

Determining the solubility of haematin and haem–drug complexes at relevant pH in aqueous medium *via* spectrophotometric titration.



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UNIVERSITY OF CAPE TOWN

GHANNISH SOOGARY

SUPERVISORS

Professor Timothy J. Egan, Dr Kathryn J. Wicht,
and Dr John G. Woodland

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Declaration

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I, Ghannish Soogary, hereby declare the following:

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Abstract

For centuries, quinoline-based compounds have formed the mainstay of treatment against the pervasive and deadly infectious disease of malaria. While the ongoing emergence of resistance to existing chemotherapies has driven efforts to discover new antimalarial chemotypes, interest in the quinoline drugs has not waned. The two most important classes of quinoline-based drugs, namely the 4-aminoquinolines and the quinoline methanols — represented in this study by chloroquine (CQ) and quinidine (QD), respectively — are widely accepted to act against the most virulent malaria parasite, *Plasmodium falciparum*, via the haemozoin inhibition pathway within the acidic digestive vacuole (DV) of the pathogen. However, mechanistic details of this mode of action are still unresolved. Following the release of toxic ferriprotoporphyrin IX (Fe(III)PPIX, or ferrihaem) in the DV upon haemoglobin degradation by the parasite, the formation of a drug–ferrihaem complex has been hypothesised to be responsible for the parasitocidal effects of various haemozoin inhibitors. Information on the solubilities of drug–ferrihaem complexes and of ferrihaem itself is scarce, and would enrich the arsenal of knowledge toward resolving the intricate mechanism of parasite death caused by these complexes.

As the concentration of the drug–ferrihaem complex relies on the solubility of ferrihaem itself, the latter was investigated in this research at pH values approximating those of the parasitic DV, in exclusively aqueous medium. Based on the methodology employed in a previous study, UV–visible spectrophotometric titration experiments of haematin with sodium hydroxide were performed. The optimal incubation time periods allowing for complete sample equilibration were determined to be 16 days at both 10°C and 20°C, 12 days at 25°C and 8 days at both 37°C and 50°C. The recrystallisation of haematin was determined to be unnecessary since indistinguishable titration and solubility data were obtained from the use of recrystallised haematin *versus* commercially-sourced haematin without further purification. However, at each of the five temperatures, erratic data points were observed at low pH values in the spectrophotometric titration data, possibly due to solid haematin not dissolving. As a result, reliable and reproducible thermodynamic parameters for ferrihaem solubility could not be determined under these conditions. Nevertheless, the solubility of π – π dimeric ferrihaem at the parasitic DV pH of 5.0 was predicted to be $2.6 \pm 1.3 \mu\text{M}$ at 37°C via extrapolation from the remaining data points.

In an alternative approach, spectrophotometric titrations of haematin with perchloric acid were conducted to directly investigate ferrihaem solubility at a more relevant pH range. The

optimal incubation time to ensure sample equilibration was determined to be 24 hours. However, as pH was lowered, the UV-vis spectra exhibited the collapse of the ferrihaem *Q* band and the concomitant appearance of a charge-transfer band due to porphyrin aggregation, making it impossible to obtain solubility data. Consequently, a pyridine-ferrihaemochrome method was employed to enable the quantification of free ferrihaem, yielding a reliable solubility value for dimeric ferrihaem of $0.24 \pm 0.08 \mu\text{M}$ at a pH of 5.0 and a temperature of 37°C. The solubility product constant for the dimer was computed to be $(1.9 \pm 0.4) \times 10^{-42}$.

The pyridine-based acid titration methodology was also utilised to determine the solubilities of two drug-ferrihaem complexes. First, the optimal sample incubation time was determined to be 48 hours for each of the CQ-ferrihaem and QD-ferrihaem complexes, however, consistent scatter in the UV-vis spectrophotometric titration data was observed due to extremely poor solubilities of the complexes at pH values close to 5.0. Consequently, *ranges* of values were reported within which the true solubility of the complexes would most likely lie: for the dimeric CQ-ferrihaem complex, lower and upper bounds of 0.5 nM and 1.8 nM with a 95% confidence interval of 1.0 nM – 1.1 nM, and for the QD-ferrihaem complex, lower and upper bounds of 0.2 nM and 18.0 nM with a 95% confidence interval of 2.0 nM – 2.9 nM.

Overall, this dissertation has employed spectrophotometric tools to provide physiologically-relevant solubility data for ferrihaem and, for the first time, the drug-ferrihaem complexes of the clinically-relevant antimalarials CQ and QD. These represent a crucial set of information to bolster an *in silico* model aiming at elucidating the haemozoin inhibition pathway and the modes of action of existing and experimental antimalarials — paving the way for the development of new, life-saving medicines against this devastating infectious disease.

Conference Proceedings

Parts of this work have been presented at the following scientific meetings:

- **2022:** South African Chemical Institute (SACI)/Royal Society of Chemistry (RSC) Regional (Western Cape) Youth Chemists' Symposium, Stellenbosch University, September 2022 (Flash talk presentation).
- **2022:** Holistic Drug Discovery and Development Centre H3D Symposium, "A Spectrum of Opportunities in Infectious Disease Drug Discovery to Enhance Global Health", Webersburg Estate, Stellenbosch, October 2022 (Poster presentation).

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The late Professor Timothy J. Egan. Our father figure, who sadly passed away following a brave fight against cancer. I will forever be grateful to you for taking me under your wing in the Egan stable; it is something I will always wear proudly, like a badge. You offered to me the opportunity of working among brilliant peers. My project was your brainchild, and you always spurred me on to give my best, to tackle research-related issues head-on, and to keep the faith despite the clouds of doubt that rose when things would not work out in the laboratory. It has been a privilege to learn from someone as knowledgeable and disciplined as you. Thank you for being an excellent scientist and lecturer, and a supervisor par excellence, but more importantly, a genuinely nice person, which means a lot in today's world. I hope I have made you proud.

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To Nirvashi, for being in my life.

To my family: Mom, Dad, Shruti, and my late grandparents, Dada and Dadi: Everything I do, I do it for you.

Abbreviations list

ACT	Artemisinin-based combination treatment	ICP-MS	Inductively coupled plasma mass spectrometry
AQ	Amodiaquine	IR	Infrared
AS-AQ	Artesunate–amodiaquine	K76T	Lysine 76 mutation to threonine
AS-PY	Artesunate–pyronaridine		
ATPase	Adenosine triphosphatase	K_{sp}	Solubility product constant
COVID-19	Coronavirus disease 2019	MCD	Magnetic circular dichroism
CQ	Chloroquine	MD	Molecular dynamics
CQR	Chloroquine-resistant	MF	Mefloquine
CQS	Chloroquine-sensitive	NADPH	Nicotinamide adenine dinucleotide phosphate
CSD	Cambridge Structural Database		
CT	Charge transfer	NMR	Nuclear magnetic resonance
DHA-PPQ	Dihydroartemisinin–piperaquine	PfCRT	<i>Plasmodium falciparum</i> chloroquine resistance transporter
DLS	Dynamic light scattering		
DMSO	Dimethyl sulfoxide		
DNA	Deoxyribonucleic acid	PfMDR1	<i>P. falciparum</i> multi-drug chloroquine resistance
DV	Digestive vacuole		
Deuteroferrihaem	Iron(III)deuteroporphyrin IX	Por	Porphyrin
EPR	Electron paramagnetic resonance	PV	Parasitophorous vacuole
ESI	Electron spectroscopic imaging	QD	Quinidine
Ferrihaem	Iron(III)protoporphyrin IX	QN	Quinine
Haem	Iron(II)protoporphyrin IX	RBC	Red blood cell
Haematin	HO–iron(III)protoporphyrin IX	ROS	Reactive oxygen species
HRP	Histidine-rich protein	SA	Simulated annealing
IC₅₀	50% inhibitory concentration	SDS	Sodium dodecyl sulfate
Haemin	Cl–iron(III)protoporphyrin IX	SEM	Standard error of the mean
HDP	Haem detoxification protein	UV–vis	Ultraviolet–visible
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	v/v	Volume/volume
HF	Halofantrine		

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CHAPTER 1

Introduction and Literature Review

1.1. History of malaria and its prevalence

Malaria is a devastating infectious disease having a history spanning several centuries. It is a mosquito-borne and acute febrile disease which has plagued every continent, bar Antarctica.¹ In classical antiquity, a deadly upsurge of casualties due to Roman fever — the illness caused by a particularly lethal malaria strain that struck the city of Rome — has been linked to the collapse of the Roman Empire;² interestingly, the term ‘malaria’ is derived from ‘*mal aria*’ meaning ‘bad air’ and was coined by the Romans who misconceived that the disease came from pernicious swamp fumes.³ The classical and ensuing medieval ages failed to deliver any potent antimalarial, relying mostly on herbal medicine to treat symptoms, such as the *Belladonna* plant to alleviate pain and the bark of the *Cinchona* tree to calm febrile symptoms.⁴⁻⁵ However, the nineteenth century marked a paradigm shift in malaria treatment: While analysing the red blood cells (RBCs) of an infected patient in 1880, French physician Alphonse Laveran observed moving flagella and posited that the latter were the parasitic protozoa that cause malaria.⁶ In 1886, Italian biologist Camillo Golgi propounded that malaria existed in at least two forms based on their periodicities: tertian malaria, causing fevers on every third day, or quartan malaria where the patient would be febrile on every fourth day.⁷ Concurrently, comprehensive pathological work by Italian scientists Marchiafava, Celli and Bignami oversaw the naming of the *Plasmodium* genus and the description of important morphological differences between the malaria parasites *P. vivax*, *P. malariae* and *P. falciparum*.⁸

