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## Hemozoin: a Biocrystal Synthesized during the Degradation of Hemoglobin

**Prof. Dr. David J. Sullivan Jr.**

The Malaria Research Institute, W. Harry Feinstone Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe St. Baltimore, MD 21205, USA. Tel.: 410 614-1562; Fax: 410 955-0105; E-mail: dsulliva@jhsph.edu

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DMSO	dimethylsulfoxide
EPR	electron paramagnetic resonance
FePPIX	iron protoporphyrin IX
FTIR	Fourier transform infrared
ICAM-1	intracellular adhesion molecule-1
IL	interleukin
MHC	major histocompatibility complex
MOG	Mono-oleoylglycerol
PECAM	platelet endothelial cell adhesion molecule
<i>Pf</i> HRP II	<i>Plasmodium falciparum</i> histidine-rich protein II
RecHRP II	recombinant histidine-rich protein II
SDS	sodium dodecylsulfate
SQUID	superconducting quantum interference device
TNF $\alpha$	tumor necrosis factor $\alpha$ .

## 1 Introduction

The protozoan *Plasmodium* parasite, the agent of malaria, produces a brown birefringent heme crystal while inside the hemoglobin-laden erythrocyte. The parasite ingests 5 mM hemoglobin as a source of amino acids. Free toxic heme, which is capable of generating oxygen radicals, is released after hemoglobin catabolism in an acidic lysosome-like organelle. The principal essential first step in detoxification of the almost 0.4 M heme is incorporation into an intracellular crystal called malarial pigment or hemozoin. A unique iron–oxygen coordinate bond links the central iron to the

oxygen of the carboxylate side chain of the adjacent heme. The exact mechanism of crystal initiation is still the subject of debate. Lipids and *Plasmodium falciparum*-derived histidine-rich proteins (*Pf*HRP) can accelerate the chemical conversion of heme monomers to hemozoin crystals *in vitro*. Much of the heme may be degraded by glutathione in the cytoplasm rather than by heme oxygenase. Because the widely used quinolines such as chloroquine and quinine inhibit hemozoin formation and glutathione degradation to kill the *Plasmodium* parasite, the mechanism of drug interaction with heme and determinants of quinoline resistance, is important. Although hemozoin appears to be inactive with regard to biological conse-

quences inside the parasite, the heme crystal has diverse modulatory effects on the immune and endothelial cells.

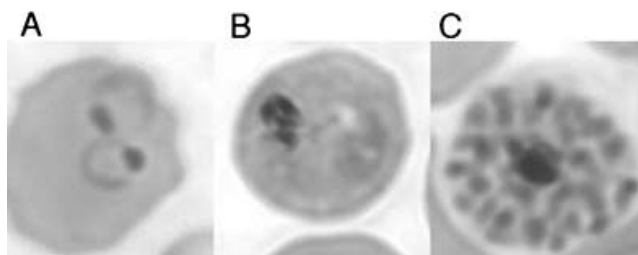
### 1.1

#### Malaria

Each day, malaria disease claims the lives of over 3000 people, 90% of whom are children in sub-Saharan Africa. Among the more than 120 *Plasmodium* species that infect reptiles, birds, and mammals, four protozoan species of *Plasmodium* infect humans. While *Plasmodium vivax* is more geographically widespread, *P. falciparum* is more common, causing over 90% of the deaths. *Plasmodium ovale* is limited to tropical Africa, while *Plasmodium malariae* has the same distribution as *P. falciparum* but is far less common. Humans are the only host for all four of these species, with the exception of the rare *P. malariae* which is transmitted to chimpanzees in Africa. More than 300 million cases of malaria occur each year, with over 2 million deaths (WHO, 1998).

