

Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

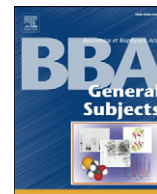
In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/authorsrights>



Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Malarial hemozoin: From target to tool

Lorena M. Coronado^{a,b}, Christopher T. Nadovich^c, Carmenza Spadafora^{a,*}^a Centro de Biología Celular y Molecular de Enfermedades, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología, Ciudad del Saber, Panama^b Department of Biotechnology, Acharya Nagarjuna University, Guntur 522 510, A.P., India^c Electrical and Computer Engineering, Clarkson University, Postdam, NY 13699-5720, USA

ARTICLE INFO

Article history:

Received 27 August 2013

Received in revised form 26 January 2014

Accepted 10 February 2014

Available online 17 February 2014

Keywords:

Hemozoin synthesis

Hemoglobin degradation

Hemozoin structure

Hemozoin biophysical properties

Plasmodium falciparum

ABSTRACT

Background: Malaria is an extremely devastating disease that continues to affect millions of people each year. A distinctive attribute of malaria infected red blood cells is the presence of malarial pigment or the so-called hemozoin. Hemozoin is a biocrystal synthesized by *Plasmodium* and other blood-feeding parasites to avoid the toxicity of free heme derived from the digestion of hemoglobin during invasion of the erythrocytes.

Scope of review: Hemozoin is involved in several aspects of the pathology of the disease as well as in important processes such as the immunogenicity elicited. It is known that the once best antimalarial drug, chloroquine, exerted its effect through interference with the process of hemozoin formation. In the present review we explore what is known about hemozoin, from hemoglobin digestion, to its final structural analysis, to its physicochemical properties, its role in the disease and notions of the possible mechanisms that could kill the parasite by disrupting the synthesis or integrity of this remarkable crystal.

Major conclusions: The importance and peculiarities of this biocrystal have given researchers a cause to consider it as a target for new antimalarials and to use it through unconventional approaches for diagnostics and therapeutics against the disease.

General significance: Hemozoin plays an essential role in the biology of malarial disease. Innovative ideas could use all the existing data on the unique chemical and biophysical properties of this macromolecule to come up with new ways of combating malaria.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Malaria is a disease caused by *Plasmodium* parasites, resulting in approximately one million deaths every year around the world. Regions and countries affected by poverty are most at risk of infection. One of the greatest obstacles in the control of malaria has been the spread of drug resistance almost worldwide. Currently, the standard treatment of uncomplicated malaria in these regions consists of artemisinin-based combination therapies (ACTs), while chloroquine combined with primaquine is the treatment of choice for chloroquine-sensitive infections. For the treatment of severe malaria there are two classes of drugs available: the cinchona alkaloids (quinine and quinidine) and artemisinin derivatives (artesunate and artemether) [1]. Resistance to chloroquine and sulfadoxine–pyrimethamine fueled the ongoing scourge of *Plasmodium*

falciparum, the principal cause of malaria morbidity and mortality in the world. In response to the increased number of infections, the World Health Organization (WHO) has recommended the use of combinatorial therapies that include artemisinin derivatives as first-line therapy. Nevertheless, even combinations with new drugs have the potential to create resistance [2–4]. Research efforts are needed to find alternative treatments for malaria that avoid the problem of drug resistance altogether.

One approach for the development of new treatments against malaria is to study the *Plasmodium* distinctive molecule hemozoin and to try to target this vital pathway of the parasite [5]. Hemozoin (HZ) is a metabolically crystallized byproduct of the digestion of hemoglobin by the parasite during infection of the red blood cells (RBCs). The formation of hemozoin from heme residues is common among diverse hematophagous organisms without phylogenetic relation to *Plasmodium spp.*, such as *Schistosoma mansoni* and *Rhodnius prolixus* [6]. Since sequestration of heme into hemozoin is an essential process for the survival of the malaria and other apicomplexan parasites, this molecule has become an attractive target for new drugs that could interfere with the biocrystallization of hemozoin and would help fight diseases caused by these pathogens, especially malaria [7–10].

Abbreviations: HZ, hemozoin; RBC, red blood cell; FV, food vacuole; HDP, Heme Detoxification Protein; NMR, nuclear magnetic resonance; PPIX, protoporphyrin IX; CQ, chloroquine

* Corresponding author at: CBCMe, INDICASAT AIP Edificio 219, Ciudad del Saber Panama, Republic of Panama.

E-mail address: carmenzaspadafora@gmail.com (C. Spadafora).

2. Hemozoin synthesis

2.1. Hemoglobin degradation

Hemoglobin is the principal component of red blood cells, composing approximately 95% of the proteins of the cytosol, reaching a concentration of about 5 mM inside the cytoplasm [11].

During the intra-erythrocytic stages of malaria infection, up to 80% of the cytoplasm of the host is consumed. Although during the ring stage some hemoglobin degradation is detectable, the major part of this process takes place during the trophozoite and schizont stages, which perform most of the metabolic activity of the parasite [12].

The hemoglobin degradation process occurs mostly inside the digestive vacuole of the parasite (Fig. 1). These organelles in *P. falciparum* are acidic structures with an estimated pH of 5.0 to 5.4 [13,14]. The acidic pH in these vacuoles is maintained by a proton gradient activated by an ATPase pump. It is thought that the digestive vacuole is a vesicle dedicated almost exclusively to hemoglobin degradation because of the lack of the characteristic lysosomal phosphatase and glycosidases present in other organisms. Hemoglobin degradation was believed to play a vital role as an amino acid source for malaria parasites because it has been observed that they have a limited capacity to synthesize their own. Some have alleged that the parasites do not need to degrade other macromolecules to sustain growth and development because they obtain most of the nutrients directly from the digestion of hemoglobin [15]. But, given that hemoglobin is a poor source of methionine, cysteine, glutamine and glutamate, and completely lacks isoleucine, parasites must be degrading hemoglobin for purposes other than just nutrition or detoxification. Such an idea was tested in experiments where parasites were placed in a culture medium that provided them with the 20 essential amino acids; it was shown that the parasites were still degrading hemoglobin [16]. Several studies demonstrated that some protease inhibitors block the proteolysis of hemoglobin, and the result was that the parasite development was interrupted, even with all nutritional resources available [17–20]. Therefore the idea of hemoglobin being digested mainly for nutritional purposes was not completely accurate. An equilibrium system exists inside the parasite between host hemoglobin degradation, efflux of the amino acids produced by hemoglobin degradation, and influx of extracellular amino acids and subsequent incorporation into parasite proteins. While the parasite grows and develops, the amount of hemoglobin degradation increases but not the

amount of parasite protein: even though malaria parasites digest more than 65% of the host cell hemoglobin, they only use approximately 16% of this digestion to synthesize proteins for their own needs [21]. The membrane of the infected erythrocyte becomes more permeable to amino acids as it develops, allowing for large amounts of them to exit from the cell [21]. Later findings evidenced that the parasite digests the erythrocyte hemoglobin in order to prevent it from early lysis, which could take place if the parasite did not offset the increase in cell volume [22]. This colloid osmotic hypothesis was corroborated with mathematical models, which accurately fit the experimental data [23].

Most of the enzymes implicated in the hemoglobin degradation pathway have been elucidated. Two aspartic proteases (plasmepsins I and II) and a cysteine (falcipain) protease have been isolated and purified from the digestive vacuole of *P. falciparum*. All of these proteases function optimally at a pH of around 5, coinciding with the digestive vacuole acidity [24,25]. Aspartic proteases account for 60–80%, and cysteine protease for 20–40% of the globin-degrading activity of purified digestive vacuoles [11].

Plasmepsin I makes the initial attack between the α 33Phe and 34Leu residues of hemoglobin and then the subsequent cleavages take place elsewhere. The 33–34 bond is located in a specific region that plays an essential conformational role in the hemoglobin molecule, acting like a hinge in the tetramer. Apparently, cutting the molecule in this specific site allows for the uncoiling of hemoglobin, exposing other sites for later proteolytic attacks by cysteine and aspartic proteases of the degradation pathway [25].

For the hemoglobin degradation process, the parasite needs both plasmepsins I and II. The reasons for the parasite needing two different enzymes for a single function are still unknown. In any case, different enzymatic expression patterns of these molecules have been detected along the intraerythrocytic stage of the parasite [26]. During the ring stage only plasmepsin I is expressed, and its expression continues during the following stages of the parasite. In contrast, plasmepsin II is mainly expressed during the trophozoite stage, which is the most metabolically active. Not surprisingly, almost all of the host cytoplasm is consumed during this stage. In fact, both enzymes are active during the trophozoite stage and are necessary to fulfill the massive catabolic processes taking place in this period of growth of the parasite [11].

In studies performed by Mueller and colleagues, exploiting the difference in sensitivity of both plasmepsin enzymes to the plasmepsin inhibitor SC-50083, they determined their role in the survival of the parasites, and the team concluded that blocking the activity of plasmepsin I leads to the death of parasites. Surprisingly, although the amount of plasmepsin II

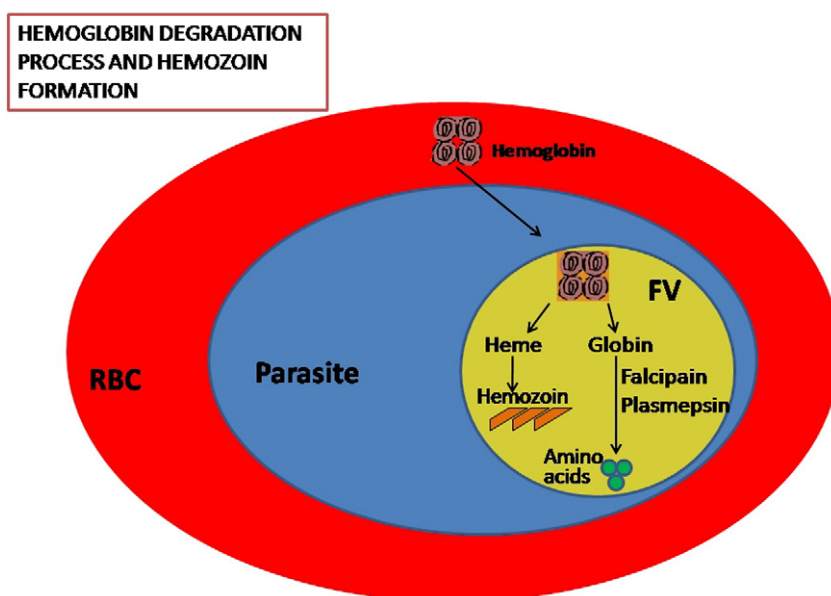


Fig. 1. Diagram of hemoglobin degradation and formation of hemozoin inside *P. falciparum* infected erythrocytes. Hemoglobin is degraded to obtain amino acids and to regulate osmotic pressure, and the parasite converts the toxic heme part into harmless hemozoin inside the parasitophorous food vacuole. RBC: red blood cell; FV: food vacuole.

is higher during the trophozoite stage than the amount of the enzyme during the ring stage, this stage abundance was not enough to compensate for the absence of plasmepsin I in the trophozoites [17].

