Investigation of Blood Flow Analysis and Red Blood Cell Aggregation

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Abstract

This work aims to develop a method for quantitatively analyzing red blood cell (RBC) aggregates under controlled flow conditions. Images of experiments are captured and processed in order to quantify aggregation. The experimental setup consists of RBC suspensions in blood plasma entrained by a phosphate-buffered saline solution in a 110 × 60 μm polydimethylsiloxane microchannel. The experiments are performed by varying the hematocrit (5, 10, and 15%) and the flow rate (Q = 5 and 10 μl/hr) in order to observe the effect of shear rate on RBC aggregation. Microchannel dimensions as well as fluid flow rates are determined using numerical simulations. The flow is visualized using a high-speed camera coupled to a micro particle image velocimetry system. Videos obtained with the high-speed camera are processed using a MATLAB program, with each frame analyzed separately. RBC aggregates are detected based on the image intensities and the connectivity between RBCs, using image processing techniques. The average aggregate size and distribution of RBCs for various aggregate sizes are determined for each of the shear rates and hematocrits. These aggregates are shown to be larger at low flow rates where the shear rate is small. Results from tests performed at high hematocrits also show larger RBC aggregates.

Keywords: Red blood cells (RBCs), Micro particle image velocimetry (μPIV), Hematocrit (H), Image processing, Aggregation

1. Introduction

Red blood cells (RBCs) play an important role in the determination of the rheological properties of blood. They are almost singularly responsible for the particular behavior of blood in vitro and in vivo. Under physiological conditions, RBCs are able to deform and adapt to the smallest vessels in the body, i.e., the capillaries (4-10 μm), for gas exchange. At low shear rates, these cells have the unique ability to assemble and form three-dimensional aggregates, thereby contributing to the non-Newtonian behaviour of blood. The formation of these aggregates can be altered by several factors, such as the RBC membrane elasticity, suspending medium composition, the hematocrit of blood (volume of RBCs in blood), vessel diameter, and shear rate [1-3]. Under physiological conditions, RBCs occupy 40 to 45% of the blood volume. In microcirculation, RBCs occupy only up to 20% of the blood volume due to smaller vessel diameters and the plasma skimming effect [4]. This phenomenon of plasma reduction in microcirculation is known as the Fåhræus effect.

Typically, RBC aggregates form at low shear rates, approximately under 10 s⁻¹ for human blood [5]. Above this range, RBCs tend to disaggregate and return to their natural state. These aggregates are often seen at the macrocirculation level (diameter: >300 μm). At this scale, blood is considered as a Newtonian fluid and a homogenous mixture. However, these aggregates are rarely seen at the capillary level (diameter: 4-10 μm) and are usually an indication of pathological conditions such as diabetes [6] and obesity. Other pathological conditions that could change RBC aggregation include inflammatory or infectious conditions, cardiovascular diseases such as hypertension or atherosclerosis, genetic disorders, and chronic diseases [7].

Therefore, understanding the RBC aggregation mechanism and analyzing these entities (by defining a relationship between the size of these aggregates and the flow conditions) could lead to an understanding of the microrheological behaviour of blood and hence relate it to clinical applications. Several methods can be used to analyze aggregate formation and provide relevant information about blood behaviour by defining an aggregation index.

Dusting et al. investigated the effect of RBC aggregates in a shearing system on micro particle image velocimetry (μPIV) velocity measurements. By observing the RBC aggregates under various shear rates (from 5.4 to 252 s⁻¹) and defining several aggregation indices, they showed that the velocity measurement depends on the degree of aggregation [8]. This
measure of aggregation was assessed based on the microscopic images obtained, dividing the experimental cell free layer detected from the images by the theoretical cell free layer based on the hematocrit. For this purpose, a two-dimensional (2D) correlation coefficient was determined and used to investigate the effect of the degree of aggregation on the accuracy of the velocity measurement.

Kaliviotis et al. [9] investigated the spatial variation of blood viscosity in a shearing system. For this purpose, a constitutive equation was coupled to shear rates determined based on μPIV velocity measurements. The authors were able to show the spatial variation of the viscosity in the shearing system based on the degree of RBC aggregation, hence accounting for the time-dependent behaviour of the aggregates.

