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Maria D. Khokhlova
Eugeny V. Lyubin
Alexander G. Zhdanov
Sophia Yu. Rykova
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Maria D. Khokhlova,[a] Eugeny V. Lyubin,[a] Alexander G. Zhdanov,[a] Sophia Yu. Rykova,[b] Irina A. Sokolova,[c] and Andrey A. Fedyanin[a]
[a]Lomonosov Moscow State University, Faculty of Physics, Moscow 119991, Russia
[b]Lomonosov Moscow State University, Faculty of Basic Medicine, Moscow 119991, Russia
[c]Lomonosov Moscow State University, Institute of Mechanics, Moscow 119991, Russia

Abstract. Direct measurements of aggregation forces in piconewton range between two red blood cells in pair rouleau are performed under physiological conditions using double trap optical tweezers. Aggregation and disaggregation properties of healthy and pathologic (system lupus erythematosis) blood samples are analyzed. Strong difference in aggregation speed and behavior is revealed using the offered method which is proposed to be a promising tool for SLE monitoring at single cell level. [DOI: 10.1117/1.JBO.17.2.025001]

Keywords: erythrocyte aggregation mechanisms; optical trapping; pathologically enhanced aggregation.

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1 Introduction

Hemorheological properties of blood are mainly determined by rheology of red blood cells (RBCs) as the main blood fraction. A lot of works were focused on studying the elastic properties of RBC membranes.[1–5] Another fundamental property of RBCs is their ability to aggregate,[6] which is a reversible process of mutual attraction between cell membranes. Formation of RBC rouleaux exerts a considerable influence on the hydrodynamic parameters in venules, promotes the displacement of leukocytes toward the vessel wall and therefore allows them to perform their protective function. Development of various multifactor diseases, for example, system lupus erythematosus (SLE), are aggravated by pathologically enhanced RBC aggregation.[7] SLE is a chronic autoimmune disease characterized by genetically induced disruption of immunoregulatory mechanisms. One of the SLE clinical signs is a considerable enhancement of RBCs aggregation forces.

During the last years, RBCs aggregation has been studied quite extensively, but the physical mechanisms responsible for RBC aggregation still remain controversial. Two major theories for the biophysical basis of this process are bridging[8,9] and depletion.[10–12] The bridging theory proposes that the macromolecules from plasma are absorbed onto the surface of RBC membranes forming the bridges between adjacent cells. According to the depletion theory, protein concentration is reduced near the surface of the RBCs comparing to the surrounding medium. An osmotic pressure occurs which tends to withdraw the water from between the RBCs creating the force that brings and holds the cells together.

The majority of available methods for RBC aggregation research, such as light-transmission and backscattering techniques, deals with an averaged response of a large number of RBCs.[13,14] Recent development of optical tweezers has led to the widespread dissemination of this technique in biomedical research due to the ability to trap and manipulate single objects at cellular and subcellular level.[15–18] This technique is finding an increasing application in study of the elastic properties of individual RBCs, which determine the ability to withstand large deformations while passing through small capillaries. Recently, qualitative measurements of RBC properties such as membrane viscosity, adhesion and zeta potential have been performed at individual cells utilizing double optical tweezers.[19] The use of optical tweezers was also proposed for qualitative analysis of red cell (dis)aggregation.[20,21]

In this paper, quantitative determination of force parameters of normal and pathological RBC pair aggregation utilizing double-trap optical tweezers is performed. This experimental approach provides a method to probe RBC aggregation mechanisms between individual cells and can offer insights into the connection between two theories of RBC aggregation. The relative statistical analysis is presented for healthy and SLE blood samples by measuring and analyzing aggregation forces and aggregation velocities in RBC pairs. All measurements were held in autologous plasma to provide physiological conditions for RBC aggregation and to achieve the most accurate level of quantitative experiments. The measurements have been performed on a significant number of RBCs.

