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An Immunosensor for Parasite Lactate Dehydrogenase Detection as a Malaria Biomarker- Comparison with Commercial Test Kit

Aver Hemben, Jon Ashley* and Ibtisam E. Tothill*

Cranfield University, Cranfield, Bedfordshire MK43 0AL, England, UK

jash@nanotech.dtu.dk

i.tothill@cranfield.ac.uk

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*Corresponding author. I.E. Tothill Tel: +44 (0) 7500766487

*Corresponding author.Jon Ashley Tel: +45(0) 45258162

Abstract

This paper describes the development of an affinity sensor for the detection of *Plasmodium falciparum* parasite Lactate dehydrogenase (pLDH) as one of the biomarkers used for malaria detection. The gold sensor was functionalised with anti-pLDH after cleaning the electrode surface to remove impurities (120 ^{o}C, 1 hour). The sensor was then treated to block unreacted groups on the surface and minimise matrix interference, before applying it in a sandwich assay to detect pLDH in buffer samples using dose concentration assay. The sensor was optimised to achieve the best detection sensitivity before using it for pLDH detection in serum samples. The developed sensor achieved a limit of detection (LOD) of 1.80 ng mL^{-1} and 0.70 ng mL^{-1} for the detection of pLDH in buffer and in serum samples respectively. The sensor sensitivity was enhanced further with the use of AuNP conjugated to the detection anti-pLDH-enzyme, achieving an LOD of 19 pg mL^{-1} in buffer and 23 pg mL^{-1} in serum

samples. The performance of the sensor was compared to commercially available *Plasmodium* immunochromatographic (ICT) malaria kits. The developed sensor was able to detect pLDH in the Dd2^{luc} culture medium supernatant at 0.002 % parasitaemia without the use of AuNP signal enhancement when compared to the OptiMAL-IT ICT kit (detect pLDH) and the BinaxNOW ICT kit (detection of both pLDH and *Pf*HRP 2) samples. Therefore, the sensor developed in this work is highly sensitive and can be used for pLDH detection for on-site diagnosis of malaria. A cheap and simple device as developed in this work is required to tackle malaria detection.

Keywords: Malaria; pLDH; *Plasmodium falciparum*; parasites; biosensor, biomarker, AuNP.

1. Introduction

Malaria is an acute febrile illness and a life-threatening disease caused by *Plasmodium falciparum* that is transmitted to people through the bite of an infected female Anopheles mosquito. According to estimates released by the World Health Organisation [1], there were ~ 198 million cases of malaria in 2013 with ~ 584,000 deaths globally. While in 2017, a new report [2] gave an estimate of ~ 445,000 deaths for 2016, compared to ~ 446,000 deaths for 2015. However, malaria mortality rates have fallen by 47% globally since the year 2000, and 54% in the African region, due to human intervention and efforts to eradicate the vector [3]. In 2015, global malaria burden was high in Sub-Saharan Africa (89% malaria cases and 91% of malaria deaths). Of the deaths caused by malaria, under-fives made up 65% of deaths [4].

It is further stated that in 2015, 97 countries had on-going malaria transmission and an estimated 3.2 billion people (nearly half the world's population) were at risk of contracting malaria. In 2016, most cases were reported in the African regions (90%), followed by South-East Asia (7%) and the Eastern Mediterranean region (2%), while the rate of incidence of malaria is estimated to have decreased by 18% globally, from 76 to 63 cases per 1000 population at risk, between 2010 and 2016 [2]. Despite these efforts, malaria continues to be a global problem [5] and imported malaria cases are a potential threat [6]. *Plasmodium falciparum* is the most prevalent malaria parasite in sub-Saharan Africa, accounting for 99% of estimated malaria cases in 2016 [2]. The African region also accounted for 91% of all malaria deaths in 2016, followed by the South-East Asia Region (6%) [2]. However, between 2015 and 2016, mortality rates stalled in the regions of South-East Asia, the Western Pacific and Africa, and increased in the Eastern Mediterranean and the Americas.

Malaria can be both cured and prevented by the use of appropriate measures. Prompt diagnosis and treatment is by far the most effective means of preventing mild cases of malaria from developing into severe disease and death [2]. The gold standard for malaria detection is light microscopic identification of the malaria parasite on a fixed, stained thin film of patient whole blood. Microscopic detection of malaria was first performed by Alphonse Laveran who identified *Plasmodium sp*. as the pathogen for malaria around 1800 [7]. Since then, a range of malaria detection methods have been developed and the most commonly used include: quantitative buffy coat method [8], immunochromatographic tests [9], indirect fluorescent test and Enzyme linked immunosorbent assays [10-11], molecular methods of polymerase chain reaction [12-13], and DNA microarray [14]. Many other methods have also been developed such as; laser desorption mass spectrometry [15], flow cytometry [16], Raman spectroscopy [17-19], magnetic resonance imaging [20-21], and Near infrared imaging for triaging and prognostication in cerebral malaria [22-27]. These methods can be costly, mostly laboratory based and require skilled users. Hence, the requirement for easy to use sensors able to detect the disease in field settings with high sensitivity.

Biosensors have shown great potential for the detection of various malaria biomarkers showing various levels of sensitivity using different transducer platforms and detection of different biomarkers [28-29]. The advent of nanoscience and nanotechnology has led to the development of various nanomaterials which explore the inherent advantages of nano regime which have been applied in biosensor developments for malaria [30-31]. *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP 2), is a biomarker specific for the detection of

Plasmodium falciparum, while parasite lactate dehydrogenase (pLDH), and parasite aldolase are biomarkers common to all *Plasmodium species* [32]. pLDH is produced in high levels during the intraerythrocytic stage of the Plasmodium parasite life cycle [33] with levels in blood reported ~ 4.9 ng mL^{-1} [34-35]. Parasite LDH gene is known to possess global genetic variations [36]. To date, only a few examples of biosensor for the detection of parasite lactate dehydrogenase have been developed. Lee et al. [37] developed an electrochemical impedance spectroscopy (EIS) sensor based on ssDNA aptamer which showed specificity toward pLDH with detection limit reported around 1 pM. Markwalter et al. [38], reported the use of magnetic bead-based colorimetric enzyme assay for *Plasmodium falciparum* lactate dehydrogenase detection (*Pf*LDH) in which the biomarker is extracted from parasitized whole blood and achieved an LOD of 21.1 ± 0.4 parasites/ml. The same group [39], also reported an LOD of 2.6 ± 1.5 pM for pLDH detection using the same assay when two biomarkers were detected at the same time including *Pf*HRP2. From our previous studies, an immunosensor was developed for the detection *Pf*HRP 2 which demonstrated a LOD of 2.14 $ng{\cdot}mL^{-1}$ in buffer samples and 2.95 $ng{\cdot}mL^{-1}$ in 100% spiked serum samples [40].

Encouraged by our previous results, we set out to develop, a pan malaria amperometric immunosensor for the detection of the malaria biomarker, parasite lactate dehydrogenase (pLDH). A gold sensor was functionalised with anti pLDH and demonstrated low limit of detections (LOD) towards pLDH in serum samples. When the sensor was used in combination with gold nanoparticles for signal amplification, pg ml^{-1} detection limits were obtained in spiked serum samples. The developed sensor demonstrated superior performance when compared to OptiMAL-IT ICT kit (detect pLDH) and the BinaxNOW ICT commercial kits for the detection of pLDH in the $Dd2^{luc}$ culture medium supernatant at 0.002 % parasitaemia. With a global focus on malaria elimination, the proposed sensor will provide a sensitive, low cost, easy to use detection platform suitable for use in the developing world at the point of care. To the best of our knowledge, this is the first reported immunosensor which utilizes colloidal gold nanoparticles for signal amplification of a amperometric immunosensor for the detection of pLDH, although a recent report on an immunochromatographic assay used in conjunction with colloidal gold for the detection of *Plasmodium falciparum* malaria showed 86.6% sensitivity and 96.4% specificity [41].