There is some evidence for the possible involvement of a third aspartic protease, but it has not been confirmed either by purification or by cloning [27].

The last enzyme involved in hemoglobin degradation is a cysteine protease called falcipain. Falcipain does not cleave the hemoglobin molecule unless it is previously denatured either by reducing conditions, acid-acetone treatment, or previous plasmepsin cleavage [24].

Chugh et al. recently reported the presence of a protein complex of about 200 kDa in the food vacuole of the parasite that is needed for the processes of hemoglobin degradation but also for hemozoin synthesis. This complex is formed by the most important enzymes involved during these two important processes: falcipain 2, plasmepsin II, plasmepsin IV, histo-aspartic protease and a protein called Heme Detoxification Protein (HDP) [28]. The existence of this complex has been proven by several techniques such as coimmunoprecipitation followed by mass spectrometry, coelution from a gel filtration column and *in vitro* protein interaction analyses [29].

2.2. Hemozoin biocrystallization

The proteolytic process of hemoglobin degradation releases free heme that would be toxic to the malaria parasite. The lack of heme oxygenase in *Plasmodium spp.*, the enzyme present in all vertebrates and responsible for the degradation of the heme moiety, means that the parasite must provide for another method of detoxification, which is achieved by converting the heme monomer into the inert biocrystal called malarial pigment or hemozoin.

α -Hematin (ferriprotoporphyrin IX), which is toxic to the parasite, is released during hemoglobin digestion [30]. However, and most possibly as a protection strategy, the parasite transforms α -hematin into hemozoin (chemically identical to β -hematin), a molecule with paramagnetic properties. The magnetic properties of this ferriheme were extensively studied by Pauling et al. by the middle of last century [31,32]. By determining the magnetic susceptibilities of ferriheme it was concluded that it contains a five coordinate high spin trivalent iron compound [33]. It was shown that in this arrangement the iron atom is attached to the four adjacent nitrogen atoms of the porphyrin, not by covalent bonds but by ionic bonds. In ferriheme the iron is present essentially as Fe(III).

The term β -hematin was coined by Hamsik (in the 1920s) [34] for sodium bicarbonate insoluble hematin. The facts that β -hematins are insoluble in sodium bicarbonate solution, that hematin esters are not transformed into β -hematin compounds, and that β -hematins are reconverted into hemin esters by esterification indicate that the carboxylic acid groups of hematin are involved in β -hematin formation. Lemberg & Legge (1949) [35] used the term β -hematin to allude to the presence of a specific linkage in which iron of one hematin unit is bound to the propionic acid group of another; β -hematin can either be a cyclic dimer or a linear polymer [5].

It is important to note that synthetic β -hematin crystals can be made *in vitro* with the same NMR and X-ray diffraction pattern as parasitic hemozoin by adding the whole trophozoite lysate to heme acidified to a pH between 5 and 6 [36]. However, there are differences between synthetic (sHZ) and natural hemozoin (nHZ). The size and shape of synthesized β -hematin crystals may not be identical as a result of differences in the purification process used. While natural hemozoin is composed of smaller crystals measuring between 50 and 500 nm, synthetic β -hematin crystals can range from 50 nm to 20 μ m, depending on the solvent used to form them. This is an important consideration to take into account, as different sized hemozoin crystals may possess diverse immunomodulatory responses, as explained elsewhere [37].

Studies to elucidate the mechanism of hemozoin formation can be divided into two main categories: First, those that investigated its formation under entirely non-biological conditions; and a second group

of studies that focused on analyzing the formation of β -hematin in the presence of biological material, either in the form of trophozoite extracts, histidine rich protein (HRP) or lipids.

The first group concentrated on β -hematin formation carried out in acetate solution, and suggested that a complete β -hematin formation was possible at a moderately low pH [38,39]. These observations led to the proposal that the reaction might be spontaneous in the parasite, without any initiator or catalyst.

Concerning the second group of studies, three important hypotheses have been postulated [40]:

1. Hemozoin biocrystallization is an autocatalytic process: recent studies have obtained microscopic images of hemozoin crystals growing on the surface of pre-existing hemozoin crystals [41].
2. Biocrystallization is due to the action of enzymes: this presupposes the presence of proteins. One possible candidate is HRP1. This protein is ingested by the parasite in the process of hemoglobin degradation and it was demonstrated that it can promote *in vitro* hemozoin synthesis [42], but the involvement of another HRP could not be ruled out. In *Plasmodium* parasites there are many processes with a redundancy. Examples are found in the invasion process [43,44] and in the hemoglobin degradation process [45]. In the same way, there was also evidence of common redundancy in the existence of at least two HRPs that could be involved in hemozoin formation [42]. In 2007, E. Hempelmann convincingly discussed how the unfruitful search for a heme polymerase responsible for forming hemozoin in HZ-producing species ended up in the realization that hemozoin is not a true polymer and thus established "biocrystallization" as the best term to describe the process that forms hemozoin [5]. In fact, in light of this, and of the observation that double mutants for HRPII and HRPIII still form hemozoin [46], Egan concluded that HRP is probably not involved in hemozoin formation at all [47].
3. Biocrystallization is catalyzed by lipids: The third hypothesis attributes hemozoin formation to the action of lipids [48–50]. With the evidence obtained in recent investigations there is no doubt that some lipids promote the formation of β -hematin crystals. The product of this synthesis has been well characterized by spectroscopic techniques [51,52]. These lipids have been described as a neutral lipid blend (NLB) and monopalmitoylglycerol (MPG), which are synthesized by the parasite and packed in the FV. These lipids associated with the FV are thought to come from the digestion of the transport vesicles. It was shown that these lipids are highly efficient at converting Fe(III) PPIX to β -hematin with yields at or above 80% [53] just as was suggested by Sullivan in 2005 [54].

While several lines of evidence point to involvement of acylglycerol lipids in the nucleation process [53,55,56], a recent study focused on the location and orientation of hemozoin crystals. To address this, they applied cryogenic soft X-ray tomography and three-dimensional electron microscopy. They found that hemozoin nucleation occurs in an oriented manner at the inner surface of the digestive vacuole, with crystallization, surprisingly, occurring in the aqueous phase rather than in the lipid phase [57].

Finally, concerning the hemozoin formation processes, and as mentioned in the hemoglobin degradation section, Rathore et al. identified and characterized another protein that converts heme into hemozoin in a very powerful manner. This protein is the HDP [28].

To conclude, at present it is still unclear which and if any of these mechanisms is actually involved in hemozoin crystallization processes *in vivo*. More studies in this direction are needed to elucidate the complete mechanism of HZ production in *Plasmodium* parasites.

3. Hemozoin structure

The structure of hemozoin has been elucidated by X-ray diffraction, infrared spectroscopy, Raman spectroscopy and chemical synthesis. The molecule was first determined to consist of an unusual polymer of heme

groups linked by bonds between the oxygen from the carboxylate of one heme and the central ferric ion of the next heme. This unusual linkage allows the heme units to be aggregated into an ordered insoluble crystal (Fig. 2), representing a unique way of heme processing [58].

In the early nineties, Slater et al. used purified hemozoin and the hemozoin forming unit (hematin) to address the hemozoin structure through differences in solubility and spectroscopic properties. This was a better way to elucidate the structure because the one for hematin was known, and a comparison of the behavior of these two molecules could infer similarities and differences. First, hematin is soluble in weakly basic bicarbonate or borate buffers and in aprotic solvents. In contrast, hemozoin is insoluble in both kinds of solvents [58].

The solubility difference could be explained if there is a modification in the environment surrounding the carboxylate side groups and the iron of heme in hemozoin. Concerning the hemozoin and hematin IR spectra, it has been determined that they are also quite different. Hemozoin is more sharply resolved than hematin, suggesting a reduction in the hydrogen bonding. Also, the IR spectrum of hemozoin contains additional major features at 1664 and 1210 cm^{-1} absent in the spectra of hematin. As absorbances between 1720 and 1650 cm^{-1} indicate C=O stretching, this suggests the presence of only one bond between the carboxylate side chain and the metal iron in hemozoin.

Solubility data and IR spectra of hemozoin lead to a possible structure of hemozoin where it displays a direct coordination between a carboxylate of one heme and the iron of the following [58].

Additionally, since axial Fe–C (O) coordinations produce very strong C–O stretching frequencies around 1900 cm^{-1} [59], and they are absent in the IR spectra, a direct iron–carbon linkage can be ruled out in the structure of hemozoin. This fact, in turn, led researchers to propose that the oxygen and carbon stretch probably belongs to a carboxylate group which has one oxygen directly coordinated to the central iron of the molecule. This statement has been evidenced by infrared and Raman spectroscopy [60].

