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Electrochemical Biosensors and its Applications for Malaria Diagnosis: a Review

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Abstract

Malaria infection is a disease that affects many countries in the world, mainly the poorest population, where a high numbers of cases and deaths are reported. Carrying out tests for the early diagnosis of malaria is one of the guidelines of the World Health Organization (WHO) for the adequate control and treatment of the disease. Currently, the tests for malaria detection (conventional microscopy, rapid diagnostic tests (RTDs), nucleic acid amplification tests (NAATs) or enzyme-linked immunosorbent assay (ELISA)) possess some disadvantage such as high-cost techniques, specialized laboratories, and skilled personnel. In the last 20 years, the research related to the development of biosensors with biomedical application has increased considerably, becoming an important area within materials science, where sensorial devices are manufactured to detect biomarkers of different diseases. Surface functionalization allows the development of simple methods, low-cost and with high sensitivity and specificity during detection. In this paper we present a review about electrochemical biosensors ant its applications for detection of malaria biomarkers.

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1. INTRODUCTION

Malaria is a severe disease caused by protozoan parasites of *Plasmodium sp.* and transmitted to humans by *Anopheles sp.* female mosquitoes. The disease is characterized as infectious, non-contagious and transmitted by vectors [1]. It is believed that malaria outbreaks began approximately 2700 years BC, being very widespread due to the high number of deaths that occurred at the time and was considered the cause of great military defeats [2, 3].

Malaria is distributed in six regions defined by the WHO, with prevalence in tropical and subtropical countries. In 2019, an estimated 229 million cases were reported in 87 countries where the malaria is endemic, which nineteen countries belong to sub-Saharan African and India and registered approximately 85% of the total cases. The number of deaths by malaria is significantly high, with 409 000 deaths recorded in 2019 (approximately 94% in Africa). Exposure to infection in pregnant women and children is significant in many regions and has fatal consequences, affecting the health of the mother and fetus, leading to premature births and leading to high infant mortality [4]. There are five species associated with human malaria: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovalae e Plasmodium knowlesi.* The typical symptoms of the disease are fever, chills, profuse sweating, weakness and headache, which occur in cyclical patterns depending of the infecting species [5].

Currently, the microscopy and RDTs are the two main laboratory techniques used to detect the malaria infection [6]. The first is performed through microscopic observation of blood samples. This approach requires trained professionals, specialized facilities, expensive equipment and reagents, and long relatively analysis times [7]. On the other hand, RDTs are based on detection of biomarkers. The method constitutes a good alternative and presenting advantages such as: low cost, short analysis times, possibility of in situ detection of several species and do not require qualified work, but their performance and sensitivity are determined by some factors like conditions of temperature, humidity, storage, and execution [8]. Also, other advanced techniques are used for detecting the parasites nucleic acids, including quantitative or real-time polymerase chain reaction (PCR) and isothermal loop-mediated amplification (LAMP). The advantages of NAATs include high sensitivity, ability to detect one parasite per μL of blood, detection of multiparasitic infection, ability to process simultaneously many samples and to detect drug-resistant strains. However, these techniques are

time consuming, expensive, have low reproducibility at low concentrations, require modern laboratory facilities and qualified personnel [9].

2. BIOMARKERS OF MALARIA

Biomarkers are biochemical or molecular changes measurable in biological media such as tissues, cells or fluids [10]. In addition, the measurement and evaluation of biological characteristics as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention may be included in the definition [11]. The use of biomarkers for early detection of *Plasmodium* infections is a very important diagnostic tool, which can provide dynamic and powerful approaches to understanding the disease [12]. The main biomarkers associated with malaria are: histidine-rich proteins (HPPs), lactate dehydrogenase (LDH), aldolase, glutamate dehydrogenase (GDH), hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and hemozoin [13].

