Microdevices for Hemozoin-Based Malaria Detection

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Malaria, which is transmitted by the bite of female Anopheles mosquitoes infected with Plasmodium parasites, is one of the most life-threatening infectious diseases worldwide, with a significant impact on human lives. Optical microscopy and immuno-rapid tests are the standard malaria diagnostic methods in the field. However, these are time-consuming and fail to detect low-level parasitemia. Biosensors and lab-on-a-chip devices, as reported to different applications, usually offer high sensitivity, specificity, and ease of use at the point of care. Thus, these can be explored as an alternative for malaria diagnosis. Alongside malaria infection inside the human red blood cells, parasites consume host hemoglobin generating the hemozoin crystal as a by-product. Hemozoin is produced in all parasite species either in symptomatic and asymptomatic individuals. Furthermore, hemozoin crystals are produced as the parasites invade the red blood cells and their content relates to disease progression. Hemozoin is, therefore, a unique indicator of infection, being used as a malaria biomarker.

biosensor diagnosis hemozoin lab-on-a-chip malaria microdevices

1. Introduction

Most malaria cases occur in tropical and sub-tropical developing countries, where poverty limits access to proper healthcare conditions and infrastructures [1]. The use of insecticide-treated nets and artemisinin-based combination therapies allowed noticeable progress to be made in the face of the burden of malaria [1]. Nevertheless, this progress levelled off in recent years. Malaria incidence and mortality decreased by 27% and 52%, respectively, from 2000 to 2015, and then around 2% and 16%, respectively, from 2015 to 2019 [1]. This decline most likely results from an increase in disease transmission due to mosquitoes and parasites growing resistance to insecticides and antimalarial drugs, respectively [1]. In fact, in 2020, the World Health Organization (WHO) still reported 241 million global malaria cases and around 627,000 deaths [2]. The COVID-19 pandemic, which undermined prevention, diagnosis, and treatment, is likely a major contributor to these devastating numbers, and there are calls for the approval of the first malaria vaccine, despite its modest efficacy [2][3].

Malaria may manifest as symptomatic or asymptomatic, despite parasites circulating in the patient bloodstream. Typically, symptomatic malaria includes fever, tiredness, digestive symptoms and shaking chills, which may progress, in severe cases, into a coma, seizures, cerebral malaria and even death [4]. Malaria symptoms are related to the intraerythrocytic stage of infection in the human host and, thus, this stage is the target for infection detection. Nevertheless, since these symptoms are common to other febrile illnesses, they are often neglected or misdiagnosed. Therefore, sensitive and specific malaria diagnostic techniques, able to strengthen the disease

surveillance for better management and control, are a crucial step to achieve the United Nations Sustainable Development Goal 3 towards malaria elimination [5][6].

The ability to quantify and detect low-level infections (ideally less than 5 parasites/μL of blood) is of utmost importance, as it: (1) prevents the progression into severe disease and even death of the patient, (2) allows patients to be cleared after treatment and to identify emerging drug-resistant strains (identified by the inability of the drug to clear the parasites in three days) and (3) decreases inadequate treatment and prevents the emergence and spread of antimalarial drug resistance [7]. The conventional malaria diagnosis methods rely on optical microscopy of Giemsa-stained blood smears and rapid-diagnostic tests (RDTs) [1][8]. Optical microscopy allows parasite species and stage to be identified, and parasitemia quantification up to detection limits of 50–200 parasites/μL of blood. However, it is time-consuming, requires infrastructures that are not easily accessible in endemic areas and is highly microscopist-dependent. RDTs, which work on the principle of the detection of specific antigens produced by the malaria parasite, are portable and easy to use at the community level, reaching more patients. Nevertheless, these do not allow parasitemia quantification nor present a better limit of detection than microscopy, achieving only 100–200 parasites/μL of blood [9]. The most sensitive malaria detection (around 5 parasites/μL of blood) is achieved by nucleic acid-based detection methods. However, these are only performed in research settings since they require skilled personnel and high-rate equipment, difficult to reach in malaria-endemic regions [10]. Thus, the lack of on-field sensitive methods able to detect malaria and quantify parasitemia, coupled to rapid, easy to perform and low-cost detection, mean that there is a need for new diagnostic approaches for proper malaria control, the performance of which must be compared with the one of the gold-standard optical microscopy (below 50–100 parasites/μL of blood). In fact, the need for such a device has already led to the development and improvement of many novel technologies [11][12][13][14]. However, to date, none fulfil all the critical requirements regarding detection limits, sensitivity, specificity, portability, low cost, ease of use and, ideally, non-invasiveness [11][12][13][14].