The infection begins with the bite of an infected female mosquito, which injects a few hundred sporozoites of which only one or two infect, within hours, the liver cells or hepatocytes. Within 10 days to 2 weeks the parasite has produced thousands of daughter merozoites in a single liver cell. The cell

bursts, releasing the infective merozoites into the bloodstream where a rapid asexual replication begins with the invasion and reinvasion of erythrocytes. Within 48 h after invasion, the intraerythrocytic parasite generates 16 to 32 progeny by a binary nuclear fission called schizogony. Within 7–10 days of release from the liver, the human host can have trillions of circulating infected erythrocytes. The release of daughter merozoites from a population of erythrocytes correlates to the symptoms of fever, sweats, rigors and chills called malaria. The newly infected erythrocyte contains a ring stage; this is named after the signet ring appearance of the chromatin dot and blue circle of parasite cytoplasm. The ring stage changes into the trophozoite stage at the point that microscopically visible hemozoin or malarial pigment appears amidst the increasing cytoplasm. The schizont stage commences when the nucleus replicates. The erythrocyte ring, trophozoite and schizont stages are shown in Figure 1. The schizonts release merozoites, which infect a new crop of erythrocytes. Less than 1% of the asexual stages will differentiate into male and female gametocytes. *P. vivax* develops gametocytes before symptoms, in contrast to *P. falciparum*, which develops gametocytes after causing the disease malaria. Only gametocytes are infective to the female *Anopheles* mosquito, which



**Fig. 1** Modified Giemsa stain of intraerythrocytic *P. falciparum*. The stages include (A) a ring stage; (B) a trophozoite stage with visible malarial pigment; and (C) a schizont stage with more than one nucleus. The erythrocyte diameter is  $\sim 6 \mu\text{m}$ .

ingests a mere 5  $\mu$ l of blood containing 25 million erythrocytes of which approximately 250,000 may be infected, correlating to 250 gametocytes. Within the mosquito stomach, the male gametocytes will agitate wildly, sending out male flagellum, akin to spermatozoa. The flagellum fertilizes the female gametocyte to form an ookinete that will penetrate the stomach epithelium to form an oocyst next to the basement membrane. Soon, thousands of sporozoites are released from the oocyst into the hemocoel of the mosquito. The sporozoites attach and invade the salivary glands in preparation for injection into the warm-blooded host upon the second blood meal about 10–15 days after the first. The sporozoites soon invade the liver cells to begin the next cycle. Both *P. vivax* and *P. ovale* have the ability of a protracted dormant phase (of months to years) in the liver (called hypnozoites) before rapid replication begins to release merozoites into the bloodstream (Gilles and Warrell, 1993).

## 1.2

### Malaria Pathogenesis

In the case of non-lethal *P. vivax* or tertian malaria, the bloodstream broods take a few cycles to synchronize, and this is responsible for the 48-h periodic onset of the classic malaria paroxysm of cold, hot and sweating stages that last for 12–18 h. The symptoms are often severe and incapacitating, especially for the nonimmune host, and many have described the exhaustion as like ‘completing a marathon’. *P. vivax* parasitemias have a built-in ceiling of less than 5% infected erythrocytes because this species only invades the young reticulocytes. The classic malaria disease triad is fever, splenomegaly, and anemia.

In addition to the classic triad, *P. falciparum* malaria takes two major life-threatening

disease forms of cerebral malaria and severe anemia. The intraerythrocytic *P. falciparum*, unlike the other three human species, forms adhesive knobs on the surface of the erythrocytes that stick to endothelial receptors such as intracellular adhesion molecule (ICAM)-1, CD-36 or chondroitin sulfate (Miller et al., 2002). In lethal *P. falciparum*, rings predominate in circulation, because older trophozoite forms are adhering to tissue capillary beds to cause hypoxia and end-organ damage, including coma. *P. falciparum* can invade all ages of erythrocytes, reaching parasitemias of over 50% which cause severe anemia. Other complications of *P. falciparum* malaria may include lactic acidosis, shock, lung or kidney failure, hypoglycemia, or placental malaria. In geographic areas where transmission occurs all year round, children aged under five years account for most of the morbidity and mortality, despite peak prevalence for parasitemia occurring at about age 10 years. A partial humoral immunity protects older children and adults from severe disease. In subtropical geographic areas where the malaria transmission is seasonal, severe malaria morbidity and mortality affect all ages, from children to adults. Human genetic balanced polymorphisms such as sickle cell disease, thalassemias, glucose 6-phosphate deficiency and erythrocyte membrane defects protect from severe disease, but not infection (Miller, 1994; Weatherall, 1997). An absence of Duffy antigen receptor chemokine provides sterile protection for *P. vivax* infection in most of West Africa (Miller et al., 1975, 1976).

