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Abstract. Kinetics of optical tweezers (OT)-induced spontaneous aggregation and disaggregation of red blood cells (RBCs) were studied at the level of cell doublets to assess RBC interaction mechanics. Measurements were performed under *in vitro* conditions in plasma and fibrinogen and fibrinogen + albumin solutions. The RBC spontaneous aggregation kinetics was found to exhibit different behavior depending on the cell environment. In contrast, the RBC disaggregation kinetics was similar in all solutions qualitatively and quantitatively, demonstrating a significant contribution of the studied proteins to the process. The impact of the study on assessing RBC interaction mechanics and the protein contribution to the reversible RBC aggregation process is discussed. [DOI: 10.1117/1.JBO.21.3.035001]

Keywords: red blood cell; aggregation; blood microcirculation; cells interaction; optical tweezers; fibrinogen; albumin.

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1 Introduction

Red blood cell (RBC) aggregation is the reversible clumping of cells under low-shear forces or at stasis. RBCs have a tendency to spontaneously form aggregates that initially consist of face-to-face linear structures that resemble a stack or roll of coins. Under *in vivo* conditions, RBCs spontaneously aggregate and disaggregate depending on the blood vessel geometry and the shear rate. In the present work, we distinguish the "RBC spontaneous aggregation" and "RBC disaggregation" processes, and "RBC aggregation" refers to the whole reversible aggregation process. RBC aggregation is one of the main determinants of blood viscosity at low-shear stress.[1] It was first observed by William Hewson in 1773, who wrote: "It is necessary to remark, that in a few minutes after particles are spread out on glass, they run in clusters and stick to each other. . . ." A detailed study of RBC aggregation was first performed by Fåhraeus.[2] Recent studies on the clinical and physiological significance of RBC aggregation have shown that this process is related to functional capillary density.[1–3] Other roles of RBC aggregation involve the complex regulation of blood viscosity, hematocrit, and blood cell (leukocyte and platelet) localization along blood vessels.[4,5] Therefore, RBC aggregation can be considered a determiner of blood microcirculation. In most pathologies, RBC aggregation is significantly altered, along with other blood property alterations,[4,6–8] resulting in blood circulation complications that are known to be one of the major causes of mortality in cardiovascular diseases.[9] Therefore, because RBC aggregation has a significant impact on microcirculation, it is important to understand RBC interaction mechanics and the contribution of proteins to this process to allow monitoring and therapy of blood microcirculation in the future.

1.1 *Methods Used to Study Red Blood Cell Aggregation*

RBC aggregation has been studied for several decades and a number of methods have been developed to measure RBC aggregation parameters.[4] The majority of experimental results were obtained using optical aggregometry, a method that involves laser beam scattering by blood suspensions along with RBC spontaneous aggregation *in vitro*.[6,10,11] Recently, a similar method made it possible to also measure some of the dynamic characteristics of cell aggregation.[12,13] Other methods used to study RBC aggregation are microscopy and image analyses, photoacoustics, and sedimentation.[4,14,15] However, these methods are not suitable for studying cell interaction mechanics, as they do not provide detailed information about individual cell-to-cell interactions. Methods such as micropipette aspiration, scanning electron microscopy (SEM), atomic force microscopy (AFM), and optical tweezers (OT) have been used to study RBC aggregation at the single-cell level and to assess the dynamics/mechanics of RBC interaction. Studies using micropipette aspiration and SEM enabled the first approximate estimates of the interaction energy and speculations about the mechanism of RBC interaction.[16–18] The interaction energy density between RBCs was found to be on the order of a few $\mu J/m^2$. However, as with other methods, the main results were obtained for neutral (nonionic)

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macromolecules that mediated RBC aggregation, and almost no data were obtained for plasma or protein mediated aggregation. During last two decades RBC interactions been studied in plasma and protein solutions using AFM and OT.[19–23] The interaction forces between RBCs were found to be on the order of dozens of pN. In particular, OT offers the possibility to assess the aggregation mechanics further by allowing the study of interaction kinetics with a large degree of freedom that is challenging to achieve using other methods.[21–23]

1.2 *Optical Tweezers to Probe the Red Blood Cell Aggregation Process*

OT is the method first proposed by Ashkin.[24] The description of the operating principle of OT can be found elsewhere.[25] OT can be used to trap and manipulate single microparticles using a tightly focused laser beam. If appropriate calibration is performed, OT allow precise measurement of forces ranging from femtonewtons to hundreds of piconewtons with essentially no harmful heating or mechanical effects, despite high light intensities. A breakthrough in the field of biophotonics, biophysics, and biotechnology was achieved in the last few decades, when OT became widely used as a tool for measuring the forces of cell and macromolecule interaction.[26]

Bronkhorst et al.[23] were the first to apply OT to study RBC aggregation. Although force measurements were not obtained, it was shown qualitatively that interaction is dependent on the suspension media, interaction time, and interaction area. The latest studies with OT have shown that precise quantitative measurements of the RBC aggregation kinetics can be performed.[21,22,27] The interaction forces between RBCs measured by disassembling an RBC pair were found to be approximately 10 pN.[21,22] Khokhlova et al.[22] showed peculiarities of cell disaggregation using OT: the cell interaction force increases as the cells are slowly separated, which underlined the perspectives of using OT to assess the cell interaction dynamics. In comparison with other single-cell methods, OT features a number of clear advantages: it produces negligible mechanical and heating effects, freely manipulates multiple cells, and allows for higher precision in force measurements up to the sub-pN range. Compared with whole-blood methods, such as optical aggregometry, the OT method yields clear advantages for precisely assessing the dynamics of cell interaction. These advantages are especially important in studying RBC aggregation, as RBC interaction forces range from a few to dozens of pN, and exhibit peculiarities that could not be observed with other methods.