2. Materials and methods

2.1 Materials

Phosphate buffered saline tablets (PBS, pH 7.4), PBST (0.05 v/v Tween-20, phosphate citrate buffer tablets, sodium hydroxide, potassium chloride (KCl), sodium carbonate, sodium bicarbonate, 3,3´5,5´-tetramethyl benzidine hydrochloride hydrate (TMB) (powder), colloidal gold, hydrogen peroxide, 95% ethanol, potassium ferricyanide [$K_3Fe(CN)_6$] and human serum (P2918) were purchased from Sigma Aldrich (Dorset, UK). Milk concentrate blocking solution was purchased from KPL (USA). Commercial AuNPs (40 nm) were purchased from BBI. All chemicals and solvents were analytical or HPLC grade and were used without further purification. For the development of pLDH immunosensor, HuCAL monoclonal capture antibody (*Plasmodium falciparum* HCA 158), and detection antibody (horse radish peroxidase-conjugated *Plasmodium falciparum* HCA 157 P) were purchased from AbDSerotec, now Bio-Rad, (Kidlington, UK) while recombinant *P. falciparum* pLDH (CSB-EP632935PLS) was purchased from 2B Scientific (Oxford, UK). Roswell Park Memorial Institute (RPMI) 1640 culture medium; Lot no. RNBF4698, HEPES buffer, glucose solution, NaOH, glutamine solution, 1000x hypoxanthine solution, pooled human serum, Albumax-II, BSD medium and gentamycin sulphate solution were obtained from Sigma (Dorset, UK). Giemsa stain (20 %), 100% methanol, 70% alcohol, 5% sorbitol, passive lysis buffer, luciferase substrate (luciferin) and pH test Strips Lot 010B164786, were purchased form Sigma (St. Louis, USA). OptiMAL-IT malaria kit (containing dipstick, buffer ampoule, reaction / wash wells, with Pasteur pipette) and positive control were purchased from Bio-Rad (Oxford, UK). BinaxNOW Malaria test kit (lateral flow device and wash buffer) was purchased form Alere (Bedford, UK).

2.2 Apparatus

Electrochemical measurements were made using a computer-controlled four channel Autolab electrochemical analyser multipotentiostat (Metrohm, The Netherlands). Gold based screen-printed electrodes comprising of a gold working electrode (0.226 cm^2 planar area) as the anode, carbon counter to balance three electrode system and Ag/AgCl reference electrode were used for all electrochemical measurements and were supplied by DuPont Microcircuit Materials (JD 2b, DuPont Microcircuit Materials, Bristol, UK). Electrodes were printed onto 125 µm thick polyethylene tetraphthalate (PET) sheets. Suspended particles in Giemsa stain solution were removed using 0.45 nm syringe-driven filter unit, Millipore (Dublin, Ireland). GloMax luminometer, Promega (Madison, USA) and autoclave from Priorclave (London, UK) were used in cell culture and luciferase assay. Light microscope, immersion oil and

microscope slides purchased from Olympus (Essex, UK). A stainless steel hand counter (available at miniscience.com) were used in calculating percentage parasitaemia. Experiments were conducted in NuAire DH Autoflow, microbial safety cabinet (ON, Canada).

2.3 Capture and detection antibody optimization

Capture antibody optimization was carried out using HuCAL parasite L-LDH, a ubiquitous enzyme detectable in serum during active infection. To control cost of HuCAL reagents, assay optimization procedures and blocking reagents were adopted from the previous work on *Pf*HRP 2 immunosensor [40]. Prior to the immobilization of antibodies, the sensors were cured at 120°C for 30 minutes. The electrodes were then washed twice in 1000 µL of DI water and dried in gentle nitrogen stream. The working gold electrode was coated with 20 µL pLDH capture antibody (HCA 158) at concentrations of 2.5 µg mL^{-1}, 5.0 µg mL^{-1}, 10 µg mL^{-1} and 20 µg mL^{-1} using sodium carbonate-bicarbonate buffer (1 M, pH 9.6). After the incubation step (overnight at 4 ^{o}C), the electrode was washed two times using 1000 µL PBST (phosphate buffered saline with 0.05 % Tween - 20) and twice with 1000 µL de -ionised water then dried in gentle nitrogen stream. The sensor surface was then treated with a 100 µL blocking buffer solution containing milk protein concentrate (KPL Inc., Gaithersburg, USA) diluted 1:10 milk concentrate / PBS buffer (0.01 M, pH 7.4.) to block the surface and reduce non-specific binding (1 hour at 37°C). After repeating the wash step, 20 µL of 30 µg mL^{-1} of recombinant L-LDH antigen prepared in 1: 10 milk PBS then was pipetted on the working electrode and incubated for 1 hour at 37°C. Wash step was repeated to remove unbound material with 1000 µL PBST (0.05 v/v) followed by 1000 µL de-ionized water (18.2 mΩ). The purified detection antibody HCA 157 P was diluted to a working strength of 30 µg mL^{-1} in 1:40 milk concentrate / PBST (0.05 v/v). A 20 µL volume was applied to the working electrode and incubated for 1 hour at 37°C. The sensor surface was then washed with buffer before the addition of 100 µL of the TMB / H_2O_2 freshly prepared solution.

For optimization of detection antibody, the above procedure was repeated using the best concentration of capture antibody immobilised on the sensor surface. A 20 µL volume of parasite L-LDH capture antibody (10 µg mL^{-1}) in sodium carbonate-bicarbonate buffer (1 M, pH 9.6) was immobilized on the WE overnight at 4ºC. The sensor was then blocked and washed before the addition of pLDH analyte following the above procedure. The purified detection antibody (HCA 157 P) which is attached to the enzyme HRP was diluted to working strengths of 10, 20 and 30 µg mL^{-1} in PBST. A 20 µL volume was dropped on the

working electrode and incubated for 1 hour at 37°C. Chronoamperometry detection was then conducted by adding 100 µL of 4 mM TMB and 0.06% H_2O_2 in 50 mM phosphate citrate buffer, 0.1 M KCl (pH 5.0) onto the electrode and measuring the current at -0.2 V.

2.4 pLDH detection in buffer and serum samples

To determine the limit of detection of the sensor for recombinant *P. falciparum* parasite L-LDH, 20 µL of 10 µg mL^{-1} parasite L-LDH capture antibody (HCA 158) in sodium carbonate-bicarbonate buffer (0.1 M, pH 9.6) was immobilized on the working electrode and incubated overnight at 4°C. After incubation, washing, and blocking steps, recombinant L-LDH (CSB-EP632935PLS) antigen was serially diluted in 1:10 milk / PBS (0.01 M, pH 7.2) from 0.1, 1, 10, 100, 1000, 10,000 and 100,000 ng mL^{-1}. A 20 µL volume was dropped on the working electrode and incubated at 37°C for 1 hour. The wash step was repeated and the detection antibody (HCA 157 P) was diluted to a working strength of 10 µg mL^{-1} in 1: 40 milk concentrate / PBS 0.01 M). Chronoamperometry detection was performed in the same manner as described for the recombinant protein standard in buffer. Lowest detection of antigen (LOD) was calculated as (3 x SD of the blank measurement) + (average blank measurement) and interpolated using GraphPad prism (version 5).

To assess the electrochemical response in serum samples, the assay was conducted as described above using 100% commercial Human serum (P2918) from Sigma (Dorset, UK). A serial dilution of L-LDH recombinant protein from 0.1, 1, 10, 100, 1000, 10,000 and 100,000 ng mL^{-1} was prepared in the serum samples and used on the sensor. All experiments were performed in triplicate.

Amplification using Gold Nanoparticle assays were conducted after attaching the detection antibody-HRP to the particles. Gold nanoparticles (40 nm) were conjugated to the detection antibody (HCA 157 P) - HRP according to the modified method of Salam and Tothill [42].

2.5 Culture of malaria parasites Dd2^{luc}

The media preparation and *in-vitro* culture of *P. falciparum* cells was conducted in a level 3 laboratory. For over 40 years, Roswell park memorial institute (RPMI) 1640 medium has been commonly used for *in-vitro* parasite culture preparation using a well-established protocol [43]. A transgenic *Plasmodium falciparum* clone expressing luciferase under the control of *Pfpcna* flanking sequences [44] Dd2 ^{luc} was cultured in RPMI medium. A 500 mL volume of RPMI 1640 medium was taken in a tissue culture bottle and supplemented with 1

M HEPES buffer (pH 7.4, 18.75 mL), 45% glucose solution (2 mL), 1 M NaOH (2.5 mL), 200 mM glutamine solution (5 mL), 1000x hypoxanthine solution (0.1 M solution in NaOH); (500 µL), pooled human serum (20 mL) 5% Albumax-II (20 mL) BSD (125 µL), 10 mg mL^{-1} gentamycin sulphate solution (1.25 mL) and 8% v/v human serum) at the desired haematocrit in an atmosphere of 1% Oxygen / 3% CO_2 / N_2 ; in a modified method according to Hasenkamp et al., [45]. RPMI medium prepared in tissue culture flasks, using 0.5%, 1%, 2% and 4% early trophozoites and 0.5%, 1%, 2% and 4% mixed stage cultures in a second group. Both groups had culture media (plain and containing uninfected RBC) as control. Following inoculation, the samples were incubated at 37°C for 48 hrs.

The supernatant was obtained from the tissue culture flask collected in falcon tubes. The tubes were spun at 3,500 rpm for 5 minutes at room temperature (18-25°C). The supernatant now devoid of plasmodial cells (6% parasitaemia) was separated from the pellet and stored in aliquots of 1 mL at -20°C to be used for subsequent immunoassay tests and the biosensor development.

