Dynamic characterization of red blood cells (RBC) deformability in microchannels

António Simões Maximiano

1 Department of Mechanical Engineering, Instituto Superior Técnico, Lisboa, Portugal

Abstract

The potential use of red blood cell (RBC) deformability as a diagnostic tool for several diseases is well known, but, in order to establish this type of diagnosis in a miniaturized environment, a better understanding of the erythrocyte deformability in a microchannel flow is needed.

The present work aims to characterize the cellular deformation of RBC in cell-sized constriction microchannels (7µm), as well as microchannels whose size is 17 (97µm) and 130 (700 µm) times bigger than the cell. Measurements of an elliptical deformation index (DI), cell orientation to streamlines, diameter and wall distance were taken in predefined regions at different flow rates, storage time and hematocrit. Microchannels were built using soft lithography principles and a custom digital image processing method was developed in Matlab®. Defibrinated horse blood sample is used.

The results show an increase in DI and streamline orientation in the high shear stress area. Higher storage times show a more rigid cell population. The transverse pressure gradient was shown to have an important role in the RBC deformation in a rectangular nozzle geometry. Hemolysis is visualized and quantified in terms of impaired deformability versus the normal RBCs.

Keywords: red blood cell deformability, microchannel, Hemorheology, Elliptical deformation index, erythrocyte, red blood cell, blood storage time.

Introduction

Each RBC goes through the microcirculation over 1000 times a day. During each passage the RBC is exposed to high shear stress and forced to pass through capillaries that can be as small as one quarter of the cell diameter [1]. To these cells, in order to survive and accomplish its goal, high deformability is a must [1, 2]. The importance of RBC deformability can also be considered in terms of diagnosis, since many pathologies such as hypertension, diabetes or malaria, can cause changes in this property [3]. There are three main factors responsible for RBC deformation: cytoplasm viscosity; cell geometry; and the membrane skeleton [1, 2, 4-6], each one related to complex biomechanical and biological phenomena. Due to this underlying complexity, deformability measurements are only an oversimplified view of the myriad of factors contributing to its value [3].
The assessment of deformability using a microchannel coupled with a microscope for direct visualization of the flow, also called a rheoscope setup, has the advantages of allowing to experiment for different geometries as well as direct visualization of the cell while being deformed [3]. Several studies using this setup are found in the literature: measuring wall distance [7], DI [8], RBC orientation to streamlines [9] or the dynamical pressure drop variations along a small constriction as a RBC deforms to cross it over [10, 11]. Some investigation was made to find the relation, if any, between parameters like RBC volume and DI [12] or wall distance and DI [13]. Despite the number of publications in this field, the need for a comprehensive simultaneous measurement of all this parameters is in order. This work aims to provide a simultaneous measurement of the wall distance to the RBC, DI, cell orientation to the channel axis and it’s diameter, providing a comprehensive view of the RBC deformability.

Materials and Methods

Two types of microchannels were used, one containing a microvalve, and another one with two cell-sized parallel constrictions, both presented in Figure 1. The later (right side image of Figure 1) was designed and built for this work, being the first channel under 10 µm produced in our laboratory, and as such, a new procedure for the microfabrication was developed and tested during this work.

![Figure 1 – Left image: Microvalve dimensions and regions of interest, height=100 µm. Right image: Constriction channels fabricated in our laboratory, height=4 µm. Flow direction is downwards in both cases.](image)

Microvalve channel

In this geometry there are four defined regions of interest (ROI): A, A2, B and C. The B region is used for individual manual tracking, to understand how the parameters change as the same RBC goes through nozzle type geometry. The other regions are used for bulk analysis varying storage time and hematocrit (ROI A vs A2) as well as cross sectional area (ROI A vs C), for statistically inference purposes.
ROI A2 is tested with 1µL/min flow rate, blood with high hematocrit and a 3 days storage time, while ROI A is tested with the same blood but with low hematocrit, 1µL/min flow rate and 18 days of storage time. ROI C is tested for the exact same conditions as ROI A.

Although no measurement of the shear stress is made in this work, we can make some comparative analysis to conclude that ROI C must have, for the stated conditions, higher shear rate than ROI A, or that ROI A2 and ROI A must have shear rates of the same order of magnitude.