2. Biosensors for Hemozoin-Based Malaria Diagnosis

In a general view, biosensors are sensing devices that comprise a bio-recognition element and a transducer. The bio-recognition element (e.g., enzymes, antibodies, microorganisms, DNA) identifies and interacts with the analyte/target of interest, and alterations in its physicochemical properties (e.g., optical, piezoelectric, magnetic, electrochemical) are converted into a quantitative or semiquantitative measurable electrical signal by a transducer [15]. In recent years, due to their sensitivity, specificity and high-throughput screening, biosensors have had significant growth, aiming at a vast range of applications [16]. Specifically, in diagnosis, biosensors have been exploited for integration into point-of-care devices. The most commonly known biosensor is the glucometer, which measures the glucose levels in the blood and has greatly contributed to diabetes management [17]. Given its success, this technology has been widespread to other diseases, including malaria, which is the focus of this manuscript. The current major challenges of malaria screening are the need for point-of-care sensitive detection of low parasitemia. Ideally, such challenges and limitations can potentially be overcome with properly designed biosensors, that must fill the gap of high sensitivity and specificity, as well as being easily miniaturized for point-of-

care diagnosis. Although there are several reports of biosensors for malaria diagnosis, these are mainly based on the use of enzymes as a target [11][18][19][20][21][22], i.e., RDTs. Nevertheless, as mentioned above, these have been threatened by parasite genetic evolution, and they do not detect low-parasitemia (100 parasites/µL of blood far from the ideal less than 5 parasites/µL of blood) or quantify infection. On the other hand, hemozoin: (1) is present in all parasite species, (2) is a crystal not being prone to genetic modifications, (3) is formed as parasites invade the patient's RBCs and (4) its content relates to disease progression, i.e., increase in parasitemia, and hence disease severity. Thus, a specific bio-recognition element for hemozoin detection incorporated in biosensors can be a starting point to future point-of-care malaria diagnostic devices. Preferably, this device should not only detect hemozoin but also quantify it in an amount equivalent to 1 parasite/µL (0.4512 pg of hemozoin [23][24]).

2.1. Electrochemical Biosensors

Electrochemical biosensors detect an electrical signal when a biological analyte reacts with the surface of the sensor. The amplitude of the electrical signal correlates with the concentration of the analyte. These biosensors have the advantage of being highly sensitive and specific, low-cost, presenting a rapid response, allowing quantification and performing a simple assay [25]. However, these are thermo-sensible, with a narrow temperature range, and may present a short life span, with limited shelf time, due to the nature and stability of their biological analytes or number of interactions with their targets. The probability of nonspecific binding of the analytes continues to be one of the main limitations of these biosensors [26]. Due to their temperature sensitivity, these sensors typically include internal circuits for temperature compensation, which may increase their complexity.

Regarding the development of electrochemical biosensors aiming malaria detection, recently, Obisesan et al. [27] developed an electrochemical nanosensor for the detection of β-hematin, the synthetic hemozoin. The authors, chemically and by using a microwave, synthesized metal oxide nanoparticles of copper, iron and aluminium, and deposited them on a gold electrode by using the drop-dry method [27]. A metal oxide electrode disk was used as the working electrode, a platinum disk was used as the counter electrode and an Ag/AgCl, saturated KCl was used as the reference electrode, at a constant pH of 9.0 [27]. The electrochemical sensor was tested in human non-malaria-infected urine samples, human malaria-infected serum, as well as mice non-infected and infected serum, all mixed with β-hematin. Additionally, the behavior of each metal oxide-coated electrode was explored by a cyclic voltammetry experiment. The report shows that the gold-coated electrode with metal oxide nanoparticles, preferably with copper, presented improved electrode catalysis, high stability and sensitivity of high reduction current and lower energy towards malaria detection, thereby supporting the potential of these sensors for detection and quantification of malaria parasite in biological fluids [27]. In fact, the authors were able to detect and consistently quantify 3.50–4.8 mM and 0.65–1.35 mM of β-hematin in mice and human serum samples, respectively [27]. Considering the conversion of units as proposed by other literature reports (such as [23][24]), the amount detected by this system might not present a better detection limit than the standard methods (approximately 1.77×10^9 – 3.68×10^9 parasites/µL of blood for the blood samples quite far from the 50–200 parasites/µL and 100–200 parasites/µL of blood of microscopy and RDTs, respectively). Furthermore, stable electricity for the operation of devices (including microwave, sonicator, magnetic stirrer, centrifuge) is required for the preparation of nanoparticles and the electrode and processing of the sample before use. It is noteworthy that

the authors show that the stability of the electrodes decreases due to an increase or decrease in current response at the electrode after 20 cycles. These, coupled with the expensive technology, might compromise the applicability of the method.

