Probing the Red Blood Cells Aggregating Force With Optical Tweezers

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Abstract—The red blood cells (RBC) aggregation is of current basic science and clinical interest, as a determinant of blood microcirculation. Thus, the measurement and assessment of the RBC aggregation property (aggregability) and aggregation state at different physiologic conditions of a human individual or laboratory animal are an important issue. In this paper, in order to assess the dynamics of RBC interaction, optical tweezers were used to probe the forces during the RBC doublet formation or disruption. We show that in autologous plasma, RBC aggregating and disaggregating forces have different absolute values, ca 2–4 pN and dozens of piconewton, correspondingly. We speculate that in plasma, RBC aggregation and disaggregation processes have different driving forces.

Index Terms—Blood, biophotonics, biological processes, biological interactions, biophysics, cellular biophysics.

I. INTRODUCTION

T H E red blood cells (RBC) aggregation is a reversible process of cells clumping under low shear rates. In conditions of permanently changing blood vessels geometries and shear rates the RBCs in a fixed volume of the flow permanently form aggregates that are reversibly dispersed. This process is one of the main determinants of blood viscosity and, therefore, of microcirculation of the blood [1]. RBCs tend to form face-to-face linear structures—rouleaux under low shear forces or at stasis. The RBC aggregation is a clinically and physiologically significant process, which has complex functions [1], [2]. RBC aggregation takes place in the regulation of blood viscosity, changes of a functional capillary density [3], regulation of a peripheral hematocrit and blood cells localization [4]. RBC aggregation is also closely related to thrombosis and hemostasis. During most of pathologies, the RBC aggregation is significantly altered along with other blood properties [5], [6]. It leads as consequence to blood circulation disorders, which are known to be one of the major reasons of mortality during cardiovascular diseases [7].

Studies of the RBC aggregation were carried out intensively during last few decades as a significant diagnostic parameter that allows for monitoring the state of blood microcirculation. Different methods were developed and approbated to measure RBCs aggregation [2]. Currently, the most widely used method is optical aggregometry. This method is based on analyzing the laser beam scattered on blood suspension referred to the RBC aggregates formation in vitro [5], [8]. Optical aggregometry yields a number of parameters related to RBC aggregate size, formation time and strength [8], [9]. Diffusing Wave Spectroscopy was applied to monitor structural characteristics of blood elements during the aggregation for healthy donors and patients with cardiac ischemia [10]. Another widely used method is the traditional light microscopy coupled with image analyses [11], [12]. To study RBCs interaction mechanics, the single cell level methods like micropipette aspiration technique (MAT) [13], atomic force microscopy [14] and optical tweezers (OT) [15], scanning electron microscopy (SEM) [16], and interference reflection microscopy [17] were applied. Pioneer studies using MAT and SEM methods, yielded the first estimates of RBC interaction energy and speculating about the mechanism of RBCs interaction [18], [19]. Based on MAT measurements, the interaction energy density was calculated for autologous plasma and found to be about 2 μJ/m² [13]. During the last decade, with the appearance of OT the interaction dynamics of RBCs was studied in more details. OT is a powerful tool that allows for trapping and manipulating microparticles like single cells or molecules, and measuring the piconewton (pN)-ranged forces [20]. It has been intensively used for studying cellular and molecular interactions [21]. The force required to separate the aggregated RBC pairs was found to be of order of dozens of pN [22], [23]. The detailed dynamics of RBC disaggregation assessed with OT showed a number of contradicting results with predictions made basing on the existing interaction models [15].