## 1.3

### Hemoglobin Degradation

Within 24 h of invasion, the erythrocyte has been remodeled both on the outside, with the *P. falciparum* knob proteins decorating

the surface to cause adhesion to endothelial beds (Miller et al., 2002), and also on the inside, with an efficient conversion of more than half of the entire 30-pg hemoglobin-rich erythrocyte cytosol into a proper eukaryotic protozoan (Krugliak et al., 2002). With a 10% parasitemia, the average 6-L blood volume of an adult can contain  $5 \times 10^{12}$  infected parasites. The 20-pg (two thirds of 30 pg) digestion of hemoglobin equals  $3 \times 10^{-11}$  g. This  $\sim 100$  g catabolism of hemoglobin means that almost a “quarter-pounder” (113 g) of protein is consumed by the circulating parasites in less than 24 h. Specialized organelles, termed the cytosome and digestive vacuole, accomplish the efficient ingestion and subsequent digestion of the hemoglobin-containing cytosol (Olliaro and Goldberg, 1995). Double-membrane vesicles emerge from the cytosome to route to the acidic digestive vacuole. After fusion and release of vesicle contents, hemoglobin-degrading proteases – principally plasmepsins and falcipains – cleave hemoglobin into small fragments that are transported to the parasite cytosol for final degradation to amino acids that are utilized by the growing parasite (Kolakovich et al., 1997).

During the catabolism of hemoglobin, heme or iron protoporphyrin IX (FePPIX) is released along with oxygen. Iron is a metal that can be either oxidized to a ferric ( $\text{Fe}^{3+}$ ) state or reduced to a ferrous ( $\text{Fe}^{2+}$ ) state. Only reduced ferrous heme can transport oxygen. Upon hemoglobin degradation, reduced heme is released that oxidizes to ferric heme. Because of the high oxygen content and acidic pH, oxygen radical production via the Fenton and Haber–Weiss reactions is ideal in the digestive vacuole (Wardman and Candeias, 1996; Koppenol, 2001). Molecular oxygen accepts the iron electron to initiate the chain of oxygen radical generation and protein and lipid alkylation (Minotti and Aust, 1989; Atamna and Ginsburg, 1993). In

order to remove the reactive FePPIX species from solution, the *Plasmodium* parasite creates a FePPIX crystal known as hemozoin or “malarial pigment”. The presence of this brown birefringent crystal (as seen by light microscopy) demarcates the transition from ring stage to the pigmented trophozoite stage (Gilles and Warrell, 1993). The pigment persists until the schizont ruptures, releasing daughter merozoites from a residual body composed of membranes surrounding the pigment-laden digestive vacuole. The gametocytes also catabolize hemoglobin during the first few days in the erythrocyte. The gametocyte hemozoin is carried into the mosquito where the fertilized macrogamete transforms into the ookinete that still carries the intracellular pigment across the stomach epithelium while forming the oocyst. Here, the sporozoites leave behind the hemozoin. Therefore, only the sporozoites in the salivary gland of the mosquito and later the liver cells and merozoites in the liver and later erythrocytic cells lack hemozoin. All other stages contain the FePPIX detoxification crystal.

## 2 Historical Outline

Extracts of cinchona bark that contains quinine, quinidine and the cinchona alkaloids were used as a specific antimalarial agent for over 300 years before the etiologic agent, *Plasmodium*, was identified. Following the trail of malarial pigment more than 100 years ago enabled researchers to pinpoint the cause of malaria as a protozoan infection of the human erythrocytes, transmitted by the mosquito. The debate lasted almost 80 years as to whether the biochemical composition of hemozoin was protein, heme, or both. Although much progress has been made during the past decade regarding

hemozoin, the exact mechanism of intracellular formation, the arrangement of heme in the crystal, and the exact mode of inhibition by the quinoline class of drugs has not been fully resolved.