In an opinion article, Moutaouakil and colleagues dissect the properties of graphene, more specifically its electrical and optical properties, and propose the use of a graphene-based biosensor for malaria diagnosis [28]. The authors suggest that the flexible nature of graphene allows it to be integrated into PCBs to offer different commercial applications, including in RDTs and thick blood films [28]. Graphene is able to monitor the electronic transfer reactions of hemoglobin and, thus, may detect malaria infection [28]. This is because the conversion of hemoglobin in hemozoin causes oxidation of the iron from its ferrous state Fe^{2+} into its ferric state Fe^{3+}, leading to electrons transfer [29][30]. In fact, it was demonstrated that RBCs are immobilized on a glassy carbon electrode surface and that, by cyclic voltammetry, a reduction peak was observed for hemoglobin [31]. However, despite the promising results, it remains unknown whether this can be applicable for hemozoin-based malaria detection.

2.2. Optical Biosensors

Optical biosensors are based on detecting changes in light upon the interaction between the bio-recognition element and the target and they include reflection, fluorescence, luminescence, optical fiber, photonic and surface plasma resonance (SPR) biosensors [15]. Among these, fluorescence and SPR biosensors are the most popular. Fluorescence sensors are based on the fluorescent light emission of fluorophore molecules at a specific wavelength, after the radiation absorption at a different energy level (lower wavelength). The fluorescence intensity is proportional to the concentration of the analyte and the sensor response can be measured either through intensity or decay-time sensing [32]. Although these sensors assure high sensitivity and specificity and are immune to light scattering, they are limited by the short life span of the fluorophores and their photostability, and they are susceptible to pH and oxygen interferences [32]. SPR biosensors measure alterations in the refractive index of the plasma resonance material, in the SPR angle and reflectance intensity, caused by the interaction between the bio-recognition element and the target [33]. These biosensors are highly sensitive, present high resolution, can be label-free and allow real-time measurements, as they are adequate for point-of-care applications. However, SPR sensors are motion-sensitive and depend on the development of light detectors with a high signal-to-noise ratio [34]. They require precise alignment between light and the sensing area, regarding both distance and angle, and their signal is dependent on the molecular size and concentration of the analyte. Additionally, when not fully automated and integrated, optical measurements need long calibration processes and are mainly limited by the time required for precise sample and setup preparation in order to avoid light interferences [32][35].

Regarding optical biosensors for malaria detection, Briand and co-workers used hemoglobin-polyacrylic acid as a bio-recognition element for a gold-coated SPR-based sensor for rapid heme detection [33]. This biosensor acts by removing heme from hemoglobin, followed by heme-free hemoglobin exposure to samples containing heme that interact with the bio-recognition element. The authors were able to rapidly (less than 10 min) detect the presence of heme with a detection limit of 2 µM or 1.30 µg/mL with high selectivity, proving the method applicability [33]. As the authors measured heme concentration (an intervenient prior hemozoin formation), the values cannot be converted

into parasite/µL. Furthermore, this device presents a good performance and good reusability, shown by the fact that it was used 12 times [33]. Additionally, for on-field applicability, other polymers in the SPR surface and an increase in the concentration of the biorecognition element might improve specificity as well as sensitivity [33].

Taking advantage of the different refractive index of infected and non-infected RBCs, Sharma et al. proposed a biosensor based on 2D photonic crystal, using a linear waveguide with a nanocavity to trap RBCs and detect shifts in the transmission peak at 1550 nm [36]. Bendib S. and Bendib C. also designed and simulated a 2D photonic crystal biosensor [37]. This simulated biosensor uses a sensitive increaser ring resonator based on GaAs rods of a rectangular lattice suspended in air background and was investigated by using plane wave expansion and finite difference time domain methods [37]. The authors relate the refractive index with the bandgap of infected (in ring, trophozoites and schizont stages) and non-infected RBCs to improve the sensitivity of the biosensor [37]. However, the authors do not specify the parasitemia of the infected samples used in the performed assays. Recently, Rashidnia and co-workers used the same principles and designed and simulated a 2D photonic crystal biosensor with a rectangular geometry of gold rods [38]. Ankita et al. proposed a simpler 1D PC photonic crystal biosensor with a defect layer also able to detect changes in the transmission peak, according to the concentration of hemoglobin in infected and non-infected blood samples [39]. While the fabrication of photonic crystals might be a challenge due to their precise structure and dimensions, there are several fabrication methods and materials that make them inexpensive.

Quite recently, Chaudhary et al. joined the technology of photonic crystals and SPR to develop a gold-immobilized photonic crystal fiber-based SPR biosensor for malaria detection [40]. This system measures changes in the RBCs refractive index, not specifically hemozoin [40]. Briefly, the sample is added into the photonic crystal fiber and an SPR shift in resonance wavelength, which is dependent on the refractive index, and is detected between healthy and infected RBCs at different stages [40].