Cell-sized constriction microchannel

This channel was designed and fabricated for this work, using the principles of soft-lithography. The fabrication procedure is a great contribution for the future of this research group since it is the first procedure to be tested and upgraded to obtain channels under 10 µm using the current available facilities. Briefly, a darkfield photomask is placed on a silicon wafer, previously spin-coated with a 4µm thick layer of negative photoresin, and exposed to a pulsing UV light source. After the development a positive mold is obtained, on which a liquid solution of polydimethylsiloxane (PDMS) is poured. This PDMS is cured and peeled of the mold, and after punching the inlets and outlets holes, this negative mold of the channels is irreversibly bounded to a glass slide by a hand held corona treatment, finishing the device.

The visualization of the RBC flowing in the constriction is meant to provide more information on the cellular aspects of the deformability, opposed to bulk behavior in the former microchannel. This understanding is crucial to the correct interpretation of deformability results.

Experimental Setup

![Experimental setup](image)

Figure 2 - Experimental setup

The results were obtained using the experimental setup in figure 2. The channels were placed on a custom built adapter on the stage of a microscope (Olympus® CX41 UIS2). Horse difibrinated blood was maintained at 4°C, and for each experiment a sample was loaded into a gastight syringe. The flow rate was controlled using a syringe pump (Chemyx Nexus 6000) allowing the examination of two different flow rates for the microvalve channel (1µL/min and 5µL/min) and another flow rate for the constriction microchannel.
(0.01\mu L/min). The image acquisition was obtained by a camera CMOS (Optronis® CR600x2) mounted on the previous microscope. The image acquisition was accomplished using the software Timebench 2.3.1.

**Measured parameter**

In the microvalve channel the parameters measured for the statistical characterization (regions A, A2 and C) are:

- \( DI = \frac{A-b}{A+b} \) where A is the major axis and b the minor axis of the RBC ellipse fitting;
- Cell circular diameter;
- Orientation to the channel axis;
- Distance from the centroid of the RBC to the wall (except for region A2).

**Digital Image Analysis**

In order to extract information from the raw images obtained, a custom digital image processing routine was developed in Matlab®. This routine shares some similarities with other works found in the literature [8, 12, 13], and was another meaningful contribution to the research group.

The objective of this analysis is to correctly identify and segment the RBCs contained in each image, fitting an ellipse to each of them and extract the parameters mentioned earlier.

For individual tracking the user is required to choose the same RBC in each frame. This method also supports an editing stage where the user is presented with the ellipse fitting of the RBC, allowing him to accept it or to edit it as required. Other than this, the methodology used is the same for all digital image analysis performed, and can be divided in three stages: obtaining a binary image, filtering it and finally ellipse fitting. In order to obtain a binary image, depending on the hematocrit, one of two techniques is applied, background subtraction or thresholding. The first one is used for low hematocrit blood, establishing a background image from the average of all images, which is subsequently subtracted from each frame, resulting in a binary image of the foreground objects. The latter is used for high hematocrit blood on the contrast stretched image of the original frame, defining a range of grey level intensity, outside of which the information is considered noise.

The filtration of the binary image is done by evaluation the area and roundness of each blob, eliminating any one that is outside of user defined limits. After this stage each blob is considered to be a RBC. An ellipse is fitted to each blob and its parameters of interest are extracted.

These filters are required to measure a minimum of false positive RBCs, and they force a compromise between a low number of false positives and the maximum DI detected as well as the bigger and smaller diameter captured, as seen in Table 1.
Table 1 – Limits of the diameter and DI measured with the parameters used to digitally analyze each frame.

<table>
<thead>
<tr>
<th></th>
<th>ROI A</th>
<th>ROI A2</th>
<th>ROI C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum diameter [µm]</td>
<td>3.18</td>
<td>2.78</td>
<td>2.76</td>
</tr>
<tr>
<td>Maximum diameter [µm]</td>
<td>5.74</td>
<td>7.37</td>
<td>5.95</td>
</tr>
<tr>
<td>Maximum DI</td>
<td>0.23</td>
<td>0.23</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Error of the digital image analysis

The three dimensional effects of the flow occurring for ROI A, A2 and C are not accounted for. These include tumbling of the RBCs as well as out of focus RBCs that appear blurred in the frames. These out of focus artifacts are excluded by a sufficiently small signal to noise ratio, while the tumbling of RBCs represents a bigger challenge to correctly identify and exclude from this analysis, and is not yet done. In the manual tracking the user selects which RBC to follow, and excludes tumbling RBCs from the start, while in the smaller microchannel its height (h=4 µm) is sufficient to prevent any tumbling.