2.6 Blood film preparation

After a 48 hr incubation period, plasmodium parasite went through asexual developmental stages [46]. To observe the parasites by microscopy, the culture was removed from incubation at 37°C between 26-38 hours to target the trophozoites [47]. A drop (~20 µL) of the sediment containing cultured parasites was transferred to a clean, dry microscope slide. The film was made by spreading the drop on the slide. The film was gently blow dried. The film was fixed for 1 minute using methanol and washed in tap water. Giemsa stain (20%) was filtered using syringe-attached microfilter (0.45 nm) to remove particles. The filtered stain was applied to cover the dry film and allowed to stain for 10 minutes. The slide was then washed in tap water and gently blow dried. The dried film was then immersed in oil and observed using a microscope. The estimation of percentage parasitaemia was determined from counting infected red blood cells (parasitized cells). The percentage parasitaemia was then estimated by the following equation [48]:

$$\%\, parasitaemia = \frac{Average\ 10\ parasitized}{Average\ 3\ parasitized + non\ parasitized}\, x\ 100$$

Where the average of 10 counted fields of parasitized cells is divided by the average of 3 counted fields of the total visible cells i.e. parasitized and non- parasitized cells. When not in use, the samples were stored at -20°C. To determine viability of the culture *P. falciparum* ($Dd2^{luc}$ cells), luciferase assay was conducted on the cultured $Dd2^{luc}$ cells, following culture of the parasites, at 0 h and again at 48 hr [47].

2.7 Sorbitol lysis to obtain developmental stage parasites

Developmental stages were separated using staging. Briefly, samples were synchronised (lysis of non-target developmental stages) at 0 h and again at 48 hr incubations at 37°C. Parasite stages were then separated by adding 5% sorbitol (five times the pellet) to each pellet and incubated at 37°C for five minutes. The pellet was then centrifuged at 1,800 rpm for 5 minutes. Following incubation, the supernatant was removed and the pellet re-suspended in RPMI 1640 culture medium and then transferred to a sterile tissue culture flask. The medium was gassed in 1% Oxygen / 3% (CO_2 / N_2) and incubated for 48 hrs at 37°C to obtain a pure culture of *P. falciparum* trophozoites.

2.8 Comparison of the Developed Sensor against Commercial kits

The developed sensor was compared against both the OptiMAL-IT and *BinaxNOW* commercial malaria kits by determination of recombinant LDH in 100% commercial serum.

The pOptiMAL-IT assay was performed according to the manufacturer's instructions. Briefly, 1 drop (20 µL) from the provided buffer ampoule was added to the conjugate well already coated with pLDH antibody, and 4 drops (80 µL) were added to the wash well then allowed to stand for one minute. A concentration of 100 µg mL^{-1} and 0.8 mg mL^{-1} (20 µL) recombinant pLDH in 100% serum was drawn up using the pipette. The entire volume was added to the conjugate well. The wells were allowed to stand for 10 minutes so the sample / conjugate mixture was completely soaked up. The dipstick was transferred to the wash well and allowed to stand for 10 minutes so that the reaction was completely cleared of unreacted serum leaving the control band clearly visible. The reaction was read immediately after following the manufacturer's instructions.

To test $Dd2^{luc}$culture medium supernatant on the OptiMAL-IT, the same process was conducted on the dipstick assay using 20 µL of 3-fold serially diluted culture medium supernatant.

2.9 pLDH detection using culture medium supernatant on the biosensor

The immunosensor for detection of pLDH was applied here using a 20 µL volume of 6% parasitaemia Dd2^{luc} culture medium supernatant. The culture medium supernatant was aspirated from the tissue culture flasks and the sample containing soluble *Plasmodium falciparum* biomarkers *Pf*HRP 2 and pLDH was then serially diluted 3-fold and used in the electrochemical sensor to detect pLDH.

2.10 BinaxNOW Malaria lateral flow assay

BinaxNOW Malaria is an *in-vitro* immunochromatographic assay using monoclonal antibody (MoAb) for the quantitative detection of Plasmodium antigens circulating in human venous and capillary ethylenediaminetetraacetic acid (EDTA) whole blood of individuals with signs and symptoms of malaria infection. The kit for pLDH and *Pf*HRP 2 malaria detection allows for the differentiation of *P. falciparum* from other less virulent species. The kit was investigated for sensitivity of the test on culture medium supernatant, according to manufacturer's instructions. The same samples used for the OptiMAL-IT assay and the biosensor were defrosted (from -20°C to room temperature, 18-25°C). A volume of 15 µL of the asynchronous and synchronous culture medium supernatant (0%, 0.5% and 4%) were applied to the bottom half of the purple reaction pad together with a blank in triplicate. Two (2) free-falling drops (~40 µL) of Reagent A were added vertically to the white pad below the purple pad. The sample ran until it almost touched the absorbent pad at the top of the strip. Just before the sample reached the base of the white absorbent pad, four (4) free-falling drops of Reagent A were applied to the pad. When the sample just reached the base of the absorbent pad, the adhesive strip was removed then the device was closed. From closing, the reaction was timed for 15 minutes. The result was read through the viewing window.

3. Results and discussions

3.1 Antibody optimization

Capture antibody optimization was conducted using a sandwich assay on the SPGE is shown in **Figure 1A**. Recombinant protein L-Lactate dehydrogenase (CSB-EP632935PLS) and detection antibody conjugated to horse radish peroxidase (HCA 157 P) concentrations were kept constant at 30 µg mL^{-1} while concentration of capture antibody (HCA 158) was varied as 2.5 µg mL^{-1}, 5 µg mL^{-1}, 10 µg mL^{-1} and 20 µg mL^{-1}. Signal is average of three sensors per concentration (embedded graph). Capture antibody optimization shows an increase in signal

until saturation at 10 μg mL^{-1}. This implies that 10 μg mL^{-1} is the optimal concentration for the assay.

To determine the most suitable detection antibody- horse radish peroxidase concentration for the sensor, sandwich procedure was conducted as above. Signal obtained from electrochemical detection of pLDH using capture antibody 10 μg mL^{-1}. Detection antibody concentrations of 10, 20, and 30 μg mL^{-1} were tested. Signal saturation was found at 10 μg mL^{-1} antibody concentration. The profile of amperometric signal is shown in **Figure 1B**.

3.2 pLDH detection using the optimised sensor

The developed immunosesor with the optimised capture (10 μg mL^{-1}) and detection (10 μg mL^{-1}) antibodies was used to quantify pLDH and conduct an analyte dose response assay. Standard curve was obtained by testing the immunosensor response to pLDH at different concentrations (0.1 ng mL^{-1} to 100 μg ml^{-1}) in buffer samples. Serum samples spiked with pLDH were prepared at the same final concentrations as described for buffer samples. Capture and detection antibody concentrations kept constant at 10 μg mL^{-1} for all measurements. Blank reading was subtracted from the signal and data plotted against pLDH concentration. All measurements were performed in triplicate. The developed assay displayed a limit of detection (LOD) of 1.80 ng mL^{-1} and 0.70 ng mL^{-1} for pLHD in buffer and spiked serum samples respectively as shown in **Figure 2A-B**. Although the serum sample showed a slightly lower detection limit, the difference in detection limits may be due to a better blocking of the electrode surface with serum proteins with this patch of sensors taking into consideration some systematic errors in the analysis, suggesting the effect of the matrix on the sensor measurement may be minimal.

3.3 AuNP signal assay amplification

The detection antibody (HCA 157 P) and horse radish peroxidase were conjugated to commercial available AuNPs (40 nm) which was used for all subsequent pLDH measurements of pLDH in buffer and pLDH spiked serum samples. The final AuNP amplified immunoassay, showed an increased sensitivity, demonstrating an LOD of 0.019 ng mL^{-1} and 0.023 ng mL^{-1} in buffer and pLDH spiked serum samples respectively. These results demonstrate the excellent sensitivity and reproducibility and robustness of the developed sensor. As reported, blood levels of pLDH of ~4.9 ng mL^{-1} have been reported in

the literature suggesting that our developed sensor is more than capable of detecting pLDH in real clinical blood samples [49, 34-35].

3.4 Estimation of percentage parasitaemia and cells viability

The percentage parasitaemia was estimated after thin film identification of parasites in infected red blood cells by microscopy slide evaluation (**Figure 4 A)**. Parasitaemia due to malaria infection has been reported to have a threshold of 2,500 parasites per microliter [50]. The number of visible parasites should be reported especially where the infection is due to *P. falciparum*. Giemsa dye commonly used for routine examination of stained blood films for malaria diagnosis [7, 51]. Levels of parasitaemia are not necessarily correlative with the progression of the disease, particularly when the parasite is able to adhere to blood vessel walls.

Luciferase assays were conducted to determine the viability of the Dd2^{luc} culture by measuring luciferase expression in cultured samples. Both groups of synchronized (synchronized samples contained parasites at same developmental stages) and asynchronized (asynchronized samples contained all stages of parasite development) trophozoite samples with parasitaemia ranging from 0.5 - 4% showed growth when compared to the control. The control contained no parasites. Two controls were used namely: RPMI medium only (M) and RPMI + uninfected red blood cells (RBC); (**Figure 4 B).** Since the transgenic strain, Dd2^{luc} expresses luciferase in the trophozoite stage, all the samples showed luminescence response. Luciferase assays have been used widely in high throughput screening for drug candidates [45] and to observe cell growth. With culture medium supernatant of known parasitaemia, the samples were then tested using the immunochromatographic tests.

