Single Cell Mechanics Study of the Human Disease Malaria*

Chwee Teck LI**
**Division of Bioengineering and Department of Mechanical Engineering, NUS Nanoscience & Nanotechnology Initiative, National University of Singapore, 9 Engineering Drive 1, Singapore 117576, Singapore
E-mail: ctlim@nus.edu.sg

Abstract
Living cells do possess structural and mechanical properties and any deviation in these properties not only results in the breakdown of their physiological functions, but may also give rise to human diseases. One such example is that of malaria. Single cell mechanics study of malaria had been done to investigate the changes in the structure-property-function relationship of red blood cells (RBCs) arising from infection by the malaria parasite, *Plasmodium falciparum*. Here, biophysical experiments using micropipette aspiration, optical or laser tweezers and microfluidics are presented to highlight some research work done to quantitatively investigate the progressive stiffening and change in biorheological properties of RBCs at the different stages of infection. This stiffening is due to the cellular and molecular changes caused by the parasite within the infected RBCs and can result in the impairment of blood flow thus leading to organ failure, coma or even death. These single cell biomechanics studies demonstrate the relevance of biomechanics in the understanding of the pathophysiology of malaria. Also, the biophysical methodologies developed may provide a suitable testing strategy to quantitatively evaluate the effectiveness of certain agents and drugs being developed to prevent or inhibit stiffening of the *Plasmodium falciparum* infected RBCs.

Key words: Nanobiomechanics, Single Cell Biomechanics, Pathophysiology, Human Diseases, Malaria Infection, Red Blood Cells, Biophysical Techniques, Micropipette Aspiration, Laser/Optical Tweezers, Microfluidics.

1. Introduction

As physical entities, the living cells in our human body do possess structural and mechanical properties. These structural and mechanical properties are critical not only in ensuring that the cells can continue to withstand the physical forces acting on them from within and outside of the body, but also in the proper functioning of the cells. For example, many biological processes, such as growth, differentiation, migration, and apoptosis (programmed cell death) are affected by the cell shape change, cell mechanical properties and their physical interactions with their neighboring cells and structures. Any change in the structural and mechanical properties of cells can give rise to the breakdown of their physiological functions and this can inevitably lead to human diseases. Examples of such diseases are cancer and malaria.

Here, I will focus on the human disease malaria and will review some of the work done...
to investigate the changes in the structure-property-function relationship of red blood cells (RBCs) arising from infection by the malaria *Plasmodium (P.) falciparum* parasite. This will enable us to establish some connections between single cell mechanics and the pathophysiology of the disease.

2. Pathophysiology of Malaria

Malaria is one of the most deadly human diseases on earth. It infects about 500 million people worldwide and results in more than 2 million deaths each year\(^5\). Of the four different species of malaria parasites which infect human RBCs, *P. falciparum* and *P. vivax* are the most common, with the former being the most widespread in humans and giving rise to the severest form of malaria\(^6\). Transmission of malaria parasites occurs when a female *Anopheles* mosquito feeds on human blood. During this process, millions of *P. falciparum* sporozoites are injected into the human body along with the saliva of the mosquito which contains an anticoagulant. The sporozoites then travel to the liver via the blood stream where they invade and reside in the hepatocytes. One sporozoite can literally develop and multiply into tens of thousands of merozoites (*P. falciparum* parasites) in the course of about 7 to 9 days. Thereafter, the millions of merozoites get released into the blood stream. The disease state occurs when a merozoite invades a RBC and causes significant structural changes to the cell\(^7\),(8) at both the cellular and molecular levels.

The erythrocytic developmental stages of the parasite\(^6\),(7),(9) begins with the ring stage at about 30min after invasion of a RBC. It then progresses to the trophozoite stage where the parasite continue to grow inside the red cell as well as export parasite proteins, some with knob-like features, to the membrane of the cell at about 20hours after invasion. It finally reaches the last schizont stage where the nuclear division of the parasite results in the production of more merozoites as well as further export of parasite proteins to the membrane at about 25 to 40hours after invasion. This results in the distortion of the cell cytoskeleton and membrane and the infected RBC appears more spherical than biconcave in shape. Hence within a period of 48hours, the parasite multiplies inside each RBC producing up to about 20 merozoites per parasite. At the end of the schizont stage, the merozoites break out of the cell and invade other healthy red cells.

