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Detection of Malaria Parasites in Blood by Laser Desorption Mass Spectrometry

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A novel method for the in vitro detection of the protozoan *Plasmodium*, the causative agent of malaria, has been developed. It comprises a protocol for cleanup of whole blood samples, followed by direct ultraviolet laser desorption (LD) time-of-flight mass spectrometry. Intense ion signals are observed from intact ferriprotoporphyrin IX (heme), sequestered by malaria parasites during their growth in human red blood cells. The LD mass spectrum of the heme is structure-specific, and the signal intensities are correlated with the sample parasitemia (number of parasites per unit volume of blood). Parasitemia levels on the order of 10 parasites/μL blood can be unambiguously detected by this method. Consideration of laser beam parameters (spot size, rastering across the sample surface) and actual sample consumption suggests that the detection limits can be further improved by at least an order of magnitude. The influence of experimental factors, such as desorbed ion polarity, laser exposure and fluence, sample size, and parasite growth stage, on the threshold for parasite detection is also addressed.

Despite intense efforts to combat it, malaria still afflicts more than 500 million people, with socioeconomic consequences (mostly for developing countries) being next to impossible to estimate.[1] Each year, malaria infections kill between one and two million people, predominantly children. In humans, malaria is caused by four different protozoa species of the genus *Plasmodium*, with *P. falciparum* being the most lethal. The *Plasmodium* parasite life cycle is complex and proceeds through several asexual and sexual stages.[2] *Plasmodium* sporozoites, transmitted by female *Anopheles* mosquitoes, are injected in the blood of a human host, together with mosquito saliva. After initial proliferation in the liver, parasites in the merozoite stage are released back into the blood stream. A single merozoite then invades a red blood cell (RBC) and matures by forming a ring-shaped cell. In ∼24 h, the matured parasite enters the trophozoite stage, during which most of the RBC cytoplasm is catabolized. Through the final (schizont) stage in the RBC, the parasite undergoes several divisions to produce up to 32 new merozoites that burst the host RBC and invade new erythrocytes.

Rapid, sensitive, and reliable methods for malaria detection are a factor that determines the ultimate success in controlling, restricting, and eradicating this disease. Moreover, accurate parasitemia quantitation is indispensable in malaria treatment as well as a tool in screening new drug and candidate vaccine efficacy and the emergence of drug-resistant parasite strains. Although optical microscopy of Giemsa-stained blood smears is still considered the "gold standard" for malaria detection, several new diagnostic techniques have been developed in recent years.[3−6] These include fluorescence microscopy, PCR-based assays, serological ("dipstick") antigen detection, and flow cytometry (with or without laser light depolarization monitoring). Currently, most such techniques are either time-consuming, have low sensitivity or specificity, or are expensive for mass screening. These features are potential drawbacks in efforts to design field-portable systems for monitoring malaria infections in large populations.

Here we present a novel physical method for detection of malaria in blood that is based on direct ultraviolet laser desorption (LD) time-of-flight (TOF) mass spectrometry. Mass spectrometry has been developed in recent years into a viable technique for intact microorganism characterization.[7−9] Laser desorption TOF mass spectrometry, in particular, has enhanced the prospects for field-deployable, robust, automated, and miniaturized detection systems for applications in a variety of areas from microbiology to bioterrorism counter-measures.[10−12] Our approach for malaria detection in blood is based on LD-TOF mass spectrometry detection of heme (Scheme 1), hyper-accumulated inside the parasite during its intraerythrocytic growth.[13] To this end, a protocol for sample cleanup, preceding LD mass spectrometry, has been developed (Figure 1). After initial lysis of the cholesterol-

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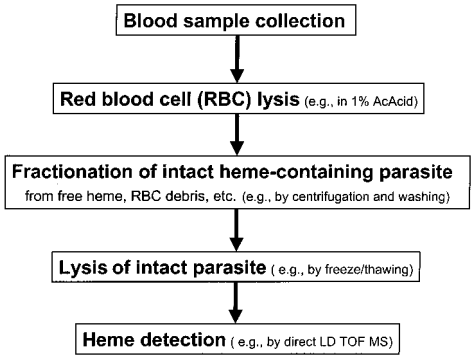
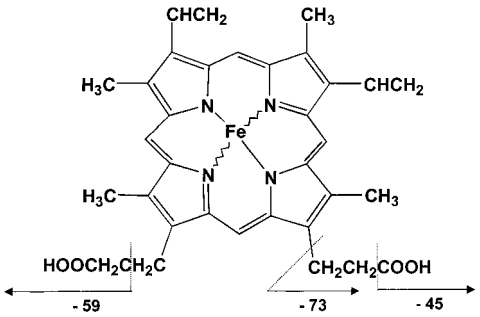