3.7 LDH detection using immunochromatographic tests kit

The test was carried out to examine pure recombinant pLDH antigen (10 µg mL^{-1} and 0.8 mg mL^{-1}) in 100% serum samples first on the OptiMAL-IT LFD kit. The results showed negative readings at both concentrations while the internal control (Positive pLDH control from the kit) confirmed the test validity and gave positive readings. These results confirmed the kit manufacturer's assertion that the test kit is only valid when live parasites are present in the sample. A comparison was then made with the *P. falciparum* culture medium supernatant to assess the validity of the results. The culture medium supernatant was diluted 3-fold in blank RPMI 1640 medium from 6% parasitaemia sample and tested until no band was seen on the

LFD. The culture medium supernatant showed a progressively lighter band with serial dilution of the sample as shown in (**Figure 5A)**. A band was seen at 2% parasitaemia, but no band in dilution number two was visible (0.007%). According to OptiMAL-IT kit manufacturer, the kit can detect parasitaemia levels of 0.001-0.002 % (50-100 parasites per μL of blood). The variation in sensitivity may be affected by dilution used as opposed to the direct supernatant obtained from a culture of specified parasitaemia. Also, it is not clear if the values are for staged parasites.

BinaxNow Malaria kit is sensitive to both histidine rich protein 2 and lactate dehydrogenase malaria biomarkers, so it combines both markers detection for the diagnosis of malaria. This kit was therefore used in this work to test the *P. falciparum* culture medium supernatant used for the OptiMAL-IT kit. The results **(Figure 5B**), showed that synchronized and asynchronized samples of 4% parasitaemia showed positive *Pf*HRP 2 / LDH. While at 0.5% parasitaemia, both synchronized and asynchronized samples gave negative readings in the culture medium supernatant. Lateral flow devices are sensitive to small volumes of malaria antigens in blood samples. The sensitivity of these devices relies on the species and number, the viability and difference in amount of parasite antigen as well as the condition (storage ≤30ºC) of the device and the efficacy of the method [52]. Previous work has been reported on the use of monoclonal antibodies in conjunction with colloidal gold in an immunochromatographic assay for *Plasmodium falciparum* malaria detection showing 86.6% sensitivity and 96.4% specificity [53]. Immunochromatographic tests may be used to complement microscopy in developing countries at the point-of-care [54-56].

The samples tested on the test kits were also tested for comparative sensitivity using the developed immunosensor for Pan-malaria amperometric detection. The biosensor developed in this work successfully detected pan malaria antigen pLDH in the sample of $Dd2^{luc}$ culture medium supernatant. Amperometric signal using culture medium supernatant shows similar results to the signals obtained in pLDH detection (**Figure 6**). Results of culture supernatant test using the immunosensor show signals were detected in samples with low paraeitemia (0.002%). The range of amperometric signal reflects the change in the analyte concentration. Smallest signal is at -0.11 μA, which was obtained after subtracting the difference of the background of blank RPMI 1640 culture medium. The results in **Figure 6** imply that the immunosensor can be used for the detection of malaria infected blood samples through the detection of parasite lactate dehydrogenase (pLDH), an enzyme common to all malaria parasites with high sensitivity.

4. Conclusion

An immunosensor for the detection of pan malaria antigen parasite L-Lactate dehydrogenase was successfully developed and compared to commercial malaria kit OptiMAL –IT and BinaxNow Malaria kit. The sensor showed high sensitivity and reproducibility, cost effective point-of-care immunoassay which can performed in < 2 hours. The developed sensor has the potential to compete against microscopy as the gold standard in malaria diagnostics. Sample Matrix effects were found not to be significant in the assay, a good indication that the limit of detection within real clinical samples will be within the range detectable. The sensor could potentially be used with a mobile networked device and hence, evaluation and impact assessments can be conducted in a point-of-care manner. The sensor was compared to commercially available malaria assays using *Plasmodium falciparum* culture medium supernatant. The immunoassay was able to detect the antigen in samples of buffer, commercial serum and culture medium supernatant below the detection limit of both the OptiMAL-IT and BinaxNow Malaria kits. Future experiments will be performed on clinically relevant blood samples.

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References

[1] World Health Organization. 2014. Malaria fact sheet on malaria reduction (http://www. who.int/).

[2] World Health Organization, World Malaria report 2017, Geneva: World Health Organization; 2017. Licence: CC BY-NC-SA 3.0 IGO. http://www.who.int/malaria/publications/world-malaria-report-2017/en/

[3] World Health Organization. 2015. Projected changes in malaria incidence rates from 2000 to 2015 / Malaria world map, taken from the World Health Organization website http://www.who.int/gho/malaria/en/.

[4] World Health Organization Malaria fact sheet 2016. http://www.who.int/malaria/media/world-malaria-report-2016/en//.

[5] Jain, P., Chakma, B., Patra, S. and Goswami, P. 2014. "Potential Biomarkers and Their Applications for Rapid and Reliable Detection of Malaria", BioMed research international, Article ID 852645, 20. http://dx.doi.org/10.1155/2014/852645

[6] Castellanos, M.E., Diaz, S., Parsons, E., Peruski, L.F., Enriquez, F., Ramirez, J.L. and Padilla, N. (2015). "First imported *Plasmodium ovale* malaria in Central America: case report of a Guatemalan soldier and a call to improve its accurate diagnosis". Military Medical Research 2: 1, 3. doi: 10.1186/s40779-015-0030-9

[7] Fleischer, B. 2004. Editorial: 100 years ago: Giemsa's solution for staining of plasmodia. Tropical Medicine & International Health 9, 755-756. doi:10.1111/j.1365-3156.2004.01278.x

[8] Adeoye, G. and Nga, I. 2007. Comparison of Quantitative Buffy Coat technique (QBC) with Giemsa-stained Thick Film (GTF) for diagnosis of malaria. Parasitology International. 56, 308-312. doi: 10.1016/j.parint.2007.06.007

[9] Mubi, M., Janson, A., Warsame, M., Martensson, A., Kallander, K., Petzold, M. G., Ngasala, B., Maganga, G., Gustafsson, L. L. Massele, A. 2011. Malaria rapid testing by community health workers is effective and safe for targeting malaria treatment: randomised cross- over trial in Tanzania PloS one. 6, 7. https://doi.org/10.1371/journal.pone.0019753

[10] Merwyn, S., Gopalan, N., Singh, A. K., Rai, G. P., Agarwal, G.S. 2011. Monoclonal antibodies against recombinant histidine-rich protein 2 of *Plasmodium falciparum* and their use in malaria diagnosis. Hybridoma. 30, 519–524. doi: 10.1089/hyb.2011.0061.

[11] Thongdee, P., Chaijaroenkul, W., Kuesap, J. and Na-Bangchang, K. 2014. Nested-PCR and a New ELISA-Based NovaLisa Test Kit for Malaria Diagnosis in an Endemic Area of Thailand. The Korean journal of parasitology. 52, 377-381. doi: 10.3347/kjp.2014.52.4.377

[12] Scholl, P. F., Kongkasuriyachai, D., Demirev, P. A., Feldman, A. B., Lin, J. S., Sullivan, D. J., Jr and Kumar, N. 2004. Rapid detection of malaria infection in vivo by laser desorption mass spectrometry. The American Journal of Tropical Medicine and Hygiene 71, 546-551.

[13] MaIERA Consultative Group on Diagnoses and Diagnostics. 2011. A Research Agenda for Malaria Eradication: Diagnoses and Diagnostics. PLoS Med. 8, 1. https://doi.org/10.1371/journal.pmed.1000396

[14] Grubaugh, N. D., Petz, L. N., Melanson, V. R., McMenamy, S. S., Turell, M. J., Long, L. S., Pisarcik, S. E., Kengluecha, A., Jaichapor, B., O'Guinn, M. L. and Lee, J. S. 2013. Evaluation of a field-portable DNA microarray platform and nucleic acid amplification strategies for the detection of arboviruses, arthropods, and bloodmeals. The American Journal of Tropical Medicine and Hygiene. 88, 245-253. doi: 10.4269/ajtmh.2012.12-0048

[15] Ter Kuile, F.O., Parise, M. E., Verhoeff, F. H., Udhayakumar, V., Newman, R. D., van Eijk, A. M., Rogerson, S. J., Steketee, R.W. 2004. The burden of co-infection with human immunodeficiency virus type 1 and malaria in pregnant women in sub-saharan Africa. Am J Trop Med Hyg. 71, 41– 54.