In another study, Researchers developed an analytical model to predict the distribution of hematocrit in a T-bifurcation channel [10]. The empirical model was then compared to laboratory experiments for aggregating and non-aggregating blood suspensions. Experimentally, the distribution of the hematocrit was determined based on image processing techniques. The authors thus obtained the hematocrit, viscosity, and velocity distribution in a T-bifurcation channel.

These studies indirectly investigated RBC aggregate sizes by determining the ratio of the occupied space in a shearing system measured based on blood microscopy images. The degree of aggregation as well as the local viscosity was also investigated. The present work provides a direct measure of the aggregate sizes in a constant shear environment in a microfluidic device. Moreover, it is important to note that in this study, the blood flow in the shear system (perpendicular to the flow direction) is directly observed, providing a different angle on flow investigation compared to previous studies and a visualization of the full domain of interest.

This study aims to develop a standard method for quantifying RBC aggregates under controlled flow conditions using image processing. The blood velocity and shear rate are determined using a μPIV system and the flow is visualized using a high-speed camera. The results obtained are then processed with MATLAB code based on the image intensities in order to detect the RBCs and determine their aggregate sizes.

2. Methodology

2.1 Experimental setup

The experimental setup comprises a μPIV device (LaVisions MITAS, Germany) in order to visualize the flow and estimate the velocity field. This μPIV setup is composed of a CCD, Double Pulsed (DP) Image Intense camera (LaVision GmbH, Germany) with a resolution of 1376×1040 pixels and a NewWave Solo-II Nd:YAG laser (New Wave Research, USA) emitting a wavelength of λ = 532 nm, and a MITAS inverted microscope (LaVision GmbH) with 20× lens magnification. In order to control the position of the microchannel, the μPIV setup uses a moving stage, which is controlled by Davis Imaging Software (LaVision GmbH). In order to determine the velocity field within the microchannel, fluorescent tracer particles (polymers) diluted at 1% in water ($d_{\text{particle}} = 0.86 \, \mu m$, $\lambda_{\text{dye}} = 542 \, \text{nm}$, and $\lambda_{\text{emission}} = 612 \, \text{nm}$) are introduced into the fluids, which illuminate when exposed to the appropriate wavelength. The fluids, contained in two 50-μl glass syringes (Hamilton, USA), are pushed into a polydimethylsiloxane (PDMS) microchannel at various flow rates using two pumps (Nexus3000, Chemyx Inc., USA and Picoplus, Harvard Apparatus, USA). The motion of RBCs in the microchannel is visualised using a high-speed camera (Grafterk Imaging, Inc., Austin, TX, USA) controlled using LabVIEW software (National Instruments, USA) coupled with the LaVision device. For the maximum field of view allowed by the camera (resolution of 2048×1088 pixels), the highest frame rate possible is 340 frames/s, which is sufficient for the ranges of flow rates used within the microchannels. The experimental setup is shown in Fig. 1.

![Figure 1. Experimental setup.](image)

2.2 Fluid sample preparation

Fresh and healthy porcine blood samples were used in this study. The blood collected from an accredited slaughterhouse was treated with 2 g/l of ethylenediaminetetraacetic (EDTA) as an anticoagulant. All samples were centrifuged three times following standard procedures at 3000 rpm for 10 min in order to separate the RBCs from the other blood constituents. Blood plasma was collected only from the first centrifugation to ensure minimum contamination with white blood cells and platelets. Removing these constituents for experiments facilitates the use of μPIV and increases the quality of image processing. The RBCs of each sample were then suspended in their original plasma at three hematocrits (5%, 10%, and 15%) to visualize and determine the effect of the hematocrit on the aggregates. Fluorescent tracer particles, used for velocity measurements, were added to each of the RBC suspensions at 0.06% of the sample volume (6% of the particle solution).

2.3 Microchannels

The microchannels were fabricated from PDMS using standard photolithography methods. PDMS is a flexible silicon-based polymer. Its transparency allows good visualisation and its flexibility mimics blood vessel elasticity, justifying its use in this case. The microchannel configuration (double Y-microchannel, as shown in Fig. 2) was transferred to a photo-mask that was used to engrave the channel geometry on the wafer to create the mold. When exposed to ultraviolet light,
the photo-resist (SU8-50 epoxy-based negative photo-resist) hardened to form the channel mold. PDMS was prepared from Sylgard-184 (Dow-Corning, USA). The channel was bonded to a glass slide using the oxygen plasma bonding method performed with the PE-50 series plasma system (Plasma Etch, USA). The microchannels had a rectangular cross section of 110 × 60 µm. In comparison, an average RBC has a diameter of 8 µm and a thickness of 2 µm. Therefore, the microchannel width was sufficient for observing proper aggregation and accurately determining the velocity profiles.