In order for RBC aggregation to start, the presence of relatively big macromolecules is necessary [12], [24], [25]. RBC aggregation does not take place in buffer solutions, but it does so in autologous plasma and serum, which are solutions of electrically charged proteins. RBC aggregation occurs also in solutions of neutral polymers like high molecular weight dextrans [26]. The RBC interaction induced by polymer solutions is
simpler to assess than that induced by plasma that contains a set of charged macromolecules. At the current stage, most of the studies of RBCs interaction mechanics are related to polymer induced aggregation, which is considered to be driven by osmotic forces. The cell-free “depletion layer” is formed between the adjacent RBCs, which results in osmotic pressure from the macromolecules outside the depletion layer acting onto the cells [17], [20]. In case of the plasma the interaction between cells might differ significantly from what is predicted for neutral polymers because of the electrostatic interactions resulting, in particular, the adsorption of the protein molecules on the cells membranes.

This consideration is well matched with direct measurements made with OT. These measurements showed that in the process of separation of a pair of RBCs, the interaction between RBCs becomes stronger even though the interaction area between RBCs is decreasing [15]. This finding contradicts with the osmotic force model, according to which the interaction should be proportional to the interaction area. On the other hand it might be a peculiarity of the disaggregation process and cannot be directly compared with a model developed to assess the aggregation process.

In this work, we analyze the differences observed in our OT measurements and the predictions of the existing interaction models. The aggregation dynamics is found to be consistent with the predictions made by the hypothesis of osmotic force driven aggregation. We indicate that there is a significant difference between aggregation and disaggregation processes and speculate about the differences in the driving forces.

II. MATERIALS AND METHODS

A. Materials

The blood samples were obtained by venipuncture from ten clinically healthy male donors of age 20–30 years. The ethylenediamine-tetra-acetic acid was used as an anticoagulant. Experiments were performed within 3 h after drawing the blood. The highly diluted suspension of RBCs in platelet free plasma was used as sample for experiments. Platelet free plasma was obtained by the following procedure: (1) the whole blood was centrifuged at 1800 g for 10 min; (2) obtained plasma was washed again of any remaining platelets by centrifuging again at 12 000 g for 10 min. The RBCs obtained at the first step of centrifugation were added to platelet free plasma to achieve the final concentration of about 0.05%.

The experimental chamber consisted of glass coverslips and microscope slide separated by a small gap of 0.1 mm made of an adhesive tape. The glass plates were cleaned with ethanol prior to their usage. Approximately 60 μl of the sample were put into a chamber and Vaseline was used to isolate the cuvette. The RBCs on the surface formed a diluted monolayer and their shapes remained discoid for 4–5 h. Prior to experiments the RBCs were let to settle down to the glass surface. Then two individual non interacting RBCs were lifted from the surface with OT and used for experiments. The experimental procedures are described further along with the measurement results. The measurements were conducted on 5–7 pairs of RBCs for every donor and 50–70 pairs of RBCs were measured in total. All measurements were carried out at the room temperature.

B. Methods

Optical tweezers are the main experimental method used in this paper. The general description about principle of optical trapping is given elsewhere [27]. Generally, the trapped particle can be considered as attached to springs (optical traps) with certain stiffness. Thus, optical traps act with a returning force depending on how far the particle is separated from the trap centers up to a certain maximum value. In order to study the force interaction between ordinary RBCs, two independent optical traps were used as shown in Fig. 1. The traps were formed by orthogonally polarized continuous wave beam by single mode diode pumped Nd:YAG lasers (Shanghai Dream Lasers Technology) with the wavelength of 1064 nm and the output power up to 1 W. The two channels of laser beam were obtained using a beam-splitter. Large numerical aperture 100× water immersion objective (N.A. 1.00, Olympus, ∞-corrected LumPlanFi) was used to focus the laser beams and form the optical traps. The RBC trapping force varied between 1 to 20 pN. The position of one of the traps inside the sample was controlled in the focal plane of the focusing objective by a conjugated beam shifting mirror. Visual control of the trapped objects was implemented in transmission configuration using the CMOS camera (Thorlabs). The calibration process for force measurements is described in details in [23]. Briefly, the escape force calibration was made matching the external force with the trapping force, and the maximum returning force was found through the calibration of OT. The same approach was used for measuring the interaction forces between RBCs.