2 Experimental Setup and Samples

In order to study 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 laser beams from two neodymium-doped yttrium aluminium garnet (Nd-YAG) lasers with the wavelength of 1064 nm, the output power of 100 mW and TEM_{00} mode structure. Large

Address all correspondence to: Andrey A. Fedyanin, Lomonosov Moscow State University, Faculty of Physics, Moscow 119991, Russia. Tel: +0074959393910; Fax: +0074959391104; E-mail: fedyanin@nanolab.phys.msu.ru

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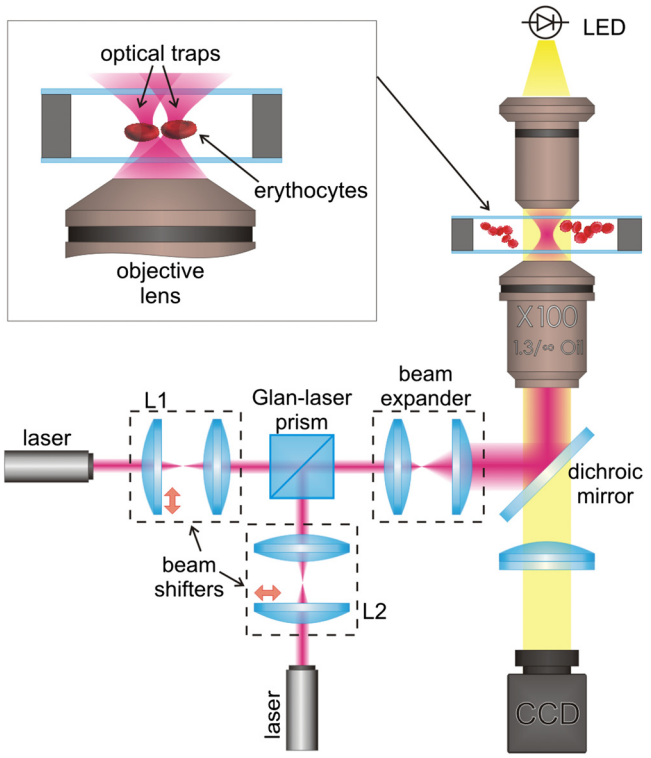

Fig. 1 Sketch of the double-beam optical tweezers setup for studying RBCs pair (dis)aggregation.

numerical aperture (N.A. 1.3) 100× oil immersion objective (Olympus, ∞-corrected Plan Semi-Apochromat) was used to focus the laser beams and form the optical traps. Laser power in each trap varied in the range between 6 and 20 mW. Two beam expanders provided the maximal optical field gradient at the beam waist for the most effective RBC trapping. The positions of the traps inside the sample were controlled within the focal plane of the focusing objective by the beam shifters consisting of two convex lenses placed in the confocal configuration. Step-motor translation of **L1** and **L2** lens perpendicular to the optical axis allowed movement of the trap with translation accuracy about 30 nm per step. Visual control of the trapped objects was realized in transmission configuration using the CCD camera.

The hermetic observation chamber was made of RBC suspension in autologous plasma placed between two coverslips separated by a gap of about 0.15 mm. The observation chamber was placed on the two-coordinate motorized stage for fine sample positioning in the focal plane. Erythrocytes obtained from the venous blood of 39 healthy volunteers and six patients with SLE were separated from plasma using protocol which consisted of blood stabilization by disodium EDTA (ethylenediaminetetraacetic acid) (0.002 g/ml) and centrifugation (Centrifuge 5417R, 2500 RPM, 25 °C, 5 min). RBCs suspension was then dissolved in pure plasma of the same patient to achieve a lower erythrocyte concentration of approximately 5×10^3 μl^{-1} convenient for handy trapping of individual RBC. Experiments were performed at room temperature ($T \sim 25$ °C).