Figure 1. Method for detection/diagnosis of heme-sequestering parasites (e.g., *Plasmodium*) in whole blood.

rich erythrocyte membrane that leaves the cholesterol-deficient parasite membrane intact, all free heme outside the parasite cell is efficiently removed. Parasite cells are still intact after this initial RBC lysis step (Scheme 1). The efficacy of residual heme removal has been confirmed by LD mass spectrometry examination of negative control blood samples (not infected with *Plasmodium*). The LD mass spectra of intact heme contain a series of structure-specific ions, and signal intensities can be quantitatively correlated with the sample parasitemia (number of parasites per unit volume of blood). We also describe the influence of experimental factors, such as polarity of desorbed ions, laser exposure and fluence, sample size, and parasite growth stage, on the threshold of parasite detection.

Scheme 1. Structure of Ferriprotoporphyrine IX (FP)



EXPERIMENTAL SECTION

Parasite Organism Growth and Culture. *P. falciparum*, strain 3D7, was maintained in culture as described by Trager and Jensen.[14] Briefly, parasites were cultured at 5% hematocrit, Hct, (5×10^5 human RBC/μL) in RPMI 1640 growth medium (Catalog no. 31800-022, Gibco-Life Technology, Rockville, MD), supplemented with 50 μg/mL hypoxanthine, 25 mM HEPES (Catalog no. 391338, Calbiochem, San Diego, CA), heat-inactivated (56 °C, 30 min) O^+ normal human sera, and 0.26% $NaHCO_3$. Parasite cultures were kept in an incubator at 37 °C with daily changes of the growth medium. When parasitemia levels reached 3−4%, as determined by Giemsa-stained thin smears and optical microscopy, parasites were subcultured by up to 10-fold dilution of the donor culture in fresh medium and fresh erythrocytes. A gas mixture of 5% CO_2, 5% O_2, and 90% N_2 was infused into the culture. For culture synchronization, a mixed stage (nonsynchronized) culture was collected by centrifugation at 3×10^3 g for 10 min. The pellet was then resuspended in 2 volumes of 5% sorbitol and incubated at 37° C for 10 min. Late-stage parasites are lysed by the sorbitol treatment, in contrast to the ring-stage parasites, which remain intact. Parasites were then washed three times with PRMI 1640 media by centrifugation at $3 \times 10^3 g$ for 10 min to remove traces of sorbitol. Parasites were returned back to culture in a fresh medium. **CAUTION:** Proper procedures ("Biohazard level 2") should be followed when handling human blood plasma.[15]

Sample Cleanup. Samples for mass spectrometry analysis were prepared according to described procedures.[16] Parasites were collected when the culture reached the desired parasitemia level (typically around 5%, or 2.5×10^4 parasites/μL). Serial dilutions of the parasite suspensions in 5% Hct and RPMI 1640 were performed. Samples (2 mL) were centrifuged at $10^4 g$ for 10 min, and the supernatant was removed. Two different agents for RBC lysis treatment were compared: 1% acetic acid solution (AcS) and 0.15% saponin solution (SS). In both cases, the parasite pellets were resuspended in freshly prepared AcS or SS on ice for 10 min. Samples were diluted with an equal volume of PBS and centrifuged at $10^4 g$ for 10 min. Pellets were washed twice with PBS. Finally, all samples were resuspended in PBS to the same initial volume. Sample suspensions were kept frozen at −20 °C before mass spectrometry. All sample preparation procedures involving live *P. falciparum* were performed in a laminar flow hood in a BL2-rated laboratory.

Mass Spectrometry and Sample Preparation. Positive and negative ion mass spectra were obtained on a Kompact MALDI 4 (Kratos Analytical Instruments, Chestnut Ridge, NY) time-of-flight instrument at (±) 20 kV nominal accelerating voltage. The N_2 laser ("VSL-337ND" Laser Science Inc., MA, provided with the instrument) had an estimated fluence of 10 mJ/cm^2 before attenuation (0.2 mJ average energy/pulse at 337 nm laser wavelength, pulse duration 4 ns). Pulsed ion (delayed) extraction was optimized for ion focusing and transmission at m/z 1200. Spectra were acquired in linear and reflectron modes. Unless otherwise stated, each spectrum was the average of 100 consecutive laser shot traces, with the beam rastered linearly across the entire sample well.

