped with piezo-driven (NS) stage to house and better manipulate the microscope slide and a digital camera (CCD) to take a live 2D bright-field contrast image of the sample in the slide using 30mW halogen lamp (HL). Both the piezo-driven stage and the digital camera are interfaced with a computer (PC).

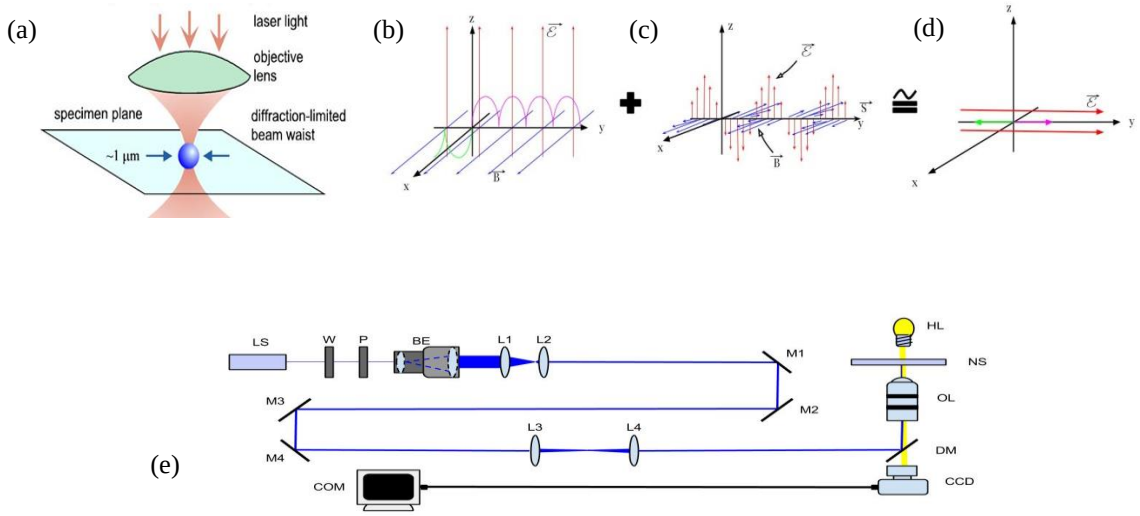


Figure 2. (a) A laser trap formation with the converging laser beam incident on a particle. (b) The paths followed by a positive (purple) and negative (green) charged particle in the presence of uniform orthogonal electric and magnetic fields. (c) The orientation of the sinusoidal orthogonal electric and magnetic fields in an electromagnetic wave. (d) The simplification of (a) and (b) combined into and electric field. (e) The experimental design with an inverted microscope.

2.4 RBC's Mechanical Properties

The mechanical properties of the RBCs studied were focused on the size changes of the cells when the RBC was trapped and isolated by the laser tweezers over different laser powers. The laser powers tested at spanned from 30mW up to 280mW in five intervals. The power of the laser was measured in between lenses L3 & L4. Note that these powers are not the powers incident on the cell. The power incident on the cell is measured to be much less due to experimental restraints with a linear ratio. But for the purpose of comparison between cells it is not necessary to know the exact power at the trap location. The power incident on the microscope will suffice. Each bloo d sample was diluted with FBS at 1:1000 dilutions. The diluted blood samples were placed on a well-slide and mounted on the piezo-driven stage (NS) of the microscope. Three consecutive images of the cell were taken when it was free of the trap at the bottom of the slide and another three images when the cell was trapped and isolated $12\mu m$ above the bottom of the slide. The individual images were measured by tracing the outer boundaries of the bright and dark regions and the middle in between the two [Fig. 3]. As in Section 2.2, the pixels inside the boundaries were counted and calibrated into an area in squared meters providing a means of obtaining the physical properties of the cell. The values obtained for each boundary (bright, dark and middle) was averaged over ten cells for each hemoglobin type at each power, i.e. the dark region of cell one while it is free is averaged with the dark region of cells 2-10 of the same hemoglobin type at the same power. This was done for each hemoglobin type at each power interval.

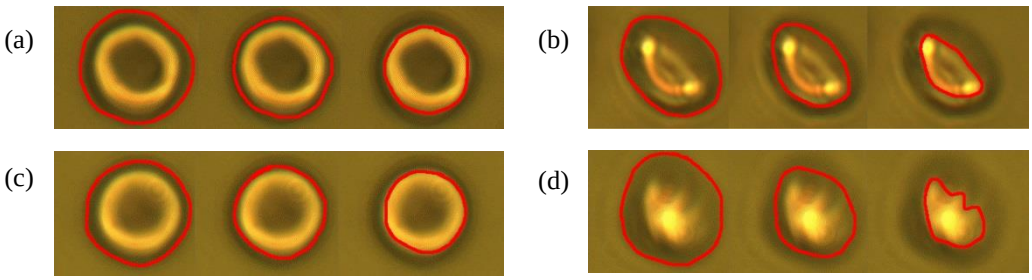


Figure 3. Three images of a free RBC [(a) & (c)] with HbAA and three images of the same RBC in the laser tweezers [(b) & (d)] with (a) and (b) being the same cell and (c) and (d) being the same cell. (b) was trapped at 30mW and (d) at 80mW. The enveloping red line around the cell is part of the measurement process. The lines also illustrate the difference in size induced by the squeezing of the laser tweezers.

