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Assessment of the "cross-bridge"-induced interaction of red blood cells by optical trapping combined with microfluidics

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Abstract. Red blood cell (RBC) aggregation is an intrinsic property of the blood that has a direct effect on the blood viscosity and circulation. Nevertheless, the mechanism behind the RBC aggregation has not been confirmed and is still under investigation with two major hypotheses, known as "depletion layer" and "cross-bridging." We aim to ultimately understand the mechanism of the RBC aggregation and clarify both models. To measure the cell interaction *in vitro* in different suspensions (including plasma, isotonic solution of fibrinogen, isotonic solution of fibrinogen with albumin, and phosphate buffer saline) while moving the aggregate from one solution to another, an approach combining optical trapping and microfluidics has been applied. The study reveals evidence that RBC aggregation in plasma is at least partly due to the cross-bridging mechanism. The cell interaction strength measured in the final solution was found to be significantly changed depending on the initial solution where the aggregate was formed. [DOI: 10.1117/1.JBO.22.9.091516]

Keywords: red blood cell; aggregation; optical tweezers; plasma; fibrinogen; albumin; force; microfluidics.

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1 Introduction

Red blood cell (RBC) aggregation is a reversible process of the cells clumping and dispersing that determines the microcirculation of blood.[1,2] Enhanced RBC aggregation is a risk factor that is observed in a number of pathologies, such as malaria, diabetes, hypertension, inflammations, and others.[3,4] This process is currently attracting a significant research interest for the purpose of clinical application and fundamental studies.

1.1 *Methods for Studying RBC Aggregation*

Intensive studies were performed during the last few decades for characterizing the RBC aggregation and using it as a parameter for diagnostics and monitoring of the pathological states. Different methods, such as laser beam scattering on blood suspension,[5,6] microscopic image analyses,[7] measurement of electrical conductivity,[8] electrophoresis,[9] and others,[10] were developed to quantify the RBC aggregation. On the other hand, for assessing RBCs interaction mechanism, methods such as micropipette aspiration technique,[11] scanning electron microscopy,[12] atomic force microscopy,[13] and optical tweezers[14] were implemented. However, even though various methods are available, the mechanism of the RBC aggregation still remains unclear.

1.2 *RBC Aggregation-Inducing Factors*

It is known that RBC aggregation in a native solution (e.g., autologous plasma) is mainly correlated with the concentration of about 10 proteins, such as fibrinogen, alpha-macroglobulin, C-reactive protein, and gamma-globulin.[15–18] While most of the studies were based on the correlation between protein concentration and RBC aggregation, only a few pointed out the possible importance of the synergetic effect of proteins. The model solutions containing just a few types of proteins were found to be insufficient for initiating the RBC aggregation.[19,20] In an artificial environment, a solution of neutral macromolecules (e.g., dextran) can be used to induce the RBC aggregation that resembles the one that occurs in plasma.[21] Use of the neutral macromolecules for studying the RBC aggregation is important, because the RBC aggregation is also determined by cellular property that is called "RBC aggregability."[3] This parameter might significantly change during pathology. Aggregability is obtained as a ratio of the RBC aggregation parameter between two groups (e.g., in normal and pathology) in a defined solution, and dextran solution is most widely used for it. Using RBC aggregability allows detecting alterations of the cellular factors affecting the RBC aggregation. Despite the vast amount of the data accumulated, understanding of the underlying processes of the RBC aggregation is still unclear and further fundamental studies of the cell interaction mechanisms are necessary.

1.3 *Interaction Mechanisms of the RBC Aggregation*

Currently, most of the studies on the cell interaction mechanism are performed in the neutral macromolecule solutions.[22] Although interaction of the cells in these solutions resembles the interaction in plasma, it is simpler because plasma is an

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electrolyte containing a variety of macromolecules. Two different models describing a mechanism of the RBC aggregation were proposed.[21,23] The first one was the "cross-bridge" model describing RBC aggregation based on adsorption of the macromolecules (e.g., dextran) to cell membranes and formation of the cross-bridges connecting adjacent cells.[23] The second one was the "depletion layer" model that describes the cell interaction using a phenomenon of the depletion layer formation.[21,24] In the depletion layer model, macromolecules are depleted near the RBC surface due to the balance of entropy, and when cells get close to each other the depletion layers overlap and osmotic pressure starts to push the cells to each other and forms RBC aggregates.

