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Continuous force-displacement relationships for the human red blood cell at different erythrocytic developmental stages of *Plasmodium falciparum* malaria parasite

John P. Mills[1], Lan Qie[3], Ming Dao[1], Kevin S. W. Tan[4], Chwee Teck Lim[3] and Subra Suresh[1,2]
[1]Department of Materials Science and Engineering, [2]Division of Biological Engineering and Affiliated Faculty of the Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.
[3]Division of Bioengineering, [4]Department of Microbiology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 117576. Republic of Singapore.

ABSTRACT

Prior work involving either aspiration of infected cells into micropipette under suction pressure or deformation in laminar shear flow revealed that the malaria parasite *Plasmodium* (*P.*) *falciparum* could result in significant stiffening of infected human red blood cells (RBCs). In this paper, we present optical tweezers studies of progressive changes to nonlinear mechanical response of infected RBCs at different developmental stages of *P. falciparum*. From early ring stage to late trophozoite and schizont stages, up to an order of magnitude increase in shear modulus was found under controlled mechanical loading by combining experiments with three-dimensional computational simulations. These results provide novel approaches to study changes in mechanical deformability in the advanced stages of parasite development in the erythrocyte, and suggest a significantly greater stiffening of the red blood cell due to *P. falciparum* invasion than that considered from previous studies.

INTRODUCTION

The architecture of the human RBC (erythrocyte), without a nucleus or internal organelles and with a well-defined biconcave shape, presents a relatively simple model system, compared to other biological cells, for the study of single-cell mechanics. The RBC membrane enclosing the cytosol consists of a phospholipid bilayer supported by a complex cytoskeletal network which comprises spectrin molecules anchored at actin nodes, and proteins 4.1, 4.2, ankyrin and adducin [1, 2]. Interactions involving ankyrin, the RBC anion transporter band 3, protein 4.1 and sialoglycoproteins such as glycoprotein A facilitate connections between phospholipid membrane and cytoskeleton. The cytoskeletal network facilitates deformability of the RBC that permits its biological function of transporting oxygen and carbon dioxide. With a biconcave or discocyte shape and a diameter of about 8 μm, the RBC passes through narrow capillaries with inner diameters as small as 3 μm. There it undergoes large, reversible, nonlinear elastic deformation with strains in excess of 100%. The RBC also severely deforms through intercellular gaps of sinusoids in the spleen where stiffened and aged RBCs are removed.

This deformability is severely hampered by the *P. falciparum* parasite, the deadliest of the four species of malaria, which results in two to three million deaths annually [1]. When an RBC is invaded by the *P. falciparum* parasite**,** it experiences increased propensity for adherence to linings of small blood vessels, thereby promoting parasite sequestration and obstruction to tissue perfusion. Consequences of these effects of *P. falciparum* include abnormal microcirculatory responses and parasite accumulation in the microvasculature of different organs. Enhanced rigidification of RBC is a key feature of the biology and pathophysiology of malaria whose

mechanisms are influenced by proteins transported from the intracellular parasite to the cell membrane and the associated alterations to the spectrin molecular network underneath the phospholipid bilayer [1-3]. Consequently, quantitative understanding of the systematic changes to deformation characteristics of parasitized RBC, that exploits latest advances in nanomechanical experimentation, would provide critical insights into possible connections among subcellular structure evolution, elastic properties of cell membrane and disease states.

Some studies have demonstrated severe stiffening of the RBC infected with *P. falciparum* by recourse to micropipette aspiration experiments [3-6]. Considerable uncertainty can arise in mechanical property extraction from micropipette aspiration experiments because of (a) feasibility to aspirate the stiffened RBC only partially into the micropipette [3], (b) geometric and mechanical artifacts induced by the stress singularity at the site of aspiration of the cell into the micropipette, (c) strong adhesion of cell membrane to the inner wall of the micropipette in the late stages of culturing [3], and (d) extraction of elastic properties based on simple analysis which do not account properly for biconcave cell shape or nonlinear deformation. In these and in a subsequent study [7] on deformability of RBCs parasitized by *P. falciparum* and *P. vivax* to different developmental stages in a laminar shear flow system, mechanical deformation characteristics in the advanced (schizont) stages of erythrocytic development could not be determined because of experimental difficulties. These two methods do not provide a direct and continuous force versus displacement measurement of the progressive stiffening with maturation of the malaria parasite inside RBCs from early ring stage to late schizont stage. This information is essential for proper interpretation of stage-specific deformation characteristics.

Using an optical tweezers technique to determine continuous force versus displacement responses of entire single cells to large deformations [8], we briefly review and summarize our recent results on progressive stiffening of *P. falciparum* infected RBCs over parasite developmental stages [9]. We also provide additional observational evidence and results on changes in red blood cell deformability due to the invasion of the cell by *P. falciparum*.