β -Hematin can either be a polymer or a dimer. Electrophoretic studies were done in 1993 to distinguish between the two models; a polymer would produce a ladder after separation by SDS electrophoresis and a dimer only a single band: a ladder was never found [46]. The inescapable conclusion was that β -hematin found in *P. falciparum* is composed of dimers.

Pagola et al. finally elucidated the structure of β -hematin using high resolution X-ray powder diffraction data and analyzed it with a simulated annealing algorithm. They solved the crystal structure by positioning

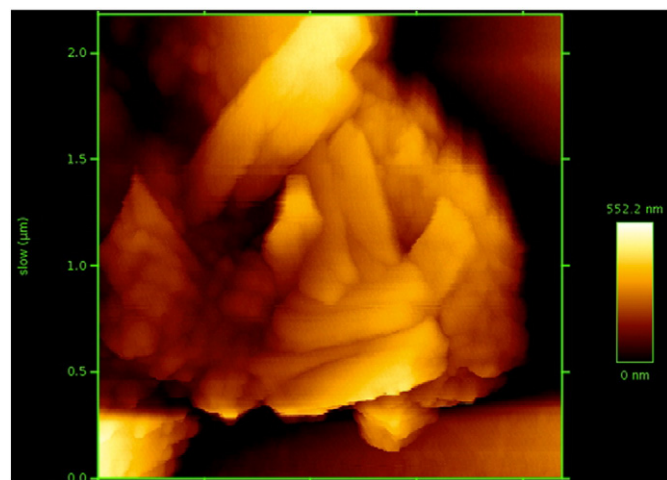


Fig. 2. Atomic force microscopy (AFM) image of *P. falciparum* hemozoin. A culture of blood stage *P. falciparum* was examined with a Nanowizard, JPK Instrument AG, Germany. An ultrasharp noncontact AFM-tip (NSG10, ATOS GmbH, Germany) was used and the AFM was operated in the instrument's intermittent contact mode (Image by Lorena Coronado at Volker Deckert Laboratory, Institute of Photonic and Technology, Jena, Germany).

the molecule and finding the torsion angles that give the best agreement between experimental and calculated powder diffraction profiles. In all of the combinations made by the software, the molecules were paired into dimers by iron–oxygen bonds. Thus, the idea that hemozoin is just a polymer was invalidated. It is now well known that it is composed of a series of molecules linked into dimers through reciprocal iron–carboxylate bonds to the propionic side chains of each porphyrin, and that the dimers form chains linked by hydrogen bonds in the crystal [61] (Fig. 3). This was concluded through photoacoustic spectroscopy (discussed later in this review) [62] and corroborated more recently by using β -hematin DMSO solvate, being this the first Fe(III) PPIX model of hemozoin studied by single crystal X-ray diffraction [63].

About the spin state of the iron center in hemozoin, the definite conclusions were given by Sienkiewicz et al. in 2006 with multi-frequency high-field electron paramagnetic resonance (HFEP) studies. Based on previous data of magnetic susceptibility and Mossbauer spectroscopy, the presence of a high spin iron seemed very likely, but there were still ambiguities in the interpretation of the data, and they persisted until the analysis, done by the team of Sienkiewicz using the same technique, showed that the spectra obtained for hemozoin and synthetic β -hematin can only correspond to a high spin Fe III ($S = 5/2$), finally giving closure to this issue [33].

4. Hemozoin immunomodulatory action

Phagocytes are the first line of defense against malaria infection in non-immune patients. There is a relationship between their capacity for phagocytosis and the parasite stage. Macrophages ingest more avidly RBCs infected with trophozoites and schizonts, rather than rings of *P. falciparum*. It is thought that, as the red blood cell membrane

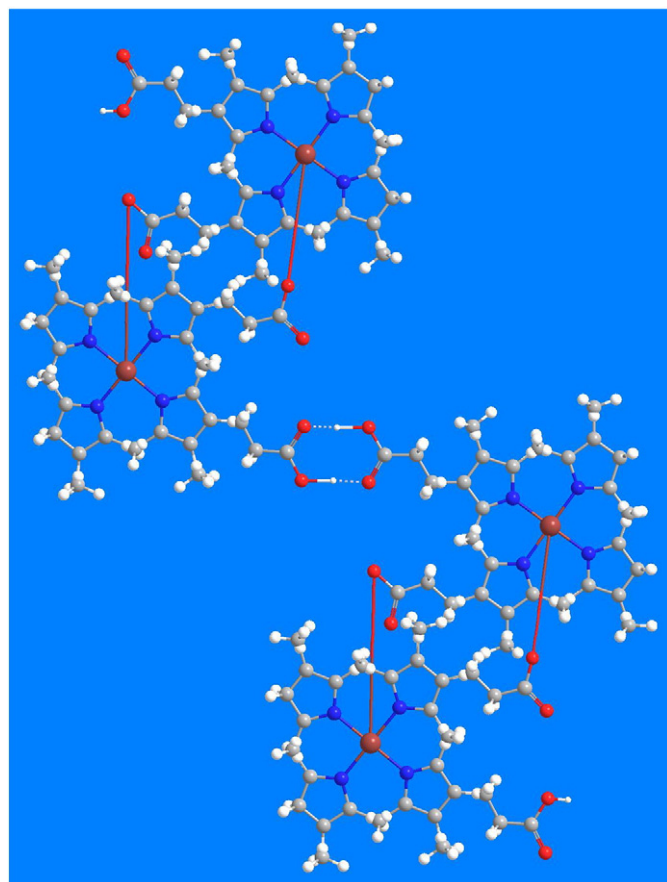


Fig. 3. Chemical arrangement of hemozoin. A) Monomers of hemozoin with iron, shown in gray, will join to form a dimer. B) Crystal moiety with the dimers forming H-bonds with oxygen atoms (red) of contiguous ones.

containing mature staged parasites has more alterations due to the size of the parasite, it is better recognized by IgG and complement components [64]. In humans, the three most common locations where the macrophages that ingest hemozoin can be found are: the spleen, liver and bone marrow [65–67]. In *Plasmodium*, hemozoin was considered a non-toxic form of digested heme from the host cell [36,68]. However, it can be seen now that hemozoin is involved in other aspects of the disease, affecting the macrophages which ingest it and contributing to the pathophysiology of the disease [69–75]. Hemozoin is released into circulation during erythrocyte lysis. After schizont rupture, crude hemozoin, composed of aggregated heme and a variety of attached components of parasite and host origin (lipids, lipid derivatives and proteins) is avidly taken up by phagocytes, both *in vivo* and *in vitro* [76]. Macrophages that have ingested infected RBCs with parasites in the ring stage are able to degrade hemoglobin rapidly and can repeat the phagocytic cycle. In contrast, when the parasites ingested are in a mature stage the macrophages are unable to degrade hemoglobin or continue with the normal phagocytic cycle [77]. These macrophages appear to lose all functionality regarding the ingestion of opsonized, damaged erythrocytes or the killing of ingested bacteria, fungi or tumor cells [78,79]. It has been suggested that this toxic effect in macrophages that have ingested latter stages of *Plasmodium* parasites is due to the HZ crystals causing a permanent damage in the normal function of these cells [77]. HZ is rapidly ingested by phagocytes and induces the production of several pro-inflammatory mediators such as IL-1b and TNF α and is also associated with cytokine production in murine models [80]. Studies in both human and murine macrophages, *in vitro* and *in vivo*, show a thermal deregulation, associated with *in vivo* fever, after injecting chemically synthesized HZ [80]. It should be stated, however, that Jaramillo et al. reported that similar immune responses with native and synthetic hemozoin can be obtained only after verifying that the crystals of the synthetic HZ (sHZ) resemble those of native HZ (nHZ) in size, morphology, and physicochemical characteristics [81,82]. Moreover, the extraction method utilized to isolate nHZ for immunomodulatory experiments must be carefully taken into account since host proteins, lipids and even DNA attached to the crystals might withstand the process of extraction and induce immune reactions [83,84] that differ from those caused strictly by nHZ. It is important to note, though, that nHZ is usually attached to other material also *in vivo*, with various immunological consequences. For example, host fibrinogen (FG) is present and stably bound to nHZ from plasma cultured parasites, and is responsible for the rapid stimulation of reactive oxygen species (ROS), due to the interaction of FG with Toll Like Receptor 4 (TLR4) and integrin CD11b–CD18 [85]. The role of the TLRs in the immune reaction to malaria is discussed further down.

Millington et al. demonstrated *in vitro* and *in vivo*, that malaria parasites induce failure in the function of dendritic cells. This, subsequently, causes a decline in T cell activation that would normally modulate heterologous and infection-stimulated immunological reactions [86–90]. Thus, hemozoin affects directly the normal function of the adaptive immune response. Some have argued that the decline in functionality of phagocytes that have ingested hemozoin could also be due to the generation of reactive oxygen species inside the white blood cell [75]. HZ has the ability to become a powerful generator of radicals due to its unique sequestration of the ferric heme in a paracrystalline compact form. Crude hemozoin, loaded *in vitro* to murine macrophages, and to macrophages isolated from malaria infected murine livers, is not capable of generating radicals by itself but it can do so in the presence of a lipid component, specifically unsaturated fatty acids; lipid removal totally abolishes radical production [71]. A lack of activation of nitric oxidase synthase (NOS) in human monocytes when challenged with HZ, in contrast with the upregulation found in murine macrophages, has been reported [91]. This observation was contrary to findings suggesting that inducible NOS (iNOS) upregulation does take place in humans and could even confer protection against malaria [92,93]. A careful analysis of the data published on this matter reveals that experimental

conditions might influence the outcome of the investigation. This fact, in conjunction with the natural differences found between the two species, should alert researchers to the need for caution when inferring similar immunomodulatory reactions through extrapolation of results obtained in murine models to humans.

