Measuring electrical and mechanical properties of red blood cells with double optical tweezers

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Abstract. Red blood cell (RBC) aggregation in the blood stream is prevented by the zeta potential created by its negatively charged membrane. There are techniques, however, to decrease the zeta potential and allow cell agglutination, which are the basis of most of antigen-antibody tests used in immunohematology. We propose the use of optical tweezers to measure membrane viscosity, adhesion, zeta potential, and the double layer thickness of charges (DLT) formed around the cell in an electrolytic solution. For the membrane viscosity experiment, we trap a bead attached to RBCs and measure the force to slide one RBC over the other as a function of the velocity. Adhesion is quantified by displacing two RBCs apart until disagglutination. The DLT is measured using the force on the bead attached to a single RBC in response to an applied voltage. The zeta potential is obtained by measuring the terminal velocity after releasing the RBC from the trap at the last applied voltage. We believe that the methodology proposed here can provide information about agglutination, help to improve the tests usually performed in transfusion services, and be applied for zeta potential measurements in other samples. [DOI: 10.1117/1.2870108]

Keywords: optical tweezers; red blood cell; agglutination; membrane viscosity; adhesion; zeta potential.

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1 Introduction

Methods related to cell agglutination caused by antigen-antibody interactions are the basis of most of immunohematologic tests used in transfusion centers. Red blood cell (RBC) membrane proteins are responsible for this agglutination that can be specific or nonspecific. When the agglutination is nonspecific, some of the membrane proteins from one cell link to some membrane proteins of the other cell. RBCs agglutinated in a nonspecific way are called RBCs rouleaux. When the agglutination is specific, it is caused by antigen-antibody interaction. There are also blood antigens on the RBC membrane, such as A antigen and D antigen. The identification of the antibodies against the erythrocyte antigens is of fundamental importance for transfusional routines, because a mistake can lead to serious hemolytic reactions. Mechanical measurements performed in agglutinated RBCs can provide valuable information on the type and number of protein bonds.

While manipulating RBCs rouleaux with double optical tweezers, we observed that RBC cells slide easily over each other but are strongly connected by their edges. An explanation for this behavior could be that when the cells are dragged in a shear movement, sliding one over the other, the force involved to drag the bonded proteins through their lipidic membrane is of a viscous nature. On the other hand, to release two RBCs it is necessary to break the protein bonds with a much stronger force perpendicular to the membrane surface. This can explain why it is easy to slide the RBCs over one another but it is hard to disconnect them. Due to the fact that viscous forces are proportional to the drag velocity, the apparent membrane viscosity can be extracted from the curve force versus drag velocity. This measurement can provide information not only on the number and type of proteins of the two

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cell that bind to each other, but also on the whole membrane protein network that are dragged together. The adhesion force, on the other hand, depends essentially on the number and nature of binding proteins. The forces involved in nonspecific bonds are sufficiently small to be measured with a double optical tweezers system, practically limited to maximum forces of the order of 100 to 200 pN, but not the stronger antibody-antigen bond type.

In addition to protein binding, however, there are a set of barriers to prevent the agglutination process happening spontaneously in the blood stream, which could cause blood clotting. The first barrier is an electric repulsion between different RBCs, which prevents them from coming close to one another. The fluid lipid bilayer is composed not only by phospholipids but also of glycolipids and cholesterols. Due to the presence of the glycolipids, the RBC membrane surface is negatively charged. This induces a cloud of oppositely charged ions surrounding the cell (the double layer) and creates a repulsive electric (zeta) potential between the cells that prevents them from coming close and, therefore, aggregating in the blood stream.[1,2] Only the cells that overcome this zeta potential barrier can become sufficiently close together to agglutinate. A common way to enhance the agglutination rate and test sensitivity is to lower the zeta potential. This can be accomplished using low ionic solutions or by the introduction of macromolecular substances, such as dextran, albumin, and PEG (polyethylene glycol), to increase the dielectric constant.[1] Another possibility is to reduce the negative charge of the RBC surface using enzymes such as papain and bromelain.[3,4]