Plasmodium falciparum synthesizes a unique set of soluble HRPs during its asexual erythrocytic development: HRP1, HRP2 and HRP3. HRP1 aids in the cytoadherence of infected erythrocytes to the venous endothelium and partially contributes to the high parasitemia and hypoxia associated with the parasite. HRP2 is a unique biomarker of *P. falciparum* and is related to binding with glycosaminoglycans causing antithrombin inhibition and heme detoxification forming hemozoin. *Pf*HRP2 is very stable, found mainly in the cytoplasm of the parasite and released in abundance into the host's bloodstream. It can be detected in red blood cells, serum, plasma, cerebrospinal fluid, and urine of infected individuals, with a positive correlation between the concentration of the protein present in the bloodstream and the parasite biomass. HRP3, also known as small protein rich in histidine-alanine, is found in a smaller proportion than HRP1 and HRP2 [12, 14].

LDH is a water-soluble enzyme produced by the sexual and asexual stages of parasites. Expressed in high concentrations, it is essential for the anaerobic generation of adenosine triphosphate (ATP) and catalyzes the parasite's glycolytic pathway, helping to convert pyruvate to lactate. *p*LDH has a 26% of identical amino acids sequence to human LDH, conserves catalytic residues for enzymatic activity, and shares approximately 90% of amino acids identity among all *Plasmodium* species [15].

Aldolase is an enzyme that plays an essential role in the parasite's glycolytic pathway, where it converts fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. Its homotetrameric, with ~160 kDa molecular size, and can be founded in the cytoplasm of the parasite in an active and soluble form or membrane-binding

in insoluble form [9, 16]. *P. vivax and P. falciparum* contain one type of aldolase isoenzyme, with a length of 36 amino acids, where the nucleotides and amino acid sequences are relatively conserved. This enzyme is considered vital for the survival of the parasite, being an important target molecule and potential biomarker in the development of biosensors for malaria diagnosis [12, 17].

GDH is a soluble metabolic protein, with a molecular size between 50 and 60 kDa, used by the parasite to obtain energy via Krebs cycle, where occurs the reversible oxidative deamination of L-glutamate into alpha-ketoglutarate and ammonia, using calcium phosphate, nicotinamide adenine dinucleotide phosphate (NADP) and releasing its reduced form (NADPH) during the intraerythrocytic phase [18, 19]. Although malaria parasites display several isoforms of *p*GDH, eg *P. falciparum* displays three types of isozymes, with a 23% sequence identical to human GDH, they act as a reliable and selective biomarker that can be used to detect the disease and in the production of antibodies to improve immunodiagnostic assays [20, 21].

HGPRT is an important enzyme involved in the purine metabolism of parasitic protozoa, converting free purine nucleobases (hypoxanthine and guanine) into nucleotides, being the phosphoribosyl group derived from phosphoribosyl pyrophosphate in a reaction that requires Mg^{2+}. *Pf*HGPRT shares 44% identical sequences with human HGPRT, and constitutes a potential therapeutic target for the development of antiparasitic drugs and diagnostic methods [22, 23].

Hemoglobin degradation plays a vital role as a source of amino acids for parasites. Due to process of digestion of hemoglobin in the red blood cells, the heme is formed and its considered quite toxic to cells, which is later converted by the parasite into a product denominated hemozoin, that is a non-toxic, yellow-red, microcrystalline, inert and insoluble [24]. It's often called malaria pigment, has unique characteristics, is used as a visible marker to identify parasites and acts as a biomarker for diagnosis because it is absent in healthy individuals [12, 25].

3. BIOSENSORS

A biosensor is a device that uses a biological component as a recognition element and emits a certain signal when it receives a stimulus. Biosensors can be classified as optical (luminescence, fluorescence, and refractive index), electrochemical (amperometric, potentiometric, impedimetric and conductometric) and piezoelectric. Among them, electrochemical biosensors stand out, which emit electrical signals (current, voltage, impedance, etc.) in response to biochemical events (enzyme-substrate reaction, antigen (Ag) - antibody (Ab) interaction, etc.). For development of these sensing platforms,

an electrode is used as a key component of the system and constitute the solid support for the biomolecules immobilization (enzymes, antibodies, proteins, aptamers or nucleic acids) and the movement of electrons [26–28].