3. Data Analysis and Results

3.1 Hemoglobin Quantitation

The results for the hemoglobin quantitation assessment by Ultra2-HPLC for each blood samples are shown in Table 1. The blood samples for individuals' with normal hemoglobin (AA) are shaded green and for individuals with SCT (AS) are shaded yellow. The last two columns represent the hemoglobin quantitation for a SCA patient treated with blood transfusion (orange) and another SCA patient treated with no blood transfusion (Red). In general in normal adults the normal hemoglobin type (HbA) is always > 96% and the fetal hemoglobin (HbF) ~0 %. For newly born infant the HbF is ~100%. The number for HbF rapidly decreases as the infant grows switching dominantly over to HbA (normal), HbS (SCA), or a co-dominance between the two (SCT) depending on the genetic inheritance of the child. This switching won't happen until six month of age[6].

Table 1. Size Measurement And Hemoglobin Quantitation For The Blood Samples.

Blood sample		N1	N2	N3	N4	N5	N6	SCT1	SCT2	SCA1	SCA2
Hb type		AA	AA	AA	AA	AA	AA	AS	AS	SS	SS
Sex		F	M	F	F	M	M	F	F	M	F
Age		22	4	20	19	24	42	20	2	20	29
Draw date (M/D/2012)		05/07	05/15	05/25	05/25	05/25	05/25	05/17	05/20	05/03	05/21
Delivery date (M/D/2012)		05/10	05/17	05/25	05/25	05/25	05/25	05/23	05/23	05/07	05/22
HPLC run date (M/D/2012)		05/12	05/22	05/26	05/26	05/26	05/26	05/23	05/26	05/07	05/23
Relative % of each Hb type	HbA (%)	97.70	96.50	96.70	97.2	97.30	97.10	54.82	52.74	20.92	02.12
	HbA_2 (%)	02.30	03.10	02.30	02.60	02.40	02.30	03.90	04.10	03.00	04.70
	HbS (%)	00.00	00.00	00.00	00.00	00.00	00.00	40.78	40.56	63.38	86.68
	HbF (%)	00.00	00.40	00.00	00.00	00.00	00.00	00.50	02.60	12.70	06.50
Size measurement date (M/D/12)		05/13	05/18	05/26	05/26	05/26	05/26	05/25	05/27	05/10	05/25
Average Diameter (μm)		06.22	05.39	06.46	06.64	06.09	06.18	05.47	04.60	06.22	07.07
Standard Deviation (μm)		00.67	01.21	00.52	00.49	00.52	00.67	0.94	00.71	01.69	00.69

In Table 1 the normal adult blood samples (N1,3,4,5,6) the HbA was >96% and HbF was 0%. For the normal infant blood sample (N2) had 0.4% of HbF. The small presence of HbF in N2 was due to the young age of the individual.

For the blood samples from the individuals with SCT there was nearly 55% of HbA, 41% of HbS, and 0.5% of HbF in the first individual (SCT1) and 53 % of HbA, 41 % of HbS, and 3% of HbF in the second individual (SCT2). The percentage of HbA and HbS in the SCT1&2 individuals demonstrates the co-dominant production of the two hemoglobin in individuals with SCT. The 3% of HbF in SCT2 was again due to age, but no speculation for the HbF in SCT1 will be made here. For both blood samples from SCA patients the percentage of HbS was much higher than HbA. In SCA1 the HbA level was slightly higher than 20% whereas in the second patient was less than 3%. On the other hand, the HbS level was about 63% and 87% in the patients, respectively. The 19% difference in HbA is suspected to be due to the blood transfusion treatment the SCA1 patient undergoes. In these two patients we also see elevated HbF (about 13% in SCA1 and 7% in SCA2) due to HU[7].