After the cleanup, the frozen sample solutions were thawed, and typically 0.3 μL from each sample was deposited in a 2×1 mm^2 well on the stainless steel sample holder. Samples were allowed to dry in air prior to introduction into the TOF instrument. Commercial samples of protoporphyrine IX (PP, composition $C_{34}H_{34}N_4O_4$, $M_{mono} = 562.257$, $M_{ave} = 562.667$ Da) and ferriprotoporphyrine IX ($C_{34}H_{32}N_4O_4Fe$, $M_{mono} = 616.176$, $M_{ave} = 616.487$ Da) were obtained from Sigma Chemical Co. (St Louis, MO) and used without further purification. These compounds were dissolved in chloroform, and 0.3 μL was also deposited on the stainless steel slide prior to LD mass spectrometry. External calibration in both polarities was performed with CsI cluster ions.

RESULTS AND DISCUSSION

Hemoglobin comprises 95% of the RBC cytosolic proteins,[13] and it serves as the major source of nutrients for immature

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intraerythrocytic trophozoites, providing amino acids needed for the de novo malarial protein synthesis.[13,17] Heme (FP, ferriprotoporphyrin IX, Scheme 1)—the hemoglobin prosthetic group—is liberated during hemoglobin proteolysis. The intraparasitic heme concentration reaches almost 0.4 M, and the heme is sequestered in the form of insoluble hemozoin crystals (malaria pigment) in the *Plasmodium* food vacuoles.[13] It was the observation of pigment granules in the digestive tract of *Anopheles* mosquitoes in 1897 by Ronald Ross that was the crucial step in establishing the missing links between parasite proliferation, spreading, and transmission.[18] Hemozoin formation alleviates the considerable oxidative stress from the extremely toxic intraparasitic concentration of free FP that otherwise will kill the parasite.[13] This detoxification mechanism is a unique evolutionary feature of *Plasmodium*, and many effective antimalarial drugs target the disruption of hemozoin formation.[19,20]

LD mass spectrometry[21−25] is particularly well-suited for the analysis of porphyrins. Both IR and UV laser desorption have been applied for structural characterization of natural porphyrins and their metabolites, synthetic monomeric porphyrins (e.g., used in photodynamic therapy), porphyrin polymers, and multimeric arrays.[26−33] The protoporphyrin molecule (Scheme 1) contains a 22 Π-electron conjugated system and is an efficient photoabsorber in the visible and near UV range. This feature, concurrently with their low ionization potential, warrants that LD mass spectrometry will possess extremely low detection limits for porphyrins.

Direct LD spectra of purified *P. falciparum*-containing blood samples and commercial FP samples are presented in Figure 2. In both ion modes, intense molecular ion peaks are observed, with masses corresponding to the heme radical ion species $M^{+\bullet}$ or $M^{-\bullet}$, respectively (experimental $M_{mono} = 615.8$ Da). No doubly or triply charged ions are detected in either positive or negative ionization mode. Under otherwise identical instrumental conditions, the signal intensity in the positive ion mode is about an order of magnitude higher, as compared to negative ions, for both PP and FP. Although the mass resolution at m/z 600 in reflectron mode

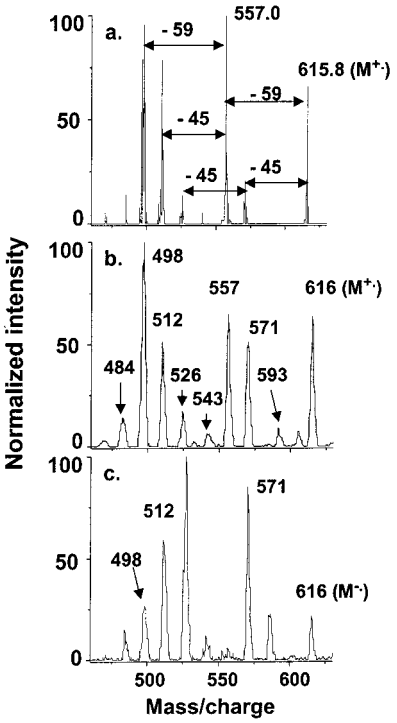

Figure 2. LD mass spectra of (a) purified *P. falciparum*-containing sample, positive ions, reflectron mode, (intensity normalized to 1.5 mV detector response value); (b) commercial ferriprotoporphyrine IX chloride sample, positive ions, linear mode (intensity normalized to 289 mV); and (c) commercial ferriprotoporphyrine IX chloride sample, negative ions, linear mode (intensity normalized to 21 mV).

