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## **A magneto-optic route towards the in-vivo diagnosis of malaria: preliminary results and pre-clinical trial data**

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### **Abstract**

We report the development of a new magneto-optic technology (MOT) for the rapid quantitative diagnosis of malaria that may also be realisable in a non-invasive format. Haemozoin, the waste product of malarial parasitic action on haemoglobin, is produced in a form that under the action of an applied magnetic field gives rise to an induced optical dichroism characteristic of the haemozoin concentration. Here we show that precise measurement of this induced dichroism may be used to determine the level of malarial infection since this correlates, albeit in a complex manner throughout the infection cycle, with the concentration of haemozoin in the blood and tissues of infected patients. Under conservative assumptions for the production of haemozoin as a function of parasitemia, initial results indicate that the technique can match or exceed other current diagnostic techniques. The validity of the approach is confirmed by a small preliminary clinical trial on thirteen patients whilst measurements on live parasitized cells obtained from *in vitro* culture verify the possibility of producing in-vivo diagnostic instrumentation.

### **Introduction**

Malaria remains a major health problem in many parts of the world (1). In regions where it is endemic effective treatment and eradication is often compromised by lack of access to rapid, accurate and affordable diagnosis since, unfortunately, the best diagnostic tools currently available require a laboratory environment. Even in Europe the number of cases and fatalities increases year on year reflecting the increasing preference of Europeans to holiday in

malarial prevalent areas coupled with a reluctance to take anti-malarial prophylaxes (2). Native Europeans have no acquired immunity so that without early diagnosis infection can, and often does, have rapidly fatal consequences (16 UK deaths in 2003). This situation is moreover only likely to worsen as global warming is now predicted (3) to facilitate the spread of malaria to areas previously free of the disease including southern Europe.

Examination by high-power microscopy of typically 200 fields of Giemsa-stained thick blood smears is still generally regarded as the so called “gold standard” for malarial diagnosis (4). Dominant for more than a century this technique can in principle attain a sensitivity of better than 10 parasite infected cells per  $\mu\text{l}$  of blood. It is however time consuming and in reality subject to significant variability in its application, particularly in respect of the number of fields examined and the methodology employed to determine parasitemia from parasite counts within the fields examined (5). The technique is also dependent on the skill base of highly trained microscopists, and consequently the highest sensitivities are rarely obtained outside specialist laboratories. Recognition of this and of the need for more rapid diagnosis has, over the last decade or so, driven the study and development of several alternative techniques. These include fluorescent microscopy, laser desorption mass spectrometry (6,7) and techniques involving DNA/RNA amplification to detect and identify nucleic acid sequences which are currently acknowledged as the most sensitive and species specific (8). In general however this emerging generation of diagnostic procedures remains time consuming and again too costly and complex for dissemination beyond specialist laboratories. For field application there are now available from a variety of manufactures rapid detection tests (RDTs) (4,9) in the form of lateral flow assays. These employ immuno-chromatographic methods to detect malarial antigens such as the histidine-rich protein II (HRP-II), aldolase (pan malarial antigen, PMA) or parasite-specific lactate dehydrogenase (pLDH) which are present in peripheral blood during infection. These tests generate results within 15 minutes and require only minimal operator training. They are however relatively expensive compared to microscopy, non-quantitative and have a limited usefulness in detecting low-level parasitemia ( $<100$  parasites/ $\mu\text{l}$ ). Despite the above developments there is demonstrably still pressing need of new diagnostic principles affording rapid yet simpler quantitative instrumentation suitable for field use or at first point symptom presentation. It would moreover be of immense benefit if new techniques could be rendered in a non-invasive form.