[16] Grobusch, M. P., Hänscheid, T., Krämer, B., Neukammer, J., May, J., Seybold, J., Kun, J. F., Suttorp, N. 2003. Sensitivity of hemozoin detection by automated flow cytometry in non- and semi-immune malaria patients. Cytometry B. Clin. Cytom. 55, 46–51. doi: 10.1002/cyto.b.10039

[17] Wood, B.R. Bailo, E., Khiavi, M. A., Tilley, L. Deed, S., Deckert-Gaudig, T. 2011. Tip-enhanced Raman scattering (TERS) from hemozoin crystals within a sectioned erythrocyte Nano Letter. 11, 1868–1873. doi:10.1021/nl103004n

[18] Yuen, C., Liu Q. 2012. Magnetic field enriched surface enhanced resonance Raman spectroscopy for early malaria diagnosis J Biomed Opt, 17. 017005. doi: 10.1117/1.JBO.17.1.017005.

[19] Garrett, N. L. Sekine, R., Dixon, W. A. M. Tilley, Leann K., Bambery, R., Bayden, R., Wood, B. R. 2015. Bio-sensing with butterfly wings: Naturally occurring nano-structures for SERS-based malaria parasite detection Phys Chem Chem Phys. http://dx.doi.org/10.1039/C4CP04930F.

[20] Joshi V., Agurto C., Barriga, S. Nemeth, S., Soliz, P., MacCormick, I., Taylor, T., Lewallen, S. and Harding, S. 2016. Automated detection of retinal whitening in malarial retinopathy. Proc. SPIE 9785, Medical Imaging: Computer-Aided Diagnosis 97852G. doi:10.1117/12.2217188.

[21] Portnoy, E., Vakruk1, N. Bishara, A., Shmuel. M., Magdassi, S. Golenser, J. and Eyal, S. 2016. Indocyanine Green Liposomes for Diagnosis and Therapeutic Monitoring of Cerebral Malaria. Theranostics 6, 167-176. doi: 10.7150/thno.13653.

[22] Luo, S., Zhang, E., Su, Y., Cheng, T., Shi, C. 2011. A review of NIR dyes in cancer targeting and imaging. Biomaterials. 32, 7127-7138. doi: 10.1016/j.biomaterials.2011.06.024..

[23] James, M. L., Gambhir, S.S. 2012. A molecular imaging primer: modalities, imaging agents, and applications. Physiol Rev. 92, 897-965. doi: 10.1152/physrev.00049.2010

[24] Nacer, A., Movila, A., Sohet, F., Girgis, N. M., Gundra, U. M., Loke, P. 2014. Experimental cerebral malaria pathogenesis-hemodynamics at the blood brain barrier. PLoS Pathog. 10, 10. https://doi.org/10.1371/journal.ppat.1004528

[25] Pai, S., Qin, J., Cavanagh, L., Mitchell, A., El-Assaad, F., Jain R. 2014. Real-time imaging reveals the dynamics of leukocyte behaviour during experimental cerebral malaria pathogenesis. PLoS Pathog. 10: e1004236. https://doi.org/10.1371/journal.ppat.1004236

[26] Mann, A., Semenenko, I., Meir, M. Eyal, S. 2015. Molecular imaging of membrane transporters' activity in cancer: a picture is worth a thousand tubes. The AAPS journal. 17, 788-801. doi: 10.1208/s12248-015-9752-6

[27] Zhao, Y., MacCormick, I. J, Parry, D. G. Beare, N. A., Harding, S. P., Zheng, Y. 2015. Automated Detection of Vessel Abnormalities on Fluorescein Angiogram in Malarial Retinopathy. Sci Rep. 5, 11154. doi: 10.1038/srep11154

[28] Ittarat, W., Chomean, S., Sanchomphu, C., Wangmaung, N., Promptmas, C. and Ngrenngarmlert, W. 2013. Biosensor as a molecular malaria differential diagnosis, Clinica Chimica Acta. 419, 47-51. doi: 10.1016/j.cca.2013.01.010

[29] Brince, K. P., Kumar, S., Tripathy, S., Vanjari, S. R. K., Singh, V. and Singh, S.G. 2016. A highly sensitive self-assembled monolayer modified copper doped zinc oxide nanofiber interface for detection of *Plasmodium falciparum* histidine-rich

protein-2: Targeted towards rapid, early diagnosis of malaria. Biosensors and Bioelectronics. 80, 39-46. doi: 10.1016/j.bios.2016.01.036.

[30] Gikunoo, E., Abera, A., Woldesenbet, E. 2014. A novel carbon nanofibers grown on glass microballoons immunosensor: a tool for early diagnosis of malaria. Sensors, 14, 8, 14686-14699. doi: 10.3390/s140814686

[31] Figueroa, M. G., Feng, L., Shiu, S. C., Marshall D, R., Cheung, Y., Tanner, J., Josef S., M., Offenhäusser, A., Mayer, D. 2018. Aptamer-Based Electrochemical Biosensor for Highly Sensitive and Selective Malaria Detection with Adjustable Dynamic Response Range and Reusability. Sensors and Actuators B: Chemical. 255, 235-243. https://doi.org/10.1016/j.snb.2017.07.117

[32] Rock, E.P., Marsh, K., Saul, A.J., Wellems, T.E., Taylor, D.W., Maloy, W.L., Howard, R.J. Comparative analysis of the Plasmodium falciparum histidine-rich proteins HRP-I, HRP-II and HRP-III in malaria parasites of diverse origin. Parasitology 1987, 95, 209–227.

[33] Frank, R. and Hargreaves, R. (2003). "Clinical biomarkers in drug discovery and development". Nature Reviews Drug Discovery 2: 566-580. doi:10.1038/nrd1130

[34] Dzakah, E.E., Kang, K., Ni, C., Tang, S., Wang, J. and Wang, J. 2014. Comparative performance of aldolase and lactate dehydrogenase rapid diagnostic tests in *Plasmodium vivax* detection. Malaria journal. 13, 272. https://doi.org/10.1186/1475-2875-13-272

[35] Dirkzwager, R. M., Kinghorn. A. B., Richards, J. S. 2015. APTEC: aptamer-tethered enzyme capture as a novel rapid diagnostic test for malaria. Chemical Communications. 51, 4697-4700. doi:10.1039/c5cc00438a

[36] Phumin, S., Sittiporn, P. and Pongchai, H. 2018. Global sequence diversity of the lactate dehydrogenase gene in *Plasmodium falciparum. Malaria Journal* 17, 16. doi: 10.1186/s12936-017-2157-5.

[37] Lee, S., Song, K., Jeon, W., Jo, H., Shim, Y., Ban, C. 2012. "A highly sensitive aptasensor towards Plasmodium lactate dehydrogenase for the diagnosis of malaria". *Biosensors and Bioelectronics.* 35, 291-296. doi: 10.1016/j.bios.2012.03.003

[38] Markwalter, C.F., Davis, K.M., Wright, D.W. 2016. Immunomagnetic capture and colorimetric detection of malarial biomarker *Plasmodium falciparum* lactate dehydrogenase. Analytical Biochemistry 493, 30-34. doi: 10.1016/j.ab.2015.10.003

[39] Markwalter, C.F., Ricks, K.M., Bitting, A. L., Mudenda, L., Wright, D.W. 2016. Simultaneous capture and sequential detection of two malarial biomarkers on magnetic microparticles. Talanta, 161, 443–449. doi: 10.1016/j.talanta.2016.08.078.

[40] Hemben, A., Ashley, J. and Tothill, I.E. 2017. Development of an Immunosensor for *Pf*HRP 2 as a Biomarker for Malaria Detection, Biosensors (MDPI), 7, 28; doi:10.3390/bios7030028.

[41] Lee, N., Baker, J., Andrews, K. T., Gatton, M. L., Bell, D., Cheng, Q., McCarthy, J. 2006. Effect of sequence variation in *Plasmodium falciparum* histidine-rich protein 2 on binding of specific monoclonal antibodies: Implications for rapid

diagnostic tests for malaria. Journal of clinical microbiology. 44, 2773-2778. doi: 10.1128/JCM.02557-05

[42] Salam, F., Tothill, I.E. Detection of *Salmonella typhimurium* using an electrochemical immunosensor. Biosens. Bioelectron. 2009, 24, 2630–2636. https://doi.org/10.1016/j.bios.2009.01.025

[43] Desai, S. A. 2013. Insights Gained from *P. falciparum* Cultivation in Modified Media. The Scientific World Journal. 10, 1155. doi: 10.1155/2013/363505

[44] Wong, E.H., Hasenkamp, S., Horrocks, P. 2011. Analysis of the molecular mechanisms governing the stage-specific expression of a prototypical housekeeping gene during intraerythrocytic development of *P. falciparu*. J. Mol Biol 2011, 408:205-221. doi: 10.1016/j.jmb.2011.02.043.