is a factor of 4 higher (1200, fwhm), as compared to linear mode (300), the signal in linear mode is a factor of 10−100 more intense. To achieve lower detection limits, positive ion linear mode spectra have been acquired from parasite-containing samples. Characteristic (heme "signature") fragment ions at m/z 571, 557, 526, 512, 498 are observed. Their masses correspond to consecutive losses of 45 ($COOH^{\bullet}$), 59 ($CH_2COOH^{\bullet}$) or 73 ($CH_2CH_2COOH^{\bullet}$) from $M^{+\bullet}$ or $M^{-\bullet}$, respectively, and are directly correlated with the heme structure (Scheme 1). Ions at m/z 593 and 607 in the spectrum of the commercial FP chloride sample (Figure 2b) originate through a loss of 59 or 45, respectively, from a precursor ion at m/z 652, corresponding to $(M + Cl)^{+\bullet}$. Ions corresponding to PP or FP dimers, $(2M)^{+\bullet}$, and dimeric fragments are observed in positive ion spectra as well, with signal intensities varying as a function of laser fluence, sample concentration, etc. Systematic investigation of factors influencing dimer formation from synthetic porphyrins, believed to occur in the plume of laser-ablated material, is being pursued.

Mass spectra from a purified nonsynchronized *P. falciparum* culture and a negative control (purified blood sample not infected with *P. falciparum*) are compared in Figure 3. Although almost no ions are observed in the range from m/z 400 to 650 from the control, all peaks, characteristic for FP, are clearly detected in the freeze−thawed *P. falciparum*-infected sample. The spectral signal variation as a function of sample parasitemia is presented in Figure 4. A linear correlation between signal intensity and amount of parasite material deposited is observed for samples containing from 500 to 1.55×10^4 parasites/μL.

The suitability of the method for in vivo detection of *P. falciparum* infection in patients was examined using a synchro-

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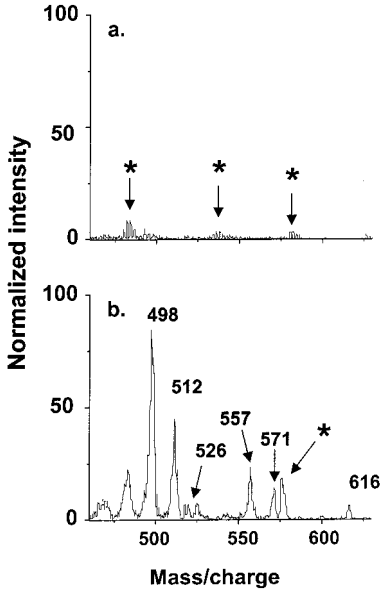

Figure 3. Positive ion LD mass spectra of (a) purified blood sample not containing *P. falciparum* and (b) purified blood sample containing *P. falciparum*. Peaks, marked with an asterisk originate from contaminants unrelated to the heme. The same experimental procedure—cleanup, mass spectrometry, sample processing—is applied to both samples. The two spectra are normalized to the same (13 mV) detector response value.

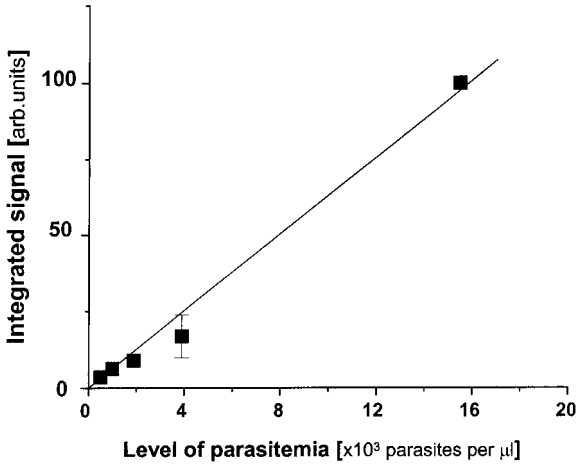

Figure 4. Integrated mass spectral signal intensity (in the range from *m*/*z* 460 to 630) as a function of parasitemia for a set of five samples containing nonsynchronized *P. falciparum* culture (0.3 μL constant volume deposited in each case).