### **Diagnostic Methodology**

During infection malarial parasites invade erythrocytes, and digest the protein (globin) part of the haemoglobin molecule. The haem component, which is toxic to the parasite, is converted into insoluble haemozoin in the form of rod-like crystals; the transformation of low spin ( $\text{Fe}^{2+}$ ) diamagnetic oxyhaemoglobin into high spin ( $\text{Fe}^{3+}$ ) paramagnetic haemozoin produces a change in magnetic state and susceptibility which has previously been used to concentrate parasitized cells to improve detection (10). Initially deposited in vacuoles within the erythrocytes the haemozoin is subsequently released into suspension in the plasma on rupture of the parasitized erythrocytes from where it is ultimately scavenged by leukocytes (11,12). Detection of the presence of haemozoin in blood or tissue is therefore positively indicative of malarial infection and its concentration might be expected to correlate in some as yet unknown manner with the level of parasitemia. Scanning electron micrographs (SEM) show that the malarial parasite produces haemozoin crystals in a distinct rectangular form within a narrow size spectrum (13). As a result of their shape these crystals are found to exhibit a high level of both magnetic anisotropy and optical dichroism and it is these properties that are identified for exploitation as the basis of a new diagnostic technique. When suspended in a fluid, such as blood, the long axes of the haemozoin crystals are randomly orientated

throughout three dimensions and so the suspension expresses no preferred direction of optical absorption on interrogation using linearly polarised radiation. However, on application of a magnetic field the paramagnetic crystals will become weak bar magnets experiencing a torque seeking to orientate them along the applied field direction. This is opposed by the thermal energy of their environment that constantly acts to randomise the assembly. At any non-zero field value, the total component of the crystals' long axes resolved along the field direction will be greater than that along the orthogonal to the field direction when integrated over all crystals in the dispersion. Optically this manifests as an induced dichroism and therefore under the action of an applied magnetic field any dispersion of haemozoin crystals behaves increasingly like a weak dichroic polarizer similar, when all crystals are fully orientated with the field, to Polaroid<sup>®</sup>. Magneto-optically the phenomenon is directly analogous to the Cotton–Mouton effect. Interrogation of dispersions of magnetically aligned haemozoin crystals with optical beams in which the polarisation is modulated between linear states aligned along and orthogonal to the applied field direction therefore produces an optical modulation signal which, when expressed as a fraction of the sample transmittance (or absorption) is directly proportional to the crystal concentration affording, in principle, a simple and precise optical technique for the diagnosis of parasitemia.

The validity of this mechanism and its potential as a technique for the detection of malaria and quantification of the level of parasitemia is initially explored by studying the optical response of suspensions of  $\beta$ -haematin crystals in phosphate buffered saline (PBS) to the imposition of an applied magnetic field.  $\beta$ -haematin is a readily synthesised analogue of haemozoin that all references cite as its complete equivalent (14,15,16). In our hands, the method of Slater et al (14) yields  $\beta$ -haematin crystals some 0.78 $\mu\text{m}$  in length with a cross section about 0.22 $\mu\text{m}$  square. This is comparable with haemozoin crystals sourced from cultured parasitic cells although the standard distribution of these dimensions is currently somewhat larger for the synthesised crystals. Fig. 1a shows the increasing dichroic signal imposed on a beam of 660nm optical radiation during transmission through a 20 $\mu\text{g}/\text{ml}$  dispersion of  $\beta$ -haematin crystals in PBS when an applied magnetic field is cycled between values of  $\pm 1$  Tesla (T).

Equivalent concentrations of purified haemozoin extracted from parasitized cells give an identical response to within 10%. It is clear that at the maximum field values the mechanism is approaching saturation as the crystals come into near full alignment with the field. Moreover, following a formalism analogous to that employed by O'Konski et al. (17) to describe the orientation of molecules in a strong electric field, the form of the behaviour observed in Fig. 1a may be modelled in terms of an order parameter of the crystals which has a value of "0" when crystals are randomly dispersed and "1" when fully aligned along the field as shown in Fig. 1b. The experimental and theoretical plots shown in Figs. 1a & b respectively are obtained in the configuration shown in Fig. 1c with the magnetic field applied in the plane of the liquid surface and the optical polarisation modulated electro-optically at frequencies of either 10Hz or 44kHz. If, however, the field is applied perpendicular to the liquid surface, then whilst the absorption for both polarisation states decreases equally with field until full alignment is obtained, no response under polarisation modulation is observed because the random in-plane distribution of crystals is maintained as they orientate with the field. Further evidence confirming the nature of the conjectured mechanism underlying the observed changes in dichroism is provided by suspending  $\beta$ -haematin crystals at the same 20 $\mu\text{g}/\text{ml}$  concentration in gelatin (10%, w/v) rather than PBS and then allowing it to set from its liquid state at about 60°C. This produces samples in which the orientation state of the crystals is fixed. Samples setting in an applied in-plane magnetic field subsequently behave as weak polarisers as shown in Fig. 1d where the transmission as a function of azimuth angle is plotted as such a sample is rotated in a beam of linearly polarised