2.4 Flow conditions

To observe the effect of shear rate on RBC aggregation, it is desirable to create a constant shear rate experimentally. For a constant shear throughout the blood layer, a linear velocity profile is required. Theoretically, this could be obtained by initially maintaining the blood layer stationary and entraining it using a moving wall. However, these conditions cannot be obtained experimentally with the channel configuration used. Thus, a second fluid was used to entrain the blood layer, which was pumped into the channel at a much lower speed. Using proper viscosity and speed ratios between the two fluids, a quasi-linear profile was obtained and the shear rate can be considered constant. Furthermore, with a thin blood layer, the linearity of the velocity profile was easily obtained. The design of the system is based on a numerical model and experimental confirmation [11]. Figure 3 illustrates the velocity profile with respect to channel width for RBCs suspended at 5%, 10%, and 15% hematocrit. Simulations and experiments were performed to help choose the best channel geometry and the input velocities for each fluid entering the channel. Numerically, the Reynolds number calculated for the blood layer was found to be 3.2 10^3.

2.5 Flow visualization and data analysis

μPIV is a particle-based flow visualization technique that can provide a 2D velocity field within a microchannel. This technique requires tracer particles to be seeded within the fluids. The double-pulse CCD camera associated with the system captures sets of double frames (100 frames) at high speed separated by a dt dependent on the flow rate. The frames that are captured are synchronized with the laser pulse to illuminate the tracer particles that fluoresce under the microscope. The sets of frames are then cross-correlated and post-processed to obtain 2D velocity vectors. The double frames are discretized into interrogation windows with a size set by the user. The choice of the interrogation windows is crucial since it should take into consideration the full velocity distribution. Therefore, the window size should be proportional to the displacement of the particles. For each correlation window, a correlation map is plotted to determine the velocity vector by relating the highest point of this correlation map. The results are then averaged in time and space to obtain the experimental velocity profiles. The averaging technique is used in order to reduce the effect of the Brownian motion of the particles and reduce the experimental error. In order to obtain the velocity values for each specific case, a calibration of the double-pulse camera is required to determine the scale factor corresponding to the lens magnification used.

The methodology used to determine the velocity is detailed elsewhere [12]. The root mean square (RMS) error is calculated by averaging instantaneous velocity measurements for n_image as follows:

\[
\text{RMS}_{\text{error}} = \sqrt{\frac{n_{\text{image}} \sum \text{pixel}_{i}^2 - (\sum \text{pixel}_{i})^2}{n_{\text{image}} (n_{\text{image}}-1)}}
\]

where n_image represents the total number of images and pixel_i is the intensity of a pixel in image i. The results obtained are in terms of pixels which are then scaled to the units of velocity (mm/s) using the timing between the frames dt and the scale factor set when calibrating the double-pulse camera.

Once the velocity profile is obtained, the shear rate can be calculated. By approximating the shear rate in the blood layer to be linear, it is possible to calculate a constant shear rate. With a linear velocity profile, the shear rate can be obtained by calculating the average slope of x-velocity with respect to the channel width.

2.6 Video processing and aggregation analysis

Aggregate detection was performed using a MATLAB program based on the pixel intensities of the images. A flow chart of the program is shown in Fig. 4. The frames of the captured videos are averaged for background subtraction, which allows better image quality by lessening the effect of RBCs in the background. Thus, a 2D analysis can be better
Figure 4. Flow chart of MATLAB program used for aggregation detection.

performed on each frame. The result is then converted into a binary image. A function converts each pixel of the image to black or white depending on its intensity in the original grayscale image by assigning a specific conversion parameter to each video. The image is then dilated in order to better distinguish the connections between RBCs. Each RBC in the image is approximated as a disk with a predefined radius chosen according to the lens used for the video capture and pixel density of the image. Connected cells are then detected by determining neighboring objects in the binary image. In this way, adjacent cells are associated as aggregates. These cell combinations are then labeled and the image is converted into a red-green-blue (RGB) image for better visualization. This processed image is coupled with the original video in order to verify the efficiency of the image processing. The aggregate sizes, in pixels, are determined based on the RBC size and later converted into µm². Using this methodology, the distribution of the percentage of RBCs within each aggregate can be obtained as a function of the aggregate size (represented by the number of cells in each aggregate).