A complete understanding of the agglutination of RBC would involve, therefore, the measurement of intrinsic (membrane viscosity and adhesion) and extrinsic (zeta potential and double layer thickness) parameters in each individual cell. In this paper, we demonstrate the use of an optical tweezers setup in an electrophoretic cell to measure the following mechanical and electrical properties of the same individual red blood cell: (1) the apparent membrane viscosity (η_m), (2) the adhesion force (α), (3) the zeta potential (ζ), and (4) the double layer thickness of charges (d). This setup was used to test the viscous force hypothesis by measuring the shear force as a function of the relative velocity between the cells. From these measurements, we are able to extract the apparent membrane viscosity.[5] We also measured the force to break RBCs bonds (the cell adhesion). The terminal velocity of the particles under the influence of an external electric field is the usual way to measure the zeta potential, which could be done without the use of the optical tweezers setup. However, if we measure the value of the force of a trapped RBC as a function of the electric field and then release it to measure the terminal velocity, we can obtain the thickness of the double layer and the zeta potential at the same time for each individual RBC. The zeta potential and the double layer depend on the cell charge and the electrolytic environment, including the ionic concentration and the dielectric constant of the medium around the RBC. These parameters can change from cell to cell, especially the charge. The simultaneous measurement of the two parameters would enable one to extract complementary information about the relation among the charge, ionic solution, and dielectric constant. The knowledge of complementary parameters would enable us to obtain the others.

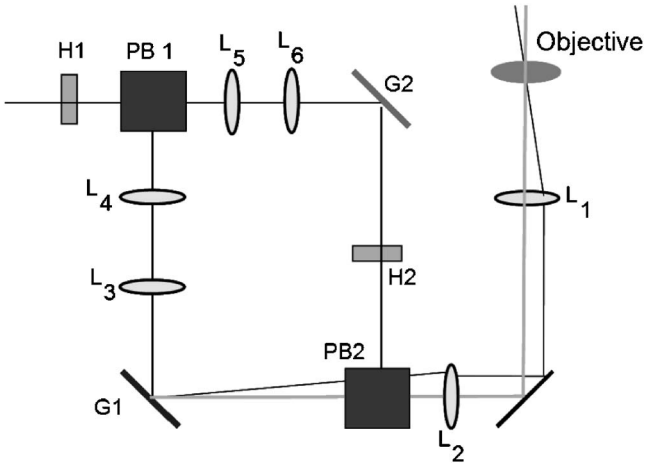

Fig. 1 Double optical tweezers system.

2 Materials and Methods

The RBC units were obtained from Hematology and Transfusion Center, Universidade Estadual de Campinas (UNICAMP). Samples were diluted in ABO compatible plasma (0.5:1000 μL) with known refractive index (Abbe refractometer) and viscosity (Ostwald viscometer). Silica beads (Bangs Laboratories, Fishers, Indiana, USA) diluted in physiological serum was added to 10 μL of RBC solution. These silica beads act as a force transducer. After a calibration, assuming a geometrical optics model, by measuring the displacement of the bead from the equilibrium position, it is possible to determine the numerical values for the optical force. The displacement of the center of the bead under the presence of external forces was quantified with the software Image Pro Plus (Media Cybernectics, Baltimore, Maryland, USA). Previous calibration of this procedure against hydrodynamic force showed good results.[6] All the measurements were carried out at room temperature (25°C). All the measurements were recorded in real time and captured by the computer. At least 10 measurements of each property (ζ, d, α) and more than 40 membrane viscosity (η) measurements were done. For the experiments, the optical tweezers trapped a silica bead that bound strongly and nonspecifically to RBCs. The laser power after the microscope objective was 60 mW for adhesion experiments, 30 mW for viscosity experiments, and 15 mW for zeta potential and double layer thickness experiments.