3.2 Physical Properties of Free RBC's

The mean diameters of ~5000 cells were analyzed. The distributions of these measurements for each sample is shown in Fig. 4 where the normal adult sample shown is N3. The individual mean diameters along with standard deviation for each sample are also given in Table 1. The mean diameter for the normal adult samples ranged from 6.10-6.60μm with a small standard deviation (0.5-0.8 μm). The normal infant blood sample (N2) had a mean diameter (5.2+/-1.2 μm) smaller with higher standard deviation compared to the normal adult samples. In contrast, the SCT adult samples were approximately the same size as the SCT infant, both being significantly smaller than the normal adult. But the SCA cells were larger by ~1μm than the normal adult cells and they displayed comparatively higher standard deviation. The mean diameter for SCA1 is about 6.2+/-2.0 μm and SCA2 is 7.1+/-0.7 μm. This shows that SCA1 has mean diameter close to the values for normal with very high standard deviation while SCA2 has a higher with comparatively with a small standard deviation. The mean diameter of SCA1 is smaller than that of SCA2. This is suspected to have been due to the blood transfusion to the SCA patient which increases the number of normal RBCs measured. For SCA2 compared to SCA1, the mean diameter is higher and the standard deviation is smaller because it consists of predominantly the abnormal RBCs with SS hemoglobin type which generally has bigger size compared to normal RBCs[13].

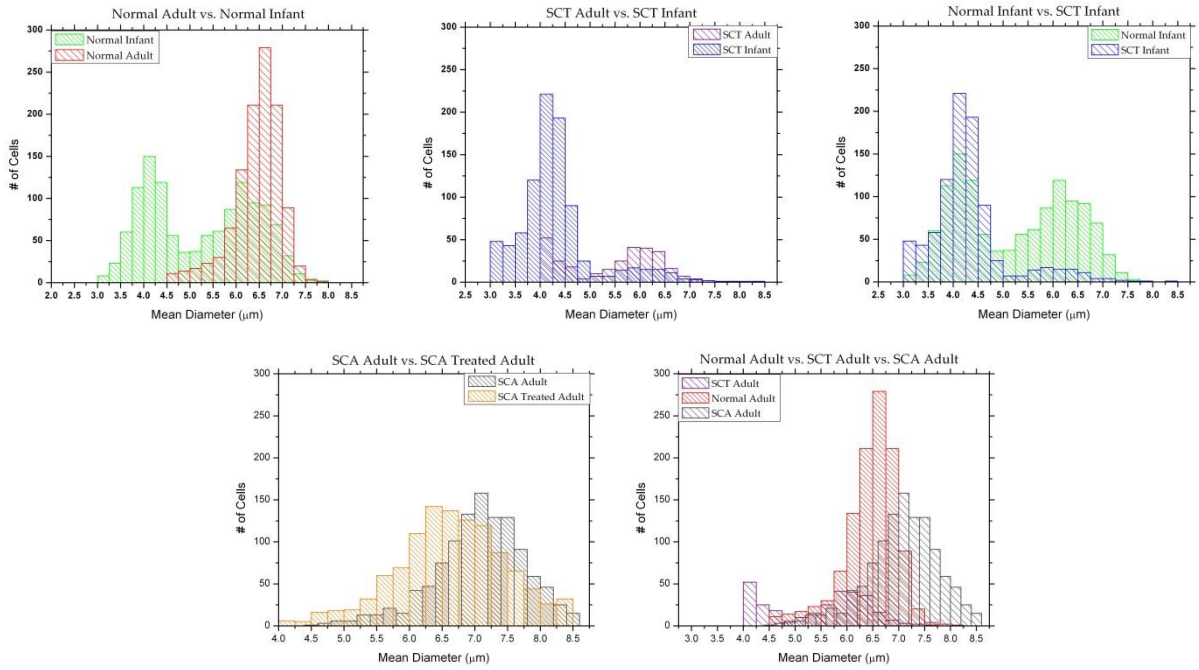


Figure 4. Histograms comparing the mean diameter distributions of the blood samples.

3.3 Mechanical Properties as Measured by the Response of the RBCs in the Laser Trap

Using the mean diameter (and area) of each cell when it is free prior to trapping, d_0 (A_0), and inside the trap, d (A), from the corresponding images, the relative change in the mean diameter (area) of the cell at a given power was determined using *% change in diameter= ((Free-Trapped)/Free)*100%*. The average values for the percent difference of the ten different cells of a blood sample were plotted against the corresponding power of the laser trap. The results are shown in Fig. 5a for the mean diameter and Fig. 5b for the area. Fig. 5a reveals that at lower laser trap power the RBCs from the SCA2 patient had the highest percent difference from the samples taken while those from the normal infant (N2) and adult (N1) showed the lowest. On the other hand those RBCs from individuals with SCT the percent difference are found to be between these two extreme ranges with those from adult (SCT1) higher than those from infant (SCT2) at lower powers. These properties could mainly be two properties related to the sizes and biochemical composition differences among the cells that influence its mechanical response. The RBCs from SCA2 as we have discussed earlier are bigger than from the rest which makes them heavier. The heavier the cell the more likely its center of gravity to be displaced below the center of the trap compared to smaller and lighter cells. This would make the maximum diameter measured for the image of the larger and heavier cells (SCA2) inside the trap to be much smaller than the actual diameter for the corresponding free cells resulting in highest relative difference of trapped with respect to the free compared to smaller and lighter cells (N1 and N2). This also justifies the high relative difference in SCT1 (adult) which are bigger and heavier than SCT2 (infant). At higher power the RBCs from the normal adult (N1), SCT infant (SCT2), and SCT adult (SCT1) show significant contraction in size that results in higher