1.3 *Factors Affecting Red Blood Cell Aggregation*

The factors affecting aggregation of RBCs can generally be separated into two parts. The first part consists of cellular factors, an intrinsic property of RBCs that affects the aggregation level, and the second part is the nature of the suspension media. The first property is called "aggregability"; it can be assessed by comparing the measurements of aggregation of RBCs of different donors using a well-defined medium.[28] Solutions of neutral (nonionic) macromolecules are generally used for aggregability measurements. Such measurements are important, as the plasma composition may be donor-dependent and it is necessary to distinguish cellular factors affecting RBC aggregation from factors associated with the medium. The details of the suspension media are no less important. RBC aggregation occurs only in the presence of macromolecules (i.e., blood proteins) and depends on their concentration and type.[29,30] Aggregation also occurs in the presence of neutral macromolecules.[31,32] In our work, we focused on accessing the influence of media factors in the aggregation of RBCs.

In protein solutions such as blood or plasma, the level of RBC aggregation is dependent on the concentration of different proteins such as fibrinogen, gamma-globulin, albumin, or acute phase proteins (haptoglobin, C-reactive protein), and others.[29,32] However, a majority of publications report the results of statistical analyses of only a few proteins in whole blood, and limited experimental data are available for model solutions of proteins. This is possibly the reason behind the diverse effects of macromolecules on RBC aggregation found by different authors. In particular, there are results indicating both a decrease and elevation of the aggregation level after albumin is added to the solution.[14,29] Among blood plasma proteins (macromolecules) affecting RBC aggregation, the main role is attributed to fibrinogen. Schechner et al.[30] showed that fibrinogen is the most significant determinant of RBC aggregation. As fibrinogen is also a determinant of the blood coagulation process, it might be considered the most significant protein for blood circulation.

1.4 *Fibrinogen-Induced Red Blood Cell Aggregation*

Aggregation of RBCs in solutions of fibrinogen with immunoglobulin and albumin was observed by Ben-Ami et al.[14] using a flow chamber augmented with computerized image analysis. However, RBC aggregation was observed to increase when the concentrations of all proteins (fibrinogen, albumin, and Ig) were increased. This might be a limitation of the flow-chamber experiments, as in the latest work using an AFM, it was shown that RBCs do interact with each other in a solution of fibrinogen alone.[19] A monotonic increase in interaction energy along with an increase in fibrinogen concentration was observed. The interaction between RBCs in a fibrinogen solution (4 mg/ml) was also qualitatively observed by Bronkhorst et al.[23] using OT. The use of specific fibrinogen-related inhibitors also decreases RBC aggregation.[20,33,34] Therefore, fibrinogen molecules influence RBC aggregation through their specific binding to RBCs. In a number of investigations, an AFM with a fibrinogen-coated tip was used to study fibrinogen interaction with RBCs.[20] The interaction between RBCs and fibrinogen was shown to be a specific one with a mean interaction force of approximately 79 pN. The inhibition of different fibrinogen binding receptors resulted in a decrease in interaction forces. Therefore, one might speculate that there are multiple fibrinogen binding sites, as the inhibition of one or another does not abolish RBC interaction. Different types of inhibitors such as eptifibatide (inhibitor of the glycoprotein αIIbβ3 platelet receptor), F(ab')2 fragments of monoclonal antibody CRC64 (inhibitor of the glycoprotein IIb-IIIa platelet receptor), and Arg-Gly-Asp-Ser (integrin receptor antagonist) were found to considerably decrease fibrinogen-induced RBC interaction.[20,33,34]

1.5 *Red Blood Cell Interaction Mechanism*

The model of RBC interaction is well developed for the case of RBC aggregation induced by neutral macromolecules in solution.[4,31,35,36] In contrast, there is a limited amount of data available for blood plasma or protein solutions that incorporate the mechanism of interaction. For RBCs aggregation induced by neutral macromolecules, the interaction mechanism is generally

considered to be depletion mediated.[35] Most of the results are based on studies of RBC aggregation kinetics in neutral macromolecule solutions.[22,23] However, as shown in a number of recent reports and in this work, this interaction mechanism cannot be directly applied to plasma. Therefore, the RBC interaction mechanism in protein solutions and plasma should be considered still unclear.[20,21,23,33,34] In particular, the RBC disaggregation kinetics obtained using OT appear to require a different interpretation from those predicted by existing interaction models.

There are currently two main hypotheses of RBC interaction: the "cross-bridge" model and the "depletion layer" model. The "cross-bridge" model describes the interaction between RBCs because of adsorption of macromolecules to the membranes of adjacent RBCs, forming "cross-bridges." RBCs aggregate by gradually forming "cross-bridges," and the strength of interaction is proportional to the interaction area and hence to the number of "cross-bridges."[36–38] In the "depletion layer" model, the interaction between RBCs is described in the opposite way, implementing the concept of depletion interaction introduced first by Asakura and Oosawa.[39] This model predicts that RBCs aggregate because of the formation of a polymer-free "depletion layer" between cells, and the osmotic pressure pushes cells toward each other. Here, again, the strength of cell interaction is proportional to the interaction area between cells, as the forces are osmotic.[28,,31] Therefore, the interaction force between RBCs in the existing models is considered to be proportional to the interaction area. The measurements made using OT displayed an increase in the interaction force with decreasing interaction surface; this finding is incompatible with existing models.[22]

At present, a sufficient amount of data exists regarding plasma- and fibrinogen-mediated interaction between RBCs. However, the kinetics of RBC aggregation induced by fibrinogen or other proteins composing the plasma is not sufficiently understood, and the interaction mechanism remains unclear. In our work, we used OT to study the kinetics of RBC aggregation in model solutions of fibrinogen and albumin and in plasma. The aim of this work was to assess how RBCs interact in protein solutions compared with plasma. RBC disaggregation kinetics in protein solutions was found to resemble that in plasma; however, aggregation kinetics was found to be different in these solutions.