The biorecognition element influence the good performance of a biosensor, which is why some requirements must be taken into account to select the layer that will be used as receptor, such as: possess a reactive site with good availability to interact with the analyte, enabling immobilization on certain supports through chemical methods without affecting their performance and present stability in the medium and measurement conditions [29]. Furthermore, the response of the device will be determined by other factors such as: diffusion of the analyte, reaction products, co-agents or interfering species and kinetics of the recognition process. Various materials with large surface area are used with porpoise of favor these processes. The improve of the charge capacity and mass transport of reagents increase the analytical sensitivity and performance of the biosensors [30].

3.1. Electrochemical Biosensors for Malaria Diagnosis

In the last two decades, the development of electrochemical biosensors applied to the detection of malaria biomarkers has been widely studied and different construction methodologies explored [9, 31]. Some of biosensors reported in the literature for the detection of malaria biomarkers are described below:

A disposable amperometric immunosensor for detection of Ag-*Pf*HRP2 in human serum samples from malaria patients was developed by Sharma et al. For which, screen-printed carbon electrodes (SPCE) were modified with multi-walled carbon nanotubes (MWCNTs) and gold nanoparticles (AuNPs). For the first time, rabbit Ab-*Pf*HRP2 were immobilized onto surface of electrodes and used as a capture element in an electrochemical device. After incubation in serum samples containing Ag-*Pf*HRP2, a sandwich immunoassay format was set up by successive incubations in mouse Ab-*Pf*HRP2 and rabbit anti-mouse immunoglobulin G-alkaline phosphatase conjugate. The device showed good performance, with limit of detection (LOD) of 12 ng mL^{-1}. The results using the a commercial Paracheck Pf kit showed a sensitivity of 79% and specificity of 81%, while the developed immunosensor showed a sensitivity of 96% and specificity of 94% [32].

De Souza-Castillo et al. constructed a magnetic electrochemical immunosensor for detection of Ag-*Pf*HRP2 using a magnetic nanoparticle-based immunoassay strategy. First, IgM type commercial monoclonal antibodies (Ab-*Pf*HRP2) were covalently immobilized on magnetic beads as well as on magnetic nanoparticles. The immunological reaction was performed by a sandwich immunoassay using a second monoclonal antibody labeled with horseradish peroxidase. The modified magnetic particles were

captured by magneto electrodes made of graphite-epoxy. The immunosensor showed a high sensitivity, with a LOD of 0.36 ng mL^{-1}. The results were compared with a novel magneto-ELISA based on optical detection. Both strategies recorded similar LODs, low nonspecific adsorption values and good reproducibility. The immunosensor is a rapid, simple and cost-effective strategy for on-site detection of malaria caused by *Plasmodium falciparum* [33].

Sharma et al. developed a piezoelectric immunosensor for direct determination of Ag-*Pf*HRP2. The surface of a gold-coated quartz crystal was modified with a mixed self-assembled monolayer (SAMs) of thioctic acid and 1-dodecanethiol. The rabbit Ab-*Pf*HRP2 were covalently coupled to the SAMS via NHS/EDC activation method. The immunosensor registered a linear response at 15 - 60 ng mL^{-1} concentration rang, with LOD = 12 ng mL^{-1}. Tests were performed on clinical samples (human serum) and the results confirmed with those obtained using a commercially available ICT kit (NOW® Malaria). In addition, it was found that after 2 weeks of device storage, 50% of the activity was still recorded. The study demonstrated that it is feasible to detect the *Pf*HRP2 malaria antigen with the proposed immunosensor [34].

Paul et al. reported a sensing platform based on electrospinning of copper-doped zinc oxide nanofibers. For the SAMs formation and subsequent covalent binding of antibodies, the surface was functionalized with mercaptopropylphosphonic acid. Copper doping in zinc oxide increases the conductivity of the nanofibers and pre-concentrates the target analyte onto the mercaptopropylphosphonic acid treated nanofiber surface due to inherent electric field generated at the copper/zinc oxide heterojunction interface. The impedimetric response of the immunosensor showed excellent sensitivity in the detection range of 10 ag mL^{-1} to 10 mg mL^{-1}, with LOD of 6 ag mL^{-1}. Moreover, the proposed biosensor is highly selective for the Ag-*Pf*HRP2, with a relative standard deviation of 1.9%, without interference from other components present in the real samples [35].