3 Force Calibration

The force exerted on a trapped object depends on its size and shape, and on the refractive indices of the object and the surrounding medium. In order to use optical tweezers as a quantitative instrument, preliminary force calibration is necessary. Traditional way is the use of spherical polystyrene beads for the trapping force calibration.[2,4,5,22] Isotonic buffer (phosphate-buffered saline: 10 mM disodium hydrogen phosphate, 1.76 mM potassium dihydrogen phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, osmolarity 300 mOsm/kg, pH 7.4) was prepared first. Osmolarity and pH of the buffer mimic the conditions of autologous plasma. Fresh blood (0.5 μl) obtained by a fingertip needle prick was then diluted in 2 ml of phosphate buffer using a vacutainer containing EDTA. Carboxylated polystyrene beads, 3 μm in diameter, were added to the suspension (~1 particle per RBC) and then washed three times by centrifugation (Centrifuge 5417R, 3000 RPM, 25 °C, 5 min). After that, RBCs and polystyrene beads were suspended in 1 ml of autologous plasma. The coverslips for observation chamber were treated by serum bovine albumin (BSA) solution to prevent RBCs from sticking to the glass. One bead and one RBC were trapped by two independent laser beams with some distance between them and were moved to 10 μm above the lower coverslip. By varying the distance between the traps the particle and the cell were brought together until they contacted. The bead stuck irreversibly and nonspecifically to the RBC membrane in plasma within 1 to 2 min. The trap power for the RBC edge was set at 20 mW and the trap power for the bead varied in order to find the laser power conditions which would equalize the trapping force of the microbead and the RBC edge. The cell was then stretched by increasing the distance between the traps until the bead or the RBC edge slipped out from the traps. Escape trapping force for the bead and for the RBC edge was the same when the trap power for the bead was of 17 ± 1 mW, which was measured for five different samples. In order to obtain the value of this force, standard escape force method was used.[3,4] Aqueous glycerol solution (15%wt.) was chosen as a calibration medium. Its index of refraction appeared to be 1.351 with Abbe number $V = 42$, which was measured using the Abbe refractometer. The same index of refraction value was obtained for plasma. As far as optical characteristics of both media were the same the trapping force of the calibration beads was expected to be the same in plasma and in glycerol. Provided by these characteristics, the same polystyrene particles as used in the previous measurements were suspended in glycerol solution and then trapped by optical tweezers at fixed height of $h = 10$ μm above the surface of the lower coverslip. As the optical trap moved, solution exerted a viscous drag force on the trapped bead which is given as[4,22] $F = \beta \cdot v$, where β is a viscous drag coefficient for a spherical particle with the radius r in fluid at a height h from the surface:

$$\beta = \frac{6\pi\eta r}{1 - 9/16(r/h) + 1/8(r/h)^3 - 4/256(r/h)^4 - 1/16(r/h)^5}.$$

η was taken to be 1.32 ± 0.02 mPa · s (room temperature 25 °C). Viscous drag force was each time equal to the trapping force when the bead just escaped the trap. Provided by the value of the trap velocity v corresponding to this case, the trapping force of the bead in our glycerol solution was obtained and appeared to be 29 ± 2 pN for the laser power of 17 mW. Such approach to calibrate the optical trapping force was appropriate in our case as far as the Reynolds number was $\sim 10^{-3}$ (bead radius $r_{bead} = 1.50 \pm 0.05$, glycerol solution density $\rho = 1036$ kg/m^3—for 15% glycerol

solution). As a consequence, escape trapping force of the RBC edge in plasma was estimated to be 29 ± 3 pN.

4 Results and Discussion

4.1 *Disaggregation Experiments: Measuring Forces in RBC Rouleau*

Double trap optical tweezers were used as a tool for artificial aggregation of ordinary RBCs. Blood samples from 39 healthy and six SLE patients were selected for revealing the distinctions in aggregation properties for normal and pathological (SLE) erythrocytes. A single cell was firstly trapped near the lower coverslip. Being initially trapped at the edge, RBC becomes oriented along the trapping beam within about 2 sec. Such trapping behavior is mostly attributed to the asymmetry of the optical trap which is weaker along the laser beam than in the lateral direction.[22] After one RBC was trapped, another cell can be attached to it by means of optical tweezers. This was achieved by trapping another arbitrary erythrocyte into the second optical trap and moving it toward the position of the first trapped RBC until the cells visually touched each other. Then the traps were turned off allowing the cells to aggregate. Then the traps were turned on again and the aggregation force between individual RBCs in a pair rouleau was directly measured as a function of the distance between the RBCs centers. Averaged results for the blood samples of healthy donors and SLE blood samples are shown in Fig. 2(a).