radiation. Conversely, those setting in the absence of a field retain the thermally randomised state and as expected exhibit no such behaviour. Finally, in addition to these measurements on constrained crystals, initial studies into the dynamics of the orientating mechanism are conducted by probing the transmission of a 20 $\mu$ g/ml suspension of  $\beta$ -haematin with an optical beam in a single linear state of polarization whilst the frequency of the applied magnetic field is varied. The constancy of the dichroic signal amplitude with magnetic field frequency in Fig. 1e shows that the rotation of the crystal rods easily follows the magnetic driving and thermal restoring forces up to frequencies of 10Hz, the highest currently achievable with our electromagnets. At these frequencies viscous damping is of little importance.

## Results

Having established the nature of the diagnostic principle its potential is first evaluated by mimicking the blood haemozoin content arising from different levels of malarial infection and measuring its fractional change in transmittance at 660nm on imposition of a 1T in-plane magnetic field. Fig. 2a displays the results for whole fresh blood doped with  $\beta$ -haematin crystals dispersed by sonication whilst the results in Fig. 2b are obtained by replacing the  $\beta$ -haematin with haemozoin in the form of mature trophozoite stage parasitized red blood cells (PRBCs) grown in culture and lysed (ruptured) by freezing and thawing before diluting with whole fresh blood. In both these batches of artificially created malarial samples the uncertainty in the haemozoin ( $\beta$ -haematin) concentration is dependent on the concentration but ranges between 2.9% at the higher concentrations to  $\approx$ 10% at the lowest. The linearity of both plots is however striking and continues unshown out to beyond at least 100 $\mu$ g/ml. Note also how the data point ( $\square$ ) obtained with cells in which the infection is at the early ring stage of haemozoin formation when the crystals are believed to be much smaller, fits closely to the linear trend. In this ring stage sample the conversion level of haemoglobin haem to haemozoin is determined less than 0.5% per PRBC. This is consistent with the low levels of HZ expected in the young parasite stages, and is of the order of 100 times lower than the conversion rate determined for later trophozoite stage culture material.

The difference in the gradients of the plots is attributed a consequence of at least two factors. Firstly, haemozoin crystals *in situ* exhibit a tendency to clump (18), which reduces the dichroism when compared with an equivalent number of individual crystals, such as is the case with  $\beta$ -haematin. Secondly, cultured cells are supplied frozen to ensure all cells are at the same point in their infection cycle and to facilitate safe handling. Freezing however appears to only partially release the haemozoin so that cellular debris continues to adhere to the crystals, altering the dynamical forces acting upon them and thereby constraining the rotation achieved in a given applied field. Adherence of diamagnetic cellular material to the crystals would for example result in a small torque opposing that seeking to orientate the crystals with the field. Alternatively, adhering cellular material might simply be providing a larger interaction cross-section for the thermal restoring mechanism. Plots obtained in the same way as Fig. 1a for blood doped in this manner have exactly the same form but with reduced dichroism. Similarly, the rate of response of the dichroic signal to changes in the applied field is also observed to be reduced. In developed instrumentation a detergent will be used to completely release the haemozoin in order to restore the dichroic signal strength to that exhibited by an equivalent  $\beta$ -haematin concentration. The effectiveness of this procedure is tested by extracting the haemozoin from two of the samples of PRBCs used in producing Fig. 2b. This is detergent-washed before reintroducing it at the same concentration into whole

fresh blood. When the fractional intensity change for these samples is then plotted ( $\diamond$ ) with the free  $\beta$ -haematin data in Fig. 2a it is in very close agreement.