The parasitic food vacuole has a specific pH and environment that promotes peroxidation. Parasitized RBCs and hemozoin contain large amounts of esterified monohydroxy derivatives of polyunsaturated fatty acids (OH-PUFAS) that can result from lipid peroxidation of polyenoic fatty acids or by the action of a lipoxygenase. Schwarzer et al. pointed out that since mature RBCs do not have lipoxygenases (LPO) and no LPO isoform or activity has been found in the sequence of *P. falciparum* or the parasite, respectively, OH-PUFAS are probably the end product of a lipid peroxidation catalyzed by heme, a process already described previously for this molecule [94]. Specific components of this lipid fraction, present in infected RBCs and hemozoin, are then responsible for the decline in the oxidative burst in monocytes. In consequence, they contribute to hemozoin-induced toxicity to phagocytes after ingestion, which leads to a failure in the immune response [76].

Malarial anemia is characterized by the destruction of infected as well as uninfected RBCs. This massive destruction leads to a decrease in erythroid precursors and erythropoiesis inhibition, usually resulting in the death of the patient. Hemozoin is thought to be related to this anemia phenomenon as well as to the production of inflammatory mediators such as tumor necrosis factor (TNF- α) in murine models. In 2004, Arese et al. demonstrated that supernatants of nHZ, nHZ fed monocytes, and 4-hydroxynonenal (HNE), generated through lipid peroxidation, inhibited progenitor growth [84]. This observation indicates that toxic molecules generated by HZ may play a direct role in erythropoiesis inhibition. This was confirmed by another group in 2006 [95]. They showed *in vitro* that isolated HZ, after a process of delipidation and in complete absence of TNF- α , inhibited erythropoiesis. They also showed in children with malaria that monocytes containing HZ inhibited erythropoiesis independently from circulating cytokines. They also found indications of apoptosis in erythroid cells *in vitro* as an effect of HZ treatment. HZ was found to enhance the levels of cleaved caspases 8 and 3 and also to disturb the membrane potential of mitochondria. They concluded that HZ has an impact on the extrinsic apoptotic pathway in erythroid cells. These results were validated and reconfirmed *in vitro* by other studies where HNE was identified as an erythropoiesis inhibitory molecule generated by HZ lipid peroxidation and alteration of critical proteins of the cell cycle [96,97].

Another important group of molecules involved in the innate immune responses to *Plasmodium* infection is the Toll-like receptors, which recognize malaria parasites and derivatives [98]. Coban et al. studied the role of TLRs and their signaling molecules in cerebral malaria pathogenesis. Their results strongly suggest that immune response *via* TLR2, TLR9 and MyD88 (an essential adaptor molecule for most TLRs) is closely involved in this clinical complication of malaria [99]. Coban's findings have been corroborated in various ways. It was recently demonstrated that TLR9 and MyD88 are crucial for the development of protective immunity to malaria [100,101] to the point of them being considered as possible adjuvants in malaria vaccines [37]. Also, polymorphisms in TLR4 predispose patients to cerebral malaria [102] and TLR7 plays a central role in early immune activation during malaria infection as well [103].

Clearly, hemozoin release plays a very crucial role in the pathology of malaria, especially in what concerns the immunological response, which evidences that hemozoin is much more than just a by-product crystal from hemoglobin degradation.

5. Hemozoin as drug target

5.1. Chloroquine mechanism of action

Since hemozoin crystallization is vital for malaria parasite survival this process stands out as an attractive target in the search for new

antimalarial drugs [104]. The postulated mechanisms by which these drugs would act on hemozoin differ subtly. The best studied one has been that of chloroquine (CQ).

A first line drug against malaria for more than four decades up to the mid 1980s, chloroquine is thought to act *via* its action in the digestive vacuole related to the inhibition of hemozoin formation. It has been demonstrated that there is a relationship between the HZ inhibition effect and the antiplasmodial IC₅₀ activity of chloroquine. Also, CQ-mediated parasite death is directly related to hemozoin inhibition [105]. And although this drug kills parasites in ring better than in trophozoite stages [106], there is evidence that in trophozoite cell extracts, chloroquine also inhibits hemozoin crystallization [36].

Chloroquine enters into the parasitic digestive vacuole through simple diffusion. The acidic pH of the vacuole (pH ~ 5), however, produces a diprotonation of the drug and in this form the molecule cannot diffuse back through the membrane, remaining trapped inside the vacuole. There, diprotonic CQ interferes with the parasite detoxification process by inhibiting heme transformation into hemozoin. The accumulation of heme causes severe damage to the parasite, leading to its death [107].

Based on what little was known about the mechanism of action of CQ, several studies focused on testing its role as a possible inhibitor of hemozoin (ferriprotoporphyrin IX) crystallization [36,108,109]. They concluded that chloroquine does inhibit hemozoin formation by interfering in its assembly, be it initiated by hemozoin or by trophozoite extracts [36,109,110]. This hypothesis is now accepted as the mechanism of action of 4-aminoquinoline antimalarials. Later, in experiments performed by Sullivan et al., it was demonstrated that CQ inhibited the HRP II mediated synthesis of hemozoin [42], which is consistent with the proposed mechanism of action of this drug on heme crystallization.

Wood et al. performed a study using resonance Raman spectroscopy to monitor the effects of CQ treatment on cultures of *P. falciparum* trophozoites. They found that some characteristic bands of HZ were reduced in their intensity in the CQ treated cells. These intensity changes were attributed to intermolecular drug binding of the CQ in complex with the ferriprotoporphyrin IX dimer units. They postulated that CQ binds *via* π - π interactions (attractive, noncovalent interactions between aromatic rings) between adjacent porphyrins, thereby disrupting the hemozoin aggregate and reducing excitonic interactions between adjacent hemes [111].

While it had been generally accepted that heme was the target for chloroquine, there was no direct evidence, until recently, linking the inhibition of hemozoin formation to an increase in the amount of free heme molecules in the parasite cytoplasm when treated with chloroquine. Similar to CQ, quinine was found to inhibit heme detoxification within the food vacuole resulting in the inhibition of hemozoin biocrystallization [112]. With the use of sophisticated technology the obvious was confirmed. X-ray diffraction was used to corroborate that different concentrations of both chloroquine and quinine decrease the rate of hemozoin formation [63]. Then measurements of Fe(III)heme-pyridine using transmission electron microscopy were able to confirm the link between the decrease in hemozoin formation and an increase of toxic free heme in the parasite [112].

Few years ago it was thought that CQ could inhibit the peroxidative degradation of heme, contributing to the building-up of toxic membrane-associated heme molecules that would end up destroying the parasite membrane [113]. But Mossbauer studies demonstrated that there is a minor role of heme degradation in the parasite, so it seems unlikely that this is the mechanism of action of antimalarials such as CQ [114].

Unfortunately, malaria parasites have developed resistance to chloroquine in most parts of the world [3]. The key to this resistance lies in a specific mutation in a chloroquine transporter protein, PfCRT. This protein is present in the parasitic digestive vacuole and it is responsible for the efflux of the diprotonic chloroquine, avoiding its accumulation in the organelle [115,116].

The role of this protein in CQ resistance has been shown by several studies and was clearly demonstrated on a recent report by Martin et al. [117]. In this study, the transporter protein was expressed in the surface of *Xenopus* oocytes. The transporter activity of the sensitive and resistant PfCRT versions was measured making sure that the chloroquine was protonated. They demonstrated that chloroquine resistance was directly related to the transport of the protonated form of the drug outside of the membrane. They found that the resistance related to this protein lies in specific mutations in only two amino acids of the entire protein sequence: lysine for threonine 76 and arginine for serine 163. In sensitive strains, threonine and serine contribute a positive charge to the active site of PfCRT, and this results in a diminished transporter activity. The single change of threonine 76 by lysine in the mutated PfCRT is not enough to confer resistance, but together with the arginine mutation, it renders the parasite resistant to this amino quinoline [117]. The exact mechanism by which the PfCRT works as the transporter of CQ is still being studied. In fact, there is a debate about whether the mutated PfCRT acts as a carrier or as a channel [115,118]. What is known is that some drugs can rescue the effect of the PfCRT-mutated parasites *in vitro*. Verapamil and amantadine increase the sensitivity of CQ-resistant parasites to the drug [117]. The most effective inhibitors of resistance in these parasites were particularly endomorphin-1 molecules (endogenous opioid peptides) such as quinine [119].

The thorough study of these mechanisms is very relevant in the design of new drugs that target hemozoin and/or to modify the existing chloroquine molecule to circumvent the resistance of the parasites. A drug like chloroquine is effective, inexpensive and has a low toxicity. Rescuing its efficacy would be especially important to control the disease in regions where malaria is endemic and access to more expensive drugs is very limited.

6. Biophysical properties of hemozoin

As is the case with CQ, the greatest problem in the control of malaria has been the development of drug resistance to the most common and less expensive drugs currently used.

The need to find new alternatives to diagnose and treat malaria is enormous. Some groups have turned to the biophysical properties of hemozoin for answers [120–124].

A detailed knowledge of these properties should be basic to the discovery of new ways of taking advantage of this molecule in the fight against malaria.

6.1. Electromagnetic properties

Hematin has several electromagnetic properties that have been exploited to detect, separate, disrupt, or kill the malaria parasite. These include:

- Paramagnetism
- Optical dichroism
- Optical non-linearity.

Paramagnetic substances have a magnetic moment only in the presence of an applied magnetic field. Paramagnetism in hemozoin arises from the unpaired electrons of the Fe³⁺ species of iron. When an external field is present, the random unpaired spins line up to enhance the field. When the applied field is removed these unpaired spins are rapidly randomized by thermal agitation.

The iron in hemozoin has a direct current (DC) magnetic molar susceptibility (X_{mol}) of $11.0 \times 10^{-3} \text{ cm}^3 \text{ mol}^{-1}$ [125]. Since the measurements in Hackett were adjusted to 298 K (in CGS units), the effective magnetic moment (μ_{eff}) per iron is

$$\mu_{\text{eff}} = \sqrt{\frac{3k}{N\mu_B^2} TX_{\text{mol}}}$$

where k is the Boltzmann's constant, N is the Avogadro's constant, T is the temperature, and μ_B is the Bohr magneton. Thus, moment μ_{eff} per iron is approximately 5.1 Bohr magneton units.

