Micro-Spectroscopy of Single Erythrocytes Infected with the Malaria Parasite

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Abstract — The erythrocytic cycle of the malaria parasite Plasmodium falciparum is marked with structural, mechanical and biochemical modifications to the host red blood cell. The parasite degrades the hemoglobin of the host cell and hydrolyzes it into hemozoin. We investigate healthy and infected erythrocytes using micro-Raman and spatially resolved absorption spectroscopy. The electronic absorption spectrum of a single cell is measured and spectral changes are related to the parasite life cycle. The Soret absorption band in the trophozoite stage is shifted to higher wavelength by 3 nm. The findings are compared with micro-Raman spectra that show consistent changes in the heme vibrations. Micro-absorption may offer a potential diagnostic marker for identifying pathological states accompanying malaria.

Index Terms — Absorption microscopy, diagnostics, erythrocyte, malaria parasite, Raman spectroscopy.

I. INTRODUCTION

Red blood cells are relatively simple biological structure as they are non-nucleated and lack intra-membrane organelles. They are biconcave shaped disks which optimizes the flow properties in the vessels. They are the principle means of delivering oxygen to the organs and mainly consist of hemoglobin, a globular protein. The malaria parasite Plasmodium falciparum introduces mechanical changes in the host red blood cell [1, 2] making it difficult for the cells to pass through the vessels. This indeed affects the oxygen transporting capability.

Malaria is responsible for over a million deaths every year mostly infants, pregnant women and young children in areas endemic for the parasites [3]. Close to half of the world’s population still lives in areas with high risk of contracting malaria. According to a world health Organization report 2009 a child dies of malaria every 30 seconds. According to the U.S. Center for Disease Control and Prevention, more than 1,400 new cases are reported annually in the United States in travelers returning from malaria-endemic areas.

The human malaria parasite has a complex life cycle that requires both a vector body (female anopheles mosquito) and a host body. The sexual reproduction of the parasite occurs in the mosquito body and the resulting sporozoites are inoculated into the human host when bitten by the infection carrying mosquito. These sporozoites infect the liver cells and mature themselves into schizonts, each containing thousands of merozites, which are released into the blood stream through rupturing. These merozites invade erythrocytes and goes through another round of asexual reproduction in the erythrocytic cycle.

During the intra-erythrocytic stage of the life cycle the malaria parasite degrades the hemoglobin. Hemoglobin degradation by the parasite during the intra-erythrocytic cycle has been studied through experimental techniques and mathematical models and simulations. Studies suggest that hydrolysis of globin provides the principal source of amino acids for erythrocytic development and also provide sufficient space for the parasite growth [4]. Hemoglobin degradation is also essential to maintain osmotic stability of the intra-erythrocytic parasite [5]. Breaking down of hemoglobin is a complex process which involves transport of hemoglobin from cytosol to the parasite food vacuole, disruption of hemoglobin tetramers, removal of heme, detoxification of heme by the formation of hemozoin and the hydrolysis of globin by a number of proteases into amino acids.

We probe hemoglobin degradation due to the parasite growth in the erythrocytes employing non invasive optical techniques. Electronic absorption spectrum of healthy erythrocytes and cells infected with the parasite are presented which can be correlated to parasite multiplication cycle. Micro-Raman spectroscopy was further employed to investigate changes in the vibrational band with hemozoin formation.

II. MATERIALS AND METHODS

Parasites are maintained in human A+ erythrocytes at 5% hematocrit in complete RPMI-1640 (Invitrogen) supplemented with 0.5% Albumax (Gibco). Cultures are split every other day to maintain a parasitemia of 2-5%, as monitored by Geimsa stained smears, and freshly washed RBCs are added. A+ whole blood was obtained from Florida Blood Centers on a monthly basis. Whole blood is washed in incomplete RPMI to remove unnecessary components and RBCs are resuspended in complete RPMI-1640 to 50% (2% Dextrose, 15mg/L Hypoxanthine, 0.2% Sodium Bicarbonate, 25mM HEPES, 25ug/ml gentamycin). Parasites were synchronized on a MACs LD Separation Columns (Miltenyi Biotec) in late trophozoite stage. Columns were placed on a magnetic stand and equilibrated with 5ml of complete media. Parasite cultures were pelleted and resuspended in 5 ml fresh media and applied to the column. Flow through containing uninfected...
RBCs, ring and early trophozoite stage parasites was discarded; late trophozoites remained bound to the column. The column was then washed with 5ml of complete media. The column was removed from the magnetic stand and parasites were eluted with 5ml complete media. Freshly washed erythrocytes were added to the synchronized culture to obtain 4% hematocrit. The following day Giemsa stained smears of the culture were prepared to evaluate parasitemia.