Despite the progress, identifying the causal vector remained key, as herein lied the crux of the issue. In 1894, Sir Patrick Manson was the first to conjecture that mosquitoes carry malaria.⁹ He pitched the idea to his mentee, British army officer Sir Ronald Ross, to whom he delegated

research duties. Ross achieved momentous success in 1897 when he observed an avian malaria parasite, *P. relictum*, in the stomachs of only one group of mosquitoes — despite not knowing the species — out of several tested types of mosquitoes.¹⁰ In the same year, Canadian pathologist William MacCallum elucidated the sexual stages of a closely related haematozoan, *Haemoproteus columbae*, in the blood of infected birds.¹¹ Subsequently, in 1898, Ross discovered the transmission process of *P. relictum* in infected birds through mosquitoes, conclusively incriminating the latter as the vectors.¹² These findings were fortified by the Italian malariologists Grassi, Bignami and Bastianelli who demonstrated that human transmission of *P. falciparum* only occurs *via* female *Anopheles* mosquitoes, and methodically chronicled the blood–mosquito life cycles of *P. vivax* and *P. falciparum*.¹³ In 1948, Henry Shortt and Cyril Garnham emphasised the importance of the malarial liver stages as they discovered a replication phase in the liver preceding egress to invade RBCs.¹⁴ Finally, the dormant, exoerythrocytic stages of the parasitic life cycle in the liver were unravelled by Wojciech Krotoski in 1982.¹⁵

Humans are infected by the five species *P. malariae*, *P. ovale*, *P. knowlesi*, *P. vivax* and *P. falciparum* — the latter being the most baneful aetiological agent in terms of deaths. Common symptoms include persistent headaches and fevers which, in serious cases, may deteriorate to potentially deadly pathologies such as acute anaemia and cerebral malaria. *P. falciparum* is predominantly rife on the African continent and *P. vivax* is the most prevalent parasite in other countries.¹⁶ Despite its wide geographic spread, *P. vivax* has often been neglected when listing major health threats. Recent studies have placed emphasis on raising awareness, citing gross underestimations in deaths due to this parasite.¹⁷⁻¹⁸ The latest *World Malaria Report* reveals the overwhelming toll of malaria, with 247 million cases and 619 000 deaths in 2021.¹⁹ The African region carried a precariously high share of the global burden, accounting for 95% of all new cases and 96% of deaths; approximately 80% of the deaths occurred in children aged under five.¹⁹ A recent study reported the insidious growth of placental malaria cases in sub-Saharan Africa between 2011 and 2019, resulting in maternal illness and low birthweight in 882 000 neonates.²⁰ Between 2019 and 2021, 69 000 malaria mortalities were attributed to fragmented health systems due to COVID-19.¹⁹ Indeed, as the number of COVID-19 cases snowballed, global interest in malaria faded into the background, even though the disease has not.

1.2. Biology of the parasite

Disease evolution is influenced by the dynamic interplay and inherent features of host-pathogen interactions. To fulfil their nutritional needs and circumvent the host's immune defences, intracellular pathogens aggressively disrupt host functions and cellular machinery. Apicomplexa are single-cell eukaryotes characterised by an apicoplast — a non-photosynthetic membrane-bound organelle — and are infamous for manipulating their host cells to meet their needs. Parasites of the *Plasmodium* genus are major apicomplexan pathogens.²¹

The five species of *Plasmodium* that infect humans share similarities in their life cycles, and only that of *P. falciparum* will be described. It is an intricate and multistage life cycle which takes place in vector mosquitoes, within which sexual reproduction occurs, and vertebrate hosts which house asexual reproduction.²²

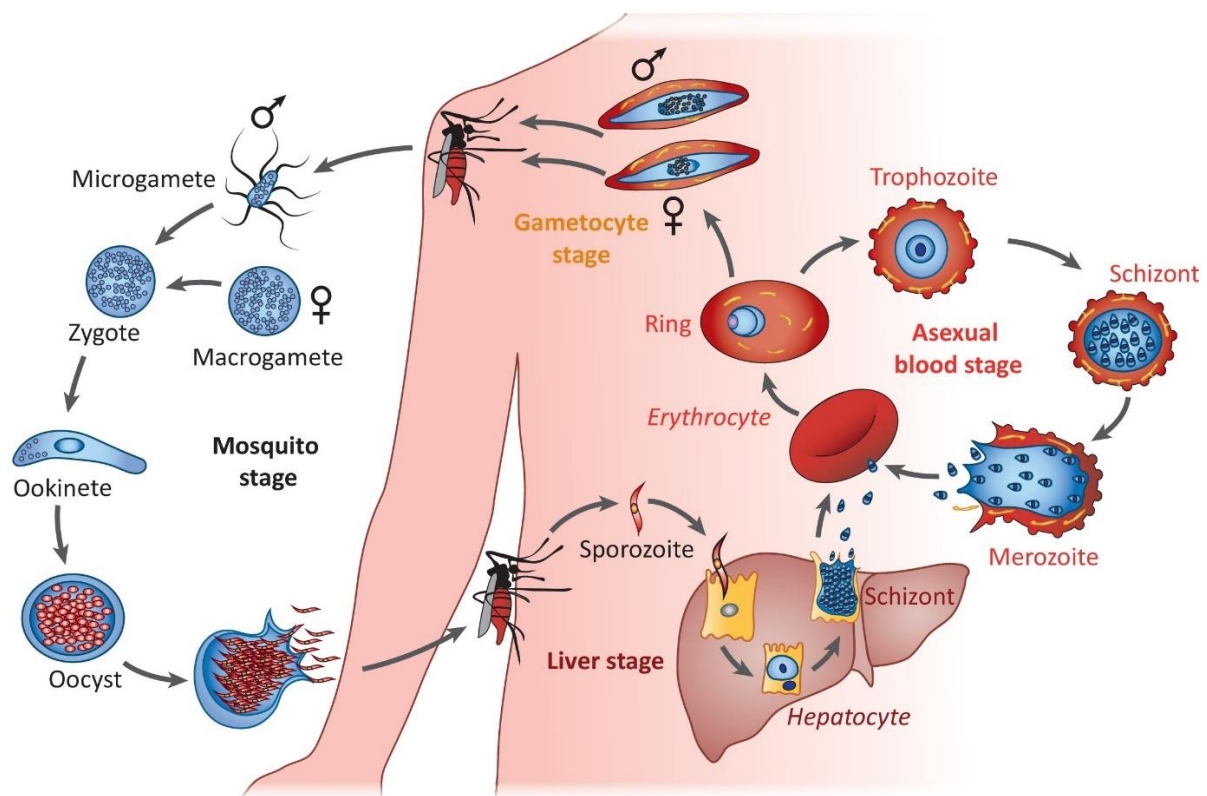


Figure 1.1. Illustration of the complex life cycle of the malaria parasite, depicting the mosquito, liver, blood, and gametocyte stages. Reproduced from Maier et al., *Trends Parasitol.*, 2019, 35, 481 with permission from Elsevier.

The obligate parasite owes its robustness to its distinctive genome which encodes more than 5 000 genes, thereby bolstering it with the required armour to survive and develop within intracellular and extracellular environments. It constitutes, to this day, a major hindrance in the establishment of a fully protective vaccine.²³⁻²⁴ As illustrated in **Figure 1.1**, transmission of the pathogen is triggered within minutes after uptake of infected blood by the *Anopheles* mosquito vector. Upon reaching the midgut, the mature ingested *Plasmodium* gametocytes undergo a rapid sequence of regulated cell division and differentiation steps — known as gametogenesis — that produce haploid gametes. Male and female gametes fertilise, combining their chromosomal sets to form a zygote, which converts into a motile ookinete. The latter transforms into a sessile oocyte, within which sporogonic replication occurs. Having matured about ten days later, the oocysts rupture, each releasing several thousand sporozoites into the hemolymphatic system of the mosquito: Sporozoites display the structural and functional characteristics of invasive Apicomplexan protozoa, and they invade the mosquito salivary glands, ready to be injected into a vertebrate host.²⁵

1.2.1. Human infection

The injection of sporozoites into the host dermis by the female *Anopheles* mosquito during a blood meal marks the onset of human infection. The motile sporozoites enter the bloodstream by penetrating a blood vessel, and quickly proceed towards the liver where they invade hepatocytes. Over the ensuing 2–10 days, the sporozoite transforms into an exo-erythrocytic schizont, concluding in the release of thousands of daughter merozoites into the hepatic circulation. The merozoites then invade RBCs, commencing the asexual intraerythrocytic stage. Interestingly, instead of forming schizonts, species like *P. vivax* and *P. ovale* can form non-replicating hypnozoites and enter a phase of latency, ergo facilitating long-term parasite survival and potentially causing relapses.²⁶

The merozoite is an important immunological target: Between egress from one RBC and invasion of the next, the merozoite is momentarily extracellular, thus imperilled by exposure to host antibodies. Following adherence, RBC invasion — which typically occurs in less than a minute — is stimulated by the merozoite orienting itself to juxtapose its apical end with the erythrocytic membrane; at the RBC surface, an invasion pit is formed which eventually engulfs the merozoite in a parasitophorous vacuole (PV) to shield it from harsh intracellular conditions.