nized culture with predominantly ring-stage parasites. In contrast to the other three human malaria species infections, erythrocytes containing *P. falciparum* in later development (trophozoite and schizont) stages adhere to endothelial cells on the inside of the blood vessels. In effect, P. *falciparum*-infected RBCs are taken out of the blood flow, and only RBCs with immature (ring-stage) parasites are circulating freely in the blood. The ring-stage culture examined had an initial parasitemia level of 2×10^4 parasites/μL, and a series of 3-fold dilutions gave a sample containing 80 parasites/μL. Mass spectra from purified ring-stage cultures with high and low levels of ring-stage parasitemia are compared in Figure 5. In this case, material from fewer than 30 ring-stage parasites deposited inside the well produces a spectrum with a signal-to-noise ratio better than 25 (Figure 5a). Moreover, the

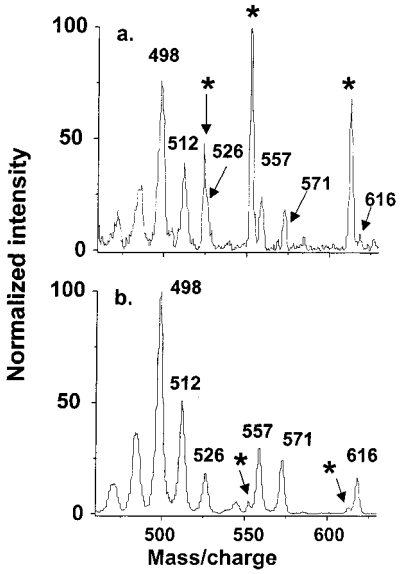

Figure 5. Positive ion LD mass spectra from purified ring-stage cultures of (a) 30 parasites deposited (intensity normalized to 17 mV detector response value) and (b) 6000 parasites deposited (intensity normalized to 225 mV detector response value). Peaks marked with an asterisk originate from contaminants unrelated to the heme.

reproducible mass spectral "signature" of the heme under a variety of LD-TOF conditions allows unambiguous parasite identification, even in the presence of contaminant peaks. Optical microscopy examination of the sample spot suggests that less than one-fifth of the surface area of the deposited sample is laser-illuminated in the current experimental setup (with linear laser beam rastering). A different experimental setup (*x*- and *y*-rastering of the beam across the sample surface and beam spot size reduction), as well as efficient control of sample material handling and consumption (e.g., preconcentration, deposition in smaller volume "nanowells"), will reduce the threshold of parasite detection at least by an order of magnitude. In terms of absolute number of parasites deposited on the sample and unambiguously detected, the method developed here is sufficiently sensitive and relevant for clinical parasitemia levels of <10 parasites/μL blood. The use for desorption/ionization of IR- or visible-wavelength lasers at resonant absorption frequencies for porphyrins is also expected to improve the threshold of detection. Additional separation protocols will combine this method with MALDI for unambiguous identification at the parasite species level using protein biomarker detection, genome and proteome databases, and bioinformatics.[8]

In experiments aimed at method optimization, two different agents for initial RBC lysis prior to cleanup were compared: acetic acid (1%) or saponin (0.15%). Higher overall sensitivity of the method was obtained using acetic acid (perhaps due to a higher lysis rate of the parasite cell membranes using saponin and a correspondingly higher loss of intraparasitic heme during the subsequent cleanup steps). Other factors examined were laser fluence and the effect of laser exposure (repetitive laser irradiation[34]) on signal duration from the same sample. The existence of a laser fluence threshold (F_{th}) for detection of PP and FP is observed. The absolute value of F_{th} is a function of the amount of parasite sample deposited; lower amounts are detected at higher F_{th}. Above threshold, the ion signal increases faster than linear as a function of fluence. This is an indication that laser desorption/