The depletion layer model is currently favored over the cross-bridging model for interpreting the cell interaction.[10,25] Few evidences were obtained to support this model such as: (1) absence of cell interaction when the RBC membrane was coated with artificial "bridges" made by covalent-bonded poly(ethylene oxide);[15] and (2) cell interaction energies calculated theoretically in the frames of the depletion layer model was found to be matching with the experimental values measured using atomic force microscopy and micropipette aspiration techniques for solution of dextran.[11,13] However, one should note that these results, as well as the models, were based on the study of the cell interaction induced by neutral macromolecules. The RBC aggregation in native medium, such as plasma or protein solutions, might have different behaviors.

Few recent studies were conducted for studying the RBC interaction mechanism in plasma and protein solutions. It was found that inhibition of fibrinogen-binding sites on the RBCs results in a significant decrease of the RBC aggregation in plasma.[26,27] The experiments with optical tweezers allowed for studying the interaction kinetics between two cells. The cells kept interacting with each other even when the interaction area was almost absent.[14,28] In model solutions of fibrinogen, it was found that even if the cells do interact strongly with each other, they might not spontaneously overlap onto each other.[20] In plasma, a significant difference between the forces that prevent RBC spontaneous aggregation and forces that separate the cells was measured.[29] It indicated a possibility that different interaction mechanisms take place at the same time. Overall results in plasma and protein solutions suggest that the interaction of the cells might be due to the cross-bridging model. However, the clear evidence for either of the interaction models is yet to be found.

1.4 *Aim of the Study*

This paper is based on the study of the RBC aggregation in plasma and model solutions while the cell suspension medium is changed. Optical trapping (OT) method was the main measurement tool. In the previous studies, OT allowed measuring the cell interaction forces with a sub-pN accuracy[30,31] while having a precise control over the process. In our recent work, we showed that parameters of the single RBC interaction measured with OT are comparable with those measured by diffuse light scattering from whole blood samples.[32] Here we present results—the evidence for the cross-bridge-induced interaction of the cells that is valuable for understanding the RBC interaction mechanism. A significant comparative difference was found between the cell interaction in (1) plasma and (2) after moving the cell doublet from phosphate-buffered saline (PBS) to plasma.

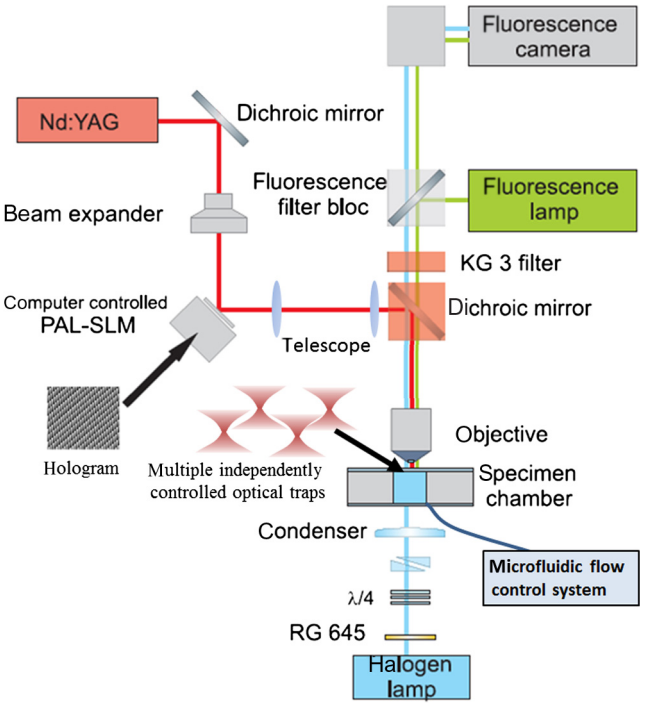

Fig. 1 Schematic layout of the holographic optical tweezers setup for measuring the RBC interaction force.