Following invasion, the pathogen flattens into a thin and discoidal structure, fittingly termed a “ring” based on its signet-like structure.²⁷⁻²⁸ It has crucially been observed to be the most prevalent parasite form in peripheral blood, and lasts for 18 to 22 hours after invasion²⁹. Besides direct exacerbation of malaria pathogenesis through cytoadhesion to endothelial receptors,³⁰ ring-infected erythrocytes may do so indirectly *via* strong retention in organs.³¹ Interestingly, the ring stage was long-thought to be metabolically nondescript until the emergence of evidence corroborating the initiation of haemoglobin degradation at this stage.³² Additionally, the parasite feeds on host cytoplasm through cytostome-derived vesicles which subsequently coalesce into a digestive vacuole (DV); electron tomography studies by Abu Bakar et al. revealed the presence of haemozoin microcrystals in the DV in as early as mid-ring stages.³²⁻³³

The next distinct stage, termed the trophozoite, is the most metabolically-active stage in the *P. falciparum* life cycle. In fact, the trophozoite differs morphologically from the ring stage only in cell shape and size, but not in intracellular composition; both feed on nutrients imported from the erythrocyte and also export proteins to it, gradually priming the RBC architecture for stronger adherence to the vascular endothelium.²⁷ The trophozoite takes up profuse amounts of haemoglobin which undergo proteolytic digestion in the DV. The rise in haemoglobin catabolism prompts the release of toxic ferriprotoporphyrin IX or Fe(III)PPIX, to which the parasite responds by triggering the haem detoxification pathway which sequesters labile Fe(III)PPIX into the inert crystal haemozoin, easily detectable in the acidic DV due to its high electron density. Moreover, late-stage trophozoites nurture the first stages of nuclear division to form multiple nuclei, setting the scene for schizogony.²⁷

The final stage of the asexual cycle entails high rates of protein and DNA synthesis to prime the parasite for schizogony. The uptake and catabolism of haemoglobin results in about 85% of host cytoplasm being enveloped, implying parasitic invasion of the majority of the erythrocyte volume.²⁷ A polynuclear schizont is formed which houses up to 30 merozoites per infected RBC.³⁴ Upon membrane rupture, the invasive daughter merozoites are freed which in turn invade other RBCs and re-initiate the asexual cycle.³⁵

However, instead of furthering the asexual cycle, approximately 1% of the parasites develop into gametocytes — sexual progenies that do not replicate in the host, and are responsible for transmission through the *Anopheles* mosquito.³⁶ Gametocytes are equipped to survive harsh conditions in the blood stream for several days, enhancing chances of transmission to the mosquito vector.

Tellingly, what separates *P. falciparum* from other malarial pathogens is its ability to induce cytoadherence of asexual parasites and gametocytes to the endothelium in several tissues, and

that of asexual parasites to placenta.³⁷ Consequently, cytoadherence is believed to exacerbate the severity of cerebral and placental malaria.³⁷

The asexual replication cycle lasts for 24 – 72 hours depending on the parasite species, at the end of which an increase in the parasite load of between eight-fold and ten-fold is observed.²⁶ The bursting of mature schizonts stimulates the host's innate immune response, which triggers the periodic malaria symptoms such as fever and chills. However, of direct relevance to this study is the trophozoite stage which highlights the processes of haemoglobin degradation followed by haem detoxification, and the iron-containing molecule haem is central to these processes. Hence, prior to delving into their mechanistic details, the next two sections outline the significant biological relevance of haem in nature.

1.3. The biological significance of haem

Fe(II)protoporphyrin IX or haem is a key and ubiquitous biological cofactor, pivotal in diverse essential biological systems.³⁸ It comprises a central iron atom incorporated in a heterocyclic tetrapyrrole ring system — termed a “porphyrin” — with two propionic acid substituents (**Figure 1.2A**). Its intrinsic absorption and fluorescence characteristics result from the conjugated framework, facilitating haem quantification. In nature, the molecule mostly functions as a prosthetic group to a multitude of haemoproteins with arguably the most prominent one being haemoglobin (**Figure 1.2B**), responsible for the storage and transfer of oxygen in erythrocytes. Between 80 and 90% of the total haem content is produced in the bone marrow and delegated towards haemoglobin formation; the remainder is synthesised in the liver and is used as a cofactor to form cytochrome P450 enzymes (**Figure 1.2C**) — crucial haemoproteins for the metabolism of many drugs.³⁹⁻⁴⁰ Besides, other important functions of haemoproteins include, *inter alia*, the movement and storing of diatomic gases such as oxygen, carbon monoxide and nitric oxide by the prototype haemoproteins haemoglobin and myoglobin⁴¹⁻⁴²; the electron transport chain mediated by cytochrome *c*⁴³; the regulation of gene transcription⁴⁴; the processing of micro-RNA and the modulation of circadian cycles.⁴⁵⁻⁴⁶

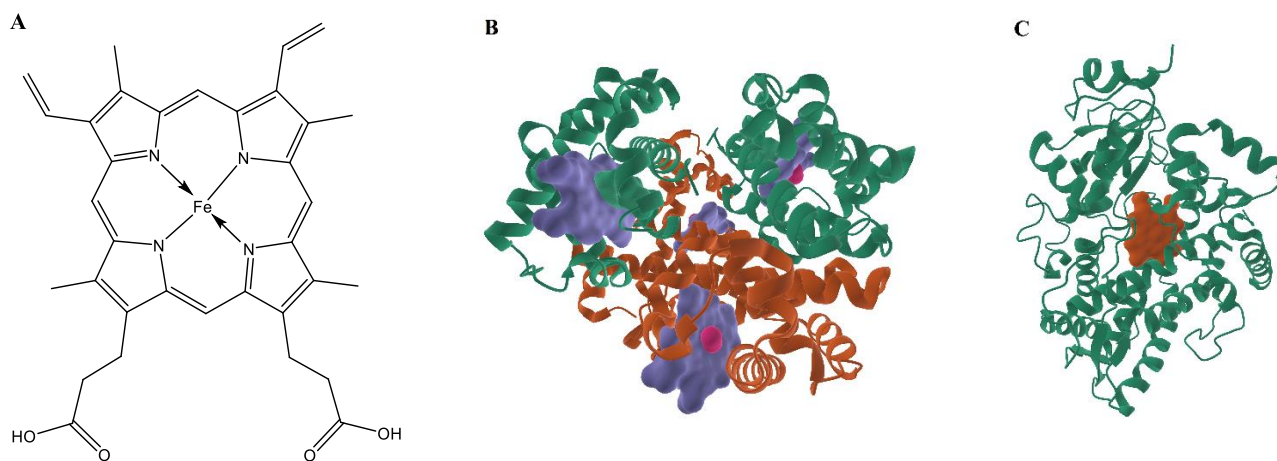


Figure 1.2. (A) Molecular structure of Fe(II)protoporphyrin IX or haem, with possible axial ligands omitted for clarity. (B) Protein structure of haemoglobin showing the four constituent haem moieties (purple) and the bound oxygen molecules (pink). (C) Protein structure of cytochrome P450, highlighting the haem group (orange). Structures were generated using crystal structure data from the PDB.⁴⁷ PDB IDs: 6BB5⁴⁸ for (B) and 1W0E⁴⁹ for (C).

Haemoglobin is composed of two α and two β subunits, each of which encloses a haem molecule which, through axial binding, associates to the protein and also binds a small molecule, such as an oxygen molecule, enabling transport of the gas. Importantly, haemoglobin displays allosteric properties: The binding of an oxygen molecule to a haem group triggers intricate structural changes with the Fe(II) ion being drawn into the plane of the porphyrin which flattens the ring. These small movements are channelled to the globin chains and the ionic interactions holding these chains together are altered; this induces relaxation of the quaternary structure as successive oxygen binding occurs, ultimately enhancing binding.⁵⁰ Furthermore, the affinity of haemoglobin for oxygen invokes an important biological phenomenon known as the Bohr effect, stating that reversible oxygen binding varies proportionally with pH, and inversely with carbon dioxide concentration.⁵¹

In addition, critical determinants of the biological functions of haemoproteins are their redox potentials which may be modulated by several factors: the range of oxidation states that the iron centre may interconvert between, with the most stable ones being +2 and +3; the substituents on the porphyrin macrocycle; hydrophobic interactions between residues in the haem-binding pocket and the porphyrin ring which may cause ring distortion, and the propensity of the propionic acid groups to form hydrogen bonds with protein residues.⁵²

1.4. Free haem

The salient features of haem have traditionally been thought to arise from binding to a polypeptide to form a haemoprotein while the unbound form of the molecule, termed “free haem”, has attracted far less attention. However, there has been an increase in the number of research studies delving into free haem in the past years, uncovering its role in fundamental biological processes such as gene transcription,⁵³ translation,⁵⁴ and protein translocation.⁵⁵ Several pathophysiological conditions such as malaria and sickle cell disease are known to trigger the release of free haem from haemoproteins, and free haem manifests cytotoxic pro-oxidant activity through catalysis of the unfettered generation of reactive oxygen species (ROS) *via* Fenton chemistry, causing oxidative stress.⁵⁶ Besides impairing complex cellular structures like DNA, proteins and lipids, the ROS may stimulate cell lysis and cause organ tissue damage.⁵⁷⁻⁵⁹ Free haem may also damage endothelial cells to provoke inflammatory lesions, causing vascular inflammatory diseases.⁶⁰ To further emphasise the toxicity of free haem, recent studies have incriminated it as a central — and currently largely overlooked — player in tumour genesis and proliferation through catalysis of the formation of carcinogenic *N*-nitroso compounds.⁶¹ Indeed, free haem is sparse in white meat or fish but present in significant quantities in red and processed meat, which have recently been categorised as “probable carcinogens” by the International Agency for Research on Cancer.⁶²

An RBC comprises approximately 1.2 billion haem moieties from haemoglobin.⁶³ With around 200 billion RBCs becoming senescent daily, haemoglobin oxidation may release deleterious levels of free haem into the vascular and, as a result, the human body has devised an elaborate haem disposal mechanism: The carrier proteins haptoglobin and hemopexin complex with haemoglobin and free haem respectively, which are mopped up by erythrophagocytic macrophages, within which haem is metabolised by haem oxygenases to form biliverdin, labile ferrous iron and carbon monoxide (**Figure 1.3**).⁶⁴⁻⁶⁶ Biliverdin is converted to bilirubin which is transported to the liver to form bile acid, whereas the reactive ferrous iron is either made innocuous in the macrophage by the iron-storage protein ferritin, or it may be ushered out of the cell by the iron exporter ferroportin.⁶⁷⁻⁶⁹ However, the haem disposal machinery may be overwhelmed by acute haemolysis caused by diseases such as malaria. Heightened haem catabolism rates may drain NADPH stores, consequently rendering the host defenceless against ROS and perpetuating the toxic effects of free haem.⁷⁰