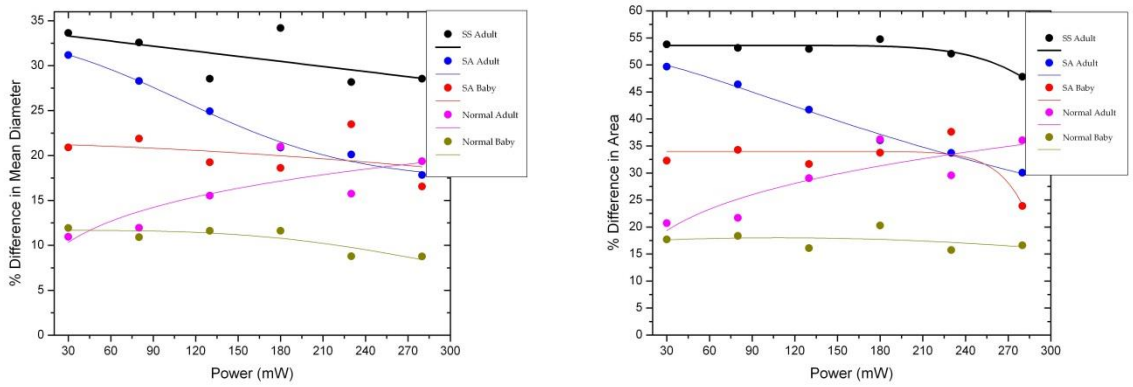


Figure 5. The percentage change in the mean diameter (a) and area (b) of the RBCs inside the trap relative to the diameter (area) of the cell when it is free as function of the laser power.

increase in percent difference compared to those RBCs from SCA patient (SCA2) and normal infant (N2). This could be because of the biochemical composition of the different types of hemoglobin that the cells predominantly made of which controls the mechanical response of the cells. For RBCs with SS hemoglobin type (SCA2) are stiffer, less elastic, and do not change significantly even when exposed to large force (high power). Those RBCs from an infant consisting of AA hemoglobin type, the fact that the size of the cells is already significantly smaller than adult RBCs and it also circulates in blood capillaries of an infant could be the reason why these RBCs do not significantly change inside the trap at high power. The percent difference in the area of the trapped relative to the corresponding free cell as function of trap power is graphed in Fig. 5b. It basically displays identical pattern as the mean diameter percent difference displayed in Fig. 5a. The only difference is the magnitude of the percent difference is much higher than the mean diameter. This is due to the flipping of the RBCs inside the trap [see in Fig. 3]. When the cell

is free the area measured is the platelet (wider side) of the cell whereas inside the trap since the cell flipped what is measured from the image of the cell is the side view area (the narrower side) and therefore the difference is higher.

4. Conclusion

We have studied the hemoglobin quantitation, the size distribution, and the relative change in the size and shape of RBCs from different blood samples. These blood samples are obtained from identified normal individuals, SCA patients under blood transfusion and HU oral medication therapies, and individuals with SCT. Our assessment in hemoglobin quantitation has shown that blood samples from normal individuals have nearly 97% normal hemoglobin (HbA), individuals with SCT have more than 50%, and untreated SCA patients have less than 2%. Depending on the age of the individual we have also verified that the individuals' blood samples could also have significant amount of fetal hemoglobin (HbF). The percentage of HbA shows a 10 fold increase in the SCA patient treated through regular blood transfusion with normal RBCs. The size distribution analyses based on the mean diameter of the RBCs have shown that the samples from normal adult individuals have fairly close size distribution and relatively very small standard deviations. The RBCs with SCA and no blood transfusion treatment displayed a similar small standard deviation, but the cells have been found significantly larger in comparison with the other blood samples we studied. A reverse of this has been observed for the RBCs from SCA patients treated with a blood transfusion. The mean size of the cells is found to be fairly close to the normal RBCs but with significantly higher standard deviations. The size distribution analyses for individuals with SCT have demonstrated that RBCs have sizes smaller than from that of the normal individuals. The relative change in the mean diameter of the trapped cells with respect to the normal cells is found to be highest for RBCs from SCA patient with no treatment and the lowest for RBCs from normal individuals. For the RBCs from individuals with SCT the relative change is found to be in between the SCA and the normal for an infant as well as an adult.

5. Acknowledgements

This material is based upon work supported by the National Science Foundation under Grant No. 0969571. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

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