The chemical formula for the β -hematin dimer is $C_{68}H_{60}N_8O_8Fe_2$ with a molar mass of 1228.9495 g/mol. Based on the basic repeating unit makeup of the crystal (crystal unit cell) dimensions [40], the unit cell volume is $1.416 \times 10^{-21} \text{ cm}^3$. The volume of a mole of hemozoin crystal without voids or imperfections would be 852 cm^3 . The density of β -hematin is therefore 1.44 g cm^{-3} .

With the molar volume known, the molar susceptibility can be converted to volumetric units. Molar susceptibility is related to volumetric susceptibility, X_v , by

$$\frac{\rho X_{\text{mol}}}{M} = X_v$$

where ρ is density, and M is the molar mass.

This calculation leads to an average SI volumetric magnetic susceptibility of $X_v = 320 \times 10^{-6}$ for the solid, perfect hemozoin crystal.

This magnetic susceptibility is very weak when compared to a ferromagnetic material, but relative to other paramagnetic materials, β -hematin shows the paramagnetic effect strongly.

Paramagnet	$X_v \cdot 10^6$
β -Hematin	320
Tungsten	68
Cesium	51
Aluminum	22

The paramagnetism of β -hematin has been exploited a number of ways, the most common being a method for separating infected red blood cells from uninfected cells [126]. Paramagnetic hemozoin, like any paramagnet, is attracted up a magnetic gradient toward the pole of a strong permanent magnet [127].

Another useful electromagnetic property of hemozoin is its optical dichroism. The anisotropic crystal structure of hematin (*i.e.* directionally dependent) leads to anisotropy in its refractive index, causing light to propagate differently through the crystal, depending on the propagation direction relative to the crystal lattice. A system that exploits both the paramagnetism of hemozoin and its dichroism was developed for detecting malaria infections [128,129]. A very strong magnetic field can orient the hemozoin crystals within the digestive vacuole of live parasites. Hemozoin crystals can be oriented to a magnetic field because they are shaped as rods, rather than spheres, and as rods they possess shape anisotropy, making the magnetic susceptibility larger (by about a factor of 2) along the long “easy” axis of the crystal. This anisotropy leads to an orienting torque that tends to align the easy axis with the field. Because of the viscosity of the surrounding fluid, crystal orientation takes some time to respond. The response time determines the highest frequency of magnetic fluctuation that can orient the crystals.

The magnetic orientation response times that appeared to the authors as “near instantaneous” were achieved [129], but close examination of the data suggests that the response time was actually near 100 μs for an applied field of 1 T, implying response up to frequencies of the order of 10 kHz.

This empirical observation of magneto-optical response time is consistent with what physics calculates [130]. Indeed the $\pi/4$ rotational orientation time constant for hemozoin is of the order of 100 μs , assuming a nano-scale hemozoin paramagnetic rod with dimensions typical for hemozoin crystals ($500 \times 300 \times 100 \text{ nm}$) oriented by a 1 tesla magnetic field in the rheology (plastic-like flow) of the food vacuole.

This orientation effect has led some authors [131] to hypothesize that rotating magnetic fields could potentially disrupt the biocrystallization of hemozoin. Although strong fields can spin hemozoin crystals, and perhaps impede crystallization, moderate magnetic fields do not induce enough torque on the crystals to overcome the rotational diffusion

caused by natural thermal agitation. Magnetic torque is proportional to the magnetic field magnitude squared, so more moderate magnetic fields have dramatically less effect on hemozoin. For example, a field of 1 mT, equivalent to a common refrigerator magnet, would generate a million times less torque on a hemozoin crystal than would a 1 tesla field. The weaker field would be unable to orient the hemozoin against thermal diffusion. The requirement of a strong magnetic field to overcome thermal rotational diffusion is evidence against the hypotheses of direct mechanical action on paramagnetic hemozoin by relatively weak magnetic forces in non-thermal RF, microwave, and optical electromagnetic wave exposures.

Another electromagnetic property of hemozoin that has been exploited is its non-linearity. A strong IR illumination was shown to induce a third-harmonic emission in the ultra-violet wavelengths [132]. This UV emission has enough energy per photon to kill nearby cells. Since the fundamental IR does not damage cells, only cells that contain the optically non-linear hematin are targeted.

6.2. Photoacoustic properties

Very recently there have been studies that suggest that hemozoin might have other physical properties that could be exploited for diagnostic or other innovative applications in the future.

A photoacoustic spectrum of a material sample is obtained by illuminating the sample with intense light and measuring the resulting acoustic vibrations in the material at different frequencies. This spectrum can be used to identify the absorbing components of the sample, and thereby detect tiny alterations in its composition.

First tried by Balasubramanian [62], photoacoustic stimulation has been used to study the malaria parasites. Simulation results [133] suggest that intraerythrocytic stages of malarial parasite may be assessed using a photoacoustic spectroscopy technique. If confirmed empirically, this technique could lead to an inexpensive detection and assay sensor for infected blood.

Interestingly, a variation of flow cytometry named photoacoustic flowmetry, where nanosecond laser pulses are aimed at the sample generating ultrasonic signals, has had effects on the absorption of electromagnetic frequencies by hemozoin and in some of its physical properties [134].

7. Conclusion

Malaria is a very complex disease. Its eradication must arise from a concerted effort to gain control over the causative agent and vectors, and success in creating awareness on how to best prevent the disease within the population that is at risk.

Science, with the convergence of biochemistry, biophysics, and engineering could make the most out of this peculiar crystal called hemozoin. Given the current state of malaria in the world, with millions of people succumbing annually to the disease, while resistance to the most common and cheapest drugs keeps developing, hemozoin could be key in the search for more sensitive diagnostics and/or alternative therapeutics to decrease both the morbidity and mortality rates of this scourge. Hemozoin is a unique molecule, a distinguishing trait of malaria parasites and other blood-feeding organisms. Its unique features should, and certainly will be exploited to make it a game-changer in the fight against this devastating disease.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

The authors would like to thank Volker Deckert and Tanja Deckert-Gaudij for the use of their facilities in The Institute of Photonics, Jena,

Germany to get the AFM picture, and Ernst Hempelmann for critically reading the manuscript. Thanks also to Ricardo Correa for creating the graphics.

Funding

This work was funded by a SENACYT/IFARHU doctoral grant to Lorena Coronado and a Bill and Melinda Gates Foundation Grand Challenges Exploration grant to Jose A. Stoute and Carmenza Spadafora. Other financial support was provided by the Fogarty International Center's International Cooperative Biodiversity Groups (ICBG) program in Panama (ICBG TW006634).