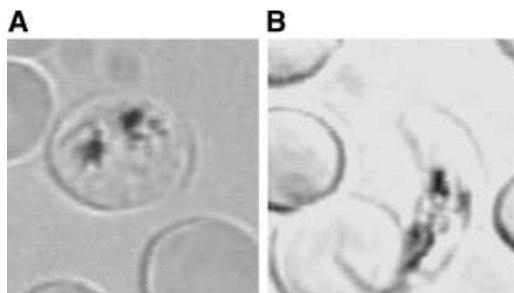
## 2.1

### Pigment Associates with Malaria

Malarial pigment has long fascinated malarialogists, with Meckel first having observed pigment in the blood and spleen during an autopsy of an insane person, though he failed to make the connection to malaria (Meckel, 1847). Meckel detailed pigment in ovoid protoplasmic bodies which he indicated were malaria parasites. Soon afterwards, Virchow detailed pigmented bodies in the blood of a patient who had died from chronic malaria (Virchow, 1849), and so first made the connection of the pigment with malaria. In 1850, Hirsch confirmed the presence of pigment with intermittent fevers (Hirsch, 1850). Together, these three workers considered that the spleen was the source of the pigment, but confused partially degraded ferritin or hemosiderin with malarial pigment. Finally, in 1880, Laveran correlated a living parasite to malaria disease after observing pigmented bodies in the blood of a soldier at Constantine, Algeria. Laveran observed thin and transparent filaments on the periphery of round pigmented bodies with filaments

oscillating so rapidly that he incontestably knew he was looking at an animated parasite. On December 24, 1880 in Italy, Laveran communicated the identification of pigmented erythrocytic cells in 26 malaria patients. His descriptions included crescents (which equate with the gametocytes), pigmented trophozoites, and the process of exflagellation. Some unstained specimens of blood with pigment granules visible are shown in Figure 2. In 1884, at the Bayview hospital in Baltimore, Maryland, Councilman performed autopsies on two fatal cases of malaria (Councilman and Abbot, 1885). He described “the brain as a dull chocolate color, lungs inflated and contained much pigment, the liver large, soft and of a dark slaty color. The spleen was very much enlarged... and of an almost black color”. A large amount of pigment in the liver and spleen was seen in two forms as irregular pigment masses outside cells and also within small hyaline bodies in red blood cells. The capillaries of the brain were filled with pigment in the small hyaline protoplasmic bodies like those seen inside red blood cells of the spleen. Pigment was the consistent pathologic link of severe fatal disease to malaria.

Whether the cause of malaria was the pigmented bodies or bacilli described by Marchiafavi and Celli was debated throughout the 1880s (Marchiafava and Celli, 1883). Until he looked at the blood of malaria



**Fig. 2** Unstained intraerythrocytic *P. falciparum*. (A) The pigmented trophozoite stage is shown as first observed over 120 years ago. (B) The crescent gametocyte pathognomonic of *P. falciparum* is shown with central pigment.

patients for himself, William Osler was skeptical of Laveran's theory (Osler, 1887), but later confirmed Laveran's findings with his own description of blood film examinations from 70 patients. He also detailed excellent drawing of parasites and related forms seen with different types of malaria fever. "The most common alteration in the blood of malaria patients is presented by a pigmented structure inside a red corpuscle." Convinced of his findings, Osler instituted routine blood smear analyses to diagnose malaria in the work-up of febrile patients at Johns Hopkins Hospital (Osler, 1889). Camillo Golgi was the first to photograph the pigmented quartan malaria parasite in 1890 (Golgi, 1890), and was also the first to indicate that the febrile paroxysms associate with segmentation of the parasite and that severity of symptoms correlates with the number of parasites in the blood (Golgi, 1886). Thayer and Barker compiled 616 well-documented malaria cases complete with fever curves and species-specific blood film microscopy, plus four autopsies of three *P. falciparum* cases and one *P. vivax* case (Barker, 1895; Thayer and Hewetson, 1895).

## 2.2

### Pigment Links Mosquito to *Plasmodium* Life Cycle

By using the pigmented Halteridian species, *Haemoproteus columbae*, two medical students at Johns Hopkins, Opie and MacCallum observed under a glass slide the penetration of a vermicule from a male gametocyte into a rounded female gametocyte, correctly naming the process sexual (MacCallum, 1898). They confirmed their findings in the blood of a *P. falciparum* patient (MacCallum, 1897). Manson communicated these findings to Ross, and finally, after exhaustive searches in mosquitoes, Ross examined a dappled wing *Anopheles* mosqui-