Because of the extensive cell modification caused by the parasite and the direct specific interaction of the exported parasite proteins with the membrane and spectrin network of the RBC, the cell becomes stiffer and sticky\(^10\). Hence, the two important pathophysiological outcomes of the *P. falciparum* infected RBC are that firstly, the infected cell becomes stiffer as the disease progresses and secondly, it becomes cytoadherent as the infected RBC becomes adhesive to the inner linings of the blood vessels and capillaries. It is this significant increase in the stiffness and adhesive response of the parasitized RBCs that cause them to sequestrate in the microvasculature of the major organs. The consequence of such sequestration results in the impairment to blood flow, especially in the capillaries and can lead to cerebral malaria, hypoglycaemia and respiratory distress\(^6\). These may result in organ failure, coma or even death. Therefore, changes in the deformability and increase in cytoadherence of single RBCs can significantly affect the biorheological properties of blood and can have adverse effects. It is these two outcomes – stiffening and stickiness of the malaria infected red cells – that justify the use of biomechanics in studying this disease, malaria.

3. Nanobiomechanics Approaches to Studying Malaria

Investigation on the pathophysiological consequences of the *P. falciparum* disease from a biomechanics perspective has been conducted using nanobiomechanics approaches. With
the recent developments and advances in nanotechnology and biophysical techniques, we can now perform direct, real-time mechanical probing and manipulation of malaria infected cells at the micro- and nanometer scale and nano- and piconewton force resolutions. At the single cell level, available experimental techniques now include the micropipette aspiration method, optical traps or laser tweezers, magnetic tweezers, atomic force microscopy and optical stretcher. These techniques have been described in recent reviews (12)-(15).

In this section, I will present some of the studies done in quantifying the stiffening of the malaria infected cells. To probe the stiffening of the infected cells and the changes in their mechanical properties at the single-cell level, techniques such as the micropipette aspiration method (25) and optical traps/laser tweezers (16) had been used. To probe the change in the biorheological properties, the microfluidic technique had been employed (17). These studies are usually conducted to obtain the biomechanical responses of Plasmodium falciparum harbored RBCs at the different stages of infection. From these studies, it is hoped that one can correlate protein-specific contributions to RBC stiffening and hence, be able to establish relation among the contributions of the parasite exported proteins, change in their mechanical properties and effects on disease progression.

3.1 Optical Traps/Laser Tweezers Studies

The optical/laser trap or laser tweezers technique uses a laser to trap, manipulate and control small transparent particles in a medium. When a laser light is shone on such a particle which has a refractive index different from that of the medium, a net light pressure or trapping force is induced which moves the particle towards the focal point of the laser. The optical trap/laser tweezers has now been increasingly used to perform single cell and single molecule biomechanics experiments (12) and has become an essential tool in conducting research in both physics and biology.

Figure 1 shows the stretching of a single healthy RBC using optical tweezers by Lim et al. (18) and Mills et al. (15) while the computational modeling has been performed by Dao et al. (19). For the stretching experiment, two silica microbeads of 4.12 µm in diameter were non-specifically attached at the diametrically opposite points of a RBC. Here, the left bead was adhered to the glass slide surface while the right bead was trapped using the laser as shown by the lighted up bead in Figure 1(b). The trapped bead was held stationary while the glass slide was moved towards the left thus stretching the cell. For the computational simulation, the RBC was modeled by a neo-hookean hyperelastic bi-concave solid membrane enclosing a fluid. From both experiments and simulations, the in-plane shear modulus was estimated to be between 3.0 to 8.0 µN/m and this was within the range of shear modulus values obtained by other researchers using either the optical traps or micropipette aspiration technique (20)-(23).