The cells interaction force was measured by matching it to the trapping force as shown in the Fig. 2 for the case of aggregating force measurements. The cells are trapped in two separate channels of OT, and then attached to each other. The aggregating force acts to overlap RBCs. OT forces are acting in the opposite direction to hold the cells from aggregation. As long as OT forces are greater than the aggregating force, the cells are
not overlapping each other (see Fig. 2 (a)). The measurement of the force is done by finding a match of the trapping force with the cells interaction force. Therefore, at a certain moment during a slow decrease in the trapping force the cells interaction overcomes it, and the cell escapes from the trap (see Fig. 2 (b)). At this moment it can be considered that the forces are matching each other. In our work, the trapping force of the unmoving trap \( F_{\text{trap1}} \) was always slightly stronger than that of the movable trap \( F_{\text{trap2}} \). The aggregating force was considered to be matching \( F_{\text{trap2}} \).

C. Data Processing

The standard deviation of the measured parameters including the calibration error is indicated by error bars in each graph of the results. The main scatter was introduced by individual differences between RBC. The interaction area between RBCs was measured by analysing the image of interacting RBCs and assuming the cells’ shape as discs and finding disks’ intersection area. Therefore the actual interaction area between the cells is expected to be slightly higher due to biconcavity of the cells.

III. MEASUREMENT RESULTS

The main aim of this study is to compare the dynamics of interaction during RBC aggregation and disaggregation. Therefore the measured parameters were separated into two parts—forces of aggregation and disaggregation correspondingly. The measured parameters were dependent on the time of interaction between RBCs during the first few seconds. Therefore in all our experiments the measurements were carried out for the interaction times longer than 20 s, during which the parameters almost did not change over time.

A. Force Measurement During the Disaggregation Process

The force measurement during the disaggregation process was made using the same method as described in the work of Khokhlova et al. [23]. Disaggregation was considered as a process of separating with OT two RBCs initially attached in a doublet. The dependence of the force required to separate the RBCs pair on the interaction area was measured. The sequence of measurement steps was as shown in Fig. 3(a): (1) two RBCs were trapped by OT; (2) the cells were brought to each other and attached with about 80% (or \( \sim 30 \mu m^2 \)) of the cells interaction area; (3) the RBCs were held still for 10 s; (4) the RBCs were pulled one from another by moving the OT position away with the velocity of 0.3 \( \mu m/s \) at a defined pulling force. The step 4 was repeated multiple times with increasing pulling force. For every step, the minimal achievable cells overlapping distance was measured for a certain pulling force.

The obtained dependence is as shown in Fig. 3(b), the force required to fully separate cells was about 20 pN. The measured dynamics of interaction shows that the force required to separate the cells was increasing along with a decreasing interaction area. The observed dependence indicates a residual effect of RBCs interaction takes place.

In order to double check the residual effect, the dependence of the disaggregating force \( F_D \) on the “initial” interaction area was measured. \( F_D \) is referred to as the force required to fully separate the overlapped cells. The sequence of steps for measuring the \( F_D \) dependence on “initial” interaction area is schematically shown in Fig. 4(a). First measurements were carried out for small “initial” interaction area: (1) two non-interacting RBC were trapped with OT; (2) the pair aggregate was formed by
attaching one RBC to another with an interaction area $\sim 20\%$ (10 $\mu m^2$); (3) the RBCs were held aggregated at low OT power during a time not less than 20 s; (4) RBC were pulled one from another by moving the OT position away with the velocity of 0.3 $\mu m/sec$ at a defined pulling force. The step 4 was repeated multiple times while slowly increasing the pulling force until the cells were fully separated and disattached. The step was about 0.5 to 1 pN and 2–3 steps were made for each measurement. This way the minimum force required for doublet disaggregation was found. For larger “initial” interaction areas, at first the RBCs were attached with an overlap of $\sim 80\%$ (30 $\mu m^2$) of cells interaction area, then they were slowly pulled with a force sufficiently high for their interaction area to reach $\sim 20\%$ (10 $\mu m^2$). Then the measurement was performed in the same way as for a small “initial” interaction area.