For the ROI affected by the three dimensional effects (A,A2 and C) there is a systematic sub estimation error of the diameter of the cell. This error can be identified by comparing the diameter results with values found in the literature as well as the results from the manual tracking, as shown in Table 2.

Table 2 – Error in diameter measurements for the ROI with 3D effects.

<table>
<thead>
<tr>
<th></th>
<th>ROI A, A2 and C</th>
<th>Manual Tracking (ROI B)</th>
<th>Constriction Microchannel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>1275</td>
<td>34</td>
<td>25</td>
</tr>
<tr>
<td>Experimental diameter (µm)</td>
<td>3.91±9%</td>
<td>5.43±3%</td>
<td>5.27±3%</td>
</tr>
<tr>
<td>Diameter from literature[14-16]</td>
<td>5.2 to 6 µm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Relative difference (%)</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results and Discussion

Microvalve channel

Comparison between ROI A and C

As shown in Figure 3, the DI (D1=0.042±0.029; n=169) measured for the ROI A with 18 days storage and Q=1µL/min is lower (t-student, p <0.001) than the one in ROI C (D1=0.121±0.088; n=133) with the same conditions. This variation is explained by the increased shear stress applied to the RBC flowing in ROI C, since its cross section is 7 times smaller than the cross sectional area of region A and flow rate is kept constant.

Another effect of the increased shear stress in ROI C is the alignment of the major axis of the ellipse fitted RBCs with the streamlines, with 70% of the RBCs showing an orientation between 30° and -30°, whereas ROI A shows only 37% of the RBCs within that range.
The formation of shifting air bubbles on the corners of the microvalve alters the RBC distribution throughout the flow, masking any natural migration patterns that might arise. Therefore no variation of parameters with wall distance was observed.

\[ \mu = 0.042, \quad \sigma^2 = 0.5 \times 10^{-2} \]

\[ \mu = 0.121, \quad \sigma^2 = 7.7 \times 10^{-3} \]

Figure 3 - Top left: DI histogram for ROI A. Top right: DI histogram for ROI C. Bottom Left: Orientation histogram for ROI A. Bottom Right: Orientation Histogram for ROI C

Comparison between ROI A and A2

Increasing storage time has a negative impact on deformability [17], whilst changing in hematocrit, being the volumetric concentration of RBC in the blood, isn’t a factor with known direct impact on RBC deformability.

\[ \mu = 0.042, \quad \sigma^2 = 0.5 \times 10^{-2} \]

\[ \mu = 0.107, \quad \sigma^2 = 4.2 \times 10^{-3} \]

Figure 4 - Top left: DI histogram for ROI A. Top right: DI histogram for ROI A2. Bottom Left: Orientation histogram for ROI A. Bottom Right: Orientation Histogram for ROI A2
ROI A2 is tested with high hematocrit blood with 3 days of storage time while roi A uses low hematocrit blood with 18 storage days.

As seen in Figure 4 ROI A2 has the higher deformation (DI=0,107±0,612; n=973) (t-student, p <0,001) comparing to ROI A (DI=0,042±0,029; n=169), suggesting that an increase in storage time impairs deformability.

When comparing the orientation of both regions it is clear that they are very similar (A: 37% between 30° and -30°; A2: 38% between 30° and -30°). This result supports the hypothesis that the storage time is the shear rate has the same order of magnitude in both ROI, since the comparison between ROI A and C allowed to see the impact of varying shear stress in orientation of the RBCs.

Region B: RBC tracking

RBCs were followed along their trajectory, measuring the change in the deformability parameters along the way. Due to the impossibility of presenting all the data individually, the RBCs within each set of experimental conditions were divided according to their trajectory (Figure 5): the ones with trajectories near the axis of channel with low curvature (low transverse pressure gradient), named central trajectories RBCs; and those with curving trajectories (higher transverse pressure gradient), called side trajectories RBCs. Within each type of trajectory the path was parameterized by the chord length, defining a new parametric variable ‘s’ that represents the distance traveled, adimensionalized by the total distance.