Fig. 3 shows the fractional change in absorption arising from magnetically induced dichroism when the experiments whose results are depicted in Fig. 2 are repeated using fresh blood containing varying concentrations of live parasitized cells. Samples for this experiment were harvested randomly from a non-synchronised parasitized culture in which the concentration of parasitized cells varied throughout the culture feeding cycle. Although in these samples all haemozoin remains secreted in the cell vacuoles, the results obtained are comparable to those of Fig. 2b for lysed blood. This demonstrates that haemozoin remains free to rotate within the vacuoles and still able to orientate either partially or fully with the applied magnetic field. Close comparison of Fig. 3 with Fig. 2b reveals however a further slight suppression of the gradient. This is because, in each sample taken, the non-synchronised nature of the culture produces parasitized cells containing haemozoin in all its developmental states. Given the finite size of the vacuoles it is unlikely that the freedom of the haemozoin crystals to rotate within them and hence contribute fully to the detected signal will remain constant as their size increases through (early) ring, trophozoite and schizont stages. The magnitude of the signal arising from schizont stages will be particularly susceptible to suppression disproportionate to their number given the known tendency of haemozoin crystals associated with this state to adhere into large clumps. Again this explanation is confirmed by subsequently lysing samples when the signal returns to correspondence with that obtained for an equivalent concentration in Fig. 2b. In all the above experiments the concentration of haemozoin in the samples was determined using spectroscopic absorption measurements.

### **Analytical performance of MOT device**

A portable demonstrator instrument operating on the diagnostic principles described was developed and evaluated in a small pre-clinical trial. After obtaining informed consent, blood samples from study cases with either confirmed malaria, undifferentiated fever symptoms, rheumatic-associated disease or haemoglobinopathies (see Table 1), were tested for *Plasmodium* infection with RDT and MOT test. The samples studied had been transported or stored frozen and were further lysed and diluted by the addition of triton detergent before being offered blind to the instrument operator. Table 1 reveals that there was an excellent correlation between MOT testing, RDT results and clinical confirmation. The ease with which the system detected a *P. ovale* infection, known for its low parasitaemia, is particularly encouraging.

### **Discussion**

The evidence presented, and the pre-clinical trial data in particular, clearly validates this new diagnostic principle when working with drawn blood. However to fully evaluate its potential worth and critically compare its ultimate sensitivity against existing techniques of this type requires making assumptions regarding the relationship between the accessible haemozoin load in the bloodstream and parasitaemia. Unfortunately reliable quantitative data that might facilitate this is scarce and even less is known about the elimination of haemozoin from the body (21). To create the comparative upper and lower axes for Fig. 2 it was assumed whole blood contains about  $5 \times 10^9$  RBCs/ml and that in mature parasitized cells conversion of 50% of the haemoglobin yields  $\approx 0.6$ pg haemozoin per cell (19,20). On this basis detecting 100