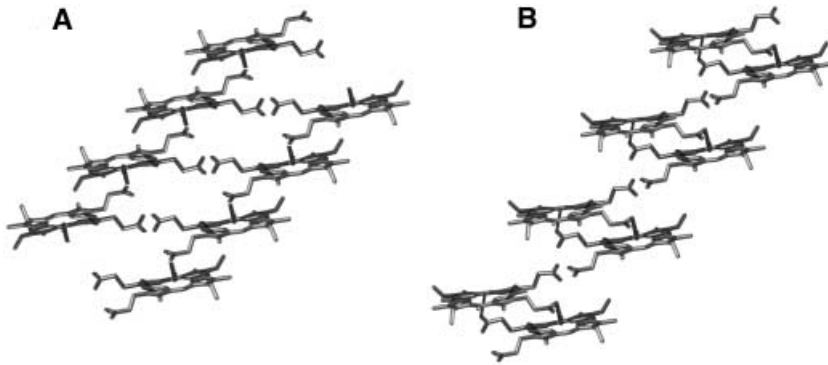
to (Ross, 1897). He comments on cells observed outside the stomach of the mosquito – "but what now arrested attention was the fact that each of these bodies contained a few granules of black pigment absolutely identical in appearance with the well-known and characteristic pigment of the parasite of malaria (large quartans and crescent-derived spheres)". Thus, following the trail of malarial pigment enabled Laveran to identify *Plasmodium* as the agent, and Ross to implicate the mosquito as the vector of malaria, and both were awarded Nobel prizes for their observations. Some 50 years later, the last part of the life cycle to be solved was the liver stage that lacks pigment, wherein Garnham described the exo-erythrocytic forms of *Plasmodium kochi* in the liver cells of monkeys in East Africa (Gilles and Warrell, 1993).

In 1911, Brown distinguished melanin from malarial pigment by deducing the heme origin of the latter (Brown, 1911), and stated that the black malarial pigment could hardly be pure heme, but should contain impurities. He astutely suggested the action of a proteolytic enzyme on hemoglobin to be the most probable mode of elaboration of the malaria pigment. Controversy on the biochemical composition of hemozoin continued for more than 80 years until the 1990s, when several workers showed hemozoin to be composed solely of heme arranged in a crystal structure. Only then was hemozoin formation proved to be the target of widely used antimalarial drugs such as chloroquine and quinine.

## 3

### Chemical Structure

The two proposed models for hemozoin are shown in Figure 3, both being based on the undisputed novel iron to oxygen bond that



**Fig. 3** Hemozoin has the central iron to carboxylate oxygen coordinate bond. (A) The original model of a true polymer of FePPIX. (B) The more recent head-to-tail dimer model. The pairs are linked to each other by hydrogen bonding. This model is representative of a biomineralization process.

joins the central iron to the side-chain carboxylate oxygen of the adjacent FePPIX. The first model is a true FePPIX polymer with coordinate bonds linking a chain of hemes (Slater, 1992). Opposite strands interact via hydrogen bonding, and the addition of hemes can occur at either end. Only a single iron is located between each porphyrin ring. The chemical formula would be  $[\text{Fe}^{+++}(\text{protoporphyrin IX-H})]_n$ , where protoporphyrin IX equals  $\text{C}_{34}\text{H}_{32}\text{N}_4\text{O}_4$ . The second model is representative of a biomineralization process with a unit reciprocal Fe1-O41 head-to-tail dimer (Pagola et al., 2000). In this situation, two iron molecules center between each pair of porphyrin rings, whilst the dimer units are stacked via hydrogen bonds in the crystal. The chemical formula for this model would be  $[\text{Fe}^{+++}(\text{protoporphyrin IX})]_2$

#### 4 Occurrence

Hemozoin occurs in the presence of large amount of heme in an acid environment where heme crystal formation outpaces heme degradation. A pH of  $< 6.0$  is an

absolute for hemozoin synthesis. Mammalian cells possess a heme oxygenase enzyme that is present in the cytosol and which degrades heme to release iron, carbon monoxide and the porphyrin ring as biliverdin. Heme is apparently transported out of the mammalian acidic lysosome to the cytosol in a manner such that hemozoin does not form in the acidic environment.