2.1 *Optical Tweezers*

The double optical tweezers consisted of an Nd:YAG laser strongly focused through a 100× oil immersion objective [numerical aperture (NA)=1.25] of an upright Olympus microscope (BH2, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a CCD camera and an *x*-*y*-*z* motorized stage (Prior Scientific, model ProScan, Rockland, Massachusetts, USA) controlled by a computer or a joystick. The laser beam was divided once and recombined using polarizing beamsplitters (BP1 and BP2), as shown in Fig. 1. The half-wave plate (H1) before the first beamsplitter was used to distribute the power to each beam and the second one (H2) to control the power of one of the beams. Two sets of telescopes were used

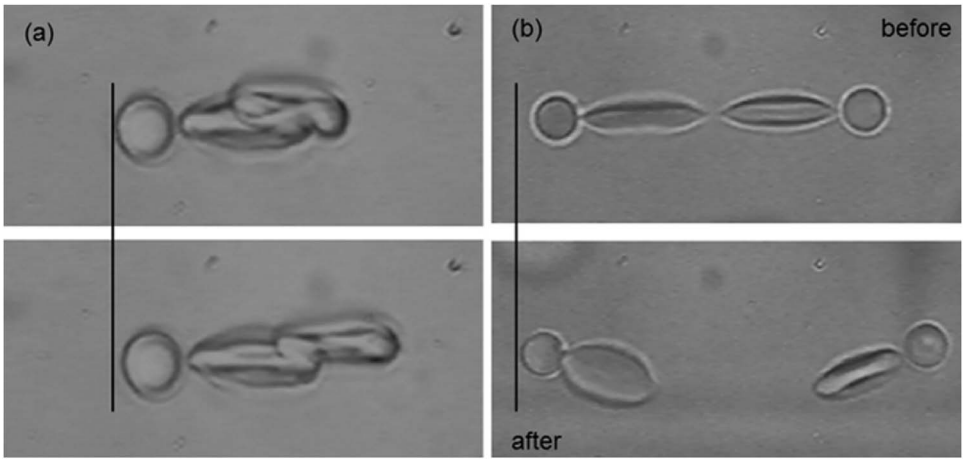

Fig. 2 Measurement of (a) the apparent membrane viscosity in a rouleaux using the double optical tweezers and (b) cell adhesion using the double optical tweezers.

in the system to capture particles in the same focal plane. Two gimbal mounts G1 and G2 were used to steer the beam position in the focal plane and the extra telescope (L_1 and L_2) was used to translate the steer pivot to the back aperture of the objective, avoiding power losses. This means that the gimbal mounts and the back aperture were conjugated optical planes. This setup enabled us to move the trap position over the whole field of view, an area of 50×80 μm, with constant trapping force.

2.2 *Apparent Membrane Viscosity (η_m) and Adhesion Measurements*

To measure the apparent membrane viscosity, the spatially fixed optical tweezers trapped a silica bead bound to one RBC of a two-cell spontaneously formed rouleaux, while the second optical tweezers trapped the other RBC directly. By moving the second optical tweezers with a computer-controlled piezoelectric actuator (New Focus) it was possible to imprint any velocity between the RBCs and, at the same time, measure the displacement of the trapped silica bead, as shown in Fig. 2(a). The relative displacement of two RBCs was roughly 1.6 μm for each velocity, therefore it was possible to obtain data for only five or six different velocities before the end of an RBC of approximately 8 μm. With this procedure, we measured the optical force as a function of the velocity between the RBCs. The force to break the bond between RBCs was much stronger than the viscosity forces, requiring much more laser power, which could damage the cells. This situation was avoided by trapping silica beads instead of the RBC directly, as the beads support a lot more power than the RBC. The only constraint to using this technique was that the bond between the RBC and the silica beads would have to be stronger than the bond between the RBCs themselves. Therefore, instead of just one bead, we used two silica beads captured by the double optical tweezers for the measurement of the adhesion force. For this adhesion experiment, one trap was fixed and the other was moved in the diametrically opposite direction, using only one of the gimbal mounts described before. We verified this movement by displacing two free beads that were not connected to RBCs. The displacement of the first bead was monitored while the second was slowly displaced. The adhesion force was then measured by the displacement of the first bead when the RBCs disagglutination occurred, as shown in Fig. 2(b).