![Figure 5- Example of RBCs grouped by trajectories.](image)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Storage time (days)</th>
<th>Flow rate (µL/min)</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Side trajectories</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>3</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>18</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>
The RBCs measured with higher flow rate showed an increase in deformation with the distance travelled with higher overall deformation. The highest DI for each set of conditions was always identified in the side trajectories, with its peak occurring in the trajectory section that shows the greatest curvature (bigger transverse pressure gradient). These results highlight the importance of the transversal pressure gradient as a cause for RBC deformation.

**Microchanel with the RBC sized constriction**

The variation of the DI obtained in these channels is not consistent with the highest shear rate that occurs in the constriction. In fact the results show that DI drops as the RBC gets closer to the constriction channel. This experiment has no reproducibility, since eventually the channel clots, rendering it obsolete. As the time for clotting is much smaller than the one needed to totally fill the outlet holes, the whole measurement occurs without a steady flow regime. This may be one of the factors explaining the decrease of deformability. Nevertheless this layout allowed to visualize some hemolysis occurring in the upper channel, as sequenced in Figure 6.

![Image sequence of the hemolysis of a RBC in the top channel. In e) occurs the collapse, with the cell releasing the cytoplasm (red arrow) through a rupture (green arrow) and emptying itself](image)

Deformability comparison between RBCs that didn’t collapse while passing the channel and the RBCs that did, allows us to establish an impaired deformability for the cells that suffered hemolysis, showed in Table 4.
Table 4- Impaired deformability of the RBCs that collapse as opposed to normal RBCs.

<table>
<thead>
<tr>
<th></th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DI</td>
<td>n</td>
<td>DI</td>
<td>n</td>
</tr>
<tr>
<td>Normal</td>
<td>0.169</td>
<td>10</td>
<td>0.220</td>
<td>19</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>0.084</td>
<td>7</td>
<td>0.050</td>
<td>13</td>
</tr>
<tr>
<td>Deformability reduction (%)</td>
<td>50.3</td>
<td>-</td>
<td>77.3</td>
<td>-</td>
</tr>
</tbody>
</table>

**Conclusion**

A microfabrication procedure was developed for the first time in our laboratory that successfully created channels with features under 10 µm. Also, a digital image processing routine was developed to deal with the segmentation of RBCs in the channels used. This code is the first of its kind in the research group and will allow for a continuation of the deformability of RBC studies.

The main contributions of this work is the simultaneous measurement of DI and orientation, with the distance to the wall parameter hindered by air bubbles oscillations, altering the RBCs distributions and masking any natural tendencies that might occur.

The deformation measured with high shear stress (ROI C: ID=0.121±0.088; n=133) was bigger than that measured with low shear stress (ROI A: ID=0.042±0.029; n=169) (t-student, p <0.001). Under high shear stress, more RBC orient themselves with the streamlines (ROI C: 70% within -30° and 30°) than under lower shear stress conditions (ROI A: 38% between -30° and 30°). RBCs from blood with lower storage time (3 days, ROI A2: ID=0.107±0.612; n=973) exhibit bigger deformation than those with higher storage time (18 days ROI A: ID=0.042±0.029; n=169).

The individual tracking of RBCs exposed the influence of the transverse pressure gradient in the deformability of RBCs in area contraction proximities. This influence was revealed by the higher DI occurring in the RBCs that had curved trajectories, with the DI peak occurring mostly in the part of the trajectory where the curvature was more pronounced, meaning a higher transverse pressure gradient.

A deformabilidade medida para estes eritrócitos (ID=0.03±0.08) é cerca de 50 a 83% inferior à obtida para eritrócitos que atravessa incólumes a constrição (ID=0.11±0.22).

The visualization and quantification of the hemolysis phenomena hinted at the impaired deformability (50 a 83% lower) of the cells that suffer hemolysis (DI=0.03±0.08) when compared to cells that go through the constriction undamaged (DI=0.11±0.22).
References