For each data point double RBC rouleau was being pulled apart with a particular calibrated value of the trapping force F_{trap} by increasing the distance between the traps with fixed trap speed of 0.3 μm/ sec as shown in Fig. 2(b). The possibly maximal distance between the RBC centers, ΔX, was determined for each escape trapping force value F_{trap}, which was supposed to be equal to the RBC aggregation force F_a. Duration of each disaggregation experiment was about 20 sec to make the temperature increase of the medium and the thermal fluctuations of RBCs the same for all rouleaux pairs. Although considerable increase of medium and cell temperature was not expectable since hemoglobin absorption is negligible at 1064 nm and lipid bilayer membrane has a temperature rise of $\sim 1.45 \pm 0.15\,^{\circ}\text{C}/100$ mW when trapped with 1064 nm optical tweezers.[23] Important experimental observation is a qualitative growth of the aggregation force F_a from 8 pN to 30 pN with the increase of the distance between the centers of the trapped RBCs. Moreover, it was observed that this dependence has threshold behavior. In case of successful rouleaux break after reaching a certain threshold value of the RBCs, contact area further separation of RBCs happened without applying greater forces from the traps which indicates the existence of the decaying part of the $F_a(\Delta X)$ dependence which is much harder to measure using disaggregation approach. Measurements were carried out for 77 pairs of RBCs among 39 different blood samples from healthy patients and for 17 pairs of RBCs among 6 SLE blood samples. The difference in force interactions between healthy and SLE erythrocytes can be easily seen: aggregation force of SLE cells is higher than that for the normal cells if considering the same value of overlapping. For the center to center distance of 3 μm, the ratio $F_a^{\text{SLE}}/F_a^{\text{norm}}$ was observed to be equal 2.1, which makes it reasonable to consider offered technique as a handy tool for of SLE effects investigation at single cell level.

Formation of double RBC aggregate is not a momentary process. Kinetics of the aggregate formation is considerably determined by the biophysical mechanisms of the RBCs aggregation. Studying of this kinetics on the pairs of erythrocytes could give a deeper understanding of the RBCs aggregation mechanisms. Optical tweezers were used for revealing the dependence of the aggregation force upon the time of aggregate formation t_a. In the experiment, two erythrocytes were trapped independently and then brought together to the full overlap. Then the traps were turned off for certain time called as aggregate formation time t_a. After that, optical tweezers were turned on and the RBC rouleau was tried to be pulled apart. The parameter characterizing the interaction force was the minimal RBCs overlapping value dX which could be achieved by pulling the erythrocytes in the opposite directions by the optical traps with fixed stiffness. Figure 3 demonstrates the increase of the minimal value of RBCs overlapping, and hence the maximal force of interaction, with the increase of aggregate formation time.

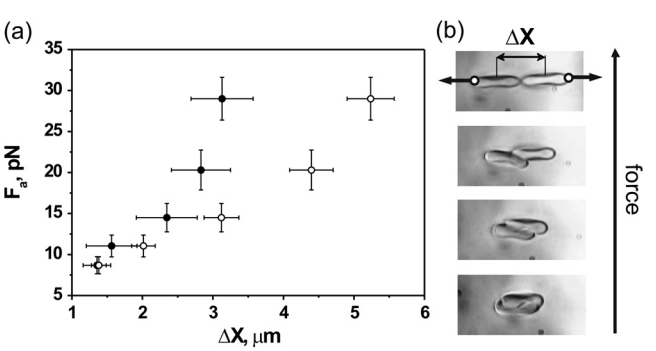

Fig. 2 (a) Dependence of the RBC (dis)aggregation force on the distance between RBC centers. The results are averaged over 39 blood samples from healthy donors and six samples from SLE patients. White dots correspond to the force measurements for the healthy blood samples while black dots correspond to the SLE blood samples. *X*-error bars correspond to the averaging over all the samples inside one group (normal or SLE erythrocytes). *Y*-error bars correspond to the calibration error bars. (b) Set of frames demonstrating the increase of the distance between cell's centers with the increase of the trapping force during artificial RBC disaggregation. Optical traps are shown by empty points.