Suresh et al. (16) investigated the change in shear modulus of the membrane of RBCs infected with the virulent Plasmodium falciparum parasite by the optical tweezers stretching of infected cells at their different stages of infection. Figure 2 shows the gradual stiffening of the infected cell when stretched at forces of 68 and 151 pN. As the infected RBC advanced to its final schizont stage, the infected cell was observed to be hardly stretched as compared to the earlier ring and trophozoite stages of infection. Computations of the shear modulus show a significant increase by about ten-fold due to the molecular and structural changes in the cell membrane at the late schizont stage of the infected cell (16). This study demonstrated a quantitative approach to measuring the change in shear modulus of the membrane of the Plasmodium falciparum parasite harbored red cell via uniaxial stretching at the different stages of infection.

However, the overall change in the mechanical properties of these infected RBCs is not
only influenced by the change in the cell membrane shear modulus but also by the more rigid parasites and their associated intracellular contents such as the food vacuole. In fact, the multiplication of the merozoites in the infected cell will further increase its rigidity. To probe the overall decrease in deformability of the infected cell, the micropipette aspiration technique which will be covered in the next section will be a suitable technique to use.

Fig. 1 Using optical trap or laser tweezers to stretch a healthy RBC. Here, two silica microbeads of 4.12 µm in diameter are non-specifically attached at diametrically opposite points of a RBC. (a) For this unstretched RBC, the left bead is adhered to the glass slide surface. (b) As the right bead is trapped using the laser as shown by the lighted up bead, it is held stationary while the glass slide is moved towards the left thus stretching the cell. (Reprinted from Mills et al. (15))

Fig. 2 Images of healthy RBC (H-RBC), uninfected control RBC (Pf-U-RBC), *P. falciparum* Ring stage parasitized RBC (Pf-R-pRBC), *P. falciparum* trophozoite stage parasitized RBC (Pf-T-pRBC) and *P. falciparum* schizont stage parasitized RBC (Pf-S-pRBC) (left column) before stretching, (middle column) stretched at force of about 68 pN (middle column) and stretched at force of about 151 pN (right column). (Reprinted from Suresh et al. (16) with permission from Elsevier)

3.2 Micropipette Aspiration Studies

The micropipette aspiration method was first developed by Mitchison and Swann (24) in
1954 to measure the elastic properties of sea urchin eggs. Since then, this method has been used to measure the membrane elasticity of many types of cells including RBCs and leukocytes (see review by Hochmuth\textsuperscript{(23)}). The micropipette aspiration method uses a suction pressure to partially or wholly suck a single cell into a micropipette whose diameter may range from less than 1 to 10 µm. By measuring the elongation of cell being aspirated into the pipette as a result of this suction pressure as shown in Figure 3, we can evaluate the shear modulus of the membrane of a cell.

![Fig. 3](image_url)  (a) Schematic of the micropipette aspiration method, and (b) optical image showing aspiration of a RBC. (Reprinted from Lim et al. \textsuperscript{(12)} with permission from Elsevier)

Zhou \textit{et al.}\textsuperscript{(11)} and Lim \textit{et al.}\textsuperscript{(25)} used the micropipette aspiration to examine the progression of the disease state of a \textit{P. falciparum} infected red cell from the early ring form stage to the late schizont stage (see Figure 4). Using a constant suction pressure, the amount of cell aspirated into the pipette was observed at each different stage of infection. One interesting finding is that while the healthy and early stage infected red cell was found to behave like a “liquid drop” and can be easily aspirated, the late schizont stage infected RBC tended to exhibit a viscoelastic solid-like behavior and can hardly be aspirated into the pipette. This arises due to the gross molecular and cellular structural changes of the infected RBC as well as the multiplication of the parasites within the cell. Due to the solid-like behavior of the late stage infected cell, Lim \textit{et al.}\textsuperscript{(25)} modeled the deformability of the whole cell using a homogeneous incompressible standard neo-Hookean solid model. The apparent bulk shear modulus of the schizont stage infected red cell was calculated to be about 50 Pa.