References

- [1] World Health Organization, Guidelines for the Treatment of Malaria, WHO Press, 1211 Geneva 27, Switzerland, 2010.
- [2] M.A. Travassos, M.K. Laufer, Resistance to antimalarial drugs: molecular, pharmacologic, and clinical considerations, *Pediatr. Res.* 65 (2009) 64R–70R.
- [3] M. Enserink, Malaria's drug miracle in danger, *Science* 328 (2010) 844–846.
- [4] J.F. Garcia-Bustos, F.J. Gamo, Antimalarial drug resistance and early drug discovery, *Curr. Pharm. Des.* 19 (2013) 270–281.
- [5] E. Hempelmann, Hemozoin biocrystallization in *Plasmodium falciparum* and the antimalarial activity of crystallization inhibitors, *Parasitol. Res.* 100 (2007) 671–676.
- [6] K.A. de Villiers, T.J. Egan, Recent advances in the discovery of haem-targeting drugs for malaria and schistosomiasis, *Molecules* 14 (2009) 2868–2887.
- [7] D. Monti, B. Vodopivec, N. Basilio, P. Oliario, D. Taramelli, A novel endogenous antimalarial: Fe(II)-protoporphyrin IX alpha (heme) inhibits hemozoin polymerization to beta-hematin (malaria pigment) and kills malaria parasites, *Biochemistry* 38 (1999) 8858–8863.
- [8] J. Ziegler, R. Linck, D.W. Wright, Heme aggregation inhibitors: antimalarial drugs targeting an essential biomineralization process, *Curr. Med. Chem.* 8 (2001) 171–189.
- [9] R. Agrawal, R. Tripathi, B.L. Tekwani, S.K. Jain, G.P. Dutta, O.P. Shukla, Haem polymerase as a novel target of antimalarial action of cyproheptadine, *Biochem. Pharmacol.* 64 (2002) 1399–1406.
- [10] V.R. Solomon, W. Haq, K. Srivastava, S.K. Puri, S.B. Katti, Synthesis and antimalarial activity of side chain modified 4-aminoquinoline derivatives, *J. Med. Chem.* 50 (2007) 394–398.
- [11] S.E. Francis, D.J. Sullivan Jr., D.E. Goldberg, Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*, *Annu. Rev. Microbiol.* 51 (1997) 97–123.
- [12] R.G. Ridley, Haemozoin formation in malaria parasites: is there a haem polymerase? *Trends Microbiol.* 4 (1996) 253–254.
- [13] D.J. Krogstad, P.H. Schlesinger, I.Y. Gluzman, Antimalarials increase vesicle pH in *Plasmodium falciparum*, *J. Cell Biol.* 101 (1985) 2302–2309.
- [14] N. Klionis, O. Tan, K. Jackson, D. Goldberg, M. Klemba, L. Tilley, Evaluation of pH during cytosomal endocytosis and vacuolar catabolism of haemoglobin in *Plasmodium falciparum*, *Biochem. J.* 407 (2007) 343–354.
- [15] D.E. Goldberg, A.F. Slater, A. Cerami, G.B. Henderson, Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle, *Proc. Natl. Acad. Sci. U. S. A.* 87 (1990) 2931–2935.
- [16] S. Zarchin, M. Krugliak, H. Ginsburg, Digestion of the host erythrocyte by malaria parasites is the primary target for quinoline-containing antimalarials, *Biochem. Pharmacol.* 35 (1986) 2435–2442.
- [17] S.E. Francis, I.Y. Gluzman, A. Oksman, A. Knickerbocker, R. Mueller, M.L. Bryant, D.R. Sherman, D.G. Russell, D.E. Goldberg, Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase, *EMBO J.* 13 (1994) 306–317.
- [18] P.J. Rosenthal, J.H. McKerrow, D. Rasnick, J.H. Leech, *Plasmodium falciparum*: inhibitors of lysosomal cysteine proteinases inhibit a trophozoite proteinase and block parasite development, *Mol. Biochem. Parasitol.* 35 (1989) 177–183.
- [19] P.J. Rosenthal, *Plasmodium falciparum*: effects of proteinase inhibitors on globin hydrolysis by cultured malaria parasites, *Exp. Parasitol.* 80 (1995) 272–281.
- [20] N. Yadav, S.K. Dixit, A. Bhattacharya, L.C. Mishra, M. Sharma, S.K. Awasthi, V.K. Bhasin, Antimalarial activity of newly synthesized chalcone derivatives in vitro, *Chem. Biol. Drug Des.* 80 (2012) 340–347.
- [21] M. Krugliak, J. Zhang, H. Ginsburg, Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins, *Mol. Biochem. Parasitol.* 119 (2002) 249–256.
- [22] H. Ginsburg, Some reflections concerning host erythrocyte-malaria parasite interrelationships, *Blood Cells* 16 (1990) 225–235.
- [23] A. Esposito, T. Tiffert, J.M. Mauritz, S. Schlachter, L.H. Bannister, C.F. Kaminski, V.L. Lew, FRET imaging of hemoglobin concentration in *Plasmodium falciparum*-infected red cells, *PLoS One* 3 (2008) e3780.
- [24] I.Y. Gluzman, S.E. Francis, A. Oksman, C.E. Smith, K.L. Duffin, D.E. Goldberg, Order and specificity of the *Plasmodium falciparum* hemoglobin degradation pathway, *J. Clin. Invest.* 93 (1994) 1602–1608.
- [25] D.E. Goldberg, A.F. Slater, R. Beavis, B. Chait, A. Cerami, G.B. Henderson, Hemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic pathway initiated by a specific aspartic protease, *J. Exp. Med.* 173 (1991) 961–969.
- [26] Z. Bozdech, M. Llinas, B.L. Pulliam, E.D. Wong, J. Zhu, J.L. DeRisi, The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*, *PLoS Biol.* 1 (2003) E5.
- [27] D.L. vander Jagt, L.A. Hunsaker, N.M. Campos, J.V. Scaletti, Localization and characterization of hemoglobin-degrading aspartic proteinases from the malarial parasite *Plasmodium falciparum*, *Biochim. Biophys. Acta* 1122 (1992) 256–264.
- [28] D. Jani, R. Nagarkatti, W. Beatty, R. Angel, C. Slebodnick, J. Andersen, S. Kumar, D. Rathore, HDP—a novel heme detoxification protein from the malaria parasite, *PLoS Pathog.* 4 (2008) e1000053.
- [29] M. Chugh, V. Sundararaman, S. Kumar, V.S. Reddy, W.A. Siddiqui, K.D. Stuart, P. Malhotra, Protein complex directs hemoglobin-to-hemozoin formation in *Plasmodium falciparum*, *Proc. Natl. Acad. Sci. U. S. A.* 110 (2013) 5392–5397.
- [30] A.U. Orjih, On the mechanism of hemozoin production in malaria parasites: activated erythrocyte membranes promote beta-hematin synthesis, *Exp. Biol. Med.* (Maywood) 226 (2001) 746–752.
- [31] L. Pauling, C.D. Coryell, The magnetic properties and structure of hemoglobin, oxyhemoglobin and carbonmonoxyhemoglobin, *Proc. Natl. Acad. Sci. U. S. A.* 22 (1936) 210–216.
- [32] L. Pauling, Magnetic properties and structure of oxyhemoglobin, *Proc. Natl. Acad. Sci. U. S. A.* 74 (1977) 2612–2613.
- [33] A. Sienkiewicz, J. Krzystek, B. Vilenko, G. Chatain, A.J. Kosar, D.S. Bohle, L. Forró, Multi-frequency high-field EPR study of iron centers in malarial pigments, *J. Am. Chem. Soc.* 128 (2006) 4534–4535.
- [34] A. Hamsik, Czech medicine factors, in: M. University (Ed.), *Writings Medical Faculty of Masaryk*, vol. 200, University in Brno, Brno, 1929, p. 13.
- [35] R. Lemberg, J.W. Legge, *Hematin Compounds and Bile Pigments; Their Constitution, Metabolism, and Function*, by R. Lemberg and J.W. Legge, Interscience Publishers, New York, 1949.
- [36] A.F. Slater, A. Cerami, Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites, *Nature* 355 (1992) 167–169.
- [37] C. Coban, M. Yagi, K. Ohata, Y. Igarai, T. Tsukui, T. Horii, K.J. Ishii, S. Akira, The malarial metabolite hemozoin and its potential use as a vaccine adjuvant, *Allergol. Int.* 59 (2010) 115–124.
- [38] A.U. Orjih, T.C. Mathew, P.T. Cherian, Erythrocyte membranes convert monomeric ferriprotoporphyrin IX to beta-hematin in acidic environment at malarial fever temperature, *Exp. Biol. Med.* (Maywood) 237 (2012) 884–893.
- [39] J. Sun, B. Chen, Y.Y. Long, Conditional inhibition of hemozoin formation by chloroquine in vitro, *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 27 (2009) 120–124.
- [40] T.J. Egan, Physico-chemical aspects of hemozoin (malaria pigment) structure and formation, *J. Inorg. Biochem.* 91 (2002) 19–26.
- [41] M.M. Chen, L. Shi, D.J. Sullivan Jr., Haemoproteus and *Schistosoma* synthesize heme polymers similar to *Plasmodium* hemozoin and beta-hematin, *Mol. Biochem. Parasitol.* 113 (2001) 1–8.
- [42] D.J. Sullivan Jr., I.Y. Gluzman, D.E. Goldberg, *Plasmodium* hemozoin formation mediated by histidine-rich proteins, *Science* 271 (1996) 219–222.
- [43] L.H. Miller, M.F. Good, G. Milon, Malaria pathogenesis, *Science* 264 (1994) 1878–1883.
- [44] H.M. Müller, I. Reckmann, M.R. Hollingdale, H. Bujard, K.J. Robson, A. Crisanti, Thrombospondin related anonymous protein (TRAP) of *Plasmodium falciparum* binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes, *EMBO J.* 12 (1993) 2881–2889.
- [45] P.L. Oliario, D.E. Goldberg, The *Plasmodium* digestive vacuole: metabolic headquarters and choice drug target, *Parasitol. Today* 11 (1995) 294–297.
- [46] E. Hempelmann, H.M. Marques, Analysis of malaria pigment from *Plasmodium falciparum*, *J. Pharmacol. Toxicol. Methods* 32 (1994) 25–30.
- [47] T.J. Egan, Recent advances in understanding the mechanism of hemozoin (malaria pigment) formation, *J. Inorg. Biochem.* 102 (2008) 1288–1299.
- [48] A.N. Hoang, K.K. Ncokazi, K.A. de Villiers, D.W. Wright, T.J. Egan, Crystallization of synthetic hemozoin (beta-haematin) nucleated at the surface of lipid particles, *Dalton Trans.* 39 (2010) 1235–1244.
- [49] M.A. Ambele, T.J. Egan, Neutral lipids associated with hemozoin mediate efficient and rapid beta-haematin formation at physiological pH, temperature and ionic composition, *Malar. J.* 11 (2012) 337.
- [50] A.N. Hoang, R.D. Sandlin, A. Omar, T.J. Egan, D.W. Wright, The neutral lipid composition present in the digestive vacuole of *Plasmodium falciparum* concentrates heme and mediates beta-hematin formation with an unusually low activation energy, *Biochemistry* 49 (2010) 10107–10116.
- [51] A. Dorn, S.R. Vippagunta, H. Matile, A. Bubendorf, J.L. Vennerstrom, R.G. Ridley, A comparison and analysis of several ways to promote haematin (Haem) polymerisation and an assessment of its initiation in vitro, *Biochem. Pharmacol.* 55 (1998) 737–747.
- [52] C.D. Fitch, G.Z. Cai, Y.F. Chen, J.D. Shoemaker, Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria, *Biochim. Biophys. Acta* 1454 (1999) 31–37.
- [53] J.M. Pisciotta, I. Coppens, A.K. Tripathi, P.F. Scholl, J. Shuman, S. Bajad, V. Shulaev, D.J. Sullivan Jr., The role of neutral lipid nanospheres in *Plasmodium falciparum* haem crystallization, *Biochem. J.* 402 (2007) 197–204.
- [54] D.J. Sullivan, Hemozoin: a biocrystal synthesized during the degradation of hemoglobin, *Biopolymers Online*, Wiley-VCH Verlag GmbH & Co. KGaA, 2005.
- [55] I. Coppens, John M. Pisciotta, Abhai K. Tripathi, Peter F. Scholl, J. Shuman, S. Bajad, V. Shulaev, David J. Sullivan, The role of neutral lipid nanospheres in *Plasmodium falciparum* haem crystallization, *Biochem. J.* 402 (2007) 197.
- [56] D.J. Sullivan, Hemozoin: A Biocrystal Synthesized During the Degradation of Hemoglobin, 2005.
- [57] S. Kapishnikov, A. Weiner, E. Shimoni, P. Guttmann, G. Schneider, N. Dahan-Pasternak, R. Dzikowski, L. Leiserowitz, M. Elbaum, Oriented nucleation of hemozoin at the digestive vacuole membrane in *Plasmodium falciparum*, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 11188–11193.