PRBCs/ $\mu$ l (0.002% parasitemia) for example, requires measurement of haemozoin concentrations  $\approx 0.06\mu\text{g/ml}$ . Whilst the lowest created-to-calibration concentration of haemozoin measured to date is  $0.01\mu\text{g/ml}$  (in PBS), as shown by the linear separation of the lower data points in Figs. 2a & b the resolution of the instrumentation, even in proto-type form, is actually somewhat better than this. Moreover, in this initial work neither the sample volume nor optical path length has as yet been optimised to maximise the detectable fractional intensity change. The measured parameter ( $\Delta I/I$ ) is of course a function of the optical path length and ultimately the sensitivity achievable will for the most part be determined by the degree to which the orthogonal polarisation states used to interrogate the induced dichroism are depolarised by scattering from cellular structures and other material during their passage through the blood sample prior to interacting with haemozoin crystals. Loss of polarisation after this point is unimportant since only the resulting intensity modulation is detected. In this context, it is noted that haemozoin is itself known to depolarise light and the additional depolarisation produced by its presence in blood has been previously explored as a means of diagnosing malaria (25). In principle there is no reason why the technique cannot readily be refined to permit measurement of fractional intensity changes with more precision than the currently achieved value of about  $5 \times 10^{-6}$ . Values lower than this are regularly attained in optical and magneto-optical measurements (22,23,24). A measurement precision of  $1 \times 10^{-6}$  would, for example, render possible the rapid automatic detection of parasitemia levels approaching 0.0002 % (10 PRBCs/ $\mu$ l) by low skilled operators even in the case of *P. falciparum* when only the ring stage exhibits a significant presence in the circulatory system and for which the above assumption of haemoglobin conversion may be optimistic by up to a factor of 50. Other working methodologies may then offer yet further improvements or simplification. For example, rather than continue with the ratiometric methodology in which evaluation of the fractional intensity change for correlation with malarial parasitemia requires two independent measurements, the same level of detection sensitivity might be more readily obtained by employing a dual beam differential system in which only one of two identically sourced samples is subject to an applied magnetic field. Whilst the simplicity and rapidity of diagnosis offered by instrumentation working as described above represents a significant improvement on existing methods, the real importance of this technology lies in its potential to function in an *in-vivo* or non-invasive mode precluding the need to draw and handle blood. During infection with *P. falciparum* a certain population of the mature PRBCs sequester in the microcirculation within organs and subcutaneous tissues (26,27). A cumulative measurement of the haemozoin present in both circulating and sequestered PRBCs together with that phagocytosed in peripheral and intradermal leucocytes and liberated in the blood vessels on schizont rupture may give a better indication of the patients' actual parasite load and thus provide a better prognostic indicator (28,29). Haemozoin has been shown free to respond to the detection process whether liberated in the blood stream or confined within cell vacuoles. Similar studies seeking to confirm the freedom of haemozoin ingested by live leucocytes and its relationship to the observed fractional intensity change in an applied magnetic field have thus far proved positive but inconclusive due to the difficulty of removing the excess haemozoin not taken up by the leucocytes. Microscopic observation though confirms, as indicated elsewhere (30,31), that haemozoin is indeed free to respond in live leucocytes. Responsive to the total haemozoin load, a non-invasive measurement, across say the fingertip or earlobe in the manner of a pulse oximetry device, therefore appears to promise potentially more sensitivity than the blood sampling instrument. Unfortunately scattering, which is much more pronounced in skin and tissue than in blood and lysed blood in particular, rapidly destroys the incident state(s) of polarisation. Initially it might therefore appear that the polarisation modulation technique described previously will be unsuccessful in a non-invasive mode.

However, Pircher et al. (32) indicate that although the stratum corneum randomises the polarisation state of light backscattered from within it, contrary to previous belief it has little impact on light progressing to the lower dermal levels so that some considerable degree of incident polarisation is retained to depths of at least 800 $\mu$ m and beyond. If this is the case then sensitive non-invasive access to all haemozoin within most of the capillary rich dermis becomes possible since, as pointed out above, loss of polarisation after interaction with a magnetically orientated crystal is unimportant because only the resulting intensity modulation is detected. Moreover, if nail were to prove similarly polarisation transparent then interrogation of the nail bed may be especially sensitive particularly if operating in the configuration in which the magnetic field is applied co-linear to the optical beam when the lightwave-haemozoin interaction functions even with un-polarised light. Other changes might be necessary or desirable to optimise performance in non-invasive mode. First it may be essential to operate at the isobestic point ( $\lambda \approx 800$ nm rather than 660nm) to preclude or reduce interference from the pulse. Secondly, after a full safety evaluation, magnetic field modulation may be a preferable route to synchronous signal modulation/detection. Finally it is obvious that the relationship between  $\Delta I/I$  and haemozoin concentration for non-invasive measurements will be significantly different from those given in Figs. 2 & 3 above and will only be determinable through clinical trials.

Instrumentation operating in invasive (blood sampling) mode is now under construction and scheduled for field trials in Africa late 2008. Research continues on the possibility of implementing the diagnostic technique in non-invasive format.