2 Materials and Methods

2.1 *Method*

An in-house made holographic optical tweezers setup based on an inverted microscope (Nikon, TE 2000) was used to measure the RBC interaction (Fig. 1). Multiple optical traps were formed using a laser beam from the single-mode Nd:YAG lasers with the wavelength of 1064 nm (1 W, Ventus) reflected by the parallel-aligned liquid spatial light modulator (PAL-SLM, PPM X8267-15, Hamamatsu Photonics) and focused with large numerical aperture oil immersion objective (NA 1.25, 60×, Nikon). The position of traps was controlled within the focal plane of the objective using PAL-SLM. Visual control of the trapped objects was done in a transmission configuration using the CMOS camera (ORCA Flash 4.0 V3, Hamamatsu). The fluorescence excitation and registration were possible using a mercury lamp and appropriate filters. Microfluidic flow control system (Elveflow, MK-1) was used to pump the samples through the microfluidic device.

2.2 *Experimental Chamber*

The experimental chamber used for the experiments consisted of two vessels containing different solutions (Fig. 2). Solution 1 (S1) had a significantly larger volume with dimensions of 0.5 cm (height) × 3 cm (length) × 2 cm (width) and had a small entrance to the microchannel containing solution 2 (S2). The microchannel had dimensions of 30 μm (height) × 1 cm (length) × 40 μm (width) followed by a snaky channel with the same height, 5 cm in length, and 10 μm in width. This specific shape of the channel allowed precise control over the flow rate that is necessary for measurements, as fluctuations of the flow rate would disrupt measurements.

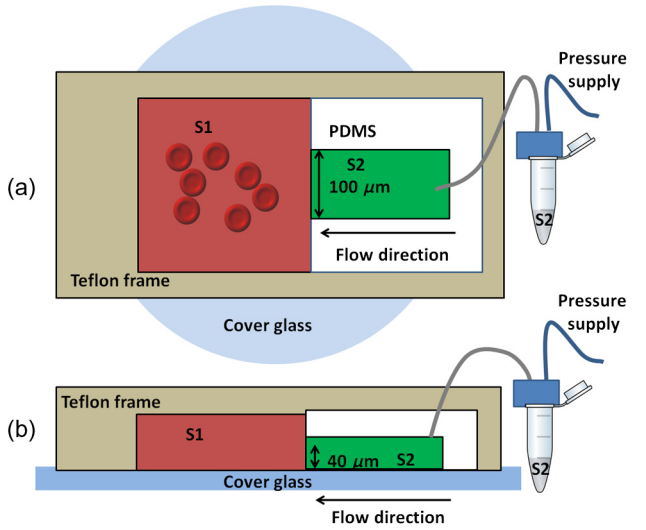

Fig. 2 Sketch of the experimental chamber (not in scale): (a) top view and (b) side view. The solution 2 is put to the microchannel (green part, right side, marked with S2) through a tube connected to microfluidic flow control system. The cells are in larger chamber (red part, left side, marked with S1) with solution 1 (S1) and moved to solution 2 (S2) using the optical traps.

The microchannel was initially filled up with S2 and then 200 μL of S1 was carefully put in a larger chamber. To prevent diffusion between solutions, S2 was continuously flushed toward S1 at flow velocity $(v_{\text{flush}}) = 20$ μm/s. v_{flush} was determined as a velocity of cell movement at the given pressure drop after being released from the trap. When a cell pair was brought close to the entrance of the microchannel, v_{flush} was reduced to 1 μm/s in order not to affect the measurement procedure. Typically, it took less than 1 min to move the cell pair into the microchannel and measure the cell interaction. After measurement, S2 was flushed toward S1 for 1 min with $v_{\text{flush}} =$ 100 μm/s to remove any diffused solution. Confirmation measurements were performed using 40 mg/ml of fluorescent albumin (Sigma-Aldrich, A9771) as S1 and PBS as S2. It was shown that with $v_{\text{flush}} = 20$ μm/s, the diffusion does not take place for at least 30 min and the fluorescent signal in S2 remains at the level of noise. When $v_{\text{flush}} = 1$ μm/s, diffusion starts to take place; however, within 1 min the diffusion is not significant. Flushing with $v_{\text{flush}} = 100$ μm/s almost immediately removed most of the solution from the microchannel.