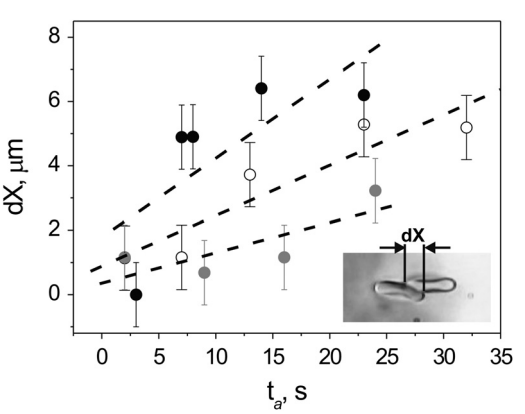

Fig. 3 Dependence of the minimal RBCs overlapping value dX upon the rouleau formation time t_a. The trapping force value was 29 ± 3 pN. Typical data from different donors are shown by different kinds of dots. Error bars correspond to the averaging over several attempts of disaggregation in one blood sample. Dashed lines are the linear approximations to the experimental data serving as the guides for the eye. Inset on the right shows a microphotograph of the disaggregation experiment.

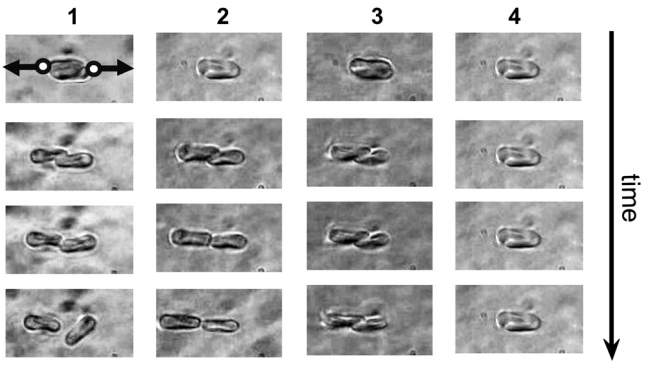

Fig. 4 (a) Set of frames demonstrating four end-points of RBC disaggregation. (b) Statistics of disaggregation end-points for healthy and SLE patients. The maximal trapping force is about 29 pN.

The more the time of aggregation formation is realized, the stronger interaction is observed. Dependence of the minimal RBCs overlapping value dX upon the rouleau formation time t_a was obtained for 12 different pairs of erythrocytes and linear approximations to the experimental data were done. The values of slopes while linear fitting are in the range of 0.14–0.72 μm/ sec which indicates strong difference in aggregation properties for RBCs even inside the same blood sample. Thus, optical tweezers technique offers an opportunity to probe aggregation properties on single cell level. For the same group of 39 healthy patients and six SLE patients, numerous experiments of RBCs rouleaux breakings by means of optical tweezers were performed with 10 to 20 attempts in each blood sample. Aggregate formation time was 3 to 5 sec. Then each cell in the pair rouleau was trapped by the focused laser beams and then the distance between traps was increased with a constant speed of about 0.4 μm/ sec.

Figure 4 shows the sets of consequent images taken with the fixed time value of approximately 20 sec during rouleau disaggregation by optical tweezers with the trapping force about 29 pN. Four different end-points of disaggregation were revealed. The first end-point was a successful break of the rouleau. The second one was the rouleau break until two RBCs were connected by small tethers. The third end-point of the rouleau break was a finite contact area of RBCs. Finally, the end-point of the fourth case was an abortive break of tight rouleaux. This scenario corresponded to the case when RBCs interacted with higher forces than the maximal forces of optical traps. Table 1 shows the statistics of disaggregation end-points averaged over all blood samples. RBC rouleaux are, on average,

Table 1 Statistics of disaggregation end-points for healthy and SLE patients. The values are averaged over 30 normal blood samples and 10 SLE blood samples.