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Patient Characteristics	Result Rapid Diagnostic Test for malaria	Final clinical diagnosis based on extensive diagnostic procedures	MOT diagnosis	MOT Fractional Change $\Delta/I$
Nigerian child with fever	Positive	<i>P. falciparum</i> infection	Positive	8.2E-5
Nigerian child with fever	Positive	<i>P. falciparum</i> infection	Positive	4.7E-5
Nigerian child with fever	Positive	<i>P. falciparum</i> infection	Positive	7.8E-5
Nigerian child with fever	Positive	<i>P. falciparum</i> infection	Positive	9.5E-5
Nigerian child with fever	Positive	<i>P. falciparum</i> infection	Positive	5.8E-5
Dutch returned traveller	Positive	<i>P. Ovale</i> infection	Positive	4.4E-5
Dutch returned traveller	Positive	<i>P. falciparum</i> infection	Positive	5.9E-3
Tanzanian adult; asymptomatic for malaria	Negative	Sickle cell anaemia	Negative	-
Tanzanian adult; asymptomatic for malaria	Negative	Beta-Thalassemia	Negative	-
Tanzanian adult; asymptomatic for malaria	Negative	Rheumatic-associated disease	Negative	-
Dutch returned traveller with fever	Negative	Undifferentiated fever	Negative	-
Dutch returned traveller with fever	Inconclusive	Undifferentiated fever	Negative	-
Dutch returned traveller with fever	Negative	Visceral leishmaniasis	Negative	-

**Table 1.** Results of evaluating a small set of clinical samples of individuals with fever or no fever but from a disease endemic country with health complaints.

**Figure 1.** (a) Difference in transmission (normalised to value at 1T) for orthogonal polarised beams as a result of magnetically induced dichroism in a  $\beta$ -haematin (BH) suspension (20 $\mu$ g/ml). (b) Magnetic orientation of crystals suspended in fluid modelled following formalism described in text, (c) Experimental configuration. (d) Magnetically aligned 20 $\mu$ g/ml concentration of BH set in 10% w/v gelatin gel behaving like a dichroic polarizer and (e) Dynamic response of dispersion of BH to alternating magnetic field.

**Figure 2.** The fractional change in transmittance  $\Delta I/I$  associated with magnetically induced dichroism in (a) differing concentrations of  $\beta$ -haematin dispersed in whole fresh blood and (b) differing concentrations of haemozoin in the form of parasitized red blood cells (PRBCs) grown in culture and lysed by freezing before diluting with whole fresh blood. **Insets** electron micrographs of synthesised  $\beta$ -haematin (BH) and haemozoin (HZ) extracted from cultured cells. **Legends** #1, #2 and #3 refer to samples mixed and measured on different occasions. Correlation between the haemozoin concentration and parasitemia scales on both the upper and lower graphs is made as described in the text.

**Figure 3.** The fractional change in transmittance  $\Delta I/I$  associated with magnetically induced dichroism arising from rotation of haemozoin crystals within the cell vacuoles of live parasitized cells grown and sampled from non-synchronous culture.