METHODS

Details of *in vitro* culturing of *P. falciparum* strains *3D7* and Gombak, used in this study, can be found in Suresh et al. [9]. Identification of erythrocytic developmental stage of *P. falciparum* could be assessed from optical microscopy images (Figure 1). The five different red blood cell test cases are healthy (H-RBC), uninfected but exposed to parasite culture (*Pf*-U-RBC), ring stage (*Pf*-R-RBC), trophozoite stage (*Pf*-T-RBC) and schizont stage (*Pf*-S-RBC) erythrocytes.

Figure 1 Optical micrographs of healthy (left), uninfected but exposed (second from left), and various developmental stages of *P. falciparum* parasitized human blood cells. Note the presence of the parasite in the infected cells (the three images on the right).

Force-displacement responses of RBC test cases were performed by optical tweezers stretch tests. In this technique, laser traps control the position of two silica beads which are attached to opposite ends of a red blood cell. Moving the laser traps, and therefore the silica beads, leads to the stretching of the cell. Force calibration is typically done by a standard Stokes' flow method [10]. Early studies of healthy RBC deformability were capable of only inducing small RBC deformations with maximum forces around 60 pN [11, 12]. Our recent optical tweezers work on healthy RBC stretches cells with maximum forces of approximately190 pN, which is sufficient to induce large, non-linear deformations [8]. We use this system to investigate the force-displacement response of RBCs at various stages of infection with *P. falciparum*. Details of experimental technique can be found in Mills et al. [8] and Suresh et al. [9].

Computational simulations based on finite element modeling (FEM), using the force-displacement data from optical tweezers stretch tests, provide a measure of the in-plane shear modulus. Details of the full three-dimensional continuum level model of RBC stretch testing used to extract properties in this study can be found in Mills et al. [8] and Suresh et al. [9]. A more general review of the FEM technique [13] and a spectrin-level simulation [14] contain specific computational details.

RESULTS

The differences in deformability of the various developmental stages of *P. falciparum* in the RBC are clearly illustrated in the optical micrographs of Figure 2, which are taken from the video images at a force of 109 ± 15 pN. Also shown in this figure for comparison purposes are the images of the control conditions: H-RBC and *Pf*-U-RBC. It is evident that the ability to deform decreases significantly from the *Pf*-R-RBC to the *Pf*-T-RBC to the *Pf*-S-RBC stages. The extent of deformation at the higher load level is also noticeably higher for all the cases except *Pf*-S-RBC where very little deformation occurs.

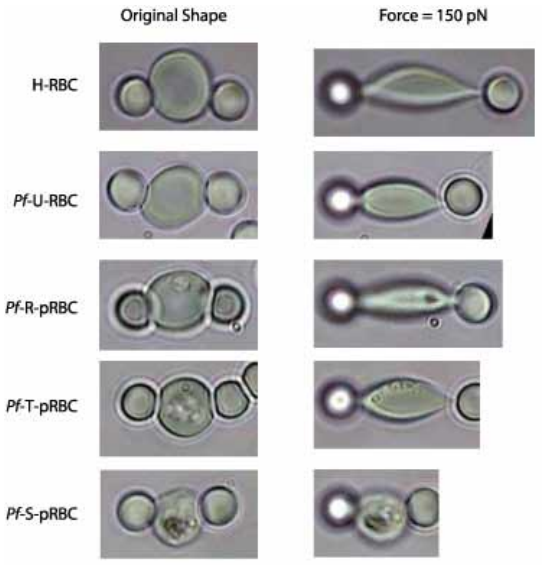

Figure 2 Experimental observations showing the effects of *P. falciparum* parasitization on RBC deformability. Optical micrographs show images of cell deformation at a fixed stretching force of 150 ± 15 pN and in the undeformed reference configuration as well.