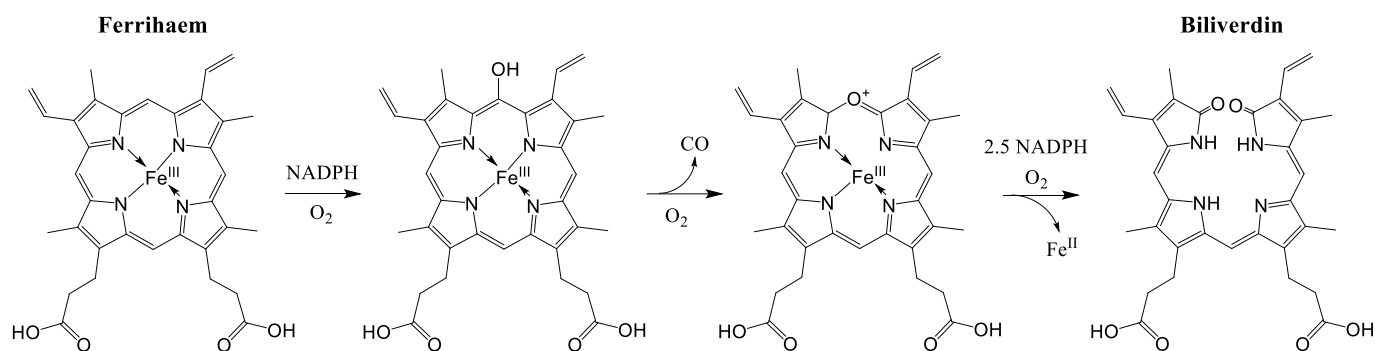


Figure 1.3. Mechanistic scheme for haem degradation. The term “ferrihaem” is employed when iron is in its oxidised Fe(III) form. Redrawn from Ortiz de Montellano, P. R., *Curr. Opin. Chem. Biol.*, **2000**, 4, 222 with permission from Elsevier.

1.5. Haem detoxification by *P. falciparum*

The human RBC offers a suitable niche for *P. falciparum* by shielding the invading pathogen from the host immune system. As mentioned, profuse haemoglobin ingestion and catabolism occurs in the acidic DV of the parasite. The DV is regulated to a mean acidic pH of 5.0 by the combined action of two proton pumps; namely, a V-type H⁺-ATPase and a H⁺-pyrophosphatase.⁷¹⁻⁷² Haemoglobin degradation is mediated by the concerted action of two crucial acid protease families in the DV; namely, plasmepsins and falcipains. The process constitutes an ingenious survival mechanism evolved by the parasite to accommodate pathogen growth in the cell and to exploit the resulting by-products as a nutrient source.⁷³ Since the pathogen lacks the machinery for *de novo* synthesis of amino acids, haemoglobin degradation is critical as it supplies amino acids to the parasite for protein synthesis;⁷⁴ it has also been reported to be fundamental for preserving the osmotic stability of the RBC.⁷⁵ Also, haemoglobin proteolysis is crucial for RBC parasite survival as studies have reported worsened parasite development rates when haemoglobin degradation is restricted by protease inhibitors.⁷⁶

Haemoglobin catabolism entails the release of four haem molecules per protein molecule that is degraded. Once liberated, haem is promptly and irreversibly oxidised to ferrihaem, probably *via* spontaneous autoxidation by oxygen, with the concurrent formation of hydrogen peroxide.⁷⁷ The ferric iron is typically coordinated axially to a water molecule or a hydroxide ion, in which case the entity is termed haematin; however, considering the dominance of haematin in the DV, the terms “ferrihaem” and “haematin” have been used interchangeably in subsequent chapters. Unlike the export of amino acids during haemoglobin proteolysis, free

haem accumulates to concentrations of approximately 0.4 M within the 4 fL DV,⁷⁸ potentially leading to immense oxidative stress which could harm the pathogen and impair the RBC through the generation of ROS. However, the pathogen is not equipped for enzymatic disposal of free haem as do mammalian cells.⁷⁹ Furthermore, enzymatic haem degradation would also have parasiticidal effects due to the production of significant levels of toxic free iron. Therefore, in a detoxification mechanism, free haem is effectively sequestered within the acidic DV into the insoluble and relatively innocuous micro-crystal termed haemozoin, commonly known as malaria pigment.

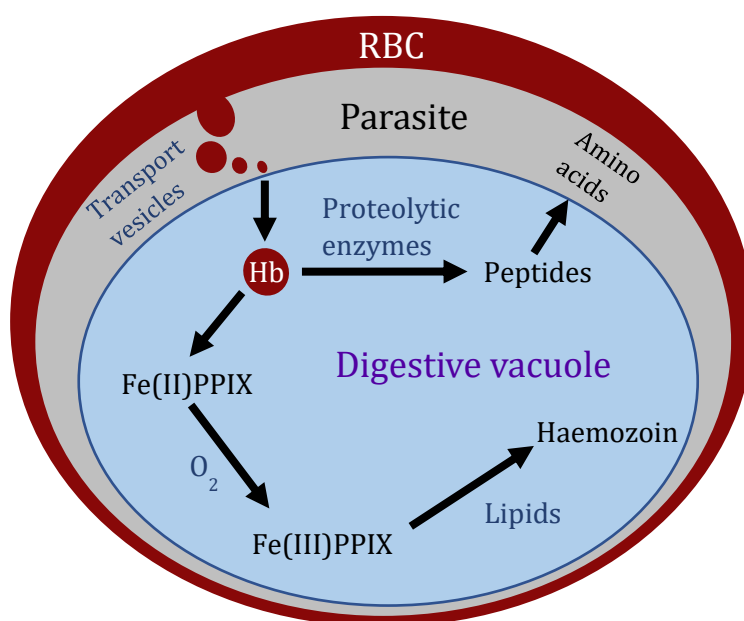


Figure 1.4. A schematic diagram of haemoglobin proteolysis and haemozoin formation in *P. falciparum*.

1.6. Haemozoin and β -haematin formation

A landmark paper towards understanding the chemical structure of haemozoin was published in 1991 by Slater et al.⁸⁰ in which they provided substantial evidence — based on infrared spectroscopy, X-ray diffraction and solubility studies — that haemozoin was structurally indistinguishable from its synthetic counterpart β -haematin. These findings were underpinned by Bohle et al.⁸¹ who reported identical powder X-ray diffraction patterns for samples of lyophilised trophozoites and synthetic β -haematin. Slater et al.⁸⁰ also proffered that haemozoin was a polymer incorporating haem molecules linked through an iron-carboxylate

bond between the propionate group of one haem unit and the central ferric iron of another, which prevailed as the dominant opinion for most of the next decade. However, this hypothesis was invalidated by Pagola et al.⁸² who employed high-resolution powder X-ray diffraction to solve the crystal structure of haemozoin (**Figure 1.5A**). It was observed to be a cyclic dimer with reciprocal iron–carboxylate bonds between the ferric centre of one haem moiety and the propionate group of the next moiety comprising the dimer, with the dimers arranged through hydrogen bonding to yield a chain-like structure (**Figure 1.5B**). In retrospect, this finding is not surprising since haemozoin crystals were observed to have well-defined faces, a phenomenon that would not occur in a polymeric compound with different chain lengths.⁸³ Controversy existed on whether haemozoin formation is the principal form of haem disposal, with earlier studies suggesting only 20–30% of the free haem being sequestered as haemozoin and the remaining being transported out of the DV and degraded by glutathione in the pathogen cytosol.^{84–85} This hypothesis would imply the presence of vast quantities of an unidentified iron-storing protein, and was hence deemed unfeasible. In a later study, Egan et al.⁸⁶ employed three independent techniques — a colorimetric ferrozine-based assay, Mössbauer spectroscopy and electron spectroscopic imaging (ESI) — to conclusively illustrate the detoxification of at least 95% of the released free haem through haemozoin formation.

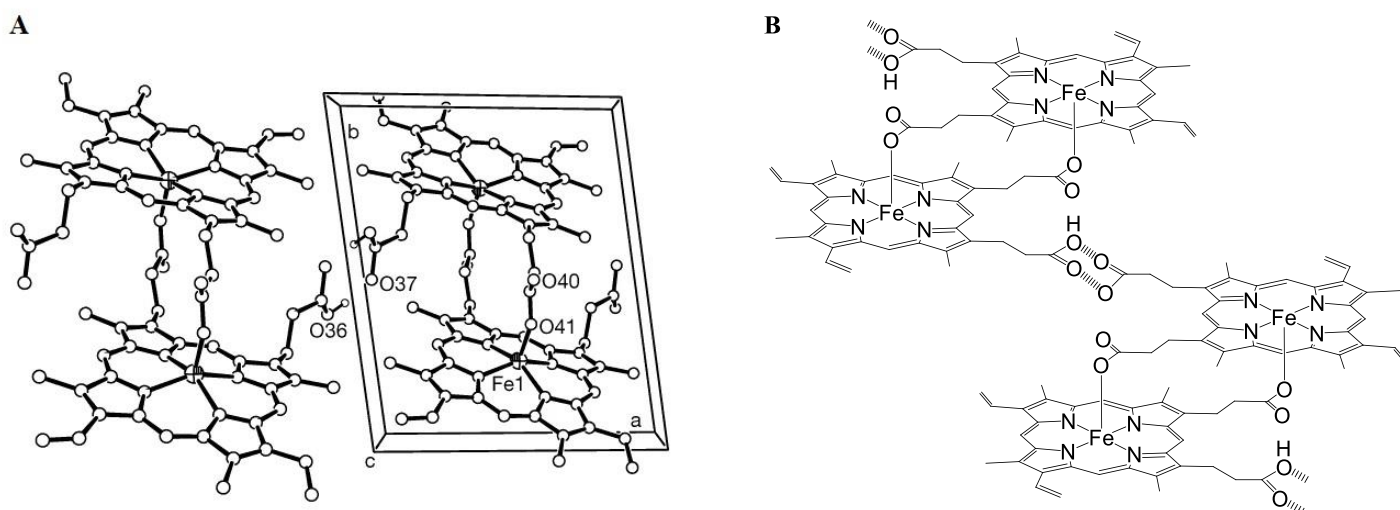


Figure 1.5. (A) The crystal structure of haemozoin, showing two unit cells. Reproduced from Pagola et al., *Nature*, **404**, 308 with permission from Springer Nature. (B) The structure of haemozoin, showing two hydrogen-bonded cyclic haem dimer units.