- [58] A.F. Slater, W.J. Swiggard, B.R. Orton, W.D. Flitter, D.E. Goldberg, A. Cerami, G.B. Henderson, An iron-carboxylate bond links the heme units of malaria pigment, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 325–329.
- [59] D. Rabinovich, *Advanced inorganic chemistry*, 6th edition (Cotton, F.A.; Wilkinson, G.; Murillo, C. A.; Bochmann, M.), *J. Chem. Educ.* 77 (2000) 311.
- [60] D.S. Bohle, R.E. Dinnebier, S.K. Madsen, P.W. Stephens, Characterization of the products of the heme detoxification pathway in malarial late trophozoites by X-ray diffraction, *J. Biol. Chem.* 272 (1997) 713–716.
- [61] S. Pagola, P.W. Stephens, D.S. Bohle, A.D. Kosar, S.K. Madsen, The structure of malaria pigment beta-haematin, *Nature* 404 (2000) 307–310.
- [62] D. Balasubramanian, C. Mohan Rao, B. Panijpan, The malaria parasite monitored by photoacoustic spectroscopy, *Science* 223 (1984) 828–830.
- [63] J. Gildenhuis, T. LeRoex, T.J. Egan, K.A. de Villiers, The single crystal X-ray structure of β -hematin DMSO solvate grown in the presence of chloroquine, a β -hematin growth-rate inhibitor, *J. Am. Chem. Soc.* 135 (2013) 1037–1047.
- [64] F. Turrini, H. Ginsburg, F. Bussolino, G.P. Pescarmona, M.V. Serra, P. Arese, Phagocytosis of *Plasmodium falciparum*-infected human red blood cells by human monocytes: involvement of immune and nonimmune determinants and dependence on parasite developmental stage, *Blood* 80 (1992) 801–808.
- [65] M.A. Levesque, A.D. Sullivan, S.R. Meshnick, Splenic and hepatic hemozoin in mice after malaria parasite clearance, *J. Parasitol.* 85 (1999) 570–573.
- [66] J.C. Silva, A. Egan, R. Friedman, J.B. Munro, J.M. Carlton, A.L. Hughes, Genome sequences reveal divergence times of malaria parasite lineages, *Parasitology* 138 (2011) 1737–1749.
- [67] R. Frita, D. Carapau, M.M. Mota, T. Häscheid, In vivo hemozoin kinetics after clearance of *Plasmodium berghei* infection in mice, *Malar. Res. Treat.* 2012 (2012) 373086.
- [68] C.A. Homewood, G.A. Moore, D.C. Warhurst, E.M. Atkinson, Purification and some properties of malarial pigment, *Ann. Trop. Med. Parasitol.* 69 (1975) 283–287.
- [69] P.L. Fiori, P. Rappelli, S.N. Mirkarimi, H. Ginsburg, P. Cappuccinelli, F. Turrini, Reduced microbicidal and anti-tumour activities of human monocytes after ingestion of *Plasmodium falciparum*-infected red blood cells, *Parasite Immunol.* 15 (1993) 647–655.
- [70] J. Prada, J. Malinowski, S. Müller, U. Bienzle, P.G. Kremsner, Hemozoin differentially modulates the production of interleukin 6 and tumor necrosis factor in murine malaria, *Eur. Cytokine Netw.* 6 (1995) 109–112.
- [71] J. Prada, J. Malinowski, S. Müller, U. Bienzle, P.G. Kremsner, Effects of *Plasmodium vinckei* hemozoin on the production of oxygen radicals and nitrogen oxides in murine macrophages, *Am. J. Trop. Med. Hyg.* 54 (1996) 620–624.
- [72] B.A. Sherry, G. Alava, K.J. Tracey, J. Martiney, A. Cerami, A.F. Slater, Malaria-specific metabolite hemozoin mediates the release of several potent endogenous pyrogens (TNF, MIP-1 alpha, and MIP-1 beta) in vitro, and altered thermoregulation in vivo, *J. Inflamm.* 45 (1995) 85–96.
- [73] D. Taramelli, N. Basilico, E. Pagani, R. Grande, D. Monti, M. Ghione, P. Olliaro, The heme moiety of malaria pigment (beta-hematin) mediates the inhibition of nitric oxide and tumor necrosis factor-alpha production by lipopolysaccharide-stimulated macrophages, *Exp. Parasitol.* 81 (1995) 501–511.
- [74] E. Schwarzer, O. Müller, P. Arese, W.G. Siems, T. Grune, Increased levels of 4-hydroxynonenal in human monocytes fed with malarial pigment hemozoin. A possible clue for hemozoin toxicity, *FEBS Lett.* 388 (1996) 119–122.
- [75] E. Schwarzer, O.A. Skorokhod, V. Barrera, P. Arese, Hemozoin and the human monocyte—a brief review of their interactions, *Parassitologia* 50 (2008) 143–145.
- [76] E. Schwarzer, H. Kuhn, E. Valente, P. Arese, Malaria-parasitized erythrocytes and hemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions, *Blood* 101 (2003) 722–728.
- [77] E. Schwarzer, F. Turrini, D. Ulliers, G. Giribaldi, H. Ginsburg, P. Arese, Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment, *J. Exp. Med.* 176 (1992) 1033–1041.
- [78] H.L. Shear, R.S. Nussenzweig, C. Bianco, Immune phagocytosis in murine malaria, *J. Exp. Med.* 149 (1979) 1288–1298.
- [79] A. Celada, A. Crucho, L.H. Perrin, Assessment of immune phagocytosis of *Plasmodium falciparum* infected red blood cells by human monocytes and polymorphonuclear leukocytes. A method for visualizing infected red blood cells ingested by phagocytes, *J. Immunol. Methods* 63 (1983) 263–271.
- [80] M.T. Shio, S.C. Eisenbarth, M. Savaria, A.F. Vinet, M.J. Bellemare, K.W. Harder, F.S. Sutterwala, D.S. Bohle, A. Descoteaux, R.A. Flavell, M. Olivier, Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases, *PLoS Pathog.* 5 (2009) e1000559.
- [81] P. Olliaro, L. Lombardi, S. Frigerio, N. Basilico, D. Taramelli, D. Monti, Phagocytosis of hemozoin (native and synthetic malaria pigment), and *Plasmodium falciparum* intraerythrocyte-stage parasites by human and mouse phagocytes, *Ultrastruct. Pathol.* 24 (2000) 9–13.
- [82] M. Jaramillo, M.J. Bellemare, C. Martel, M.T. Shio, A.P. Contreras, M. Godbout, M. Roger, E. Gaudreault, J. Gosselin, D.S. Bohle, M. Olivier, Synthetic *Plasmodium*-like hemozoin activates the immune response: a morphology — function study, *PLoS One* 4 (2009) e6957.
- [83] P. Arese, E. Schwarzer, Malarial pigment (hemozoin): a very active 'inert' substance, *Ann. Trop. Med. Parasitol.* 91 (1997) 501–516.
- [84] G. Giribaldi, D. Ulliers, E. Schwarzer, I. Roberts, W. Piacibello, P. Arese, Hemozoin and 4-hydroxynonenal-mediated inhibition of erythropoiesis. Possible role in malarial dyserythropoiesis and anemia, *Haematologica* 89 (2004) 492–493.
- [85] V. Barrera, O.A. Skorokhod, D. Baci, G. Gremo, P. Arese, E. Schwarzer, Host fibrinogen stably bound to hemozoin rapidly activates monocytes via TLR-4 and CD11b/CD18-integrin: a new paradigm of hemozoin action, *Blood* 117 (2011) 5674–5682.
- [86] O.R. Millington, C. Di Lorenzo, R.S. Phillips, P. Garside, J.M. Brewer, Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function, *J. Biol.* 5 (2006) 5.
- [87] O.R. Millington, V.B. Gibson, C.M. Rush, B.H. Zinselmeyer, R.S. Phillips, P. Garside, J.M. Brewer, Malaria impairs T cell clustering and immune priming despite normal signal 1 from dendritic cells, *PLoS Pathog.* 3 (2007) 1380–1387.
- [88] B.C. Urban, S. Todryk, Malaria pigment paralyzes dendritic cells, *J. Biol.* 5 (2006) 4.
- [89] S.M. Todryk, B.C. Urban, Dendritic cells in *Plasmodium* infection, *Future Microbiol* 3 (2008) 279–286.
- [90] S. Bousheri, H. Cao, New insight into the role of dendritic cells in malaria immune pathogenesis, *Trends Parasitol.* 24 (2008) 199–200.
- [91] O.A. Skorokhod, E. Schwarzer, M. Ceretto, P. Arese, Malarial pigment hemozoin, IFN-gamma, TNF-alpha, IL-1beta and LPS do not stimulate expression of inducible nitric oxide synthase and production of nitric oxide in immuno-purified human monocytes, *Malar. J.* 6 (2007) 73.
- [92] J.B. Weinberg, M.A. Misukonis, P.J. Shami, S.N. Mason, D.L. Sauls, W.A. Dittman, E.R. Wood, G.K. Smith, B. McDonald, K.E. Bachus, A.F. Haney, D.L. Granger, Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, bioprotein, and nitric oxide production by blood monocytes and peritoneal macrophages, *Blood* 86 (1995) 1184–1195.
- [93] J.F. Kun, B. Mordmüller, D.J. Perkins, J. May, O. Mercereau-Puijalon, M. Alpers, J.B. Weinberg, P.G. Kremsner, Nitric oxide synthase 2(Lambaréné) (G-954C), increased nitric oxide production, and protection against malaria, *J. Infect. Dis.* 184 (2001) 330–336.
- [94] J. Everse, N. Hsia, The toxicities of native and modified hemoglobins, *Free Radic. Biol. Med.* 22 (1997) 1075–1099.
- [95] C. Casals-Pascual, O. Kai, J.O. Cheung, S. Williams, B. Lowe, M. Nyanoti, T.N. Williams, K. Maitland, M. Molyneux, C.R. Newton, N. Peshu, S.M. Watt, D.J. Roberts, Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo, *Blood* 108 (2006) 2569–2577.
- [96] O.A. Skorokhod, L. Caione, T. Marrocco, G. Migliardi, V. Barrera, P. Arese, W. Piacibello, E. Schwarzer, Inhibition of erythropoiesis in malaria anemia: role of hemozoin and hemozoin-generated 4-hydroxynonenal, *Blood* 116 (2010) 4328–4337.
- [97] G.A. Awandare, P. Kempaiah, D.O. Ochiel, P. Piazza, C.C. Keller, D.J. Perkins, Mechanisms of erythropoiesis inhibition by malarial pigment and malaria-induced proinflammatory mediators in an in vitro model, *Am. J. Hematol.* 86 (2011) 155–162.
- [98] C. Coban, K.J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, T. Horii, S. Akira, Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin, *J. Exp. Med.* 201 (2005) 19–25.
- [99] C. Coban, K.J. Ishii, S. Uematsu, N. Arisue, S. Sato, M. Yamamoto, T. Kawai, O. Takeuchi, H. Hisaeda, T. Horii, S. Akira, Pathological role of Toll-like receptor signaling in cerebral malaria, *Int. Immunol.* 19 (2007) 67–79.
- [100] N.M. Gowda, X. Wu, D.C. Gowda, TLR9 and MyD88 are crucial for the development of protective immunity to malaria, *J. Immunol.* 188 (2012) 5073–5085.
- [101] N.M. Gowda, X. Wu, D.C. Gowda, The nucleosome (histone-DNA complex) is the TLR9-specific immunostimulatory component of *Plasmodium falciparum* that activates DCs, *PLoS One* 6 (2011) e20398.
- [102] F.P. Mockenhaupt, J.P. Cramer, L. Hamann, M.S. Stegemann, J. Eckert, N.R. Oh, R.N. Otchwemah, E. Dietz, S. Ehrhardt, N.W. Schröder, U. Bienzle, R.R. Schumacher, Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 177–182.
- [103] A. Baccarella, M.F. Fontana, E.C. Chen, C.C. Kim, Toll-like receptor 7 mediates early innate immune responses to malaria, *Infect. Immun.* 81 (2013) 4431–4442.
- [104] B.L. Tekwani, L.A. Walker, Targeting the hemozoin synthesis pathway for new antimalarial drug discovery: technologies for in vitro beta-hematin formation assay, *Comb. Chem. High Throughput Screen.* 8 (2005) 63–79.
- [105] A.P. Gorka, J.N. Alumasa, K.S. Sherlach, L.M. Jacobs, K.B. Nickley, J.P. Brower, A.C. de Dios, P.D. Roepe, Cytostatic versus cytotoxic activities of chloroquine analogues and inhibition of hemozoin crystal growth, *Antimicrob. Agents Chemother.* 57 (2013) 356–364.
- [106] A.U. Orjih, Heme polymerase activity and the stage specificity of antimalarial action of chloroquine, *J. Pharmacol. Exp. Ther.* 282 (1997) 108–112.
- [107] D.E. Goldberg, Hemoglobin degradation in *Plasmodium*-infected red blood cells, *Semin. Cell Biol.* 4 (1993) 355–361.
- [108] A.C. Chou, C.D. Fitch, Heme polymerase: modulation by chloroquine treatment of a rodent malaria, *Life Sci.* 51 (1992) 2073–2078.
- [109] T.J. Egan, D.C. Ross, P.A. Adams, Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment), *FEBS Lett.* 352 (1994) 54–57.
- [110] A. Dorn, R. Stoffel, H. Matile, A. Bubendorf, R.G. Ridley, Malarial hemozoin/beta-haematin supports haem polymerization in the absence of protein, *Nature* 374 (1995) 269–271.
- [111] G.T. Webster, L. Tilley, S. Deed, D. McNaughton, B.R. Wood, Resonance Raman spectroscopy can detect structural changes in hemozoin (malaria pigment) following incubation with chloroquine in infected erythrocytes, *FEBS Lett.* 582 (2008) 1087–1092.
- [112] J.M. Combrinck, T.E. Mabothe, K.K. Ncokazi, M.A. Ambebe, D. Taylor, P.J. Smith, H.C. Hoppe, T.J. Egan, Insights into the role of heme in the mechanism of action of antimalarials, *ACS Chem. Biol.* 8 (2013) 133–137.
- [113] P. Loria, S. Miller, M. Foley, L. Tilley, Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials, *Biochem. J.* 339 (1999) 363–370.
- [114] T.J. Egan, J.M. Combrinck, J. Egan, G.R. Hearne, H.M. Marques, S. Ntenti, B.T. Sewell, P.J. Smith, D. Taylor, D.A. van Schalkwyk, J.C. Walden, Fate of haem iron in the malaria parasite *Plasmodium falciparum*, *Biochem. J.* 365 (2002) 343–347.
- [115] M. Chinappi, A. Via, P. Marcatili, A. Tramontano, On the mechanism of chloroquine resistance in *Plasmodium falciparum*, *PLoS One* 5 (2010) e14064.