2.3 *Sample Preparation*

Measurements were performed using blood from a single healthy male donor to avoid possible deviations introduced by individual differences. The cell interaction was measured in different suspending media: platelet-free plasma, phosphate-buffered saline (PBS, pH = 7.4, Invitrogen), and model solutions of fibrinogen and/or albumin (in PBS). Different combinations of the solutions were used as shown in Table 1. A small amount of blood was taken by finger prick method and initially suspended in S1 to achieve a final concentration of 0.05%. Cells were allowed to settle down for few minutes and at the given concentration of RBCs formed a diluted monolayer on the bottom surface. The cell shape remained discoid for 4 to 5 h. The experiments were performed at the room temperature (22°C) within 2 h after blood withdrawal.

Table 1 Solutions used for measuring the cell interaction. Measurements were performed either in a single defined solution or by moving the cells from solution 1 (S1) to solution 2 (S2). A, F, and PBS denote albumin, fibrinogen, and PBS, respectively.

Set 1: plasma ↔ PBS	Set 2: protein solutions
Plasma	F
PBS	F-A
Plasma (S1) → PBS (S2)	F (S1) → F-A (S2)
PBS (S1) → Plasma (S2)	F-A (S1) → F (S2)
	F or F-A (S1) → PBS (S2)

Platelet-free plasma was prepared by the following procedure: (1) whole blood was obtained by venipuncture using dipotassium ethylenediaminetetraacetic acid as an anticoagulation agent; (2) whole blood was centrifuged at 1800 g for 10 min to separate plasma and RBCs; and (3) plasma was carefully collected and centrifuged again at 12,000 g for 10 min to remove any remaining cells. Model solutions were prepared in PBS (ThermoFisher Scientific) and contained fibrinogen (Sigma-Aldrich, F3879) and/or albumin (Sigma-Aldrich, A3782) with a concentration of 1.7 and 35 mg/ml, correspondingly. These concentrations of proteins were within the physiological range. Solutions were stored at 4°C and used within a week.

2.4 *Calibration*

Calibration of the OT force (F_{trap}) was done by comparing it with the viscous drag force (F_{drag}) applied to a trapped RBC in PBS. Calibration was performed simultaneously for all the measurements. Flow was introduced by moving the piezoelectric stage. Flow velocity was stepwisely increased until the trapped cell escaped from the optical trap. At the moment when the cell escapes from the trap, F_{drag} is considered to match F_{trap}. The procedure was repeated on 10 different cells. F_{drag} on the trapped RBC was calculated by considering the RBC as a prolate ellipsoid of equivalent volume with the ratio of 1:3 using

$$F_{\text{drag}} = 6\pi\eta r v K,$$
$$K = \frac{\frac{4}{3}(\beta^2-1)}{\frac{(2\beta^2-1)}{(2\beta^2-1)^{1/2}}\ln[\beta+(\beta^2-1)^{\frac{1}{2}}]-\beta},$$
$$\beta = \frac{a}{b}, \tag{1}$$

where η is a dynamic viscosity, r is a radius of sphere with the volume equivalent to the RBC, v is a velocity of the movement of the piezoelectric stage, K is a coefficient for a prolate ellipsoid, and β is a ratio between the major (a) and the minor (b) axes of the ellipsoid.

In order to avoid the influence of the zero-order trap formed by PAL-SLM, we trapped the cells 20 μm away from the zero-order trap position. The positions of the traps were moved ~2 μm from the initial setup position to separate the cells. The change of the trapping force due to this movement was less than 5%.