However, despite irrefutable knowledge about its composition, the mechanism of *in vivo* haemozoin formation is yet to be elucidated. Also, a majority of previous research which

shaped the current general consensus on haemozoin formation has profoundly relied on *in vitro* β -haematin crystallisation assays.⁸⁷ In an early study by Slater and Cerami,⁸⁸ the authors observed the promotion of β -haematin formation by parasite extracts which led them to hypothesise the direct relevance of a so-called haem polymerase in haemozoin formation. However, no such enzyme was ever isolated. Soon after, based on observations of β -haematin crystal growth being boosted by preformed β -haematin and haemozoin from parasite extracts, Dorn et al.⁸⁹ propounded haemozoin formation to be an autocatalytic process. This was quickly disputed by Bendrat et al.⁹⁰ who demonstrated the catalysis of β -haematin formation by lipids extracted from trophozoites, suggesting that the observed catalysis of haemozoin formation by Dorn et al. was due to the preformed β -haematin and haemozoin being contaminated with lipids. While subsequent research by Dorn et al.⁹¹ corroborated these findings, the authors emphasised newfound knowledge that purified β -haematin also promoted β -haematin formation without the involvement of lipids.

In a more recent study, Egan et al.⁹² reported the spontaneous self-assembly of haematin to form β -haematin near alcohol/water and lipid/water interfaces, under acidic and physiologically relevant conditions. In the same study, molecular dynamics (MD) simulations starting with the ferrihaem π - π dimer revealed the formation of a precursor to haemozoin in vacuum (**Figure 1.6A**, structure ii). The precursor species, however, was noted not to survive in aqueous media, presumably due to competitive hydrogen bonding with water molecules which prevents interaction between the propionate side chains and Fe(III) centres (**Figure 1.6A**, structure iii). Therefore, the authors postulated that the precursor species abounds at lipid/water interfaces. Furthermore, subcellular fractionation of haemozoin isolated from *P. falciparum* clone 3D7 by Pisciotta et al.⁹³ revealed the enveloping of the haemozoin crystals by neutral lipid nanospheres inside the DV; as a result, the authors proposed a shift in the intracellular haemozoin formation microenvironment from polar aqueous to lipid-mediated non-polar, with the nanosphere hypothesised to shield free ferrihaem from water for more efficient crystallisation to form haemozoin. Besides, adding to this complex picture, a number of parasite proteins — such as the histidine-rich proteins (HRPs) II and the haem detoxification protein (HDP) — have been observed to mediate β -haematin formation *in vitro*.⁹⁴ Consequently, the lipid- and parasite protein-mediated theories of haemozoin formation have enjoyed recent primacy. The inhibition of haemozoin formation is a well-validated target in antimalarial research and has been discussed thoroughly in section **1.8.2**.

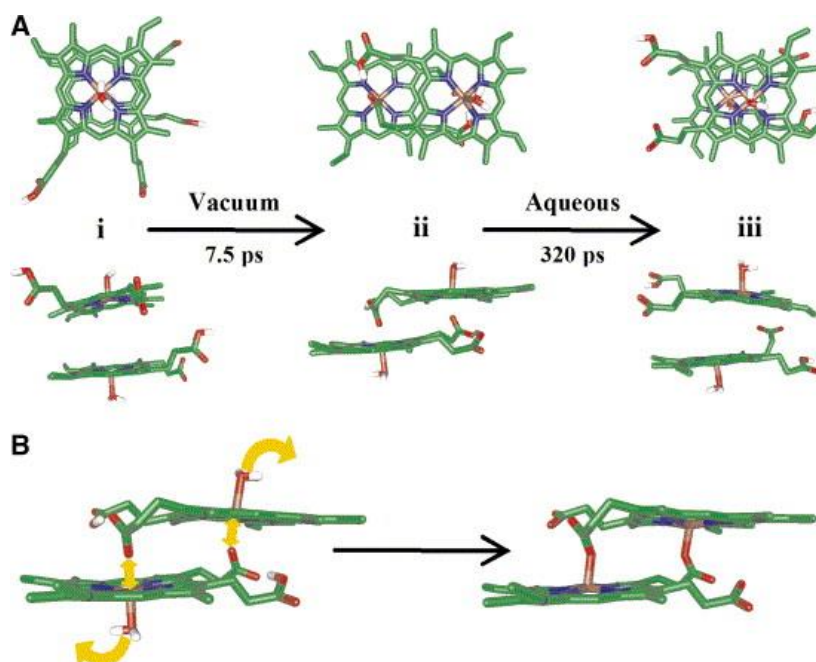
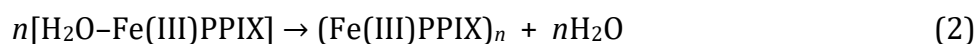
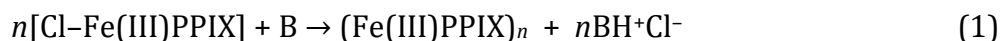


Figure 1.6. MD simulations of the π - π dimeric form (i) of ferrihaem. (A) In vacuum, the β -haematin/haemozoin precursor (ii) is rapidly formed which breaks down in aqueous medium (iii). (B) Postulated mechanism of β -haematin/haemozoin formation from the precursor, involving reciprocal coordination of the propionic acid groups to the Fe(III) centres and concurrent release of the axial water ligands. Reproduced from Egan et al., *FEBS Lett.*, **2006**, 580, 5108 with permission from Wiley.

The acidification of haemin (Cl-Fe(III)PPIX) or haematin is a long-established — albeit contentious — method of synthesising β -haematin with the mechanism of formation of the latter still unclear. Fundamentally, *in vitro* β -haematin synthesis can be divided into two alternative strategies:



where $(\text{Fe(III)PPIX})_n$ is β -haematin and B represents the organic base 2,6-lutidine.

Process (1), originally developed by Bohle et al.⁹⁵, involves the overnight reaction of haemin with 2,6-lutidine at room temperature in anhydrous conditions achieved by dry methanol. The purpose of the noncoordinating base 2,6-lutidine is to abstract HCl by deprotonating one of the propionic acid groups on a Fe(III)PPIX molecule, with the resulting propionate ligand coordinating to the Fe(III) centre of another Fe(III)PPIX molecule, yielding the μ -propionato dimeric symmetrical repeating unit of haematin.

In contrast, process (2) depicts the aqueous acid-catalysed method for β -haematin synthesis. Although the use of concentrated acetate solutions to promote the crystallisation of Fe(III)PPIX into β -haematin has been long recognised in the literature,⁹⁶ the method was rigorously described in 1991 by Slater et al.⁸⁰ involving the overnight incubation at 70°C of haematin in 4.5 M acetic acid at a pH of approximately 2, followed by water washes and the removal of unreacted haematin by dissolving in bicarbonate buffer (pH 9.1) and finished by final water washes and drying. Attempting to optimise this methodology at milder conditions, Egan et al. recorded the spontaneous formation of β -haematin — characterised using infrared spectroscopy — from haematin in 0.1 – 4.5 M acetate, between 6°C and 65°C and over a pH range of 4.2 – 5.0.⁹⁷ At low pH values, the authors observed greater solubilisation of haematin with increasing acetate concentrations.

However, these findings were contested by Pandey and Tekwani⁹⁸ who reported no spontaneous β -haematin formation at the above conditions, based on the high solubility of haematin but insolubility of β -haematin in alkaline bicarbonate buffer and 2.5% sodium dodecyl sulfate (SDS). Instead, they suggested that Egan et al. mistook the observance of a haem-acetate adduct for β -haematin. Consequently, in-depth research comprising IR spectroscopy, elemental analysis and powder X-ray diffraction studies by Egan et al. conclusively reaffirmed the formation of β -haematin in 4.5 M acetic acid.⁹⁹ Further studies by Egan et al.⁸⁷ led to the conclusion that β -haematin formation occurs during incubation in acetate solution and not during prolonged drying of the products as claimed by Blauer and Akkawi.¹⁰⁰

The process also appeared to entail an intricate chain of events conforming to Ostwald's rules of stages¹⁰¹ with acetate probably acting as a phase transfer catalyst by forming a negatively charged and more soluble haem-acetate complex: the rapid precipitation of amorphous haematin at low pH followed by its acetate-aided dissolution, before its slower redeposition as the less soluble β -haematin. This proposed description of β -haematin formation closely parallels several mineralisation reactions, leading to the authors suggesting that haemozoin formation may indeed be a biomineralisation process. Furthermore, data from Egan et al.⁸⁷ led them to infer that only monoprotonated Fe(III)PPIX crystallises to form β -haematin with slow reactions at low (<2) and higher (>5) pH values: In the former case, acetate concentrations would be low and propionate coordination to the Fe(III) centre would be reduced due to dominance of the diprotonated species, while, in the latter case, the unprotonated species would abound and β -haematin formation would be inhibited since one propionic acid group is essential to form the hydrogen bond chain constituting the β -haematin dimer, as summarised in **Figure 1.7**. Therefore, the authors suggested that β -haematin exists stably only within a specific acidic pH range.