The obtained dependence is shown in Fig. 4(b). It proves that the $F_D$ is dependent on the “initial” interaction area. For small “initial” interaction area, $F_D$ was found to be 7.2 $\pm$ 1.8 pN, however for larger “initial” interaction area it was found to be 20.0 $\pm$ 4.5 pN. This result clearly demonstrates the existence of the residual effect of RBC interaction on the disaggregation process.

### B. Force Measurement During the Aggregation Process

We considered the RBC aggregation process as a pair aggregate (doublet) formation. Two individual initially non-interacting RBCs in plasma spontaneously overlap each other after a small interaction area is created with the help of OT. In order to find the aggregating force ($F_A$), the minimum trapping force required to stop RBCs overlapping was measured. The sequences of steps for measuring the RBC spontaneous overlapping is schematically presented in Fig. 5(a): two non-interacting RBCs are (1) independently trapped with OT, and (2) brought close to each other to form a certain interaction area; they were held with OT not to allow spontaneous overlapping; (3) OT power decreased slowly until the moment when the RBC aggregating force exceeded the trapping force and the cells started to spontaneously overlap.

The measurement results are shown in Fig. 5(b). The $F_A$ measurements were made for different interaction areas and it was found that $F_A$ varies from 2 to 4 pN depending on the interaction area.

### IV. ESTIMATION OF INTERACTION ENERGY

Based on the measurement results, we calculated the mean values of interaction energy densities, a parameter commonly used for RBC interaction models. Further, in order to characterize the change in the cells interaction strength over the aggregation and disaggregation processes, the energy required to separate the cells was calculated and plotted in dependence of the interaction area. In the case of RBC disaggregation, the RBC interaction energy should match with the work required to separate the RBCs. Therefore a simple equation can be used to find the RBCs interaction energy:

$$U = F \times \Delta A.$$  

Here $U$ is the work required to move an RBC, which is equal to the cells interaction energy; $F$ is the disaggregating or aggregating force; and $\Delta A$ is the relative linear displacement of the RBCs (change in the overlapping distance). The energy was summed up for five steps of the disaggregation process. Schematic illustration of the procedure is given in Fig. 6(a). The disaggregating force and the velocity of disaggregation were assumed to be constant during each step. The resulting interaction energy was divided by the area of interaction between RBCs, and estimate of the interaction energy density of 2.7 $\pm$ 0.9 $\mu J/m^2$ was obtained. The dependence of the energy required to shift the cells by 1 $\mu m^2$ was calculated by dividing the interaction energy on each step of measurement by the corresponding change in the interaction area. The dependence is plotted as black points in Fig. 6(b).

For the aggregation process, the same approach was used. In this case, $F$ refers to the aggregating force and $\Delta X$—to the relative displacement between the measurement steps shown in Fig. 3(b). The RBC pair’s spontaneous aggregation velocity is known to be constant [23]. The mean value of interaction energy density was 0.6 $\pm$ 0.4 $\mu J/m^2$. The dependence of energy required to shift cells by 1 $\mu m^2$ was plotted as grey points in Fig. 6(b). The dependence shows that the value is almost
constant over the interaction area for the aggregation process and is about 0.4 \( \mu J/m^2 \). In the case of disaggregation process, it was increasing significantly with the diminishing interaction area starting from 2 to 6 \( \mu J/m^2 \).

V. DISCUSSION

Our measurements results show that in autologous plasma the aggregation dynamics is significantly different from the disaggregation dynamics. The \( F_A \) is gradually decreasing with decreasing interaction area (see Fig. 5) in contrast with the disaggregation process (see Fig. 3) for which the dependence is opposite and a residual effect is observed. The peculiarities of RBCs disaggregation were observed earlier using OT, first by Bronkrost et al. [15] and then quantified by Khokhlova et al. [23]. In this work, we show that the interaction energy density is significantly greater for the case of the disaggregation process compared to that of the aggregation. Here we speculate and discuss this difference.