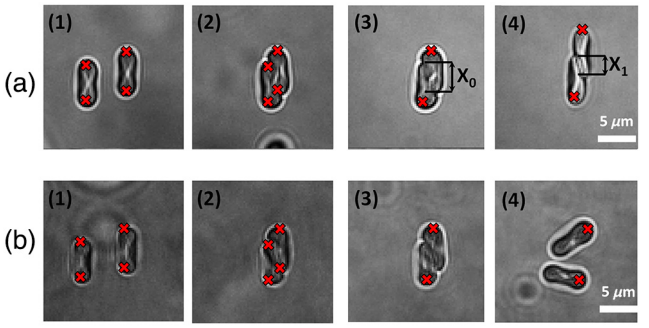

Fig. 3 Microphotographs of the measurement process in the case of (a) fibrinogen → fibrinogen with albumin and (b) plasma → PBS. The cross marks show the positions of the optical traps. (1, 2) Two RBCs are trapped and attached to one another in solution 1 (S1) with four optical traps; (3) the cells are moved to solution 2 (S2) and two middle traps are turned off remaining only two other traps on the edges; (4-a) RBCs are pulled with the defined trapping force ($F_{trap} = 12.5 \pm 1$ pN) to find the maximum achievable shift of one cell from the other. And the relative shift (ΔA) of the cells was calculated as $\Delta A = (X_0 - X_1)/(X_0)$, where X_0 is the initial linear overlap distance, X_1 is the minimum linear overlap distance; (4-b) the cells are spontaneously disaggregated as soon as they are moved to PBS.

2.5 *Measurement Procedure*

The RBCs interaction was measured as shown in Fig. 3. The procedure was as follows: (1) two RBCs were lifted from the surface and oriented parallel to each other using four optical traps; (2) the cells were attached to each other with a defined interaction surface; (3) the aggregate was moved from S1 to S2 and two middle traps were turned off remaining only two other traps on the edges; and (4-a) RBCs was pulled with $F_{trap} = 12.5 \pm 1$ pN and a relative shift (ΔA) of the cells was measured. (4-b) In the case when RBCs were moved from plasma (or protein solution) to PBS, the cells disaggregated even without moving the traps. Same measurement procedure was used for a single defined solution excluding only the step (3) where the cells were moved from one solution to another. Measurements were carried out on 10 pairs of cells for each of the solutions shown in Table 1.

Relative shift (ΔA) was a measurement of the cell interaction strength. ΔA was calculated as shown in Fig. 3(a). F_{trap} for the measurements was chosen by cross-checking ΔA value for different solutions to reflect the change of the cell interaction strength. Thus, for example, at a given F_{trap} it was $51 \pm 26\%$ in solution of fibrinogen, whereas it was only $20 \pm 17\%$ in solution of fibrinogen with albumin.

3 Experimental Results

The measurement results are presented in Table 2. The values for a single defined solution were the following: for plasma $\Delta A_P = 41 \pm 41\%$, for fibrinogen $\Delta A_F = 51 \pm 26\%$, and for fibrinogen with albumin $\Delta A_{F\text{-}A} = 20 \pm 17\%$. The cells did not interact in either PBS or albumin solution. The cell interaction changed in different ways depending on combination of S1 and S2.

3.1 *Plasma ↔ PBS*

Although the cells did not interact in PBS, we could form an artificial aggregation by pushing two cells to each other with four optical traps. We found that when the artificial aggregate is moved from PBS to plasma, the cells started to interact. The measured value of the cell interaction was $\Delta A_{PBS \to P} = 96 \pm 10\%$. Conversely, when the cells were moved from plasma to PBS, the aggregate was dispersed. As one can see, the interaction strength in case of PBS → plasma ($\Delta A_{PBS \to P}$ was $96 \pm 10\%$) is significantly weaker compared to that in plasma with $\Delta A_P = 41 \pm 41\%$.

Table 2 Relative shift (ΔA) with standard deviations, achievable with $F_{trap} = 12.5 \pm 1$ pN in different solutions as a measure of the cell interaction. ΔA is equal to 100% when the cells are separated. The calibration error is accounted in the trapping force (F_{trap}) value. A, F, and PBS denote albumin, fibrinogen, and PBS, respectively.