Abshire et al. [41] developed a heme fluorescence-based biosensor that undergoes fluorescence quenching upon heme binding. To achieve this, the authors constructed a fluorescence resonance energy transfer (FRET)-based heme sensor, in which enhanced cyan and yellow fluorescent proteins act as the donor and acceptor, respectively, and PfHRP-II as the heme-binding domain [41]. By doing so, the authors were able to identify heme pools in *P. falciparum* by fluorescence microscopy and observe alterations in heme concentrations in the presence of the antimalarial drug chloroquine [41]. Nevertheless, this biosensor is not applicable on-field since the parasite must incorporate the FRET-based heme sensor for it to be used.

Surface-enhanced Raman spectroscopy (SERS), which enhances Raman signals, is another optical technique that is becoming popular due to its high sensitivity and specificity, as it is unaffected by temperature and humidity changes. It depends on the rotational and vibrational states within the molecules, as it is used to detect the specific absorption bands of different functional groups and quantify the corresponding molecules. However, this technique is susceptible to noise interferences due to its low signal-to-noise ratio [32]. Garret et al. [42] used gold-coated *Graphium weiskei* butterfly rings for the development of a SERS biosensor for malaria diagnosis by interaction with hemozoin. By doing so, the authors were able to detect a parasitemia of 0.0005% and 0.005% of lysed samples of

early-stage *P. falciparum*-infected RBCs [42]. Assuming an RBC count of 5 × 10^6/µL of blood [43], and of that 50% corresponds to RBCs, the parasitemia range obtained within this work is equivalent to the one of microscopy (50–500 parasites/µL of blood in comparison to 50–200 parasites/µL of blood of microscopy). Nevertheless, the authors mention that this technique requires some time to collect the data and perform the assays, and that the enhancement in the SERS peak is not dependent on hemozoin concentration. Yuen and Liu used Surface-enhanced Resonance Raman spectroscopy (SERRS) with Fe_3O_4@Ag nanoparticles (nanoparticles with an iron oxide core and silver shell), following magnetic field enrichment for hemozoin-based malaria detection [44]. The magnetic field concentrates the nanoparticles and the paramagnetic β-hematin at the laser spot, which increases the Raman signal [44]. The authors were able to detect 5 nM of β-hematin, the equivalent to 30 parasites/µL [44]. Nevertheless, the magnetic field could impact the variation of SERRS readings. Thus, later, the same group tested two methods: (1) silver nanoparticles mixed with *P. falciparum* and (2) silver nanoparticles produced inside the parasites, being in closer proximity with hemozoin [45]. The limit of detection of these methods were (1) 0.01% and (2) 0.00005%, which are equivalent to 100 and 5 parasites/µL of blood, respectively, quite competitive with optical microscopy and RDTs [45]. Although highly sensitive, these methods cannot provide direct quantification, due to errors including contamination with cell debris in the lysing process in method (1) and inconsistent distribution of hemozoin inside the parasite in method (2) [45]. However, in this paper, the authors report an easier nanoparticle preparation method that is low-cost and faster since they performed the SERS measurements on random locations instead of selected hot spots and with high sensitivity, which can be further improved by using paper-based microfluidics chip for sample preparation [45]. More recently, Yadav et al. [46] enhanced the SERS signal with silver nanorods (AgNRs) on 0.3 T neodymium magnetic substrates and an externally applied magnetic field. This ultra-highly sensitive technique allows the detection limit of the equivalent to less than 10 parasites/µL [46]. Another group reported a SERS biosensor tested with β-hematin, which exploits plasmon coupling features of gold nanoparticles to enhance the Raman signals, and their tunable SPR to the near-infrared region to facilitate biological analysis [47]. In this system, 20 µL of β-hematin is deposited on a gold film in close contact with gold nanoparticles embedded in transparent and flexible polydimethylsiloxane (PDMS). A 785 nm laser irradiates the system, originating SERS signals at 1623 cm^{-1}, which are directly related to the amount of β-hematin deposited on the gold film [47]. Furthermore, the authors assured that hemoglobin cannot impact the response of SERS signals to β-hematin [47] and detected β-hematin concentrations of around 18.5 ± 4.5 and 51.5 ± 6.2 µM in healthy and sickle RBCs, respectively [47].

McBirney and colleagues designed, constructed and validated a portable, reagent-free magneto-optic technology for hemozoin detection [48][49]. This technology uses a 635 nm laser diode that emits in a 500 µL sample to a photodetector and a magnet [48]. The difference in the optical spectroscopy signal before and after applying the magnetic field indicates the level of infection [48]. This device was capable of detecting less than 8.1 ng/mL of β-hematin in 500 µL of whole rabbit blood, equivalent to less than 26 parasites/µL of blood (competitive with the 50–200 parasites/µL and 100–200 of microscopy and RDTs, respectively), and without any labelling [48][49]. Nevertheless, this system requires pre-treatment of the sample with ultrasound for blood lysis and the sample volume (500 µL) is not achievable with a finger prick [48]. The authors mention that further work will answer these

limitations through the use of other techniques for blood lysis, reconfiguration of the sample cuvette and by using alternative photodetectors [48].