![Fig. 4](image_url)  Probing the various stages of malaria infection of an infected RBC using the micropipette aspiration method. (Reprinted from Lim \textit{et al.}\textsuperscript{(25)} with permission from Elsevier)
During the development of the parasite within the cell, two proteins - the knob-associated histidine rich protein (KAHRP) and the *P. falciparum* erythrocyte membrane protein 3 (PfEMP3) - are known to have been exported from the parasite to the RBC membrane skeleton. Glenister et al.\(^{(10)}\) used micropipette aspiration to investigate the change in the shear elastic modulus of RBCs infected with transgenic parasites (with either KAHRP or PfEMP3 genes deleted) so as to determine the contribution of these proteins to the increased rigidity of parasitized RBCs. Results showed that the absence of either protein actually reduced the rigidity of the membrane than that caused by the normal parasitized cell. Also, KAHRP seemed to have greater effect than PfEMP3 on the increased rigidity in the cell. In fact, their results showed that KAHRP contributed up to about 51% of the overall increase in the rigidity of the normally infected RBCs as compared to about 15% for that for PfEMP3. For the first time, quantitative study was made on the contribution of specific parasite proteins to the changes in the mechanical properties of the *P. falciparum* harbored RBCs. This particular experiment shows how the contribution of the parasite exported proteins which cause stiffening of the infected red cell membrane can be systematically and quantitatively studied using the micropipette aspiration method.

### 3.3 Microfluidics Studies

Microfluidics involves the study of fluid flow at the microscale. The volume of fluid is normally thousands of times smaller than that of a droplet. It also involves the use of microscale systems which allows for the precise control and manipulation of such small volumes of fluids. Microfluidics research which began some two decades back has led to the development of microfluidic devices such as DNA chips as well as lab-on-a-chip technology. In fact, there has been an increase in the use of such microfluidic devices in the areas of biology, biochemistry and medicine\(^{(26),(27)}\). These microfluidic devices offer the following advantages:

(i) They provide control and can mimic microenvironments such as those found in the human capillaries.

(ii) They limit exposure to biohazardous samples such as bacteria, parasites and viruses.

(iii) They can be easily fabricated using current microfabrication and lithography techniques on silicon\(^{(28)-(31)}\) and glass\(^{(32)}\) substrates.

(iv) They allow for high-throughput testing of samples such as living cells and biomolecules.

By fabricating microscale capillary-like channels on silicone elastomer\(^{(33)}\), one can mimic the flow of living cells such as RBCs through the narrow human capillaries and observation on the deformation of these cells can then be made. Here, the silicone channels can be made to have structural properties close to that of the human capillary tissue.

Shelby et al.\(^{(17)}\) were the first to demonstrate the use of elastomeric microfluidic channels to study the flow and blockages of malaria-infected RBCs in capillaries. Here, microchannels with widths of 2, 4, 6 and 8µm were used as shown in Figure 5. It was observed that for 8µm wide channel, both uninfected and infected RBCs in the early ring stage could traverse through the channel very easily. Most of the trophozoite stage infected RBCs failed to freely traverse the 2 and 4 µm channels although some did pass through the 4µm channel. For the 2µm channel, “pitting” was observed for the trophozoites. This is a process in the body where the spleen beds expunge the parasites completely out of the RBC. For the late stage schizonts, they quickly formed a blockage for all channels except for the 8µm channel. Although there was a blockage, individual uninfected RBCs were observed to
be still able to squeeze through the blockage formed by the schizonts for the 6µm channel.

This microfluidics experiment demonstrated an excellent approach for \textit{in vitro} observation of the possible changes in the biorheological behavior of malaria infected cells as they traverse through narrow capillaries in the human body. However, two drawbacks of this experiment are that the channels used were rectangular in cross-section as compared to the more circular human capillaries and that this experiment was conducted at room temperature rather than at physiologic temperature of 37°C. It is not clear if the infected cells will behave very differently in terms of deformation and blockage of the channels arising from this difference in cross-section and temperature. Further study needs to be done using circular cross-section channels and at physiologic temperature.

4. Future Work

While the single cell mechanics experiments highlighted here sought to conduct a comprehensive study to systematically probe the stiffening of the malaria infected red cells, there are still a number of issues that need to be addressed. For example, most of these tests are performed at non-physiologic conditions such as at room temperature rather than at physiologic temperature of 37°C or at febrile temperature of 41°C. As both the healthy and infected red cells in the human body are subjected to these higher temperatures, further experiments need to be done to ascertain if the higher physiologic temperature may significantly influence the value of the stiffness of the infected red cell obtained.