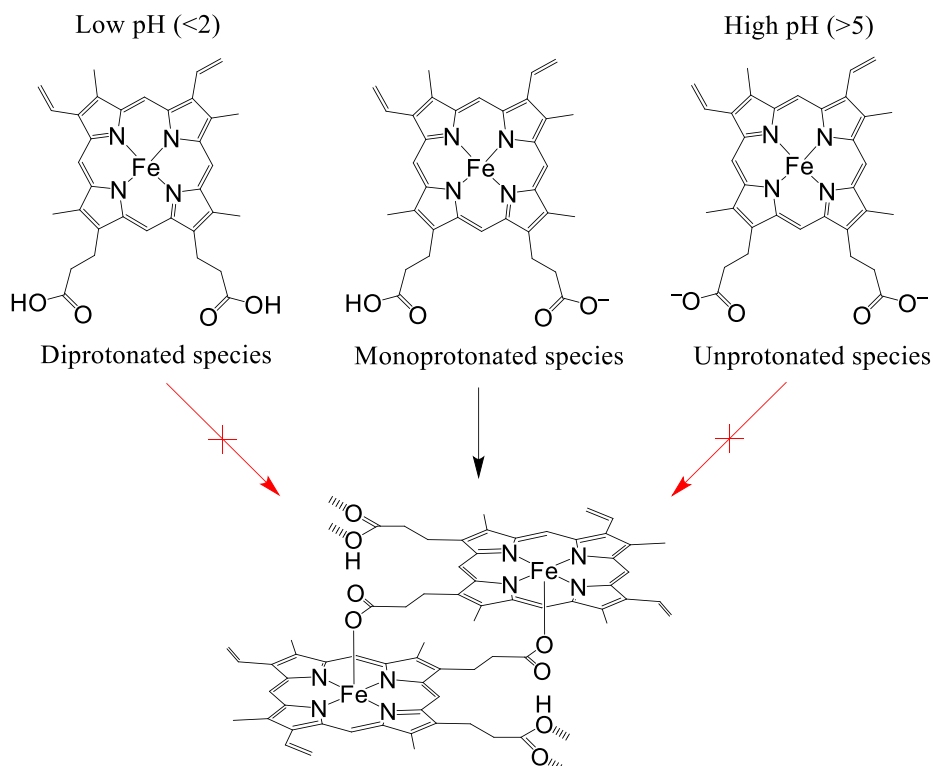


Figure 1.7. Schematic diagram to represent the hypotheses from Egan et al.⁸⁷ highlighting the formation of β -haematin from monoprotated haematin only, and not from the diprotonated or unprotonated species.

When first describing the acid-catalysed methodology, Slater et al.⁸⁰ reported benzoic, propionic, or succinic acids as possible alternatives for acetic acid. Indeed, a more recent study by Egan and Tshivhase¹⁰² at similar conditions as the aforementioned study by Egan et al.⁹⁷ revealed benzoic acid and its substituted derivatives to be stronger promoters of β -haematin formation than acetic acid, presumably through the aromatic acids π -stacking over haematin molecules, overcoming Fe(III)PPIX interactions and aiding rearrangement to β -haematin.

Therefore, under abiotic conditions, most reported methods of β -haematin synthesis involve the use of organic acids. However, Blauer and Akkawi¹⁰³ reported high yields of β -haematin formation from haemin in 43 wt% ethanol–water mixtures at 37°C in the presence of sulfuric acid, as measured by infrared spectroscopy; substituting hydrochloric or perchloric acid for sulfuric acid also resulted in moderate yields. An ensuing comprehensive study by Huy et al.¹⁰⁴ ascribed the ability of alcohols as inducers of β -haematin formation to hydrophobic alcohol–haem interactions which trigger the dissociation of aggregated haem at low pH, thus increasing haem solubility before its re-precipitation as β -haematin. However, the latter study was

conducted in acetate buffer; thus, it remained unclear whether a mineral acid would induce β -haematin formation in the absence of an organic acid or an alcohol.

Interestingly, some studies have hypothesised the formation of a short-lived intermediate solid species during the formation of β -haematin from haematin; the researchers observed the putative intermediate to be similar to β -haematin in terms of bond connectivity but amorphous morphologically, and was named 'B-haematin'.^{103, 105-106} As explanation, Buller et al.¹⁰⁷ proffered the possible formation of two β -haematin-related dimers, one centrosymmetric and the other not. The former was proposed to occur exclusively in haemozoin/ β -haematin, and the latter was suggested to be responsible for hindering the growth of crystals and forming the putative amorphous intermediate (**Figure 1.8**).

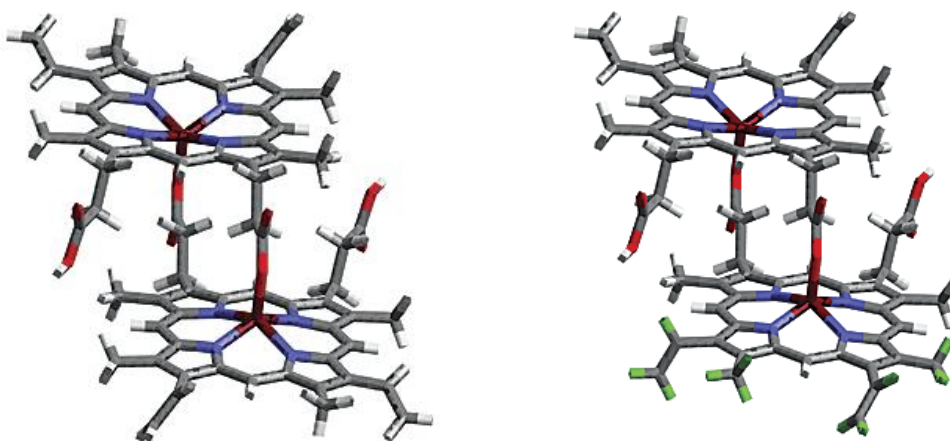


Figure 1.8. On the left, the centrosymmetric cyclic dimer occurring in haemozoin/ β -haematin. On the right, the non-centrosymmetric dimer probably responsible for the observed amorphous intermediate (B-haematin). The 'inversed' hydrogen atoms are marked in green. Reproduced from Buller et al., *Cryst. Growth Des.*, **2002**, 2, 559 with permission from ACS Publications.

Subsequently, Wood et al.¹⁰⁸ recorded the UV-vis spectra at 293 K for the transformation to β -haematin through the addition of HCl to an alkaline haemin solution: The authors attributed the observed red-shift in the Soret and Q bands (**Figure 1.9**) to excitonic coupling from Fe(III)PPIX aggregation through dimerisation; the collapsing of the Soret band — which is resolved into two bands in haemin, S and S' — into a single band, its broadening and lower intensity with decreasing pH, and the appearance of the charge-transfer (CT) band at 649 nm were all ascribed to aggregation and β -haematin formation. The spectroscopic evidence presented by the authors for Fe(III)PPIX aggregation is undeniable; however, although β -

The presence of large aggregates of aqueous Fe(III)PPIX was the dominant view in early literature. However, a lack of consensus prevailed due to starkly different findings across research studies. In the 1930s, diffusion measurements suggested the presence of units with molecular weights ranging from 50 000 g·mol⁻¹ to 145 000 g·mol⁻¹, as well as extreme values of approximately 4 000 000 g·mol⁻¹ in aqueous solution.¹¹¹⁻¹¹² Subsequently, potentiometric and spectrophotometric titration experiments in 1947 by Shack and Clark¹¹³ suggested the presence of large micelles of variable sizes comprising dimeric units of ferrihaem in alkaline solution. However, inconsistencies and highly irreproducible data meant that the authors could not provide any conclusive evidence on the exact structure of Fe(III)PPIX in aqueous solution, and they attributed the large variations to presumably weak intermolecular forces between units within the aggregates. Thereafter, ultracentrifugation studies at high salt concentrations (up to a maximum of 2.0 M, pH 11 – 12) by Blauer and Zvilichovski¹¹⁴ concluded in observed aggregates of 45 – 50 ferrihaem units.

While the assertion of large Fe(III)PPIX aggregates in aqueous solution was maintained over the decades, a seminal research paper published in 1970 by Brown et al.¹¹⁵ disputed ferrihaem aggregation beyond dimers: Upon spectrophotometric investigation of the Soret band (~ 400 nm) for aqueous ferrihaem and deuterioferrihaem (Fe(III)DPIX) solutions, the authors observed key changes in the spectra — including decreasing extinction and broadening of the Soret band with increasing Fe(III)PPIX or Fe(III)DPIX concentration — which indicated a dimerisation process. Furthermore, the researchers deduced the loss of a single proton during the dimerisation process, assuming which an appropriate model was fitted to the experimental data, yielding an association constant (pK_a) value of 7.5 for the ferrihaem dimer.

In an earlier publication, the same workers characterised the solid green precipitate formed upon addition of sodium hydroxide (NaOH) to an aqueous haemin solution in dimethylsulfoxide (DMSO).¹¹⁶ A sharp band at 903 cm⁻¹ in the infrared spectra was attributed to a characteristic asymmetric stretching frequency of an oxo-bridged species (Fe–O–Fe), following which the authors proposed a model for the μ -oxo dimeric form of ferrihaem with both six-coordinate Fe(III) centres drawn out of the porphyrin plane by 0.5 Å towards the axial oxide ligand (**Figure 1.10A**). The suggested model adhered to a range of studies reporting on the crystal structures of μ -oxo dimeric porphyrins,¹¹⁷⁻¹¹⁹ including that of the μ -oxo Fe(III)PPIX dimethyl ester crystallised from a dichloromethane–heptane solution by Cheng et al.¹²⁰ (**Figure 1.10B**). Consequently, a proposed model incorporating their findings for the dimerisation process was posited by Brown et al.¹¹⁵, although they assumed the existence of six-coordinate Fe(III) centres (**Figure 1.10C**).