First, we should mention that in our calculations of the interaction energy we did not take into account the electrostatic repulsive forces and the membrane bending forces that arise in RBCs aggregation [2], [16]. In our study, the aggregation and the disaggregation processes were studied in similar conditions, therefore we could neglect the effect of these parameters when the two processes. It is known that the aggregation process occurs if the forces acting to form RBC aggregate is greater than electrostatic forces, membrane bending forces and shear forces acting to disperse aggregates. For different interaction mechanics proposed previously, the aggregation driving forces are considered to be osmotic pressure or surface bridging forces [19], [26]. It was mentioned that earlier RBC aggregation is studied mostly based on RBCs aggregate formation process [2]. Also the majority of studies of the interaction mechanics was obtained for polymer induced aggregation. As for the RBC interaction in plasma there is a very limited amount of data available. We speculate that the interaction mechanics and therefore the driving forces might be different for aggregation and disaggregation process. This may serve as an explanation of the known contradictions between the different interaction models and the experimental results obtained using OT.

In the case of the aggregation process, the strength of cells interaction is constant over the interaction area as shown in Fig. 3(b). This character of interaction is almost matched with physical descriptions for “depletion layer” model. Osmotic pressure should be proportional to formed depletion layer. Therefore the driving force for spontaneous aggregation might be mediated by osmotic pressure as generally accepted. In the case of disaggregation, the “depletion layer” model seems to not be suitable. The interaction energy changes over the interaction area in an opposite way, increasing along with decreasing interaction area, and the residual effect can be observed. It might be that when RBCs come to a close contact via osmotic forces, they can form surface cross-bridges and stabilize the aggregates with stronger forces. This would explain that \( F_A \) is much weaker than \( F_D \). However small \( F_A \) can keep overlapping the cells despite of strong interaction between cells is unclear. To assess it a more detailed study is required.

The absolute value of the interaction energy differs significantly between the aggregation and disaggregation processes. Measurements made by Buxbaum et al., with MAT for encapsulation of RBCs, state that the energy density of interaction between RBCs is \( \sim 2 \mu J/m^2 \) [13]. Our measurement results show that the value measured in disaggregation manner for plasma is \( 2.7 \pm 0.4 \mu J/m^2 \), which almost matches the value reported by Buxbaum et al. The difference might be due to the underestimation of the interaction area in our work. Therefore we speculate that the interaction energies found in [13] for the formed aggregates are likely to be related to the forces stabilizing the aggregates. In our work, these forces are the disaggregating ones.

VI. CONCLUSION

This work denotes that in the autologous plasma, the processes of aggregation and disaggregation of RBCs are manifested differently. The force required to stop spontaneous aggregation of RBCs is about few pN, while the force required to separate the RBCs equals to about a few dozen of pN. The aggregation force is decreasing along with the interaction area. However the force required to separate the cells, in contrast, increases. The dynamics of the RBCs aggregation process can be explained using the “depletion layer” model, as the strength of cells interaction constant over the interaction area matches the physical description of the osmotic force mediated interaction. In the case of the disaggregation process, the cells interact stronger as the interaction area decreases and show a residual effect, which can hardly be explained with the same model. We speculate that this interaction can be described as a manifestation of the surface cross-bridges that stabilize the aggregates and can be formed as cells come close to each other. Therefore the higher forces are required to disaggregate RBCs. The mobility of these surface bridges might give an explanation of the residual effect. However a more detailed study should be carried out to better assess the RBCs aggregation mechanics.

ACKNOWLEDGMENT

The authors would like to thank Prof. F. I. Ataullakhanov, Prof. N. N. Firsov, Prof. V. B. Koshelev, Prof. G. V. Maksimov, and Prof. I. A. Sokolova for the valuable discussions of the obtained results.

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