2.3 *Zeta Potential (ζ) and the Thickness of the Double Layer of Charges (d) Measurements*

To measure the zeta potential (ζ) and double layer thickness (d) we built a special chamber consisting of two electrodes of platinum separated by a pool of $l=170$ mm (length), $w=30$ mm (width), and $h=75$ μm (depth). The external electrical field was applied with a voltage power supply (HP 712 C) connected to the electrodes. The double layer thickness was measured by the force on the silica bead attached to a single RBC in response to an applied voltage of 50 to 100 V. The zeta potential was obtained by measuring the terminal velocity of the RBC after release from the optical trap for the last applied voltage. The bead alone (without the RBC) did not respond to the electrical field. The RBC area used was $A=50$ μm^2 and the electrical permittivity[7] was $\varepsilon=1.06 \times 10^{-9}$ C^2/N m^2. Figure 3 shows an example of measurements done in this experiment.

3 Results

3.1 *Apparent Membrane Viscosity (η_m) and Adhesion*

The apparent membrane viscosity was determined using the Saffman theory.[8,9] Basically, Saffman modeled a protein in a membrane as a cylindrical inclusion in a continuous film and computed the drag force on the cylindrical particle undergoing translation and rotational motion. For the force of a protein in translation movement, he found

$$F = 4\pi\eta_s \frac{1}{\ln(\eta s/\eta a) - C} u, \tag{1}$$

where η_s is the intrinsic membrane shear surface viscosity, η is the fluid viscosity, a is the radius of the cylinder, $C=0.58$ is the Euler-Mascheroni constant, and u is the velocity. Consid-

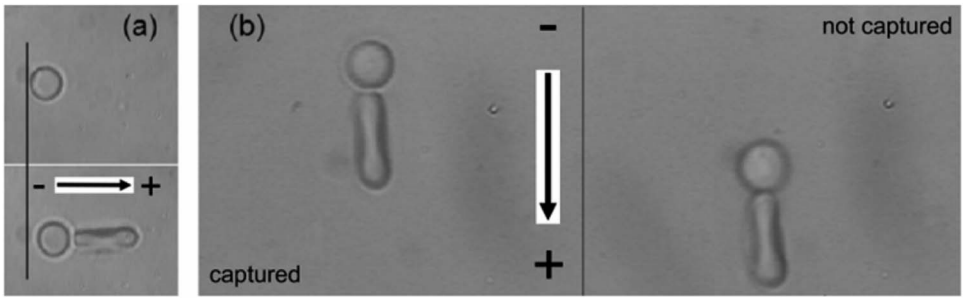

Fig. 3 Example of the measurements carried out in the same cell of (a) the size of the double layer using the force and (b) the zeta potential using the terminal velocity. The arrow shows the direction of the applied voltage. (a) Shows the bead trapped by the optical tweezers, and the line indicates the equilibrium position without any applied voltage.

ering that there are N proteins involved, we have

$$F = 4\pi\eta_s N \frac{1}{\ln(\eta_s/\eta a) - C} u = \eta_m u. \quad (2)$$

The unit of the parameter that incorporates the number of proteins and the other factors η_m, called apparent membrane viscosity, is poise. By measuring the optical force, equal to the viscous force, we obtained 19×10^{-4} poise cm. As we have two cells involved in each measurement, the apparent membrane viscosity for each cell is $\eta_m = 9.5 \times 10^{-4}$ poise cm. Figure 4(b) shows a set of results and the deviation found for the membrane viscosity. This value is of the same magnitude order as other membrane viscosity values found in the literature.[10] Figure 4(a) shows a plot of the force versus velocity for one rouleaux that confirms the expected viscous behavior for the movement of one cell on top of the other. More than 40 membrane viscosity measurements were done, and the results as well as the straight lines are very reproducible. We obtained an average value of 14 pN for the adhesion of a RBCs rouleaux of two cells (with deviation of ± 10 pN). This measurement was directly quantified using the silica bead displacements from the equilibrium position. To our knowledge, this is the first measurement of RBC cell adhesion under these conditions.