[45] Hasenkamp, S., Wong, E.H. and Horrocks, P. 2012. An improved single-step lysis protocol to measure luciferase bioluminescence in Plasmodium falciparum. Malaria journal. 11, 42. doi: 10.1186/1475-2875-11-42

[46] Coatney, G.R., Collins, W.E., Warren, M. and Contacos, P.G. (1971). The Primate Malarias. U.S. Department of Health, Education and Welfare, Bethesda, 366. doi: 10.1126/science.177.4043.50

[47] Silamut, K. 2000. *Plasmodium falciparum* staging in *in vitro* culture. Mahidol Oxford Research Unit Bangkok: 5.

[48] Neqas UK For Microbiology 2013. http:// www. ukneqasmicro. org.uk /parasitology/ index. php/ blood - parasitology.

[49] Druilhe, P., Moreno, L., Blanc C., Brasseur, P. H., Jacquier, P. 2001. A calorimetric *in vitro* drug sensitivity assay for *Plasmodium falciparum* based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. Am J Trop Med Hyg. 64, 233–241.

[50] Mwangi, T. W., Mohammed, M., Dayo, H., Snow, R. W. and Marsh, K. 2005. Clinical algorithms for malaria diagnosis lack utility among people of different age groups. Tropical Medicine & International Health. 10, 530-536. doi: 10.1111/j.1365-3156.2005.01439.x

[51] Redd, S., Kazembe, P., Luby, S., Nwanyanwu, O., Hightower, A., Ziba, C., Wirima, J., Chitsulo, L., Franco, C., Olivar, M., 1996. Clinical algorithm for treatment of *Plasmodium falciparum* malaria in children. Lancet. 347, 223–227.

[52] Bell, D., Wongsrichanalai, C., Barnell, J. W. 2006. Ensuring quality and access for malaria diagnosis: how can it be achieved? Nat. Rev. Microbiol. 4, 7-20. doi: 10.1038/nrmicro1525

[53] Lee, N., Baker, J., Andrews, K. T., Gatton, M. L., Bell, D., Cheng, Q., McCarthy, J. 2006. Effect of sequence variation in *Plasmodium falciparum* histidine-rich protein 2 on binding of specific monoclonal antibodies: Implications for rapid diagnostic tests for malaria. Journal of clinical microbiology. 44, 2773-2778. doi :10.1128/JCM.02557-05

[54] Bailey, J.W., Williams, J., Bain, B. J., Parker- Williams, J. and Chiodini, P. L. 2013. Guideline: The Laboratory diagnosis of malaria. British journal of haematology. 163, 573-580. doi: 10.1111/bjh.12572

[55] Hailu, T. and Kebede, T. 2014. "Assessing the performance of CareStart Malaria Pf/Pv Combo Test against thick blood film in the diagnosis of malaria in northwest Ethiopia". The American Journal of Tropical Medicine and Hygiene 90: 1109-1112. doi: 10.4269/ajtmh.13-0607

[56] Piper, R., Lebras, J., Wentworth, L., Hunt-Cooke, A., Houze, S., Chiodini, P. and Makler, M. 1999. Immunocapture diagnostic assays for malaria using Plasmodium lactate dehydrogenase (pLDH). The American Journal of Tropical Medicine and Hygiene. 60, 109-118.

Scheme

Scheme 1: Parasite LDH transmission via an infected female Anopheles mosquito into blood stream as a parasite metabolite which is detectable in active infections.

Figures

Figure 1: Chronoamperometric measurements for antibodies optimisation using a sandwich assay; (A) capture antibody optimization (30 µg mL^{-1} detection antibody-HRP and 30 µg mL^{-1} recombinant pLDH), (B) detection antibody-HRP optimization (10 µg mL^{-1} capture antibody and 30 µg mL^{-1} recombinant pLDH); (n=3).

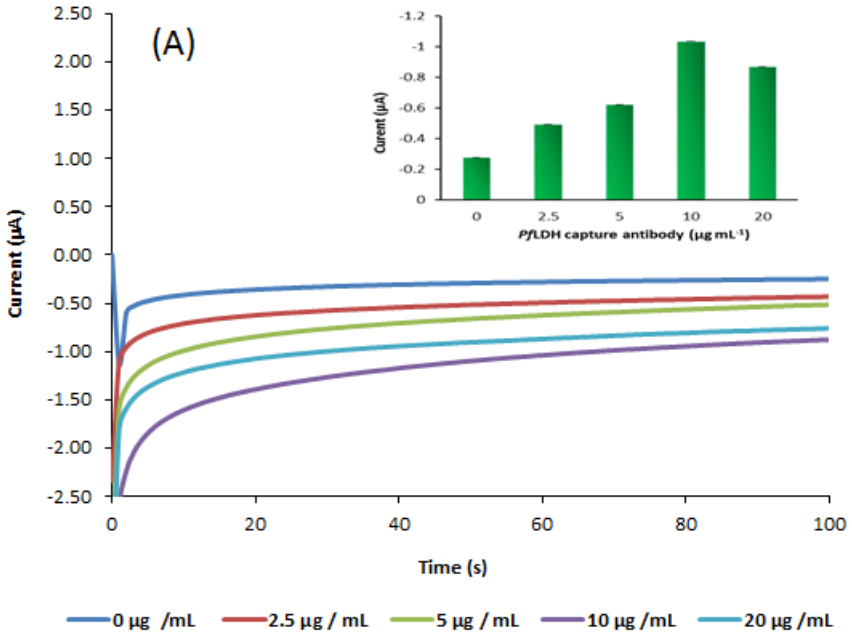
Figure 2: Linear regression analysis of chronoamperometric responses for the detection of (A) pLDH (0.1–100,000 ng mL^{-1}) in 10 mM, PBS pH 7.4 buffer ($y = -0.1269x - 0.1499$, $R^2 = 0.9916$) and (B) pLDH (0.1–100,000 ng mL^{-1}) spiked in 100% serum samples ($y = -0.1205x - 0.1518$, $R^2 = 0.9851$). All measurements were conducted at -0.2 V (n=3**).**

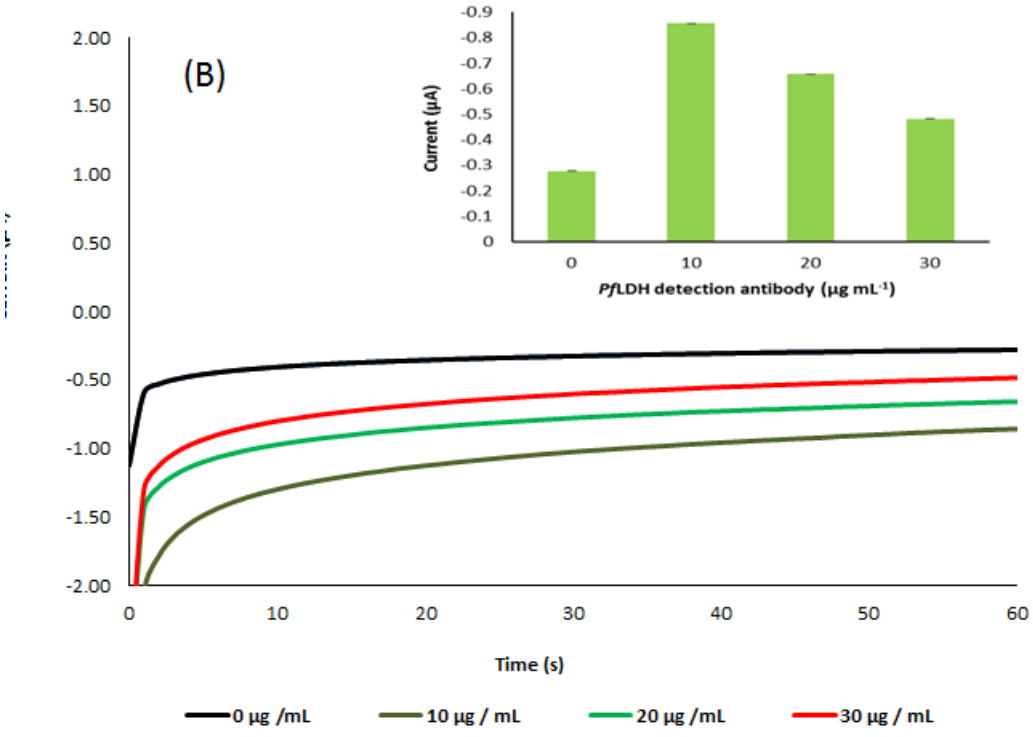
Figure 3: Linear regression analysis of the chronoamperometric response of (A) pLDH (0.1–100,000 ng mL^{-1}) in 10 mM, PBS pH 7.4 buffer ($y = -0.0461x - 0.277$, $R^2 = 0.975$) and (B) pLDH (0.1–100,000 ng mL^{-1}) spiked in 100% serum ($y = -0.0217x - 0.0725$, $R^2 = 0.9805$) using the developed sensor with AuNP-anti-pLDH-HRP at -0.2V (n=3).