2 Materials and Methods

2.1 *Method*

To study the interaction force 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 single-mode fiber pigtailed diode lasers (Lumics) with a wavelength of 980 nm and an output power of up to 330 mW. A large numerical aperture (NA 1.3) 100× oil immersion objective (Olympus, ∞-corrected Plan Semi-Apochromat) was used to focus the laser beams and form the optical traps. RBC trapping force varied between 3 and 20 pN. The position of the second trap inside the sample was controlled in the focal plane of the focusing objective by a beam shifter consisting of a lens system. The trap movement was performed by the translation of the first lens perpendicular to the optical axis. Visual control of the trapped objects was implemented in the transmission configuration using a CMOS camera (Thorlabs).

For the trapping force calibration, a carboxylated polystyrene 3 μm microbead (Kisker Biotech GmbH & Co. KG, PPs-3.0COOH, Germany) was connected to the RBC membrane. The microbead adhered irreversibly and nonspecifically to the RBC membrane within 1 to 2 min while maintaining contact. Then, the whole system was doubly trapped by the microbead and the opposite RBC edge. The trap power for the RBC edge was set at 20 mW, and the trap power for the bead was varied to identify the laser power value that would equalize the trapping force of the microbead and the RBC edge. This was found to be 17 ± 1 mW. Then, the standard escape force method was used to obtain a microbead trapping force of 29 ± 3 pN. Therefore, the RBC edge's trapping force was equal to 29 ± 3 pN at a laser power of 17 ± 1 mW at the beam waist.

It has been concluded in multiple reports that optical trapping at an appropriate laser power has a negligible heating effect on a blood cell.[21–23,40]

2.2 *Materials*

The blood used in our work was obtained from three clinically healthy male donors (age 21, 23, 24). The experiments were undertaken with the understanding and verbal consent of each donor according to the ethical policy of the Lomonosov Moscow State University. Statistical significance was obtained for only one donor; however, qualitative measurements were carried out on the other three donors to confirm the results. This allowed us to neglect the differences between donors in our measurements. The protein solutions were prepared based on phosphate buffered saline (10 mM disodium hydrogen phosphate, 1.76 mM potassium dihydrogen phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, osmolarity

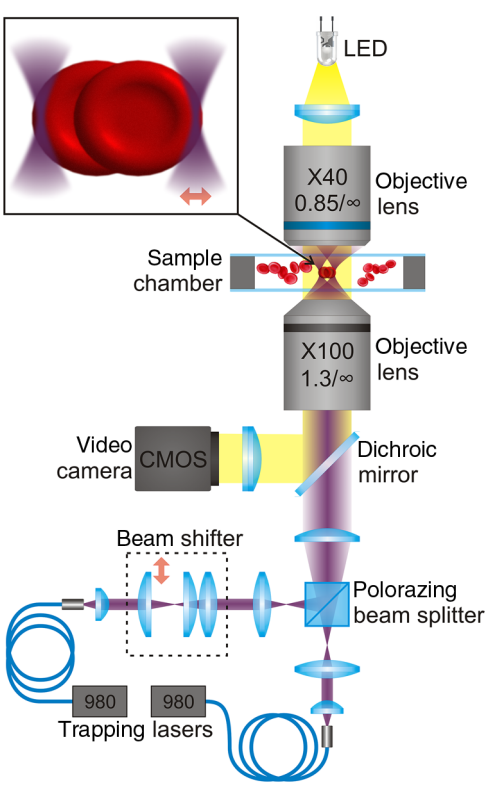

Fig. 1 Schematic layout of the dual-channel optical tweezers (see description in the text).

300 mOsm/kg, pH 7.4) containing (1) fibrinogen from human plasma (Sigma F3879), (2) albumin from human serum (Sigma A3782), and (3) fibrinogen with albumin at different concentrations. The solutions were stored at +4°C and used within 3 days. Autologous plasma for experiments was obtained with the following procedure: 4 ml of blood was drawn from a donor using EDTA as the anticoagulating agent and immediately centrifuged at 1800 g for 10 min to separate the plasma. Then, the plasma was washed from any remaining platelets by centrifuging again at 12,000 g to obtain platelet-free plasma. Plasma was stored at −20°C and used within 7 days. To prepare a sample for an experiment, a small amount of blood (0.5 μl) was taken by the fingerprick method and immediately suspended into 1 ml of the desired solution; then, 60 μl of this solution was transferred to a special chamber (cuvette) for experiments. Experiments were performed within 3 h after drawing blood. The chamber used for the measurements consisted of two glass coverslips separated by a small gap of 0.1 mm produced with adhesive tape. The glass plates were cleaned with ethanol prior to use and Vaseline was applied to isolate the cuvette. When the solution without albumin was studied, the inner surfaces were coated with 10 μl of a 1% solution of human serum albumin and dried to avoid adhesion and morphological changes in the cells caused by their interaction with the glass surface. The RBCs on the surface formed a diluted monolayer and their shapes remained discoid for 4 to 5 h.