Dutta et al. fabricated an enzyme-free immunosensor using a competitive detection scheme based on methylene blue (MB), hydrazine and platinum nanoparticles (PtNPs). The system utilizes a sandwich assay format, where the capture antibody is immobilized on an indium tin oxide (ITO) electrode, and the second antibody is labeled with MB. In presence of Ag-*Pf*HRP2, the immobilized MB-$Ab_{(2)}$/Ag/$Ab_{(1)}$ complex consume interfacial hydrazine and decrease the hydrazine electro-oxidation on the PtNPs. The quantitative measurements were performed by chronocoulometry in phosphate buffer (PBS) solution and saliva samples. The method exhibited LOD of 1 pg mL^{-1} and 2.2 pg mL^{-1}, respectively [36].

Hemben et al. developed a chronoamperometric immunosensor to detect Ag-*Pf*HRP2. The monoclonal antibodies (Ab-*Pf*HRP2) were immobilized

by physical adsorption on screen-printed gold electrodes (SPGE). For antigen detection, a format like sandwich ELISA was constructed, using horseradish peroxidase and a solution of 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide. The LOD achieved for PBS and serum samples, both enriched with the antigen were of 2.14 ng mL^{-1} and 2.14 ng mL^{-1}, respectively. Besides, the signal is amplified using AuNPs conjugated to horseradish peroxidase labeled-secondary antibody complex, recording an LOD = 36 pg mL^{-1} in PBS and LOD = 40 pg mL^{-1} in the serum samples [37].

Paul et al. reported the fabrication of a flexible, lightweight, disposable biosensor for label free detection of Ag-*Pf*HRP2. Gold electrodes were fabricated on polyethylene terephthalate and then MWCNT-zinc oxide (ZnO) nanofibers were synthesized by a simple, low-cost electrospinning technique followed by calcination process. The device exhibited good sensitivity, with a wide detection range (10 fg mL^{-1} - 10 ng mL^{-1}) and is specific for the target analyte. This methodology was the first report on flexible chemiresistive biosensor explored for the detection of malaria biomarker [38].

Chakma et al. published an aptasensor for Ag-*Pf*HRP2 detection. On this platform, monolayers were self-assembled by bonding the thiol groups (HS) of Lomant's reagent (dithiobis succinimidyl propionate) to the surface of gold disc electrodes. Following, the ssDNA aptamer against Ag-*Pf*HRP2 (previously synthesized) was immobilized on the modified electrode. The electrochemical response was studied by electrochemical spectroscopy impedance (EIS) and the increase of the charge transference resistance was proportional to the increase in Ag-*Pf*HRP2 concentration, from 1 pmol L^{-1} to 500 pmol L^{-1} with LOD of 3.15 pmol L^{-1}, being possible to detect asymptomatic, uncomplicated and complicated malaria cases [39].

Dip Gandarilla et al. described the fabrication of a one-step enzyme-free immunosensor based in Ab-*Pf*HRP2 immobilization (previously synthesized by them) on gold disc electrodes, using dihexadecylphosphate (DHP) as polymeric matrix. The device is based on a simple and low-cost method (biomolecule encapsulation through polymeric network), which allowed the detection of Ag-*Pf*HRP2 by two electrochemical techniques (differential pulse voltammetry and EIS), in the concentration range of 10 - 500 ng mL^{-1}, with a limit detection rate of 2.8 ngmL^{-1}. The results were confirmed by ELISA tests and the device exhibit be selective for the target analyte, without interference from other components present in the human serum samples [40].

Figueroa-Miranda et al. developed an impedimetric aptasensor for Ag-*Pf*LDH determination. The platform was constructed by immobilizing of the 2008s aptamer on gold electrodes. For the preparation of gold chips, a metal stack of 10 nm titanium adhesion layer and 100 nm gold layer were

deposited onto silicon wafers using electron-beam evaporation. The detection range of analyte was adaptable in different pH values around the protein isoelectric point, with a good response by EIS between 10 fmol L^{-1} and 10 nmol L^{-1}, with LOD of 0.84 pmol L^{-1}. Furthermore, the aptasensor can be easily regenerated by simply rinsing with a 6 mol L^{-1}urea solution as a denaturing agent [41].