Hemozoin occurs in all *Plasmodium* species with the exception of a single laboratory-generated *Plasmodium berghei* clone that has been cultured in the presence of continuous chloroquine selection. This isolate is believed to transport either heme or hemozoin from the infected erythrocyte (Wood and Eaton, 1993). Hemozoin is present in all *Plasmodium* erythrocyte stages—ring, trophozoite, schizont, and gametocyte—and is also present within the stages in the mosquito gut until the sporozoites leave the oocyst. The sporozoites and merozoites in the hepatocytes, as well as those released from infected erythrocytes, all lack hemozoin.

Additional organisms which have been documented to synthesize hemozoin include *Haemoproteus* sp., *Schistosoma* sp., and the Reduviid bugs. The chemical synthesis of acid hematin to produce  $\beta$ -hematin



is identical to that for hemozoin. The acid hematin pigment produced from formalin has not formally been shown to be identical to hemozoin.

Notably, neither *Babesia* species (which reside within erythrocytes) nor *Entamoeba histolytica* (which ingest erythrocytes) synthesize hemozoin. Likewise, the “professional” erythrocyte phagocyte–mammalian macrophages in the liver and spleen – do not synthesize hemozoin.

## 5

### Function of Hemozoin

The function of hemozoin is to prevent oxygen radical-mediated damage to the parasite. While no discernable effect of hemozoin on the parasite is seen, the heme crystal perturbs both white blood and endothelial cells. The formation of hemozoin essentially removes the reactive iron of heme out of solution in the oxygen-rich acidic environment of the vacuolar compartment where hemoglobin degradation occurs. The malaria protozoan parasite invades an erythrocyte that is densely packed with  $300 \text{ mg mL}^{-1}$  hemoglobin, and within 2 days the parasite has consumed more than two-thirds of the 5 mM hemoglobin-rich cytosol. In this way, the parasite remodels what is essentially a bag of hemoglobin into a functioning eukaryotic cell that is capable of exporting protein packets to the surface of the erythrocyte and preparing progeny for the next round of invasion.

The dogma concerning hemoglobin degradation is that the parasite is reliant on the amino acids in hemoglobin for incorporation into its own amino acid pool (Sherman and Tanigoshi, 1970; Theakston et al., 1970; Sherman, 1977). Indeed, the parasite is an auxotroph only for the five amino acids which are either poorly or not represented at

all in hemoglobin (Divo et al., 1985; Francis et al., 1994; Kolakovich et al., 1997). However, the parasite imports amino acids that, in hemoglobin, are both well represented and also rare to nonexistent. The most conclusive evidence of this reliance on hemoglobin degradation is that the specific inhibition of hemoglobinases is fatal for the parasite (Francis et al., 1997).

Recently, Ginsburg has carefully examined the use of protein and fate of heme in different strains of *P. falciparum*. A summary of his findings is that the parasite invades an erythrocyte containing  $300 \text{ mg mL}^{-1}$  protein, 95% of which is hemoglobin at a concentration of 5 mM; this correlates to 30 pg (or 0.5 fmol) of hemoglobin per cell. The parasite ingests more than two-thirds of the hemoglobin ( $\sim 20 \text{ pg}$ ), yet only uses 3 pg for its own amino acids (Krugliak et al., 2002). This agrees with the observation of a large efflux of amino acids from the parasite (Zarchin et al., 1986) and the low density of parasites compared to erythrocytes (Kramer et al., 1982). In addition, the FePPIX balance sheet shows that, of the 2 fmol of hemoglobin FePPIX (four FePPIX per hemoglobin molecule) contained in the erythrocyte, 1.33 fmol is ingested with hemoglobin, but only 0.55–0.6 fmol is converted into hemozoin; that is, only  $\sim 43\%$  of the FePPIX is converted into hemozoin. The authors demonstrated a role for glutathione to degrade this excess FePPIX (Ginsburg et al., 1998; Famin et al., 1999). The disposition of the resultant 5 mM iron or remnant porphyrin ring in the cytosol has yet to be elucidated. Paradoxically, the parasite is reliant on FePPIX synthesis rather than scavenging the vast amount of FePPIX from hemoglobin (Surolia and Padmanaban, 1991, 1992; Bonday et al., 1997). Likewise, low micromolar doses of iron chelators kill the parasite despite the release of almost 5 mM iron from the proposed glutathione degradation of