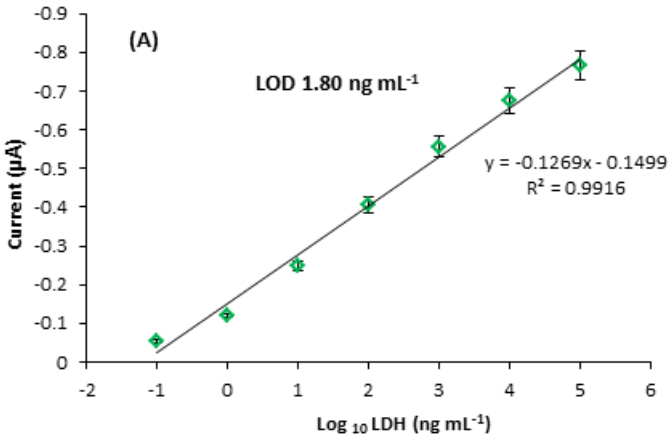
Figure 4: (A) *Plasmodium falciparum* trophozoite in red blood cells, following Giemsa staining of the thin film slide (~20 µL) and visualisation (microscopy, 100x objective lens), **(B)** Relative light unit for luciferase expression in *Plasmodium falciparum* $Dd2^{luc}$ cell culture, viability test assayed at 0 and 48 hrs growth. Both control samples are constant against 0.5%, 1%, 2% and 4% parasitaemia. Controls for the experiment comprised neat culture medium RPMI 1640 (M) and neat medium + uninfected red blood cells (RBC); (n=3).

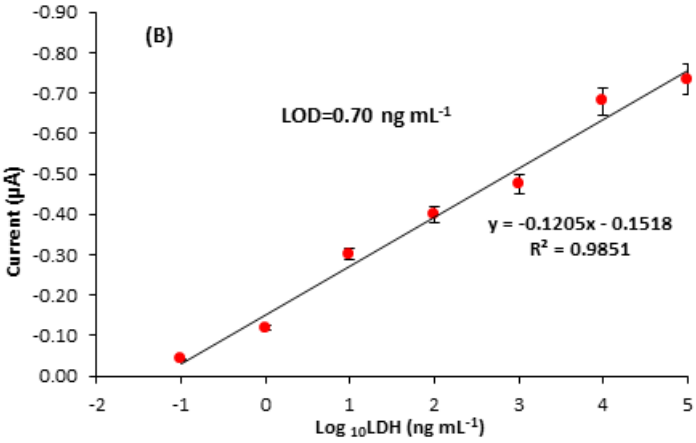
Figure 5: (A) OptiMAL-IT LFD assay for parasite pLDH detection using 3-fold dilution of $Dd2^{luc}$ culture medium supernatant. **(B)**, BinaxNOW LFD Malaria assay for *Pf*HRP 2 and pLDH detection (n=3).

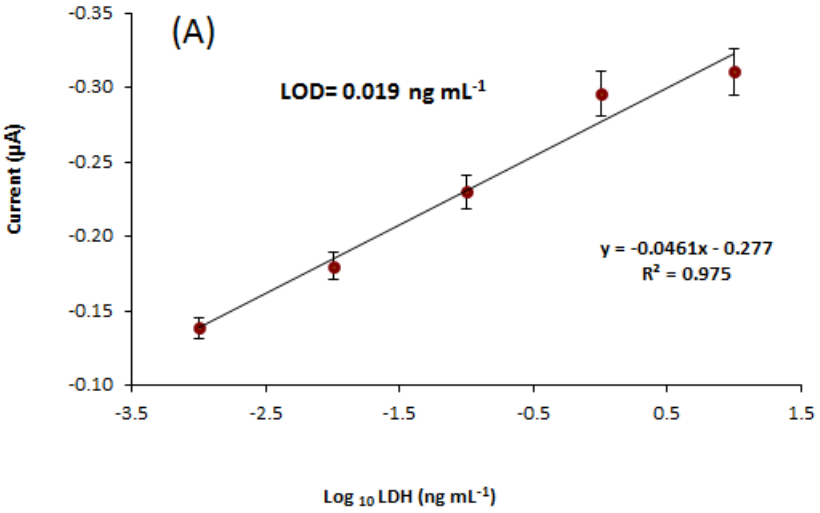
Figure 6: Amperometric signal for pLDH detection in Dd2^{luc} culture medium supernatant **(A)** using the developed immunosensor using 10 μg mL^{-1} capture antibody attached on the sensor and 10 μg mL^{-1} detection antibody-HRP for analyte sensing without the use of gold nanoparticle signal enhancement. The 6% parasitaemia was diluted in blank RPMI 1640 culture medium supernatant. Data plotted after subtracting the zero value of background signal **(B)**. Red lines show least measurement obtained at lowest parasitaemia (n=3).

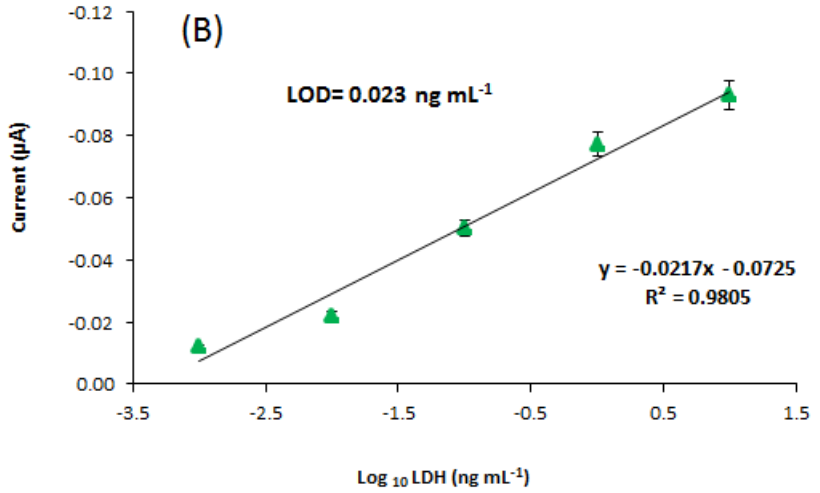

(A)
Current (µA)
Time (s)
0 µg /mL
2.5 µg / mL
5 µg / mL
10 µg /mL
20 µg /mL
Current (µA)
PfLDH capture antibody (µg mL-1)

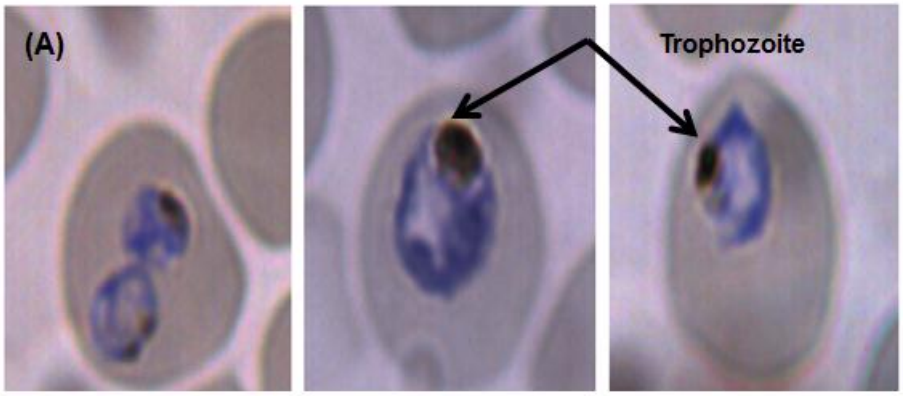

(B)
Current (µA)
Time (s)
0 µg /mL
10 µg / mL
20 µg /mL
30 µg / mL
PfLDH detection antibody (µg mL-1)