The measurements were carried out on pairs of RBCs at room temperature. Prior to experiments, the RBCs were allowed to settle on the glass surface. Then, two individual noninteracting RBCs were lifted from the surface with OT and used for experiments. The experimental procedures are described as follows along with the measurement results. The measurements were conducted on 10 to 30 pairs of RBCs for every measured parameter. The largest source of error was individual differences between RBCs. Typical deviations of the measured parameters were 20% to 30%. The parameters measured were divided into two parts: the measurement of the RBC spontaneous aggregation (as a process of doublet formation starting from a local contact) and the measurement of the OT-induced RBC disaggregation process (as the process of doublet separation).

The linear overlap distance (A) is an important parameter in our experiments. It was measured in terms of the pixel size in the micrographs recorded during the experiments. The overlap distance was the linear measure of pixels between the cell edges. In our experiments, the cells were approximately the same size and they interacted parallel to each other. The deviation in cell sizes was 10%; the deviation in the pixel measurements was negligible and is ignored here.

3 Results

3.1 *Red Blood Cell Spontaneous Aggregation*

We considered pair aggregate (doublet) formation to consist of the following process: two individual initially noninteracting RBCs spontaneously overlap each other after a small interaction area is created with the help of OT, and the cells are released from the OT. The measurement procedure and time scale of RBC spontaneous aggregation is schematically presented in Fig. 2: two noninteracting RBCs are (1) independently trapped with OT and (2) brought close to each other to form a small interaction area, after which the OT are turned off; (3) the RBCs start to spontaneously overlap at a rate dependent on the suspending medium. The RBC spontaneous aggregation process consists of three stages: compression, sliding, and creeping.[41] The spontaneous aggregation velocity is measured as the ratio of the linear measure of the cells overlapping to the corresponding time during the sliding stage when the cells are spontaneously overlapping. During the measurements, special attention was paid to minimize the effect of the OT on the measured parameters: the OT power used at the formation of the initial interaction area was as low as possible, and the cells were allowed to attach to each other slowly.

In our previous work, we showed that the velocity of RBC spontaneous aggregation in plasma for healthy donors is 0.30 ± 0.08 μm/s.[22] Here we obtained that in protein solution the RBC do not overlap spontaneously at all as shown in Fig. 2(b). Only at significantly high protein concentrations, typically twice as high as the physiological mean value, do RBCs start to slowly overlap, with a mean time of spontaneous overlapping that is longer than 100 s. These measurements indicate that fibrinogen and albumin in normal concentrations do not suffice to induce spontaneous overlapping of RBCs. However, as shown further, despite the fact that the cells do not overlap spontaneously in protein solutions, they do undergo strong interaction with each other if a local contact is made artificially using OT.

3.2 *Optical Tweezers–Induced Red Blood Cell Disaggregation*

We considered cell doublet disaggregation to consist of the following process: the RBC doublet is formed with the help of OT, as shown in the schematic sequences presented in Fig. 3. The following parameters characterizing the RBC disaggregation process were measured: the disaggregating force (F_D), which is defined as the force required to fully separate

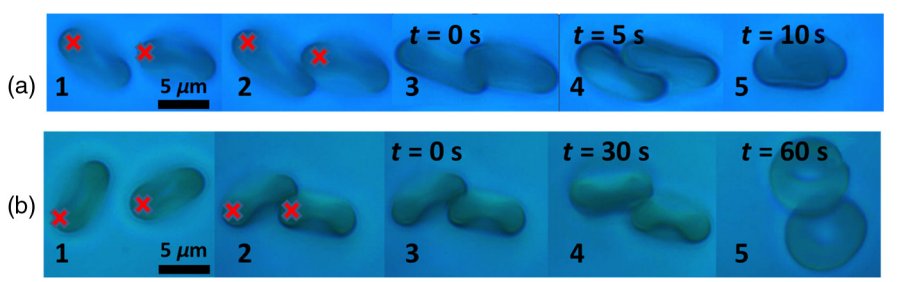

Fig. 2 (1) Two noninteracting RBCs are trapped with OT and (2) a small interaction area is formed, after which the OT are turned off. (3–5) RBCs start to spontaneously overlap and form a compact doublet in the case of: (a) plasma, or they do not spontaneously overlap in the case of: (b) fibrinogen 2.5 mg/ml + albumin 35 mg/ml solution. The cross marks show the positions of the OT.

Fig. 3 Schematic sequences of RBC disaggregating force measurement: (1) two noninteracting RBCs are trapped with OT; (2) the pair aggregate is formed by attaching the RBCs with a known interaction area; (3) the RBCs are held aggregated at low OT power during the known interaction time; (4) one of the cells is pulled with a known force. Step 4 is repeated multiple times slowly while increasing the disaggregating force until (5) the cells are separated and the minimum force required for doublet disaggregation is found. The cross marks show the positions of the OT, and the arrows show the direction of pulling.

overlapped RBCs, and the dependence of maximum achievable cell displacement on the trapping force. The latter parameter, as shown in our previous work, is an informative parameter that characterizes RBC interaction because of peculiar features of this process in plasma.[22]

The F_D was measured as a function of the concentration of protein and interaction time. The measurements resulted in the F_D dependence on protein concentration at defined initial cell interaction time and linear overlap (approximately 2 μm) are shown in Fig. 4. In protein solutions, the RBCs interacted strongly within the local interaction area formed by the OT despite the absence of spontaneous overlapping. The F_D in the protein solution was comparable with F_D in plasma. F_D was found to increase monotonically with the protein concentration for all studied proteins. The synergetic effect of albumin and fibrinogen was observed clearly: RBCs interacted much more strongly in the presence of albumin. At a fibrinogen concentration of 2.5 mg/ml, at which cell interaction was almost absent, albumin addition was followed by a significant increase in RBC interaction force. However, no interaction was observed when only albumin was present in the solution (data not shown).