3. Lab-on-a-Chip and Other Microdevices for Hemozoin-Based Malaria Diagnosis

In addition to biosensors, other novel microdevices, in particular lab-on-a-chip devices, have been drawing attention due to their potential to be used at point-of-care malaria diagnosis [50]. These correspond to miniature portable devices that integrate several laboratory techniques, allowing the screening of different features to be performed together. Usually, these are coupled with microfluidic systems with reservoirs that allow the cells to be concentrated for a more specific and sensible detection.

Taylor and colleagues reported a simple to use, plastic hydrogel chip run on a portable real-time PCR [51]. This lab-on-a-chip is thermo-stable, is low-cost (USD 1 per test and less than USD 2000 for the real-time PCR compared with microscopy USD 0.12–0.40 per test and USD 700–3000 for the instrument for microscopy), uses a small sample volume (15 µL per test), provides the result in less than 2 h and is disposable [12][51]. This microdevice was tested in clinical samples and detected a limit of 2 parasites/µL of blood with high specificity (93.8%) and sensitivity (97.4%) compared with the conventional real-time PCR [51]. When testing an instrument with a LED excitation, the authors were able to increase specificity (100%) but not sensitivity (96.7%) [51]. The real-time PCR instrument requires the equivalent of a car battery as a power supply, which can be used in areas where the electricity supply is unstable [51]. The authors mention that a battery can be incorporated in the next generation of this lab-on-a-chip [51]. Furthermore, this micro-technology makes use of primers to amplify the 18S rRNA gene from *Plasmodium*, and by doing so, it was able to distinguish *P. falciparum* and *P. vivax* infections [51]. Despite not using hemozoin as a target, this microdevice sustains the applicability of lab-on-a-chip for malaria detection and it can be adapted for hemozoin targeting by using a different set of primers [51].

Recently, Hole and colleagues proposed an inductor on an FR-4 printed circuit board (PCB) and copper as a sensor for malaria screening [23]. The principle of work of this inductive sensor is based on effective relative permeability and on the inductance value of the core at the sensing coil, which is prone to changes when in the presence of paramagnetic hemozoin [23]. In fact, in the presence of hemozoin, inductance increases while resonance frequency decreases, allowing the detection of synthetic hemozoin in 12.7–25.4 pg, which is an amount equivalent to 25–50 parasites in 0.5 µL of phosphate-buffered saline (PBS), in a one-minute assay [23]. This value is competitive with the 50–200 parasites/µL and 100–200 parasites/µL of blood of microscopy and RDTs, respectively. Therefore, this sensitivity in such a small sample volume is promising for the early detection of the disease. Furthermore, the authors explain that they fabricated the inductor in a PCB, for this sensor to be low-cost, and that added a mask on top of the inductor for reusability and as a protective layer for the sensor, avoiding any damage to it [23]. Thus, this method is promising for malaria detection in the field, assuring an economical sensitive detection. Nevertheless, the use of copper limits the applicability of this sensor due to its easy and unavoidable oxidation and consequent loss of response over time.

The relatively large paramagnetic susceptibility of hemozoin particles induces substantial changes in the transverse relaxation rate, *T2*, of proton nuclear magnetic resonance (NMR) of RBCs, which can be used to correlate with the presence of infected RBCs during malaria infection [52]. This idea was first pointed out by Karl et al. who show that it is possible to carry out NMR relaxometry on infected RBCs but concluded that it was unlikely to have enough sensitivity for malaria diagnosis in the field settings [53]. They demonstrate their studies using unprocessed raw blood [53]. Then, Peng and co-workers demonstrated that it was indeed possible to have a highly sensitive malaria diagnosis by focusing on the infected RBCs, using a simple trick of standard hematocrit centrifugation (from normal RBCs) [52][54][55][56][57]. The authors concluded this in their mouse studies where a highly sensitive detection compared to the current methods was reported (less than 10 parasites/μL versus 50–200 parasites/μL and 100–200 of microscopy and RDTs, respectively) [52]. Following this unprecedented development, several similar studies were reproduced [58][59][60][61], and new techniques were established to improve the infected RBCs separation (using microfluidics) and exploited for drugs studies [62]. In fact, Kong et al. [63] combined lab-on-a-chip microfluidics and magnetic resonance relaxometry (MRR) in order to accurately detect malaria infection. The authors used margination-based microfluidics that separates infected and non-infected RBCs based on their different deformability. By doing so, infected RBCs were concentrated, facilitating infection detection. This was followed by infected RBCs lysis and MRR detection, based on paramagnetic hemozoin detection. By doing this, the authors were able to detect as low as to 0.0005% of parasitemia of early-stage *P. falciparum*-infected RBCs [63]. Based on the same RBCs count/μL of blood [43], the parasitemia is similar to the one of microscopy, 50 parasites/μL of blood. To avoid false-positive and -negative results, it is mentioned that each sample is analyzed 5–10 times in the MRR, which takes around 5–10 min [63]. The authors believe that both microfluidics design and MRR detection might be optimized to provide more sensitive and sensible results [63]. Furthermore, there is the possibility of miniaturizing both systems in a lab-on-a-chip, and despite the elevated cost of this (several thousands of USD), the cost per assay (less than USD 0.50) would be almost comparable with microscopy (USD 700–3000 for the instrument and USD 0.12–0.40 per test) and RDTs (USD 0.55–1.50 per test) [12][63].