An average aggregate size is also determined for each of the RBC suspensions recorded with the high-speed camera. With this image processing method, the area of the detected entities (RBC aggregates) can also be determined. Averaging the aggregate areas in each frame and then averaging the results for every 20 frames, it is possible to obtain an average aggregate size.

3. Results

RBC suspensions with three different hematocrits were tested in the double Y-microchannel configuration at two flow rates. The corresponding shear rates determined using the µPIV setup are shown in Table 1.

Table 1. Shear rate values for various blood flows.

<table>
<thead>
<tr>
<th>Hematocrit</th>
<th>Flow rate</th>
<th>Shear rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>10 µl/hr</td>
<td>11.02 s⁻¹</td>
</tr>
<tr>
<td>5%</td>
<td>5 µl/hr</td>
<td>5.36 s⁻¹</td>
</tr>
<tr>
<td>10%</td>
<td>10 µl/hr</td>
<td>8.17 s⁻¹</td>
</tr>
<tr>
<td>10%</td>
<td>5 µl/hr</td>
<td>4.47 s⁻¹</td>
</tr>
<tr>
<td>15%</td>
<td>10 µl/hr</td>
<td>7.41 s⁻¹</td>
</tr>
<tr>
<td>15%</td>
<td>5 µl/hr</td>
<td>2.51 s⁻¹</td>
</tr>
</tbody>
</table>

Frames of the videos captured with the high-speed camera are shown in Fig. 5 for RBCs suspended at 10% H flowing with (a) Q = 10 µl/hr and (b) Q = 5 µl/hr. It is believed that larger aggregates result from lower flow rates and hence shear rates. The extracted distribution of the aggregate size in the sample as well as the number of RBCs in each aggregate for the RBC suspensions (5%, 10%, and 15% H) are shown in Figs. 6, 7, and 8, respectively. The average aggregate size for each of the RBC suspensions was calculated as a function of the corresponding shear rate calculated from the µPIV data; the results are shown in Fig. 9. The error bars represent the standard deviation error calculated based on the average aggregate sizes detected in each frame.

Figure 5. Captured frames of RBCs suspended at 10% H flowing with (a) Q = 10 µl/hr and (b) Q = 5 µl/hr.

Figure 6. Aggregate size distribution for blood samples suspended at 5% H flowing at Q = 10 and 5 µl/hr.

Figure 7. Aggregate size distribution for blood samples suspended at 10% H flowing at Q = 10 and 5 µl/hr.

Figure 8. Aggregate size distribution for blood samples suspended at 15% H flowing at Q = 10 and 5 µl/hr.
Figure 9. Average aggregate size as function of corresponding shear rate for RBC suspensions flowing at \( Q = 10 \mu l/hr \) at (a) 5%, (b) 10%, and (c) 15% hematocrit.

Figure 3 shows the velocity profiles for the RBC suspensions at 5% (red curve), 10% (blue curve), and 15% H (green curve) flowing at \( Q = 10 \mu l/hr \). The bars in the velocity profiles represent the velocity magnitude in each correlation window used for the cross-correlation method. The RMS errors of the velocity, also displayed in Fig. 3 for each RBC suspension, are relatively small compared to the velocity values, indicating the accuracy of the velocity measurements, and hence shear rates. The interface locations, denoted as ‘E’ and shown as solid lines in Fig. 3, are obtained from the high-speed camera recordings. By averaging all the frames of the videos captured, a background image can be obtained, where a clear delimitation of the interface can be seen.