Solution	Relative shift (ΔA)	Solution	Relative shift (ΔA)
PBS	No interaction	F-A or F → PBS	No interaction
Plasma → PBS	No interaction	F-A	$20 \pm 17\%$
Plasma	$41 \pm 41\%$	F	$51 \pm 26\%$
PBS → Plasma	$96 \pm 10\%$	F → F-A	$51 \pm 41\%$
		F-A → F	$70 \pm 34\%$

3.2 *Protein Solutions*

When the cells were moved between solution of fibrinogen and solution of fibrinogen with albumin, the results were as follows: fibrinogen → fibrinogen with albumin: $\Delta A_{F \to F\text{-}A} = 53 \pm 41\%$; fibrinogen with albumin → fibrinogen: $\Delta A_{F\text{-}A \to F} = 70 \pm 34\%$. Values are similar to the one measured in the solution of fibrinogen ($\Delta A_F = 51 \pm 26\%$), whereas it was significantly weaker in the solution of fibrinogen with albumin ($\Delta A_{F\text{-}A} = 20 \pm 17\%$).

3.3 *Interaction at the Edge and "Tether"*

It is necessary to mention that when the cells could be fully separated (e.g., in the case of PBS → plasma) we often observed a strong contact point at the edge. The similar character of the cell interaction was reported in a number of works with optical tweezers.[14,28,33] Normally, this contact can be eliminated with the same force necessary to shift the cells up to this point. However, we could rarely observe a contact point that cannot be separated within the limits of our trapping forces (up to 30 pN). Such a contact point was observed also after moving the cells from macromolecule solution to PBS. We considered it to be a "tether." Tether was reported in a number of works and is related to the cytoskeleton damage and consists of the cell membrane bilayer.[34] In our experiments, it did not hinder shifting of the cells to the edges. We considered that the cells were completely separated, if we reached the point where we could observe tether. Most of the cells were separated without it.

4 Discussion

4.1 *Hypothesis of the RBC Interaction Mechanism*

The results of our study show that RBC interaction changes significantly when the cells are moved from one solution to another. A hypothesis of our experiments was based on the

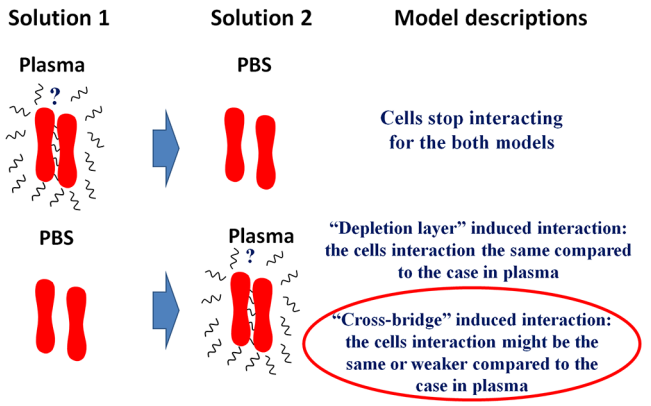

Fig. 4 Illustration of the hypotheses of the RBC interaction and description within the cell interaction models. The circle mark shows the hypothesis we have proved.

change of the cell interaction induced by change of medium. According to each model (cross-bridging and depletion layer), the cell interaction is expected to alter in different ways as shown in Fig. 4. It is expected that for the depletion layer-mediated RBC aggregation, the cell interaction should depend only on the final solution. However, results obtained in our work show that the initial solution plays a significant role, which is a clear evidence for the cross-bridge-induced RBC aggregation.