The magnetic properties of hemozoin were also explored for a magnet-based microfluidic device. Nam et al. [90] developed a PDMS microchannel with three inlets and two outlets, fabricated by soft-lithography using SU-8, coupled to a nickel wire fixed on a glass slide. In the presence of a permanent magnet, an external field of 0.6 T is created and causes the nickel wire to attract *P. falciparum* infected-RBCs, allowing their separation from the non-infected ones [90]. Thus, by using this microfluidic device, the authors were able to isolate and concentrate infected RBCs and suggest that the use of this before clinical diagnosis would increase its accuracy [90]. Nevertheless, the samples were separated with a recovery rate of approximately 73% and 98.3% corresponding to late- and early-stage parasites, respectively [90]. The authors mention that the efficiency of this device might improve by altering the distance between the nickel wire and the infected RBCs and by optimizing the microchannel outlet [90].

More recently, Milesi and co-workers developed a magnetophoretic on-chip system for malaria detection, also based on paramagnetic hemozoin detection [64]. The authors developed a silicon microchip with micro concentrators for the magnetophoretic capture of infected RBCs, and gold electrodes for measurement of the

sample electrical impedance [64]. By doing so, the authors were able to selectively detect hemozoin crystals but noticed that the system could not easily distinguish malaria infection from met-hemoglobin, a hemoglobin paramagnetic state [64]. Quite recently, the same team optimized the silicon chip with nickel microcapillars that, in the presence of a magnetic field, should interact with the hemozoin crystals of infected samples [65]. This causes infected RBCs to become stacked. The authors used met-hemoglobin, converted from hemoglobin using $NaNO_2$, to simulate infection [65]. In this model, with the proper agitation, 5 min are enough to attain 85% of capture efficiency [65]. Nevertheless, the same might not be achieved when using a real malaria sample. From a global perspective, electromagnetic sensing allows for highly sensitive and specific detection, as the use of a single excitation frequency (specific to the analyte) decreases the interferences from other molecules or media. However, the measurement of magnetic signals is also highly dependent on the temperature, so temperature compensation circuits must be taken into account [32].

Myrand-Lapierre and colleagues developed a multiplexed fluidic plunger to evaluate the deformability of RBCs through microscale funnels within a microchannel [66]. Later, the same team used this simple and inexpensive system to assess biophysical alterations in RBCs following hemin-induced oxidative stress [67]. One of the major sources of oxidative stress in the malaria parasite originates in the pathway of hemoglobin degradation to hemozoin formation as a result of iron oxidation [68][69]. Despite not measuring hemozoin directly, this system analysis an outcome of hemozoin formation and shows that hemin concentration correlates with RBCs deformability [67].

Recently Wang et al. [70] designed and fabricated in a PCB a surface acoustic wave (SAW) sensor, excited with a photo-acoustical signal. The team used a laser pulse into 2 µL of *P. falciparum*-infected RBCs and, in less than 2 min, were able to distinguish 1% of infected RBCs from non-infected RBCs [70]. The authors intend to integrate this sensor with a microfluidic system in order to increase the sensitivity through infected RBCs concentration [70]. Despite the low sample volume and rapid resolution time, considering an RBC count of 5×10^6/µL of blood, the detection limit (100,000 parasites/µL of blood) is not competitive with microscopy and RDTs yet, nor do the authors specify whether this sensor operates based on hemozoin detection. Nevertheless, the sensitivity and specificity of SAW sensors might be improved by their coating with absorptive materials [71]. Despite being thermo-stable and not requiring high energy for operation, the durability of these sensors might be a challenge [71].