The measured F_D dependence on the interaction time is shown in Fig. 5. In our previous work, we showed quantitatively that RBC interaction in plasma increases during the first few seconds of interaction.[22] In the present work, F_D values were measured as a function of the interaction time for a wider range of the interaction durations, from 10 to 90 s, to check the time course of our measurements. As shown in Fig. 5, there are no significant changes in F_D in plasma and fibrinogen solution.

The measurement process was similar to that presented in Fig. 3. However, the interaction time was controlled in a different way. First, the F_D was found for a short interaction time (10 to 20 s). Then, to achieve more precise control of RBC interaction time, a slightly overestimated value (approximately 10% to 20%) of the F_D was used in step 4. Therefore, the measurement protocol was as follows: the RBCs were (1) trapped by the independent optical trap channels, (2) attached to each other with fixed contact surfaces, (3) held at low power for 5 to 20 or 70 to 100 s, and (4) one of the cells was pulled apart from the other cell with a known F_D. The measurements were repeated with the same pair of cells, first with a short interaction time and then with a longer interaction time. Almost all (19 out of 20) pairs of RBC were fully separated using the slightly overestimated value of F_D. Therefore, we conclude that F_D does not increase over time after the first few seconds and that our measurements are independent of the interaction time, as they are performed with a mean time of longer than 10 s.

The dependence of the maximum achievable displacement on the trapping force was measured in a slightly different way. The protocol was similar to that used in our previous work, in which the force required to separate RBCs in plasma was shown to increase with a decreasing interaction area.[22] The measurement results obtained are shown in Fig. 6(a). The RBC disaggregation kinetics in the protein solution was found to be similar to that in plasma.

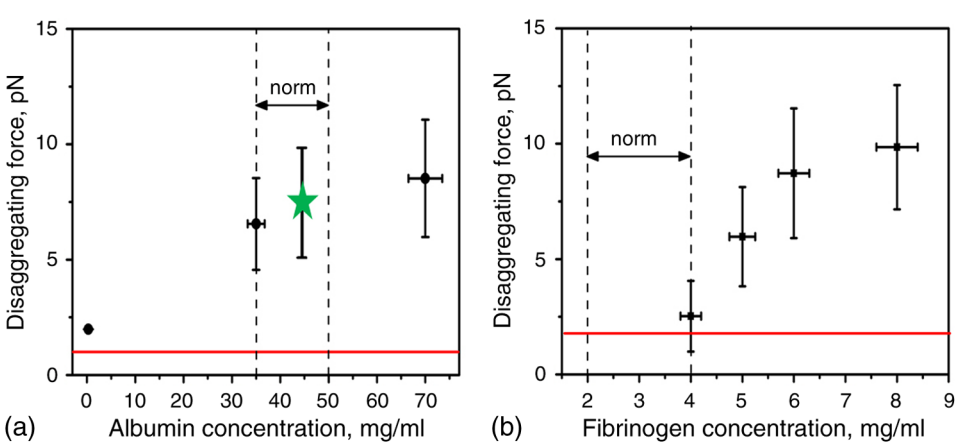

Fig. 4 Dependence of disaggregating force (F_D) on the concentration of proteins: (a) fibrinogen and (b) albumin with a fixed fibrinogen concentration of 2.5 mg/ml. The F_D increases monotonically with the protein concentration. A synergetic effect of albumin and fibrinogen can be observed. The green star symbol shows F_D for plasma with 1.9 mg/ml fibrinogen and 45 mg/ml albumin. The red line shows the minimum force that can be detected. The dashed lines delineate the normal physiological ranges of protein concentration. Note that at the concentration of fibrinogen 2.5 mg/ml, the cell interaction is weaker than the minimum force that could be measured.

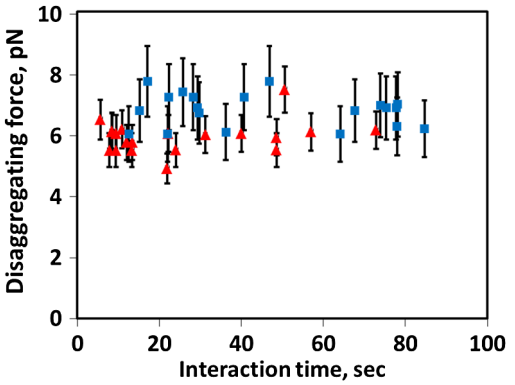

Fig. 5 Dependences of the disaggregating force on the RBC interaction time. The red triangles correspond to fibrinogen solution (5 mg/ml); the blue squares correspond to plasma. No significant changes were observed.

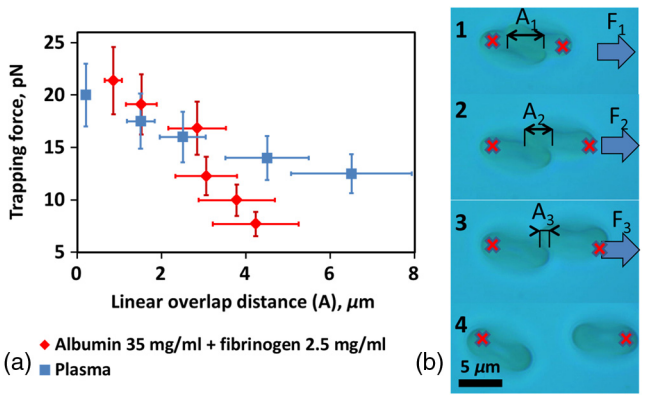

Fig. 6 (a) Dependence of maximum achievable displacement on the trapping force in plasma (concentrations of fibrinogen 1.9 mg/ml and albumin 45 mg/ml) and in the solution of fibrinogen (2.5 mg/ml) with albumin (35 mg/ml). (b) Set of frames (1–4) demonstrating the measurement process. As the linear overlap distance (*A*) decreases, a higher force (*F*) is required to further separate the RBCs. The cross marks show the positions of the OT and the arrows show the direction of pulling.