![Schematics of the micro-spectroscopy setup.](image)

**Fig. 1** Schematics of the micro-spectroscopy setup. Raman scattering is excited by a He-Ne laser and the signal is collected in a back-scattering geometry. Micro-absorption spectra are measured in transmission geometry.

Raman spectra of individual healthy and parasite infected erythrocytes were recorded on a LabRam HR 800 setup using 632.8 nm excitation from helium neon laser (4 mW). The Raman system is coupled with an Olympus BX 41 microscope with a 100x dry objective (NA = 0.9). The vertically polarized laser is directed internally using a set of mirrors and focused through a lens onto the sample. The Raman signal collected by the microscope objective in back scattering configuration through the same optical path and through a holographic notch filter to the 100 μm confocal pin hole of the spectrometer. Spectra were recorded between 1800 and 650 cm⁻¹ with a resolution of 1.5 cm⁻¹. A fused silica micro capillary with an inner bore of 50 μm and outer diameter 350 μm was used a nano liter sample holder. An optical window was created by burning the outer coating and wiping the capillary through ethanol. The sample was loaded in a micro capillary by dipping one end in the sample culture allowing capillary action to draw the cells up. The small volumes and small optical window allows us to investigate individual cells without interference from the neighboring cells.

Confocal absorption microscopy was employed to measure optical absorption spectrum with spatial resolution at the micron scale [6] to investigate the changes in the electronic absorption bands of host red blood cells after parasite infection. It couples confocal microscopy with broadband illumination in transmission geometry. It enables the measurement of the absorption spectrum of a single erythrocyte between 350 and 700 nm with a lateral resolution better than 1.5 μm.

Micro-absorption spectra were measured on red blood cells immobilized on a coverslip using standard procedures. The coverslip was rinsed with 70 % Ethanol followed by 1X Phosphate Buffer Saline (pH 7.4). Sufficient 1mg/ml poly-L-Lysine HBr was applied to coat the coverslips which were then kept at room temperature for 15 minutes. Coating solution was removed and the coverslips were rinsed with 1X PBS. The erythrocytes suspended in 1 X PBS were added to the coverslips and were allowed to adhere at room temperature for 20 minutes. Excess liquid was drained from the coverslip. The transmittance of an individual red blood cell was measured with a spectral resolution of 0.5 nm. Micro-absorption spectra were recorded of erythrocytes immobilized both on coverslips and in micro-capillaries and found to be in agreement.

III. RESULTS AND DISCUSSION

Optical microscopy images of healthy human RBC and RBC infected with malaria inducing parasite *Plasmodium falciparum* are shown in Fig. 2. The cells are contained in a micro-capillary of inner bore 50 μm. Image on the right shows the cell with the parasite in it at 24 hr post invasion. Through optical images without stains it is difficult to discern between healthy and infected cells and to correlate them to parasite multiplication cycle. The results that follow show the micro-absorption as a potential diagnostic marker for different stages of parasite multiplication cycle.

![Erythrocytes in a micro capillary: healthy (left) and infected (right).](image)

**Fig. 2** Erythrocytes in a micro capillary: healthy (left) and infected (right). The diameter of a single cell is ~ 7 μm.