3.2 *Zeta Potential* (ζ) *and the Thickness of the Double Layer of Charges* (d)

A negatively charged RBC suspended in an electrolytic solution induces a cloud of oppositely charged ions surrounding the cell. This cloud, usually referred as the electrical double layer, extends outward from the surface of the RBC with decreasing density and finally merges with the electrolytic solution arranged in such a way as to maintain electrical neutrality. Immediately next to the charged cell surface is a thin layer of counterions strongly electrostatically attached to it, called the compact layer.[11] Counterions outside this compact layer form a diffusive layer. When a voltage is applied, cells are attracted to the electrode of the opposite polarity accompanied by the compact layer and part of diffusive layer. At some distance from the cells there is a boundary beyond which the ions do not move with the cell. this is known as the shear plane and exists somewhere within the diffuse layer. The zeta potential is the electrostatic potential at the shear plane.

When an external electrical field E is applied to the cell, there is an electrical force $F_{\text{elet}} = \Delta q E = \rho(x) A \Delta x E$ acting on a thin layer of area A and thickness $\Delta\xi$. As the velocity of the ions increases, there is also a viscous force $F_{\text{vis}} = -\eta A d/dx[dv/dx]\Delta x$ balancing the electrical force and

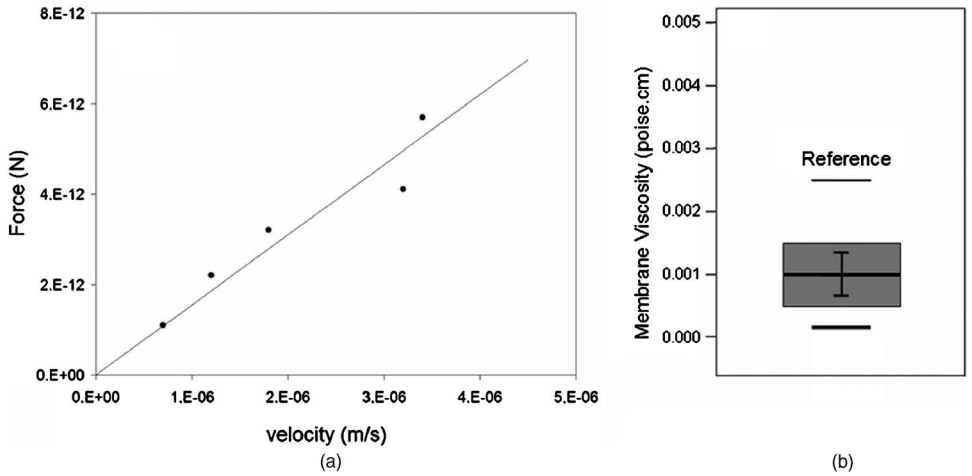

Fig. 4 Plots of (a) the optical force as function of the velocity between the cells of the one rouleaux and (b) the results obtained for membrane viscosity.

keeping the velocity of the ions constant. Thus, for the equilibrium condition,

$$\rho(x)AE\Delta x = -\eta A\frac{\mathrm{d}^2\mathrm{v}}{\mathrm{d}x^2}\Delta x \rightarrow \rho(x)E = -\eta\frac{\mathrm{d}^2\mathrm{v}}{\mathrm{d}x^2}. \quad (3)$$

Considering an RBC as a disk with the diameter much larger than its thickness, we can describe the dependence of the potential on the density of charges of an electrolytic solution by the Poisson equation in one dimension, $\mathrm{d}^2\Psi(x)/\mathrm{d}x^2 = -\rho(x)/\varepsilon$, which leads to

$$\frac{\mathrm{d}^2\mathrm{v}}{\mathrm{d}x^2} = \frac{\varepsilon E}{\eta}\frac{\mathrm{d}^2\Psi(x)}{\mathrm{d}x^2} \rightarrow \mathrm{v} = \frac{\varepsilon E}{\eta}\Psi(x). \quad (4)$$