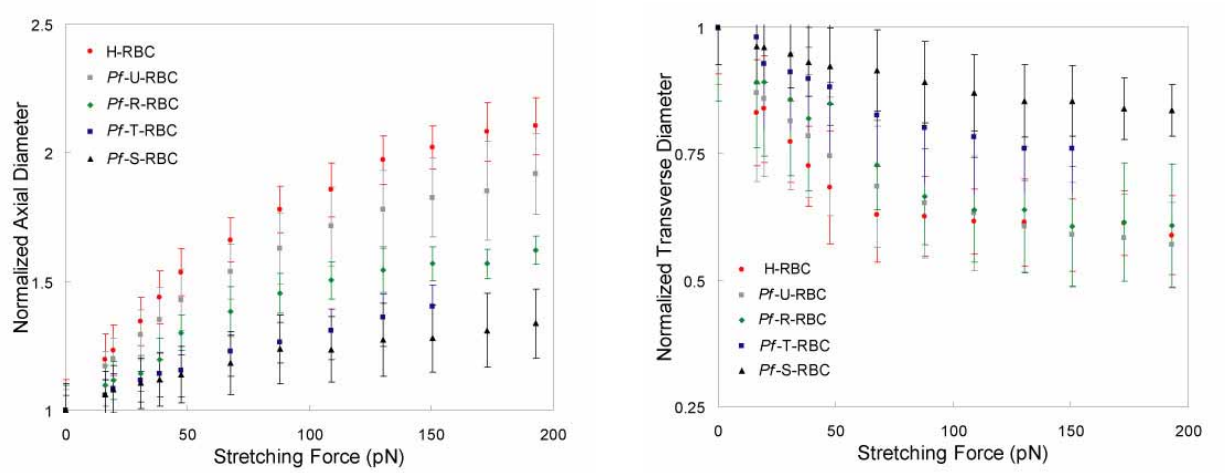

Figure 3 Effects of *in vitro* maturation of *P. falciparum* parasite inside the RBC on changes in cell geometry at different control and development stages. Plots show variations of axial and transverse diameters, normalized by initial diameter, with the stretching force. The vertical bars indicate estimated experimental scatter in the diameter change from multiple repeat experiments. The uncertainty in the reported stretching force is estimated to be less than ± 20 pN.

Figure 3 plots variations in axial and transverse diameters of the five different test cases investigated. The plot shows continuous force-displacement responses, measured by the optical tweezers technique, of an RBC over the erythrocytic developmental stages of parasite development. The ability of the parasitized cell to deform in both the axial and transverse directions is progressively reduced with erythrocytic development of the parasite. The data shown are based on repeat tests of 7, 8, 5, 5 and 23 samples for the H-RBC, *Pf*-U-RBC, *Pf*-R-RBC, *Pf*-T-RBC and *Pf*-S-RBC conditions.

The values of in-plane shear modulus of the control conditions and the infected cells, obtained by matching the three-dimensional computational results with the experimental observations shown in Figures 2 and 3, are plotted in Figure 4. The *Pf*-U-RBCs show a very small increase in modulus compared to the value of 5.3 μN/m for the H-RBCs. However, the average shear modulus values for the *Pf*-R-pRBC, *Pf*-T-pRBC and *Pf*-S-pRBC are 16, 21.3 and 53.3 μN/m, respectively.

Also in Figure 4, experimental data from micropipette aspiration [3] and laminar shear flow [7] techniques are shown for comparison. Our results imply an order of magnitude increase in the effective shear modulus of the parasitized RBC which is much greater than estimates of stiffening by a factor of two to three based on other techniques [3-7]. Also, the optical tweezers method is capable of testing the latest (schizont) stage of parasite development inside the RBC, which could not be accomplished previously.

CONCLUSIONS

We have presented direct and continuous force–displacement curves for the progressive changes to human RBC deformability for developmental stages of *P. falciparum*, including the late (schizont) stage. This method demonstrates a three times greater stiffening of the parasitized

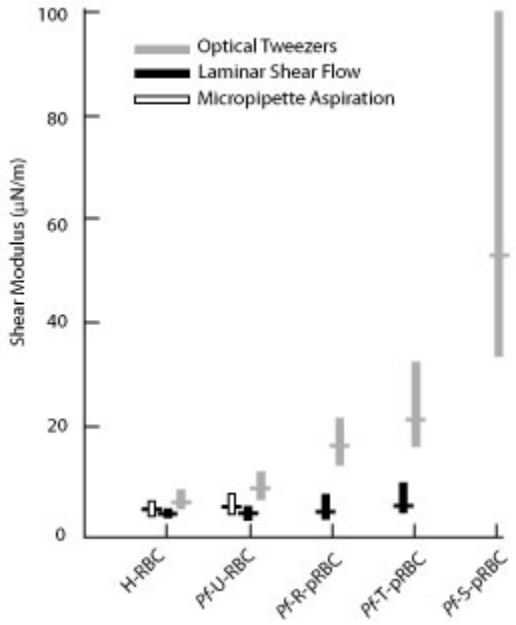

Figure 4 The range and average value for in-plane shear modulus based on optical tweezers experiments for the various stages of erythrocytic parasite development are plotted for the five test cases. Also shown are estimates of in-plane shear modulus based on other independent techniques, micropipette aspiration [3] and laminar shear flow [7].

erythrocyte than previously envisioned. The observations and images reported here complement and add to our recently published work [9].

Experimental *in vitro* measurements with optical tweezers along with full three-dimensional computational simulations [8, 9] provide a complete framework for how the mechanics of deformation can be systematically and quantitatively probed. This approach can serve as an *in vitro* benchmark for systematically assessing contributions to mechanical deformability of the RBC.

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