End-point of RBC rouleau break	Probability of the end-point (norma) (%)	Probability of the end-point (SLE) (%)
1	9	1.4
2	30.3	13.3
3	48.7	56.4
4	12.4	29

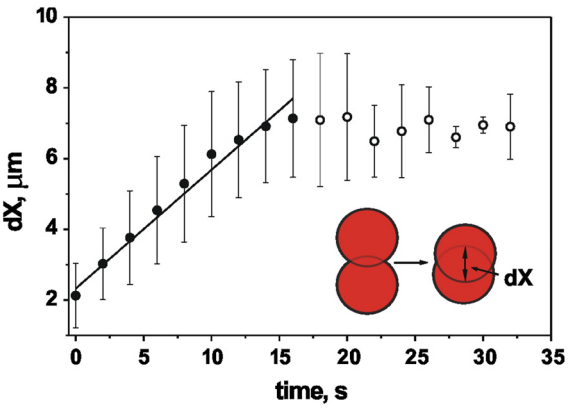

Fig. 5 Time dependence of the RBC overlap during pair aggregation. Black dots correspond to the sliding stage of aggregation, white ones —to the creeping stage. Solid line is the linear fit to the sliding stage. Inset shows schematic of the RBC overlapping increase during aggregation.

tighter for the SLE blood samples compared to those from healthy volunteers. Abortive breaks (end-point 4) are approximately 2.5 times more frequent for SLE patients than for healthy donors, while six times more successful breaks (end-point 1) are observed for normal erythrocytes.

4.2 *Comparitive Analysis of Aggregation for Normal and Pathological (SLE) Blood Samples*

Double optical tweezers technique also allows one measuring the speed of spontaneous RBC aggregation. Quantitative comparative analysis of normal and pathological RBCs was carried out by direct measurements of their aggregation speed. It was previously shown that RBC aggregation is a three-stage process of compression, sliding, and creeping.[24] The second and the third stages were measured during the experiments, and the sliding stage, which is the fastest one, was analyzed. Two arbitrary erythrocytes were trapped into two optical traps independently and then brought together into a point contact. Lasers were then turned off allowing RBCs to form double rouleaux. The time dependence of the RBC overlap dX was acquired using the CCD-camera. Measurements were performed for 39 healthy donors and for six SLE patients. Results averaged over healthy donors are shown in Fig. 5 indicating the sliding and the creeping stages. Aggregation velocity of the sliding stage was determined by the linear fit to the first part of experimental data. The value of aggregation speed for normal erythrocytes lies in the range of 0.30 ± 0.08 μm/ sec with the probability of 0.95, while for pathological ones, the value lies in the range of 0.53 ± 0.06 μm/ sec with the probability of 0.95. The mean value of aggregation speed for pathological erythrocytes is almost two times higher than for normal cells. This shows strong difference in RBCs aggregation properties for pathological (SLE) and healthy blood samples, which optical tweezers technique is able to recognize.

5 Conclusions

The force characteristics of the red blood cell (dis)aggregation were determined using the double trap optical tweezers. Disaggregation experiments revealed the dependence of the RBC aggregation force as a function of the RBC center to center distance indicating the qualitative growth and threshold behavior of this dependence. It was shown that the behavior of the force

curve is highly dependent on the type of blood studied—normal or SLE blood samples. Dependence of the RBC aggregation force upon the time of rouleau formation was obtained demonstrating strong influence of random time processes on the rouleaux formation. Quantitative comparative analysis of aggregation properties for normal and SLE blood samples at single cell level was performed. Four different end-points of RBC rouleaux breaks were revealed depending on the force of interaction between the cells. Correlation between the end-points of disaggregation and the type of blood–healthy or pathological (SLE), was obtained. Direct measurements of aggregation speed for pairs of erythrocytes shows a strong difference between normal and SLE blood samples: the aggregation speed value for the normal RBCs is almost two times lower than that for SLE ones. Experimental information provided gives an opportunity to suggest double trap optical tweezers technique as a sensitive tool for monitoring of SLE disease and its response to drug therapies on the single cell level.

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