Furthermore, Graham et al. [72] proposed an ultrasensitive polymerization-based assay that allows hemozoin detection and quantification to be integrated into a microfluidic lab-on-a-chip device. The authors reported that solubilized hemozoin catalyzes the polymerization of N-isopropylacrylamide into poly N-isopropylacrylamide, resulting in liquid turbidity that can be optically quantified at 380 nm or 600 nm for up 4 h, as an indicator of malaria infection [72]. This polymerization process requires low-cost and thermo-stable reagents and allows the detection of 10 infected RBCs/µL of parasite-spiked full-blood on a small sample volume [72]. This value is quite competitive with the current diagnostic methods. Furthermore, the turbidity rate is proportional to the concentration of hemozoin, which makes the assay quantitative. More recently, the same group optimized the reaction conditions of the assay by using pyruvate, SDS and a 7.5 pH [73]. By doing so, the authors reduced the amplification time (the time for the reaction reach its maximum) from 37 ± 5 min to 3 ± 0.5 min, while keeping around the same detection

limit and 95% confidence (1.06 ng/mL compared with 0.85 ng/mL, both equivalent to less than 10 infected RBCs/µL) [73]. It is interesting that the optimized conditions did not increase the sensitivity of the method but did significantly increase its performance time [73]. Despite requiring sample preparation for collection of blood and extraction of hemozoin, this improvement increases the applicability of the method.

Catarino and colleagues, after demonstrating that the absorbance spectra of synthetic hemozoin and hemoglobin is different, developed a first prototype of portable optical microdevice for hemozoin-based malaria detection and quantification [74][75]. The authors tested their system with 97 µL of whole blood samples mixed with a 1 µg/mL concentration of synthetic hemozoin in around 1 min analysis time [74]. Nevertheless, the need for a drop of blood is one disadvantage of this system and, thus, the authors have been exploring optical reflectance as an alternative non-invasive technique to be incorporated in a new microsystem [76][77][78].

Recently, Kumar et al. developed the magneto-optic Gazelle device for hemozoin detection [79][80]. Gazelle detects LED-emitted light into the sample in the presence and absence of a 55 T magnetic field [79]. The transmitted light is proportional to the amount of hemozoin in the sample and allows detection up to a limit of 50 parasites/µL of *P. falciparum* and 35 parasites/µL of *P. vivax* patients samples with 95% and 100% accuracy, respectively [79]. Gazelle is thermo-stable, battery operated, easy to use, low-cost (around USD 1 per test almost comparable with USD 0.12–0.40 of microscopy and USD 0.55–1.50 of RDTs) and fast (1 min in comparison with 30 min and 20 min for microscopy and RDTs, respectively) [79][80]. The device was tested on 262 patients in India and presented high sensitivity and specificity to diagnose the disease (98% and 97%, 82% and 99%, and 78% and 99% in comparison to microscopy, PCR and RDTs, respectively) [79]. Similar results were achieved in Brazilian Amazon and Peruvian Amazon Basian, which are *P. vivax*-predominant regions [81][82]. Nevertheless, Gazelle is not portable, and still requires a drop of blood for malaria detection (15 µL) and is not able to distinguish between species [79][80].

Table 1. Summarizes the main developments in biosensors, lab-on-a-chip devices and other microdevices for the detection of hemozoin and its variants.

Authors	Biosensor Type	Detection	Bio-Recognition Element	Analyte	Tested Sample	Limit of Detection	Detection Time	Ref.
Obisesan et al.	Electrochemical	3 electrode system, measured by cyclic voltammetry	Metal oxide nanoparticles of copper, iron and aluminum deposited on a gold electrode	β-hematin	Human non-malaria-infected urine samples, human malaria-infected serum, mice non-infected and infected serum, all mixed with β-hematin	*P. berghei* in infected mice's serum samples: 3.60–4.8 mM (around 1.14 × 10^{10} parasites/µL of blood) *P. falciparum* in human blood serum samples: 0.65–1.35 mM (around 2.725 × 10^{9} parasites/µL of blood)	No information	[27]