4.2 *Macromolecule Solution → PBS*

The interaction between the cells vanishes when they are moved from the macromolecule solution (either plasma or protein solution) to PBS. This can be described within the frames of both interaction mechanisms. For the osmotic nature of the interaction (depletion layer model), it is obvious that if there are no macromolecules to create an osmotic pressure, there will be no interaction. For the cross-bridging interaction, we could consider that in the conditions when no additional cross-bridge formation is possible, continuously applied weak shearing from either OT or flow is capable of breaking aggregates apart. The small osmotic pressure directed inside out due to the absence of macromolecules in solution could also contribute to the process. The last assumptions are supported by the measurements in model solutions. It should be noted that the cell interaction does not vanish immediately, but takes few seconds.

The cells attached in protein solution also disperse when they are moved to PBS. The cell interaction in these solutions exclude the depletion-layer-mediated interaction (or it is negligibly small) as shown in our previous paper.[20] The cells interact strongly when they are attached with OT and forces in range of few pN are necessary to separate them. However, when we release them after attaching with a small interaction surface, they do not overlap spontaneously. It takes more than 100 s for the cells to overlap spontaneously even with concentration of fibrinogen as high as 8 mg/ml. If the cells are suspended in plasma, they could quickly overlap with each other within a couple of seconds. Still, the force necessary to separate the cells might be of the same order of magnitude. These results allowed us to conclude that the cells in the protein solutions are attached via the cross-bridge-induced interaction as the depletion-layer-mediated interaction should have been acting to overlap the cells.

4.3 *PBS → Plasma*

Cell interaction appears as soon as the cells are shifted from PBS to plasma. At first glance, it seems more like osmotic interaction. However, the cell interaction is significantly weaker compared to one for the cells that were initially in plasma ($\Delta A_{\mathrm{PBS}\to\mathrm{P}} = 96 \pm 10\%$ and $\Delta A_{\mathrm{P}} = 41 \pm 41\%$). We consider this to be the direct evidence that shows the cross-bridge-induced RBC interaction. Osmotic forces should be the same regardless of the initial solution. Thus, we can attribute at least the difference to be cross-bridge-induced interaction.

The rest of the cell interaction cannot be distinguished between osmotic and cross-bridging forces. Considering that the intercellular distance between the cells in aggregate is about 20 to 40 nm (depending on the macromolecule size),[35] it is possible that in PBS we were not able to push the cells close enough with OT. Macromolecules could have entered between the cells after moving to plasma and thus inducing the interaction. However, as the intercellular distance is always shorter than the size of the macromolecules, it could be that not the entire cell surface is interacting. On the other hand, the osmotic forces could start pushing the cells to each other, which could mean that the both models take place during the interaction. It is difficult to distinguish the contribution of different forces, and this should be further assessed in detail.

The first assumption with the cross-bridging forces is supported by measurements in protein solutions. It is found that the cell interaction is stronger only when the cells are attached and remain in solution of fibrinogen with albumin ($\Delta A_{\mathrm{F\text{-}A}} = 20 \pm 17\%$). The cell interaction did not enhance, if the attached cells are moved from solution of fibrinogen to solution of fibrinogen with albumin ($\Delta A_{\mathrm{F}\to\mathrm{F\text{-}A}} = 53 \pm 41\%$). It could be due to the close interaction between cells formed by fibrinogen cross-bridges ($\Delta A_{\mathrm{F}} = 51 \pm 26\%$) that did not allow formation of new contacts. Similar behavior could take place in the case of PBS → plasma.

5 Conclusion

This study reveals that the assessment of RBCs interactions can be significantly improved by using optical tweezers coupled with a microfluidic system. The roles of aggregation affecting components can be carefully assessed in the well-defined conditions by moving the RBC aggregate between different solutions. Our experiments that were performed in plasma and PBS show a strong indication of the cross-bridge-mediated RBCs interaction. However, this indication does not exclude the possibility that some part of interaction is due to the depletion forces, as it was shown in the recent work based on the polymer interactions—the cross-bridging and depletion forces may coexist.[36] We expect that the cell interaction has a dual characteristic consisting of both cross-bridging and depletion forces, which should be proven with a direct experimental evidence, especially for the depletion forces in plasma. Further studies based on the proposed method could allow better assessment of the cell interaction mechanisms, e.g., by using a combination of different types of proteins and their concentrations.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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