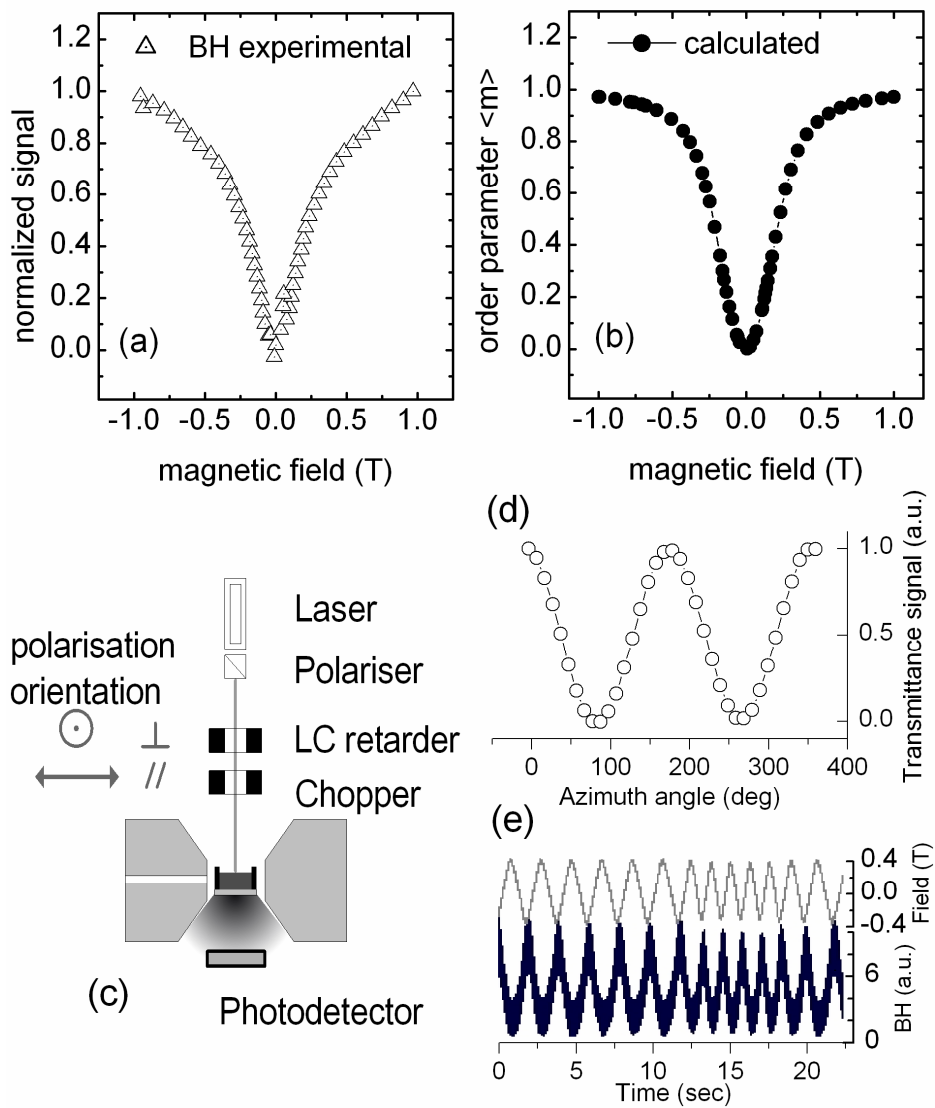


Figure 1.