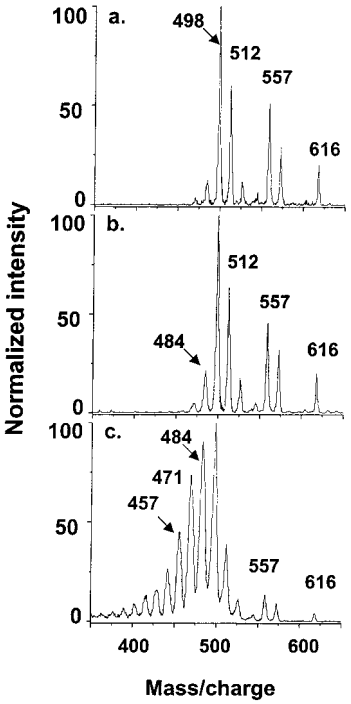

Figure 6. Effect of laser fluence, *F*, on heme mass spectra from ring-stage culture: (a) initial $F = 100\ \mu J/cm^2$; (b) $F = 160\ \mu J/cm^2$ (1.6 times higher); (c) $F = 290\ \mu J/cm^2$ (2.9 times higher).

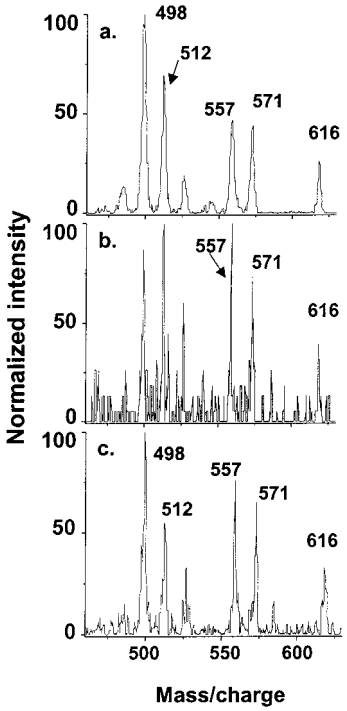

Figure 7. Dependence of the heme ion signal on laser exposure (sample, fewer than 170 parasites deposited; nonsynchronized *P. falciparum* culture): (a) spectrum (normalized to 30 mV detector response) from averaging 100 laser shots at fluence $F = 100\ \mu J/cm^2$; (b) spectrum (normalized to 1.2 mV) obtained from averaging 100 laser shots at $F = 100\ \mu J/cm^2$ after 600 laser shots at $F = 100\ \mu J/cm^2$, rastered across the sample; (c) spectrum (normalized to 7 mV) obtained by averaging of 100 laser shots at $F = 130\ \mu J/cm^2$, after exposure to 700 laser shots rastered across the same sample at $F = 100\ \mu J/cm^2$.

ionization results from multiphoton absorption processes. The degree of fragmentation of the heme molecule also increases with laser fluence (Figure 6). Increased abundance of nonspecific fragment ions (due to vinyl or methyl group losses) below m/z 498 is observed (Figure 6c). Although the signal from a sample decreases with increased laser exposure, the presence of parasites can be confirmed even after several hundred consecutive laser shots (Figure 7). The nature of this phenomenon—signal decrease as a function of exposure—is not clear, since the sample is not ablated (as revealed by optical examination). Signal decrease is probably related to photochemical processes occurring in the sample and changing its properties. Furthermore, increasing the fluence by a factor of 1.3 generates an appreciable signal, even after 700 laser shots at lower fluence levels (Figure 7c). Both laser fluence and laser exposure effects indicate that the threshold for parasite detection can be lowered further by accumulating and averaging spectra from a higher number of laser shots and at varying fluence levels. On the other hand, these instrumental factors have to be taken into account when the LD method is applied for parasitemia quantitation.

CONCLUSIONS AND FUTURE PROSPECTS

A method for detection of *Plasmodium* species in purified blood samples by laser desorption mass spectrometry of heme hyperaccumulated inside intact intraerythrocytic parasites has been developed. Parasitemia levels on the order of 10 parasites/μL of blood can be unambiguously confirmed by this method. Moreover, optimization of laser beam parameters (spot size, rastering across the sample surface), and more efficient sample handling (e.g., lower consumption, preconcentration, and deposition in smaller volume "nanowells") will enhance parasite detection by at least an order of magnitude.

The described method, based on direct LD-TOF mass spectrometry, can be multiplexed and used for rapid high-throughput screening of large batches of blood samples. Finally, its incorporation into a framework of multitiered technologies for rapid and automated malaria detection and diagnosis is envisioned. Such a framework will merge microfluidics (for efficient blood cleanup and fractionation), spectroscopic, microscopic, and PCR-based techniques for *Plasmodium* identification and characterization at the level of individual strains, both in a laboratory setting and in the field.

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