- [116] M.F. Paguio, M. Cabrera, P.D. Roepe, Chloroquine transport in *Plasmodium falciparum*. 2. Analysis of PfCRT-mediated drug transport using proteoliposomes and a fluorescent chloroquine probe, *Biochemistry* 48 (2009) 9482–9491.
- [117] R.E. Martin, R.V. Marchetti, A.I. Cowan, S.M. Howitt, S. Bröer, K. Kirk, Chloroquine transport via the malaria parasite's chloroquine resistance transporter, *Science* 325 (2009) 1680–1682.
- [118] C.P. Sanchez, W.D. Stein, M. Lanzer, Is PfCRT a channel or a carrier? Two competing models explaining chloroquine resistance in *Plasmodium falciparum*, *Trends Parasitol.* 23 (2007) 332–339.
- [119] M. Henry, S. Alibert, E. Orlandi-Pradines, H. Bogreau, T. Fusai, C. Rogier, J. Barbe, B. Pradines, Chloroquine resistance reversal agents as promising antimalarial drugs, *Curr. Drug Targets* 7 (2006) 935–948.
- [120] J. Feagin, M. Wurscher, C. Ramon, H. Lai, Magnetic fields and malaria, in: M. Holick, E. Jung (Eds.), *Biologic Effects of Light 1998*, Springer, US, 1999, pp. 343–349.
- [121] C.F. Bellows, R.M. Molina, J.D. Brain, Diminished organelle motion in murine Kupffer cells during the erythrocytic stage of malaria, *J. R. Soc. Interface* 8 (2011) 711–719.
- [122] C.C. Kim, E.B. Wilson, J.L. DeRisi, Improved methods for magnetic purification of malaria parasites and haemozoin, *Malar. J.* 9 (2010) 17.
- [123] J. Kim, M. Massoudi, J.F. Antaki, A. Gandini, Removal of malaria-infected red blood cells using magnetic cell separators: a computational study, *Appl. Math. Comput.* 218 (2012) 6841–6850.
- [124] C. Yuen, Q. Liu, Magnetic field enriched surface enhanced resonance Raman spectroscopy for early malaria diagnosis, *J. Biomed. Opt.* 17 (2012) 017005.
- [125] S. Hackett, J. Hamzah, T.M. Davis, T.G. St Pierre, Magnetic susceptibility of iron in malaria-infected red blood cells, *Biochim. Biophys. Acta* 1792 (2009) 93–99.
- [126] C. Spadafora, L. Gerena, K.M. Kopydlowski, Comparison of the in vitro invasive capabilities of *Plasmodium falciparum* schizonts isolated by Percoll gradient or using magnetic based separation, *Malar. J.* 10 (2011) 96.
- [127] F. Paul, S. Roath, D. Melville, D.C. Warhurst, J.O. Osisanya, Separation of malaria-infected erythrocytes from whole blood: use of a selective high-gradient magnetic separation technique, *Lancet* 2 (1981) 70–71.
- [128] D.M. Newman, J. Heptinstall, R.J. Matelon, L. Savage, M.L. Wears, J. Beddow, M. Cox, H.D. Schallig, P.F. Mens, A magneto-optic route toward the in vivo diagnosis of malaria: preliminary results and preclinical trial data, *Biophys. J.* 95 (2008) 994–1000.
- [129] D.M. Newman, R.J. Matelon, M.L. Wears, L.B. Savage, The in vivo diagnosis of malaria: feasibility study into a magneto-optic fingertip probe, *IEEE J. Sel. Top. Quantum Electron.* 16 (2010) 573–580.
- [130] B. Frka-Petesic, K. Erglis, J.F. Berret, A. Cebers, V. Dupuis, J. Fresnais, O. Sandre, R. Perzynski, Dynamics of paramagnetic nanostructured rods under rotating field, *J. Magn. Magn. Mater.* 323 (2011) 1309–1313.
- [131] W.M.A., J.E. Feagin, C. Ramon, H.C. Lai, Magnetic fields and malaria, in: H.M.F.a.J.E.G. (Ed.), *Biologic Effects of Light: Proceedings of the Biologic Effects of Light Symposium*, Kluwer Academic Publishers, Hingham, MA, 1999, pp. 343–349.
- [132] D. LeBlanc, R. Story, E. Gross, Laser-induced inactivation of *Plasmodium falciparum*, *Malar. J.* 11 (2012) 267.
- [133] R.K. Saha, S. Karmakar, M. Roy, Computational investigation on the photoacoustics of malaria infected red blood cells, *PLoS One* 7 (2012) e51774.
- [134] E.B. Samson, B.S. Goldschmidt, P.J. Whiteside, A.S. Sudduth, J.R. Custer, B. Beerntsen, J.A. Viator, Photoacoustic spectroscopy of β -hematin, *J. Opt.* 14 (2012) (pii 065302).