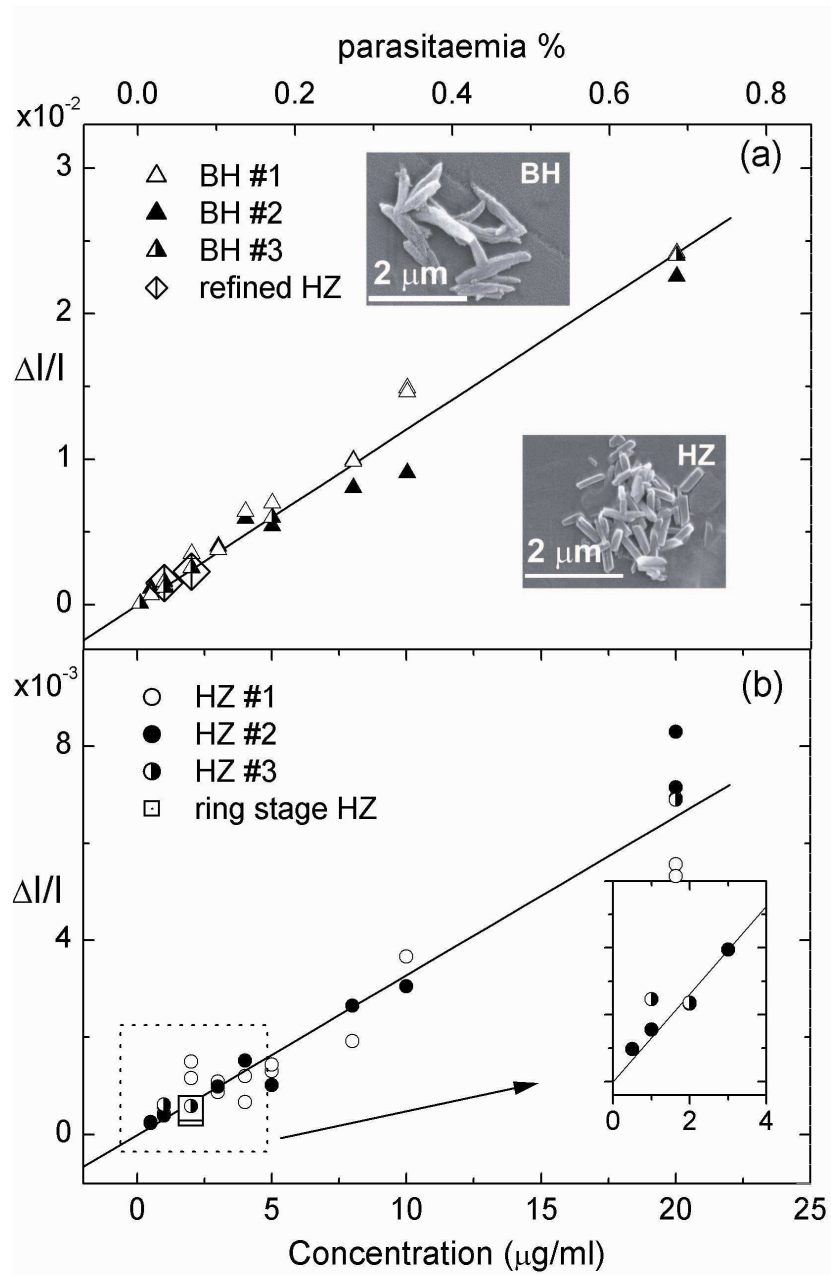
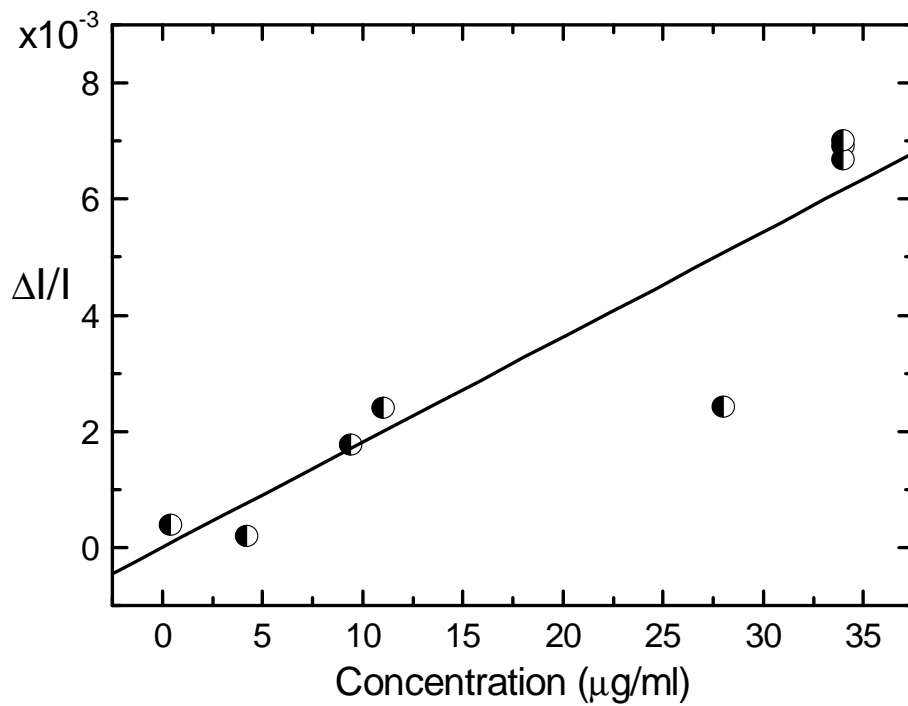


Figure 2.



**Figure 3.**