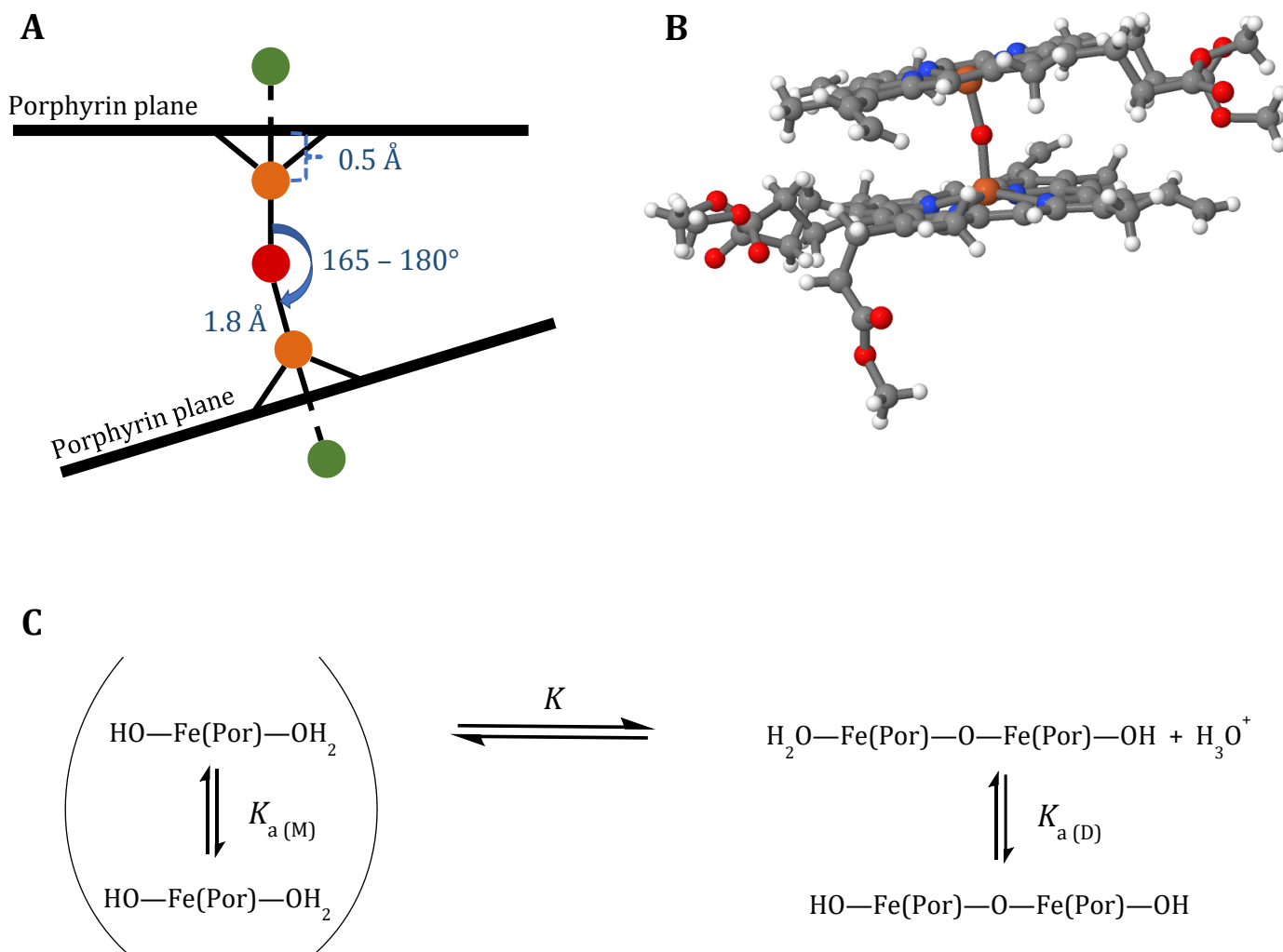


Figure 1.10. (A) Model for the μ -oxo dimeric form of ferrihaem as proposed by Brown et al., redrawn from reference 116. Red and orange-filled spheres represent the oxygen and iron atoms, respectively, and the green-filled spheres represent the water/hydroxide ligands. (B) Molecular representation of the crystal structure of the μ -oxo Fe(III)PPIX dimethyl ester reported by Cheng et al., reproduced with permission from reference 120. Red, orange, and blue-filled spheres represent the oxygen, iron, and nitrogen atoms, respectively. (C) A schematic representation of the ferrihaem dimerisation process as proposed by Brown and co-workers, redrawn from reference 115. Fe(Por) denotes the four-coordinate porphyrin ring incorporating the Fe(III) centre.

However, the dominance of the μ -oxo dimeric form of ferrihaem in aqueous solution was subsequently questioned by O’Keeffe et al.¹²¹ Mössbauer and electron paramagnetic resonance (EPR) spectroscopy performed by the authors did not yield any evidence for the presence of the μ -oxo linkage. Furthermore, based on their observed UV–vis spectra, the authors

emphasised the non-spontaneous formation of the μ -oxo dimeric species in aqueous solution, but demonstrated how it could rather be induced in aqueous alkaline media upon the addition of a weak organic base — such as 10% (v/v) pyridine or 2,6-lutidine — which probably acts as a solvating agent. In retrospect, based on the observations by O’Keeffe et al.¹²¹, the solvent conditions employed by Brown et al.¹¹⁶ (addition of NaOH to an aqueous haemin solution in DMSO) almost certainly favoured the inducement of μ -oxo dimer formation.

Interestingly, the work of O’Keeffe et al.¹²¹ was largely neglected and the misconception that the μ -oxo dimeric form of ferrihaem was dominant in aqueous solution perpetuated until recently, when De Villiers et al.¹²² convincingly demonstrated its existence as a π - π dimer instead: The NMR spectrum and magnetic susceptibility measurements by the Evans method of Fe(III)PPIX in aqueous solution displayed marked differences compared to those of aqueous Fe(III)PPIX with 10% (v/v) pyridine. Additionally, the authors observed a broad Soret band with a single Q band in the UV-vis spectrum of aqueous Fe(III)PPIX; in contrast, that of aqueous Fe(III)PPIX containing 10% (v/v) pyridine displayed a narrow Soret band with two distinct Q bands (**Figure 1.11A**). Consequently, a computational method comprising molecular dynamics/simulated annealing (MD/SA) was utilised by De Villiers et al.¹²² to propose a model for the structure of the π - π dimer (**Figure 1.11B**), comprising noncovalent interactions between the unligated faces of the porphyrins arranged in coplanar fashion; the porphyrins exhibit a lateral shift, and the axial water/hydroxide ligands are directed outwards. Besides, the authors also provided spectroscopic evidence to emphasise the adsorption of ferrihaem solutions onto glass surfaces and, more problematically, onto plastic surfaces. This phenomenon was described as possibly the principal cause for the apparent instability of aqueous ferrihaem solutions, especially at low pH values.¹²²

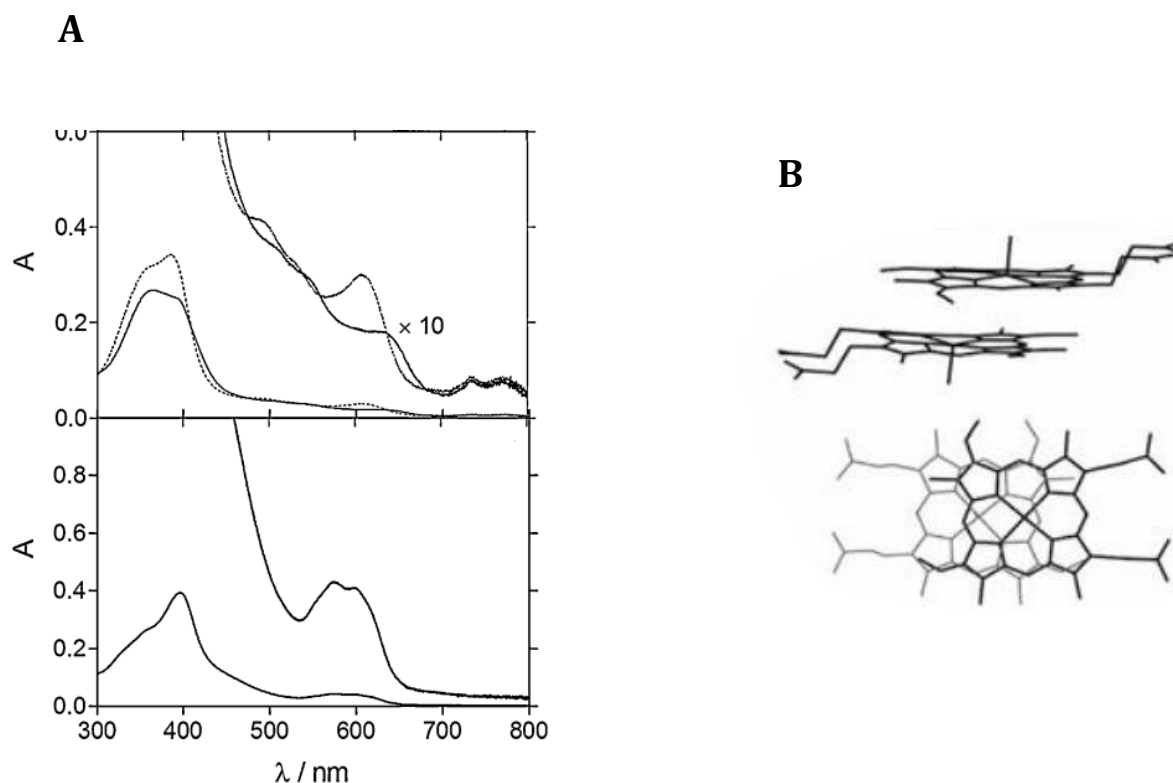


Figure 1.11. (A) In the top compartment, the UV-vis spectra of an aqueous 7 μM ferrihaem solution at pH 6.029 (solid line) and pH 9.669 (dotted line), displaying a broad Soret band and a single *Q* band, indicative of the π - π dimer. In the bottom compartment, the UV-vis spectra of a 7 μM ferrihaem solution in aqueous 10% (v/v) pyridine, displaying a narrow Soret band with two distinct *Q* bands which are markers of the μ -oxo dimer. (B) The computationally-predicted lowest-energy conformation of the π - π dimer. Adapted from De Villiers et al., *J. Biol. Inorg. Chem.*, **2007**, 12, 101-17 with permission from Springer Nature.