The sequence of the measurement process was as follows: (1) two noninteracting RBCs were trapped by different channels of OT; (2) the cells were attached to each other with a linear overlap of approximately 5 μm; (3) the RBCs were held together for 10 s; and (4) the cells were pulled apart by separating the OT positions at a velocity of 0.3 μm/s at a defined trapping force, as shown in Fig. 6(b). Step 4 was repeated multiple times by increasing the trapping force step by step, and the minimal achievable cell overlapping distance was measured for every trapping force value. Five force steps were typically used in the measurements. In the case of low fibrinogen concentration in the solution, the measurements could not be completed because the interaction force was insufficient for cells to attach.

In our experiments, we occasionally observed two cases whose results were excluded from the presented data. They were observed in approximately 1 pair out of every 10 pairs measured and were considered to be caused by individual differences between cells. In the first case, the RBCs were separated without further increase of the trapping force and/or position movement of the optical trap after reaching an interaction area of approximately 2.5 μm. The force required to fully separate these cells was typically significantly (approximately 2 times) weaker than in other cell pairs. In the second case, the RBCs could not be separated at any point, even with a significantly higher trapping force.

4 Discussion

4.1 *Fibrinogen-Induced Red Blood Cell aggregation*

In a number of works, it was mentioned that RBC aggregation can be induced by fibrinogen.[19,23,32] Our results give more specific information that most likely fibrinogen is the protein responsible for the disaggregation kinetics of RBCs, while for spontaneous aggregation, it has no effect when present alone or only with albumin in the solution. It was shown by the disaggregation force measurements that RBC interaction is strong even if fibrinogen is the sole protein in the solution. The disaggregation kinetics in a solution in which fibrinogen is present together with albumin is quantitatively similar to the disaggregation kinetics in plasma. A higher force is required to further separate the RBCs as the linear overlap distance (*A*) decreases. The dependence of the forces on fibrinogen concentration correlates with the measurements made by other authors: an increase in fibrinogen concentration led to the increased RBC aggregation level,[42,43] though measurements were carried out in plasma with an additively increased fibrinogen level and direct comparison is not available. These results support the idea that fibrinogen is the main determinant of RBC aggregation.[30]

Though just fibrinogen with albumin almost imitates cell disaggregation kinetics, more components are necessary for spontaneous aggregation. Our finding is supported by our previous studies comparing RBC interactions in plasma and serum. Interaction forces and aggregation velocities of cells were considerably impaired (~3 times) in serum compared to plasma.[44] Comparing the aggregation and disaggregation forces in plasma, a 3-fold difference was found.[45] Our results denote that OT-induced spontaneous aggregation and disaggregation are significantly different, and even the plasma component for initiation of spontaneous aggregation is still unknown.

A synergetic effect was observed in the interaction between RBCs in the solution comprising both fibrinogen and albumin. These results are in agreement with the finding of Ben-Ami et al.[14] and Maeda and Shiga.[46] In the case of plasma, in contrast, an increase in albumin concentration inhibited aggregation.[47] This might indicate that the role of albumin is complex and cannot be solely determined in solutions containing only a few proteins. On the other hand, the difference may arise from the use of different measurement methods, as the aggregation and disaggregation processes are not equivalent.

In recent experiments conducted using an AFM with a fibrinogen-coated cantilever tip, Carvalho et al.[20] measured the interaction force between fibrinogen and a receptor molecule on an RBC membrane. The authors concluded that the rupture force for a single fibrinogen-cell receptor binding event is 17 ± 4 pN. However, in our measurements, as shown in Fig. 4(a), the interaction between RBCs is significantly weaker at low concentrations of fibrinogen and is approximately 3 pN. Therefore, it may be that the interaction mechanics or the binding points were different in the AFM case and/or that a group of fibrinogen molecules binding at the same time were measured.

In our work, we observed the kinetics of cells disaggregation to be inversely proportional to the interaction area between RBCs. We hypothesize that the model of the depletion-mediated

interaction is not applicable to fibrinogen-induced aggregation because the depletion-mediated forces are osmotic and are expected to be proportional to the area of interaction. In contrast, fibrinogen molecules specifically bind to RBC membranes and cause the interaction to be distributed unevenly across the membrane. The possibility of fibrinogen binding to RBCs is supported by a number of other studies, as noted in the introduction.[20,23,33,34]

Our measurement results of the disaggregation process cannot be explained by the increasing effective interaction area while the linear overlap A between RBCs is decreasing because of the biconcave shape of the cells. RBCs are expected to reach a maximum interaction area at approximately $A = 3.5$ μm, at which point the effective interaction area is at a maximum, and then should start to decrease. However, in most cases, the force required to separate RBCs continued to increase even at the edge of interaction at approximately $A = 1$ μm.