Micro-Raman spectra of a healthy red blood cell and a cell infected with *Plasmodium falciparum* are shown in Fig. 3 (left panel). The excitation wavelength was 633 nm. The bands in the spectra mainly arise from porphyrin vibrations [7]. The Raman scattering enhancement observed at 632.8 nm may result from excitonic coupling between aligned porphyrins due to the close proximity of heme moieties [8]. The vibrational bands are indicative of hemoglobin, the major protein in the cell. They can be grouped into the following regions: 1500 – 1650 cm⁻¹: core vibrations [7]. The Raman scattering enhancement observed at 632.8 nm may result from excitonic coupling between aligned porphyrins due to the close proximity of heme moieties [8]. The vibrational bands are indicative of hemoglobin, the major protein in the cell. They can be grouped into the following regions: 1500 – 1650 cm⁻¹: core vibrations [7]. The Raman scattering enhancement observed at 632.8 nm may result from excitonic coupling between aligned porphyrins due to the close proximity of heme moieties [8]. The vibrational bands are indicative of hemoglobin, the major protein in the cell. They can be grouped into the following regions: 1500 – 1650 cm⁻¹: core vibrations [7]. The Raman scattering enhancement observed at 632.8 nm may result from excitonic coupling between aligned porphyrins due to the close proximity of heme moieties [8]. The vibrational bands are indicative of hemoglobin, the major protein in the cell. They can be grouped into the following regions: 1500 – 1650 cm⁻¹: core vibrations [7]. The Raman scattering enhancement observed at 632.8 nm may result from excitonic coupling between aligned porphyrins due to the close proximity of heme moieties [8]. The vibrational bands are indicative of hemoglobin, the major protein in the cell. They can be grouped into the following regions: 1500 – 1650 cm⁻¹: core vibrations [7].
is dominated by the core size (or spin state marker band). There are clear differences between *Plasmodium falciparum* infected and uninfected cells in this region, and in the broadening of the peaks near 1210-1230 cm⁻¹ (C-H methine deformation band) and 755 cm⁻¹ (pyrrole ring breathing mode). The spectral changes are in agreement with those reported by Wood and co-workers in independent experiments [9]. As the hemoglobin is broken down by the parasite, the protein chain fragments are transported away for further digestion. The remaining toxic heme is then oxidized to a ferric state. The release of the heme from the protein is the first step in the formation of hemozoin. The changes in the spectra could be the result of this degradation and the changes in the vibrational modes of the now free heme. As the heme rings are no longer bound within the pocket of the protein the constraints on the various bonds will be much more random which accounts for the broadening of the bands.

The right panel in Fig. 3 shows the micro-absorption spectrum of individual erythrocyte in the healthy and infected state. An individual live erythrocyte under physiological condition was illuminated using broadband excitation and the transmitted light intensity was collected using the spectrometer with 5 seconds acquisition time. The spectrum was obtained over the visible range from 350 to 700 nm. The electronic absorption spectra of porphyrins feature two weak visible transitions near 555 nm and the intense Soret transition near 400 nm [10]. The intense absorption bands result from to π to π* transitions and can be distinguished from the weak bands due to charge-transfer transitions. The spectra depend on the electronic configuration of the iron cation and can be correlated to the spin state [10]. The absorption spectrum of the healthy red blood cell is indicative of oxygenated hemoglobin with the Soret band at 415 nm and β- and α-bands at 541 and 577 nm, respectively. The ratio of relative intensities of β- and α-bands was calculated to be 0.87 as compared to the literature value of 0.92 [11].

Changes in peak positions and relative peak intensities were observed in the case of cells in pathological conditions. The Soret band was weaker than in uninfected sample and was shifted to 418 nm. The β- and α-bands moved to 543 and 576 nm, respectively. The ratio of the relative intensities of the two bands decreased to 0.67. The peaks were broader and less intense in the infected sample.

Understanding the structural changes in the degradation of hemoglobin may opens new targets for anti-malarial drug treatments. Observing the cells in a native-like environment facilitates the transfer of new diagnostics for faster detection of the parasite’s presence in the human body.

IV. CONCLUSION

Micro-Raman and micro-absorption were combined in a comparative analysis of healthy RBCs and RBCs invaded with the malaria parasite. Both techniques are sensitive to heme degradation occurring during the multiplication cycle of the parasite. The spectral changes observed in the micro-absorption spectra may enable a diagnostic probe at the single cell level.

REFERENCES