Glenister \textit{et al.}\(^{10}\) are one of the few to investigate the contributions of the parasite exported proteins, KAHRP and PfEMP3, to the stiffening of the RBC membrane. Further
study still needs to be done to examine the effects of other parasite exported proteins as well as the mechanism by which these proteins stiffen the cell membrane. One example is the Pf155/RESA protein which is known to stabilize the RBC cytoskeleton against heat-induced structural changes\(^{(34)}\).

While much work has been done to investigate the stiffening of the *P. falciparum* harboring red cells, little effort has been made to study the effects of cytoadherence. Thus far, some ligand-receptor pairs contributing to cytoadherence have been identified but the molecular interaction forces have not been well quantified to date\(^{(35)}\). Only the altered adhesive property of malaria-infected RBCs at the cellular level has been demonstrated. Evidences of this came from histological section observation of the microcirculation from cerebral malaria patients in which large amount of late stage infected cells accumulate, stick and perturb or obstruct blood flow. A close look at these sequestered cells using transmission electron microscopy revealed that the knob-like structure is the focal adherent point via which the parasitized cell sticks to the endothelial cells lining the blood vessel and capillary\(^{(35)}\).

Based on numerous studies which have examined the components of the knobs and the receptors expressing on the surface of endothelial cells in different organs, Cooke *et al.*\(^{(36)}\) sketched a comprehensive illustration to represent these array of ligand-receptor pairs involved in cytoadherence (Figure 6). Almost all cytoadherence is mediated by the parasite protein PfEMP1\(^{(37)}\) as well as various endothelial receptor molecules, including CD36\(^{(38,39)}\), intercellular adhesion molecule 1 (ICAM-1)\(^{(40)}\), E-selectin, and vascular cell adhesion molecule 1 (VCAM-1)\(^{(41)}\). Some of these ligand-receptor pairs are involved in diseases such as placenta malaria, cerebral malaria and microvascular adhesion\(^{(42)}\). There is certainly a need to perform more quantitative measurements of the cytoadherent force or stickiness of *P. falciparum* harbored RBCs with endothelial cells lining the blood vessel walls at both the cellular and molecular levels. This will help us to better understand the contributions of the specific cytoadherent binding proteins to the stickiness of the *P. falciparum* harbored RBCs.

**Fig. 6**  Schematic representation of the wide array of molecules involved in the adhesive molecular interaction between *P. falciparum* infected red blood cells and the vascular endothelial cells.  (Reprinted from Cooke *et al.*\(^{(36)}\) with permission from Elsevier)
4. Concluding Remarks

To establish connections between single cell mechanics and the human disease malaria, some research work had been done to investigate the changes in the structure-property relationship of red blood cells arising from infection by the malaria *P. falciparum* parasites. Biophysical experiments using micropipette aspiration method, optical tweezers and microfluidics were presented here to highlight the research done to investigate the biomechanical responses of *P. falciparum* harbored RBCs at the different stages of infection. Nevertheless, further work is still needed to perform these experiments at physiologic temperature, investigate contribution of parasite exported proteins and their mechanisms in the stiffening of the red cell membrane and determine the contributions of the specific cytoadherent binding proteins to the cytoadherence of the *P. falciparum* harbored RBCs. These tests will further help us to quantitatively ascertain the stiffening and cytoadherence of the infected cell arising from the cellular and molecular changes occurring within these cells.

These studies demonstrate the relevance of biomechanics in studying the pathophysiology of malaria. It is hoped that with a better understanding of the parasite-engineered structural alterations to the red cells from a biomechanics perspective, we may offer important information which may be useful to clinicians on how they may be able to interfere with these changes and reduce parasite virulence. Also, the biophysical methodologies presented here may provide a suitable testing strategy to quantitatively evaluate the effectiveness of agents and drugs being developed to prevent or inhibit stiffening of the infected cells.

References


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