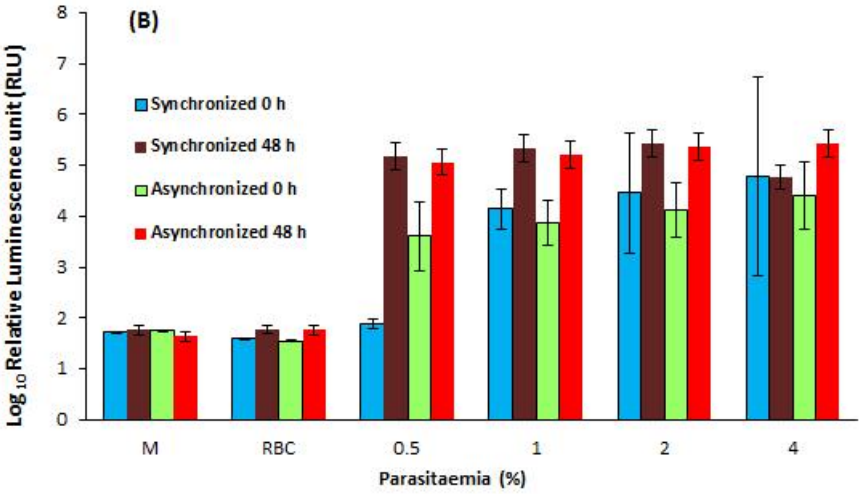


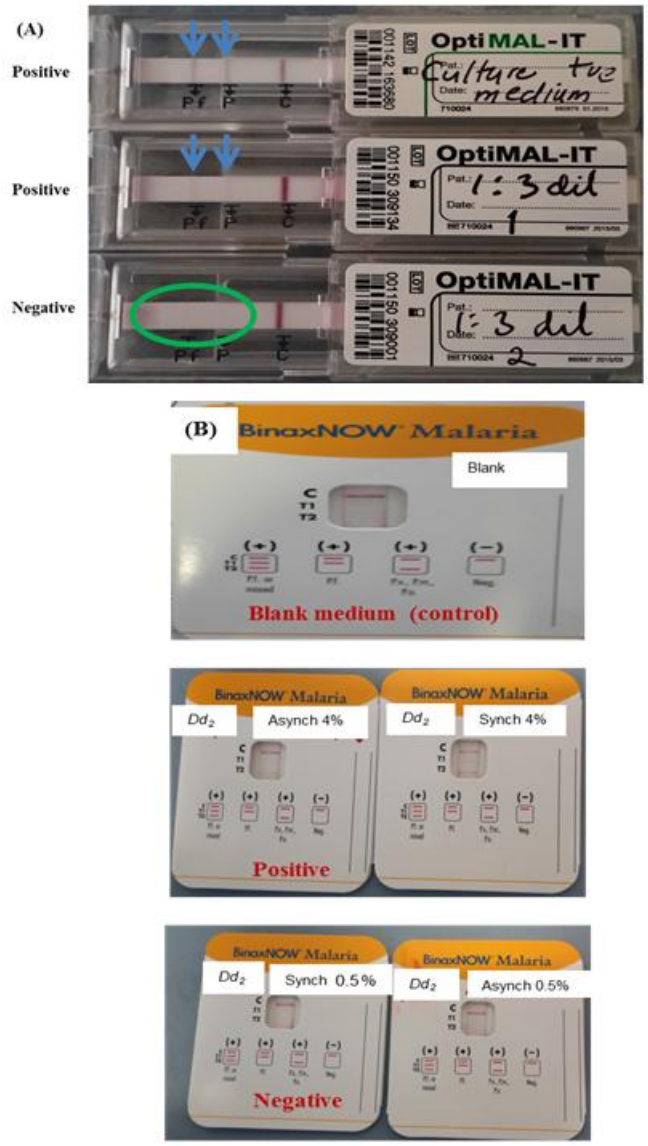



(A)
LOD= 0.019 ng mL-1
y = -0.0461x - 0.277
R² = 0.975
Current (µA)
Log 10 LDH (ng mL-1)
-0.35
-0.30
-0.25
-0.20
-0.15
-0.10
-3.5
-2.5
-1.5
-0.5
0.5
1.5

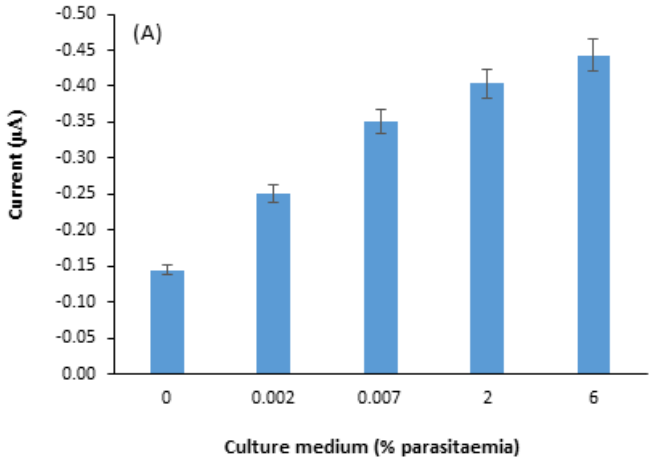

(B)
LOD= 0.023 ng mL-1
y = -0.0217x - 0.0725
R² = 0.9805
Current (µA)
Log 10 LDH (ng mL-1)
-0.12
-0.10
-0.08
-0.06
-0.04
-0.02
0.00
-3.5
-2.5
-1.5
-0.5
0.5
1.5

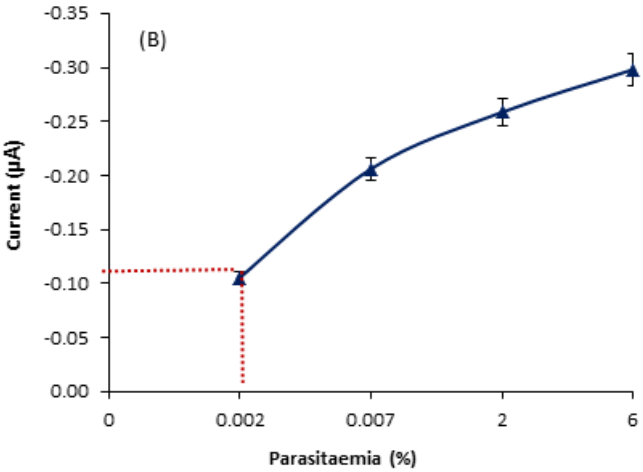


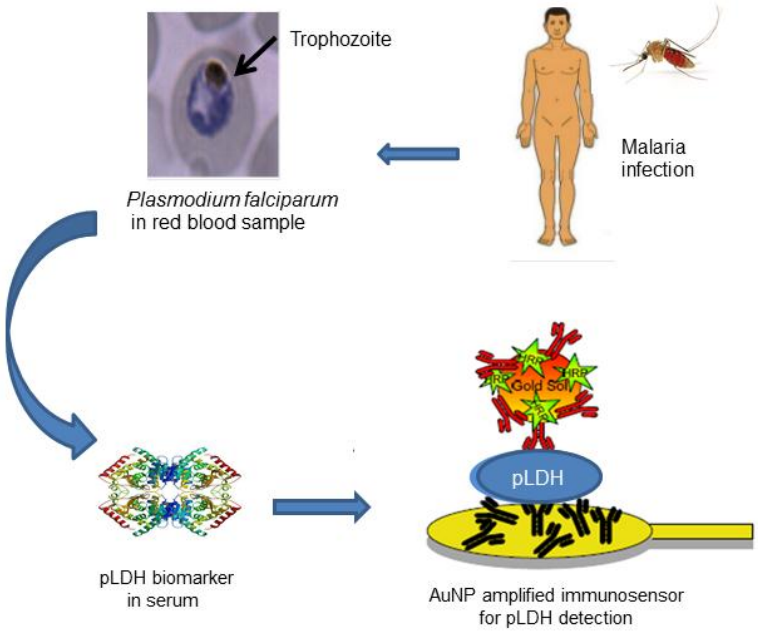




T1 = positive *P. falciparum* **T2** = positive *P. vivax*, *P. malariae*, *P. ovale* or mixed infection.

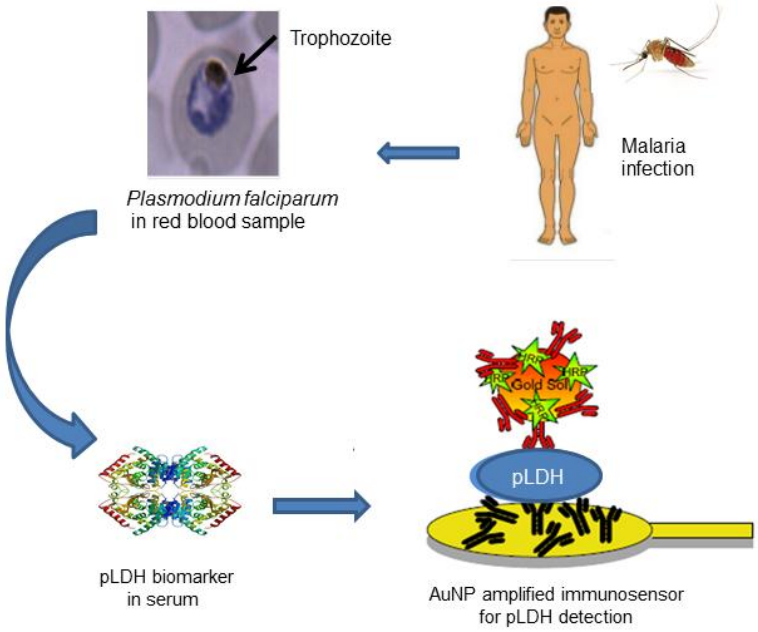






Graphical abstract

Scheme



Highlights

- First reported amperometric immunosensor for the detection of parasite lactate dehydrogenase (pLDH) which utilizes colloidal gold nanoparticles for signal amplification.
- Low pg ml^{-1} detection limits obtained in spiked serum samples.
- The immunosensor demonstrated superior performance when compared to OptiMAL-IT ICT kit (detect pLDH) and the BinaxNOW ICT commercial kits