4.2 *Models of Red Blood Cell Interaction Mechanics (Disaggregation)*

In existing models, the interaction energy is proportional to the interaction area between cells, which cannot describe the direct measurements obtained with OT. First, considering the cell interaction in protein solution, the "depletion layer" model is not applicable to describe it, as the depletion forces exclude the possibility of cell interaction without their spontaneous overlapping. On the other hand, the "bridging" model can be still used, assuming that the membranes of cells cannot be brought close enough to each other to form a bridge if there is no external force. Thus, the cells interact strongly when they are brought intact with OT. However, the ordinary "bridging" models describe the interaction such that bridges are uniformly distributed,[35] while our experimental results show that the interaction becomes stronger as cells are gradually moved to the separation point. We propose to extend the "bridging" model with a possibility of bridge mobility, which fits excellently with experimental results. If the bridges can move along with cell separation and also form new ones as the cells are moved from each other, as schematically illustrated in Fig. 7, the total interaction force will be increased. A similar approach was first introduced by Evans and Leung[48] to describe the agglutination interaction between RBCs induced by white germ agglutinin. It is necessary to mention that the agglutination interaction was about 2 orders stronger than one observed in our work. Further, the model was developed by Tozeren et al.[49] based on the interaction between a T-killer cell and its target. This model can be considered an extended "cross-bridge" model of the cell interaction, and, as illustrated schematically in Fig. 7, it assumes that binding of cells is possible via cross-bridges. These bridges are (1) formed at the initial interaction area, and (2) as cells are moved from each other, these bridges migrate along with the cell membrane (or break apart) and new bridges are formed; (3) the total number of bridges increases and so does the interaction force between the cells if migration and formation of bridges prevails over breaking of bridges. This model can also explain why the increase of interaction over cell separation is not observed in the measurements performed with whole blood aggregometry methods. In particular, in the device based on the microfluidic chamber (RheoScan), initially high and gradually decreasing shear stress is applied, which could mean that the bridges are not allowed to migrate.

In the case of plasma, spontaneous aggregation occurs. It is necessary to mention that the aggregation forces are significantly weaker than the forces required to separate the cells[43] and exhibited different dependence. It could mean that the interaction mechanisms are actually different for cell spontaneous aggregation and disaggregation. Our results support this assumption, as they show that for the interaction in protein solution, the cells can interact even without spontaneous aggregation. It might be due to both the depletion mediated (considering that there are more components in plasma and actual depletion interaction might be introduced) or cross-bridges (assuming that in fibrinogen solution, the bridges cannot be easily formed, while in plasma, there are other types of bridges that can be easily formed and thus might drive spontaneous aggregation). However, the data is lacking to assess what interaction mechanism drives the spontaneous aggregation of cells in native-like media.

4.3 *Estimation of the Energy of Interaction Between Red Blood Cells*

To further assess cell interaction, the interaction energy between cells was calculated based on OT measurements. The interaction energy between RBCs is proportional to the force required to separate the RBCs. The energy of RBC interaction was estimated using the following approach, as shown in Fig. 8. The force–displacement curve presented in Fig. 6 is used. The

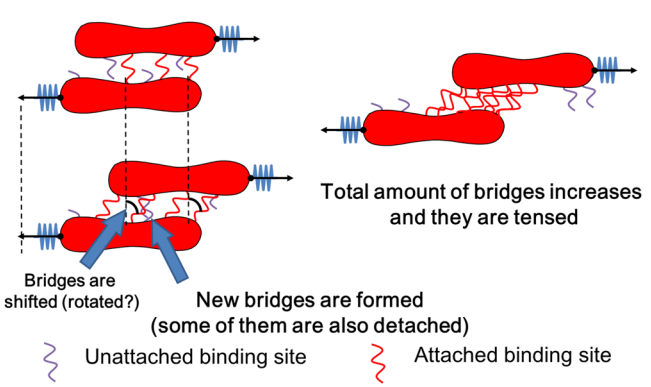

Fig. 7 Illustration of the phenomenological "cross-bridge" migration model proposed to describe RBC interaction.

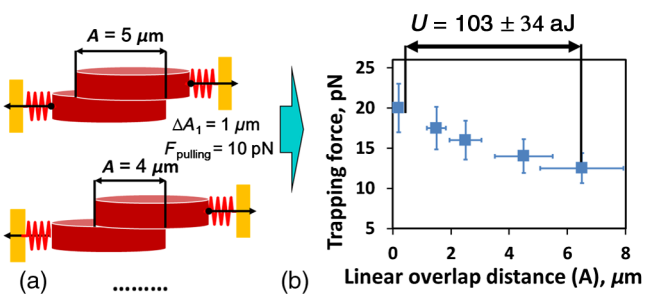

Fig. 8 (a) Illustration of the method used to calculate the energy of interaction between RBCs. The trapping force and relative displacement are known from the experiment. (b) Energy of interaction between RBCs in plasma, calculated by summing up the work required to move the cells in all steps of the disaggregation process, is approximately 100 aJ.

work required to shift the RBC position is determined using the following equation

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

Here, U is the work required to move the RBC over one step, F is the OT trapping force, and ΔA is relative change of the linear overlap distance between RBCs.

The work required to separate RBCs was integrated using our 4–5 step measurement of the force–displacement curve. The resulting work required to separate an RBC doublet was 103 ± 34 aJ, which is proportional to the RBC interaction energy. The mean interaction energy density ($\mu J/m^2$), a typical measure of RBC interaction in interaction models, was estimated by dividing the total interaction energy by the interaction area between cells. To simplify our calculations, the RBCs were assumed to be discs and the linear overlap distances were converted to the interaction area. Though the interaction areas for the biconcave shape and disc are different, this reflects peculiarities of interaction energy density during cell disaggregation. The corresponding mean interaction energy density for plasma was found to be 2.8 ± 0.5 $\mu J/m^2$, which is in agreement with the results obtained by free energy analysis.[18] However, as the interaction force is changing, the interaction energy density is expected to change along with it. For assessing this change, the interaction energy density was calculated at each step of the measurement and plotted in Fig. 9. The energy density was calculated by dividing the work required to completely separate the cells at the given force for each step and dividing it by the corresponding interaction area. The figure clearly shows that the separation of RBCs requires much more work as the interaction area decreases. These values are expected to be slightly overestimated, as the disc shapes of the cells assumed in these calculations exhibit approximately 30% less surface area than the real biconcave shapes of RBCs.