The thin layer of cations formed around the cell moves together with it with the same velocity of the cell (v_p). Therefore the zeta potential (ζ) is given by

$$v_p = (\varepsilon E/\eta)\zeta, \quad (5)$$

which is known as the Smoluchowski equation,[12] where η is the viscosity, and ε is the electrical permittivity of the electrolytic solution. It is also possible to show that the zeta potential and the electrical potential can be written as

$$\zeta = \sigma_S d/\varepsilon \quad \text{and} \quad \Psi(x) = (\sigma_S/\varepsilon k)e^{-kx}, \quad (6)$$

where σ_S is the surface charge density of the compact layer, $d = 1/k$ is the size of the double layer, $k^2 = (2n_0z^2e^2/\varepsilon k_BT)$, n_0 and ze are the concentration and the ionic force of the ion, T is the temperature, and k_B is the Boltzmann constant.[13] Using viscous force and Eqs. (4) and (6) we obtain

$$F_{\mathrm{visc}} = -\eta A[\mathrm{dv}/\mathrm{d}x] = -A\varepsilon E[\mathrm{d}\Psi(x)/\mathrm{d}x] = A\varepsilon Ek\Psi(x). \quad (7)$$

At the shear plane, $\Psi(x) = \zeta$, and in the equilibrium the optical trap force F_{op} is equal to the viscous force F_{vis}, which leads to

$$F_{\mathrm{op}} = (A\varepsilon\zeta/d)E. \quad (8)$$

Using the Eqs. (5) and (8) we managed to obtain both measurements simultaneously (ζ and d) for the same RBC. We found $d = 0.85\ \mu$m (minimum $= 0.4\ \mu$m, and maximum $= 1.3\ \mu$m) for the double layer size and $\zeta = -12.5$ mV (minimum $= -9.3$ mV, and maximum $= -15$ mV) for the zeta potential. The result obtained for the zeta potential was in accordance with the data of the literature, which uses other methods to measure the same property.[2,13]

4 Conclusion

The results presented in this paper demonstrated a new methodology using a double optical tweezers to determine RBC properties such as apparent membrane viscosity, adhesion, zeta potential, and the size of the double layer. The measurements of the zeta potential, as well as the preliminary results of the apparent membrane viscosity, are in agreement with values found in the literature. As far as we know, this is the first time that the size of the double layer and the RBC adhesion were measured using optical tweezers. The ability of the optical trap to capture and manipulate single particles enabled us to characterize cell by cell individually and also to obtain the zeta potential and the size of the double layer for the same RBC. Individual cell analysis methods are always more sensitive to small differences than those based on average values. There are various conventional electrophoresis methods to measure cell zeta potential.[14,15] However, the data acquisition of such methods are not straightforward due to the complex motion of the cells inside closed vessels, especially as the velocity of the cell depends on its distance from the walls of the vessels.

In conclusion, we believe that the methodology proposed here can provide information about cell-specific and -nonspecific agglutination because it can be sensitive to a variety of factors that can interfere in agglutination reactions performance. Better comprehension of the forces and bindings between red blood cells could improve the sensitivity and specificity of the hemagglutination reactions and also guide the development of new potentiator substances to be used in transfusion centers. In some procedures performed in transfusion centers there are low sensitivity and/or nonspecificity, which causes a great number of repetitions of the immunohematologic tests. Because we can measure the forces involved in the hemagglutination, this methodology to obtain red blood cell properties can help in the standardization of automatized routines in these hemotherapy centers.

We also believe that the methodology proposed here to measure the zeta potential using an optical tweezers can be extended and used in other types of samples where the zeta potential is an important parameter. A great number of cells in a multicellular organism are charged and embedded in an electrolytic solution. Therefore, the zeta potential is an important parameter for their adhesion to interfaces or other cells. The zeta potential can, for example, be used to differentiate low metastatic cells from high metastatic cells.[16] The stability of colloidal solution depends on the zeta potential to hold the particles apart. Therefore, measurements of zeta potential are important even for drug administration using colloids, liposomes, micelles, polymers, microspheres, and so on. In general, measurements of zeta potential are important for any kind of material where the contribution of the atoms and charges of a surface are relevant. On the other hand, it is also important to correlate the zeta potential with adhesion and viscosity. Molecular bonds are responsible for binding. The immunological system is based on molecular bonds. The surface charge, the molecular composition, and the electrolytic environment change from one biological system to another. For example, in the case of cancer, there are papers that show that both zeta potential and viscoelasticity are important cell parameters not only to study and understand the disease but also to develop new and precocious diagnosis protocols.[17]

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