Authors	Biosensor Type	Detection	Bio-Recognition Element	Analyte	Tested Sample	Limit of Detection	Detection Time	Ref.
Briand et al.	Optical	SPR-based sensor	Hemoglobin-polyacrylic acid	Heme	Heme solutions	2 µM *	Less than 10 min	[33]
Abshire et al.	Optical	FRET-based sensor	PfHRP-II	Heme	*P. falciparum*-infected RBCs	1.6 µM *	No information	[41]
Garret et al.	Optical	SERS	Gold-coated Graphium weiskei butterfly rings	Hemozoin	Lysed early-ring *P. falciparum*-infected RBCs	0.005% (equivalent to 50-500 parasites/µL of blood)	No information	[42]
Yuen and Liu	Optical	Magnetic enrichment followed by SERRS	Fe_3O_4@Ag nanoparticles	β-hematin	β-hematin resuspended in NaOH	5 nM (equivalent to 30 parasites/µL)	15 s exposure time	[44]
Chen et al.	Optical	SERS	Silver nanoparticles	Hemozoin	Silver nanoparticles mixed with *P. falciparum* and silver nanoparticles produced inside the parasites	0.01% and 0.00005% (equivalent to 100 and 5 parasites/µL of blood)	10 s exposure time	[45]
Yadav et al.	Optical	SERS and an externally applied magnetic field	Silver nanorods (AgNRs) on 0.3 T neodymium magnetic substrates	Hemozoin and Human deoxy-hemoglobin	Hemozoin and hemoglobin in PBS and deionized water; Fetal bovine seerum	equivalent to less than 10 parasites/µL	20–30 s integration time	[46]
Cai et al.	Optical	SERS biosensor	Gold nanoparticles embedded in PDMS	β-hematin	β-hematin and hemolyzed erythrocytes deposited on a gold film	18.5 ± 4.5 and 51.5 ± 6.2 µM in healthy and sickle RBCs	5 s for spectrum acquisiton time	[47]
McBirney et al.	Magneto-optic	635 nm laser diode that emits in the sample to a photodetector and a magnet	None	β-hematin	β-hematin in 500 µL of whole rabbit blood	8.1 ng/mL of equivalent to less than 26 parasites/µL of blood	No information	[48]

Authors	Biosensor Type	Detection	Bio-Recognition Element	Analyte	Tested Sample	Limit of Detection	Detection Time	Ref.
Taylor et al.		Lab-on-a-chip for DNA/RNA amplification	Master mix for amplification of the targeted DNA/RNA	18*S* rRNA gene	Frozen clinical samples of *P. falciparum*, *P. vivax* and *P. knowlesi*	2 parasites/μL of blood	Less than 2 h	[51]
Hole et al.	Inductive	Measurement of inductance/resonance frequency	None	Synthetic hemozoin	Synthetic hemozoin in PBS	12.7–25.4 pg in 0.5 μL of PBS (equivalent to 25–50 parasites/μL of blood)	No information	[23]
Peng et al.	Magnetic resonance	Magnetic resonance relaxometry detection	None	Hemozoin	early-stage *P. falciparum*-infected RBCs	Less than 10 parasites/μL in mouse studies culture	MRR detection: 5–10 min	[52] [61]
Kong et al.	Magnetic	Lab-on-a-chip with MRR detection	None	Hemozoin	early-stage *P. falciparum*-infected RBCs	0.0005% of *P. falciparum* culture (equivalent to 50 parasites/μL of blood)	Separation process: 15 min MRR detection: 5–10 min	[63]
Nam et al.	Magnetic	Lab-on-a-chip and optical microscopy detection	None	Hemozoin	*P. falciparum*-infected RBCs	No information	No information	[83]
Milesi et al.	Magnetic/Electrical	Lab-on-a-chip with magnetophoretic capture and electrical impedance measurements	None	Hemozoin	Red blood cells treated and non-treated with $NaNO_2$	No information	No information	[64]
Wang et al.	Photo-acoustic	Photo-acoust-excited surface acoust wave (SAW) sensor to be integrated with a microfluidic system	None	Not specified	*P. falciparum*-infected RBCs	1% of *P. falciparum*culture (equivalent to 100,000 parasites/μL of blood)	Less than 2 min	[70]
Graham et al.	Optical	Lab-on-a-chip with optical detection at 380 nm or 600 nm	N-isopropylacrylamide	Hemozoin	Hemozoin solutions in NaOH	10 infected RBCs/μL	37 ± 5 min	[72]
Raccio et al.	Optical	Lab-on-a-chip with optical detection at	N-isopropylacrylamide	Hemozoin	Hemozoin solutions in	10 infected RBCs/μL	3 ± 0.5 min	[73]

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Authors	Biosensor Type	Detection	Bio-Recognition Element	Analyte	Tested Sample	Limit of Detection	Detection Time	Ref.
		380 nm or 600 nm			NaOH			
Catarino et al.	Optical	Optical Absorbance	None	Synthetic hemozoin	Synthetic hemozoin diluted in whole blood	1 µg/mL	Around 1 min	[74]
Kumar et al.	Magneto-optic	Gazelle: LED-emitted light into the sample in the presence and absence of magnetic field	None	Hemozoin	*P. falciparum* and of *P. vivax* infected patient	50 parasites/µL and 35 parasites/µL of *P. falciparum and P. vivax* infected patients	Around 1 min	[79] [81] [82]
Lukianova-Hleb et al.	Photo-acoustic	Acoustic signal produced by laser induced vapor nanobubbles	None	Hemozoin	in vitro *P. falciparum*-infected RBCs and blood of *P. yoelii*-infected mice	0.0001% (in vitro); 0.00034% (in vivo); (equivalent to 10 parasites/µL and 17 parasites/µL for the in vitro and in vivo cultures)	No information	[84]

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