These results are in good agreement with the theoretical curve presented in the work of Tozeren et al.[49] Therefore, we suggest that the interaction between RBCs in native-like solutions (e.g., protein solution and plasma) could be described using the model of "cross-bridges," extended to include their ability to migrate.

As noted in Sec. 1, multiple reports support the specific interaction of fibrinogen with RBCs. However, further study is required for quantitative calculation of bridging interaction forces using the model.

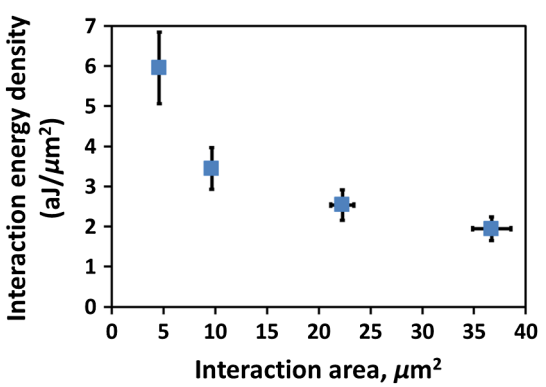

Fig. 9 RBC interaction energy density dependence on the interaction area in plasma.

The possibility of depletion-mediated interaction cannot be totally neglected either, as, to the best of our knowledge, the spontaneous overlapping of RBCs can be described well with this model. The complex effect of different proteins might increase the forces of both depletion and cross-bridges to induce RBC aggregation.

4.4 *Comparison of Optical Tweezer–Induced Red Blood Cell Spontaneous Aggregation and Disaggregation*

Even though the RBC disaggregation process in a protein solution is almost the same as in plasma, the spontaneous overlapping of RBCs was not observed in the former case. This finding emphasizes the importance of the complex role of proteins in the process of spontaneous RBC aggregation. It is in agreement with our previous study comparing cell interaction in serum and plasma for both spontaneous aggregation and disaggregation. The interaction forces were decreasing in serum compared with plasma, denoting the significant importance of the synergetic effect, especially in RBC spontaneous aggregation.

It is known that there are approximately 10 proteins that induce RBC aggregation, including fibrinogen, haptoglobin, c-reactive proteins, gamma-globulin, alpha-macroglobulin, and others.[4,50] Among them, fibrinogen is considered the most significant factor determining RBC aggregation.[30] It is worth noting that in most reports, RBC aggregation was studied based on RBC spontaneous aggregation. We showed that although fibrinogen is the main protein affecting aggregation, it is unable to initiate spontaneous aggregation. RBC spontaneous overlapping is expected to be an outcome of the presence of multiple different proteins in solution. However, fibrinogen was able to initiate a strong interaction between cells. Based on our measurements, we also speculate that different forces act during aggregation and disaggregation. RBCs quickly aggregated spontaneously in plasma, but not in protein solution, even though the interaction forces measured by disaggregating were comparable. Spontaneous RBC aggregation may be driven by osmotic force, and cross-bridges are important in stabilizing the resulting aggregates. Further study is required to assess the factors initiating spontaneous RBC aggregation and address possible differences compared with the disaggregation process.

To the best of our knowledge, the difference between the mechanisms of aggregation and disaggregation was not studied thoroughly or considered important. Here, we denote that the difference is significant and might also lead to different interaction mechanisms, and should be considered carefully. It is necessary to mention that common aggregometric methods that measure dynamic characteristics of RBC aggregation[12,13,42] do not distinguish these two phases and define the parameters as the minimum shear stress to disaggregate or to aggregate the RBCs at the same time, which in the scope of our work is not correct.

5 Conclusion

In this work, OT were used to study and compare the interaction of RBCs in plasma and PBS solutions of proteins, such as fibrinogen or fibrinogen and albumin. It is well-known that fibrinogen is the major plasma protein inducing RBC aggregation. In the PBS solution with proteins, the single RBCs interact with each other quite strongly. In spite of that, the spontaneous aggregation of RBCs was not observed. Thus, it was concluded that the presence of fibrinogen and albumin in the PBS solution

is not sufficient for initiation of spontaneous RBC aggregation; more plasma components should be considered for imitation of spontaneous RBC aggregation in laboratory conditions, which is supported by our previous works. In contrast, the kinetics of RBCs disaggregation in protein solutions was very similar to those observed in plasma. Quantitative measurements of the interaction forces between RBCs (disaggregating forces) were performed and the energy of interaction was quantitatively assessed. We found that the energy of interaction between the cells increases inversely to the area of interaction. The phenomenological model based on the "cross-bridges" hypotheses was proposed to describe the interaction between cells. The "cross-bridges" migration was incorporated into the model to describe disaggregation properly. Meanwhile, for spontaneous aggregation, a suitable model still remains an open question. To summarize the results, we denote that spontaneous aggregation and disaggregation processes in native-like media are complex processes governed by different interaction mechanisms. While spontaneous aggregation is absent, cell interaction can still persist. In particular, the spontaneous aggregation process is complex and the initiation of the process in native-like media has still not been assessed. This requires further study, while for the disaggregation process, it is becoming clear that more likely it is mainly due to cross-bridging.

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