Hemben et al. described the development of an affinity biosensor for the detection of Ag-*Pf*LDH. SPGE were functionalized with Ab-*Pf*LDH and AuNPs conjugated to a horseradish peroxidase/secondary antibody system were used to improve the electrochemical response. The LOD achieved by chronoamperometry was of 9 pg mL^{-1} in PBS buffer solution and 23 pg mL^{-1} in serum samples, both increased with antigen. The results were compared with the obtained with an OptiMAL–IT and BinaxNow Malaria commercial kits. The immunosensor showed high sensitivity and reproducibility, cost effective point-of-care immunoassay, which can be performed in less than 2 hours [42].
Low et al. manufactured an ultrasensitive impedimetric immunosensor for the detection of Ag-*Pv*LDH. Interdigitated electrodes (IDEs) were made on Borofloat glass wafers via UV-lithography. A thin film of chrome (5 nm), gold (100 nm), and titanium (5 nm) was deposited on the substrate, followed by a lift-off process to reveal the IDE pattern. The biosensor platform consisted in Ab-*p*LDH immobilizing on IDEs surface modified with 3-aminopropyl triethoxysilane and glutaraldehyde. The impedimetric response before and after Ab - Ag interaction was recorded in PBS and saliva samples, both increased with Ag-*Pv*LDH, being founded a LOD of 250 pg mL^{-1} and 2.5 ng mL^{-1}, respectively [43].

Singh et al. published a capacitive aptasensor for detecting of Ag-*Pf*GDH. A thiolated ssDNA aptamer (NG3) that binds specifically to Ag-*Pf*GDH was grafted to the gold disc electrodes by incubated overnight in an aptamer and 6-mercapto 1-hexanol (MCH) solution. The non-Faradaic EIS measurements were performed after binding with the target antigen, where the capacitive signal change was linearly correlated with the antigen concentration, from 100 fmol L^1 to 100 nmol L^1, with LOD = 0.77 pmol L^1 in undiluted serum samples spiked with Ag-*Pf*GDH. The specificity of the aptasensor was tested in presence of other proteins (Ag-*Pf*LDH, Ag-*Pf*HRP2 and Ag-*h*GDH and human serum albumin (HSA) in serum sample) and the results indicate only a minor non-specific interaction of the sensor with the Ag-*Pf*LDH. This aptasensor use a label-free method, recording a ultra-low LOD, hence it present promissory application for diagnosis of asymptomatic malaria and for monitoring during treatments with antimalarial drugs [44].
Singh et al. reported an aptamer-based field effect transistor (aptaFET) biosensor for the Ag-*Pf*GDH detection. The platform uses an extended gate field effect transistor with inter-digitated gold microelectrodes (IDµE). A

thiolated ssDNA aptamer (NG3) was immobilized onto IDμE surface. The aptamers immobilized IDμE was connected to a home-designed n-type complementary metal oxide semiconductor field-effect transistor and the aptaFET response was followed by incubation in the binding buffer or diluted human serum spiked with different concentrations of Ag-*Pf*GDH. This biosensor showed high sensitivity at 100 fmol L^{1} −10 nmol L^{1} concentration range, with LOD of 16.7 pmol L^{1} and 48.6 pmol L^{1} in spiked buffer and serum samples, respectively. The device selectivity was confirmed by testing in analogous human and parasitic proteins [45].

4. CONCLUSIONS

In this paper, we summarized about: current global situation of malaria infection, principal detection methods, biomarkers of malaria and some of the electrochemical biosensor developed ever now for determination of malaria biomarkers. The studies described involve several manufacture strategies, use of various materials and different electrochemical techniques. These sensing platforms constitute methods highly sensitive and specific, which have potential application for the diagnosis of malaria.

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