3.1 RBC suspensions with 5% H

Figure 6 shows the distribution of the aggregate sizes in the RBC suspension of 5% H flowing at \( Q = 5 \mu l/hr \) and 10 \( \mu l/hr \). The aggregate size distribution was found to be similar for both flow rates, where about 81% and 79% of the RBCs in the blood layer are flowing as small aggregates of 2 to 6 RBCs for \( Q = 10 \mu l/hr \) and 5 \( \mu l/hr \), respectively. However, slightly larger aggregates were detected for the smaller flow rate, resulting in a slightly higher percentage of RBCs flowing in larger aggregates (8 to 16 RBCs in size) for \( Q = 5 \mu l/hr \). An average aggregate size was determined based on the detected entities in the frames for both flow rates. For \( Q = 10 \mu l/hr \) in the double Y-microchannel, corresponding to a shear rate of \( \gamma = 11.02 \text{ s}^{-1} \) in the blood layer, the average aggregate size was found to be about \( 87.8 \pm 0.93 \mu m^2 \), while for \( Q = 5 \mu l/hr \), corresponding to a shear rate of \( \gamma = 5.36 \text{ s}^{-1} \), the average aggregate size was found to be about \( 95 \pm 0.97 \mu m^2 \). There was no significant variation in the aggregate size with shear rate for the RBCs suspended at 5% hematocrit.

3.2 RBC suspensions with 10% H

As shown in Fig. 5(b), for the RBC suspension flowing at 10% hematocrit, larger RBC aggregates were found for the smaller flow rate. The distribution of the aggregate sizes in the RBC suspension of 10% H flowing at \( Q = 5 \mu l/hr \) and 10 \( \mu l/hr \), shown in Fig. 7, confirms the latter observation. For \( Q = 10 \mu l/hr \), corresponding to a shear rate of \( \gamma = 8.17 \text{ s}^{-1} \) in the blood layer, about 72.7% of the detected RBCs are flowing as small aggregates of 2 to 6 RBCs, while 26.5% of the RBCs are flowing in aggregates of 8 to 28 RBCs. The corresponding aggregate size was found to be \( 100.3 \pm 1.31 \mu m^2 \). However, for a lower shear rate of \( \gamma = 4.47 \text{ s}^{-1} \) in the blood layer, corresponding to a flow rate of \( Q = 5 \mu l/hr \) in the microchannel, 63.8% of the detected RBCs are flowing as small aggregates of 2 to 6 RBCs, while 34.2% of the detected RBCs flow as larger aggregates of 8 to 28 RBCs. The remaining 2% of the RBCs form large aggregates of 30 to 44 RBCs. The average aggregate size was calculated to be about \( 133.31 \pm 2.05 \mu m^2 \).

3.3 RBC suspensions with 15% H

In Fig. 8, a significant difference in the RBC aggregate size distribution can be seen between the two flow rates tested for the RBCs suspended at 15% hematocrit. When \( Q = 10 \mu l/hr \), corresponding to a shear rate of \( \gamma = 7.41 \text{ s}^{-1} \) in the blood layer, 83.6% of the RBCs are flowing in small aggregates of 2 to 6 RBCs, while the remaining 16.4% of the RBCs are detected in aggregates of 8 to 12 RBCs. The average aggregate size was found to be about \( 107.2 \pm 1.2 \mu m^2 \). For a flow rate of 5 \( \mu l/hr \), corresponding to a shear rate of \( \gamma = 3.71 \text{ s}^{-1} \) in the blood layer, only 58.58% of the RBCs are detected in small aggregates of 2 to 6 RBCs. The remaining 41.42% of the RBCs are detected in larger aggregates of 8 to 62 RBCs. The average aggregate size was found to be about \( 159 \pm 1.83 \mu m^2 \).
107.2 µm²). However, for the lower range of shear rates (2.51 to 5.36 s⁻¹), the average aggregate sizes greatly depend on the hematocrit level (the aggregates range from 95 to 159 µm²), which is also demonstrated in Fig. 10, which shows the captured frames of the RBC suspensions flowing at Q = 10 µl/hr at (a) 5%, (b) 10%, and (c) 15% hematocrit.

5. Conclusion

RBC suspensions were tested with various flow rates and hematocrit under controlled flow conditions for a qualitative and quantitative RBC aggregate analysis. It was shown that larger aggregates of about 159 µm² were present when the RBCs were suspended at the highest hematocrit tested (15%) and flowing at low shear rates (2.51 s⁻¹). For a higher shear rate (7.41 s⁻¹), smaller aggregates of up to 107.9 µm² were found when the RBCs were suspended at 15% H.

This work quantified and characterized RBC aggregates under controlled shear rates. Future work will aim to relate the aggregate size obtained to the apparent viscosity within the blood layer and to aggregation indices calculated using standard methods. Finally, human blood will be used for further analysis and characterization of RBC aggregates.

Acknowledgments

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