Subsequently, the complexity of ferrihaem behaviour in solution was accentuated by Asher et al.¹²³ through a comprehensive study employing UV-vis and NMR spectroscopy with magnetic and diffusion measurements. The authors highlighted the identity, polarity and pH of solvents as key mediators of ferrihaem speciation: A series of aprotic solvents (e.g., 5.64 M DMSO, 2,6-lutidine, acetone) at pH 10 were observed to promote μ -oxo dimer formation while protic ones (water, ethanol) of the same concentrations yielded the π - π dimer with varying levels of the monomer, subject to solvent polarity; high salt concentrations (4.25 M NaCl) were suggested to stimulate the formation of sizeable π -stacked aggregates of μ -oxo dimeric units. Furthermore, through their findings, the authors proposed a prediction model for the speciation of ferrihaem in DMSO as a function of concentration, pH, and temperature (**Figure 1.12**).

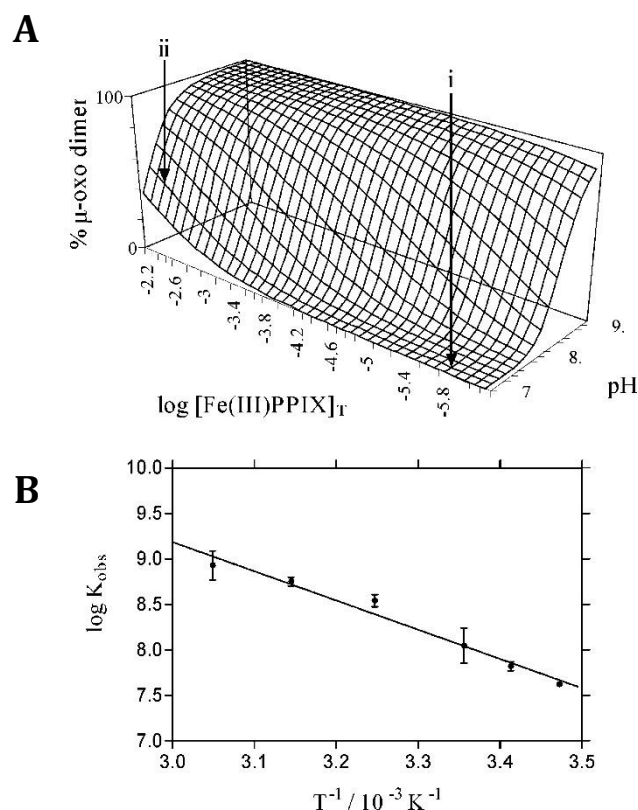


Figure 1.12. (A) A three-dimensional plot showing the percentage of ferrihaem present in the μ -oxo dimeric form in 5.64 M aqueous DMSO solution as a function of concentration (plotted as a log value) and pH. Point (i) represents a 2 μM ferrihaem solution at a pH of 7.5, and point (ii) represents a 4 mM solution at the same pH. (B) The variation of the Fe(III)PPIX dimerisation constant on the inverse of temperature, at pH 10. Adapted from Asher et al., *Inorg. Chem.*, **2009**, 48, 7994-8003 with permission from ACS Publications.

1.8. Quinoline antimalarial drugs

1.8.1. Brief history of discovery and the advent of drug resistance

It is noteworthy that since the 1600s, long before elucidation of the aetiology and pathogenesis of malaria, quinoline compounds have been used to treat the disease. They remain fundamental to contemporary antimalarial therapy. Initial forms of treatment comprised administration of concoctions made using the bark of the *Cinchona* tree, in which the quinoline-based alkaloid quinine occurs naturally.¹²⁴ Malaria treatment continued in this manner until 1820 when quinine and cinchonine were successfully isolated from the bark;

their respective stereoisomers, quinidine and cinchonidine, were present in lesser quantities.¹²⁵ Despite supply limitations due to wholesale production only possible through intensive extraction from the *Cinchona* bark, quinine prevailed as the cornerstone of malaria treatment until the 1930s, boasting cure rates of higher than 98%. However, with a remarkable revolution in synthetic organic chemistry, the onus switched towards developing new synthetic antimalarials to alleviate the burden on natural resources. Success was achieved when methylene blue — a synthetic dye used as a parasite staining agent — was observed to treat malaria, although not as effectively as quinine.¹²⁶ Subsequently, using methylene blue as prototype, a compound with a similar mechanism of action to quinine was developed and named “resochin”, but was disregarded as it was thought to have toxic side effects. However, when the supply of quinine declined during World War II, interest was revived in resochin, unequivocally resulting in substantial therapeutic effect against malaria. The compound was renamed chloroquine (CQ), and soon rose to prominence as the first-line of prophylactic malaria treatment. Several factors contributed towards its consideration as the gold standard in antimalarial therapy: its high effectiveness in treating malaria while being relatively inexpensive, very few noxious side effects, safe intake by pregnant woman, and ease of synthesis.¹²⁶⁻¹²⁸ However, the overdependence on chloroquine, and thus its massive global use, probably led to the emergence of CQ-resistant (CQR) parasite strains in the 1960s.

The inhibition of haemozoin biocrystallisation is now generally accepted as the target of quinoline drugs such as chloroquine (CQ), quinidine (QD), quinine (QN), amodiaquine (AQ), mefloquine (MF) and piperazine (PPQ) (Section 1.8.2). These drugs are known to accumulate in the parasitic DV through the pH trapping effect. In their neutral form, they are able to cross the lipid membrane to move from the parasitic cytosol (pH 7.4) into the acidic DV (pH ~5.0) where they become diprotic — or polyprotic, in the case of PPQ. As a result, they become impermeable to the enclosing lipid membrane and are unable to diffuse back to the cytosol, thus accumulating in the DV. Importantly, CQ resistance does not arise from any change in the haemozoin inhibition process, and has instead been attributed to decreased CQ accumulation in the DV of CQR parasites.¹²⁹⁻¹³⁰ Initially, the reduced CQ accumulation in the DV was ascribed to a presumably higher vacuolar pH in the CQR parasites. This hypothesis, however, was dismissed when the vacuolar pH values of CQR and CQ-sensitive (CQS) parasites were conclusively shown to be similar.⁷²

Subsequently, a panoply of mechanisms of CQ resistance have been put forward, but the chief causative factor is generally accepted to be mutations in the 424-amino acid protein *P. falciparum* CQ-resistance transporter (PfCRT) located in the DV membrane.¹³¹⁻¹³² Another key transporter protein involved in CQ resistance is *P. falciparum* multi-drug resistance transporter (PfMDR1), although the latter is believed to function as a resistance modulator in

conjunction with the PfCRT mutants rather than directly inciting resistance.¹³³ Moreover, the mechanism of CQ resistance by PfCRT was clarified by Martin et al.¹³⁴ who noted CQ transport across the *Xenopus laevis* oocyte membrane by mutant PfCRT, but not by the wild-type form of the protein. Furthermore, when the known resistance-reverser, verapamil, was added to the mutant PfCRT-containing system, the transfer of CQ was inhibited, as was observed in a prior *in vivo* study¹³⁵ in which CQR parasites became CQS upon incubation with CQ and verapamil. Hence, Martin et al.¹³⁴ concluded that CQ resistance entails the efflux of CQ from the DV by the mutant PfCRT, effectively allowing the formation of haemozoin to proceed unimpeded. Although the key PfCRT mutation responsible for CQ resistance is K76T, other mutations may occur depending on the strain, which are believed to be ancillary mutations to offset the loss of function of PfCRT potentially caused by the K76T mutation.¹³⁶⁻¹³⁷

Despite extensive early CQ resistance, the quinoline class of compounds is far from becoming obsolete: A recent study has reported the re-emergence of CQS *P. falciparum* in sub-Saharan Africa, attributed to the decreased selective pressure on CQ when it was not the first-line antimalarial treatment.¹³⁸ Furthermore, no resistance has been observed in Honduras — the highest malaria burden-bearing Central American country — where CQ is currently administered as first-line treatment.^{19, 139} The current drug regimen recommended by the World Health Organisation constitutes the artemisinin-based combination treatments (ACTs) involving an artemisinin derivative and usually a quinoline-based antimalarial.¹⁹ However, with the emergence of partial resistance to artemisinin in Africa, there is cause for concern.¹⁹ Therefore, significant research efforts are still being invested in the development of analogues or redesigned versions of quinoline antimalarial drugs in attempts to circumvent the scourge of resistance. For example, compounds from a CQ prototype with shortened side chains,¹⁴⁰⁻¹⁴² as well as side chains incorporating a ferrocene unit¹⁴³ have demonstrated encouraging signs of quelling resistance. Also, since drug resistance does not impact the viability of the haem detoxification process as drug target, an enhanced understanding of the mechanisms of action of quinoline-based drugs is therefore likely to be of great worth to the development of novel and efficacious antimalarials.

1.8.2. Mechanisms of haemozoin inhibition by quinoline antimalarial drugs

As described above, the haemozoin detoxification pathway is now conventionally accepted as the target of various quinoline antimalarials (**Figure 1.13**). A selection of proposed alternate mode of actions from the literature include the following: the inhibition of nucleic acid synthesis in mammalian cells¹⁴⁴⁻¹⁴⁵; the inhibition of haemoglobin-degrading proteases¹⁴⁶⁻¹⁴⁷; intercalation into parasite DNA¹⁴⁸⁻¹⁴⁹; and the inhibition of the peroxidase degradation of ferrihaem,¹⁵⁰ with several of these suggested pathways now believed to be unlikely. The parasitic DV was proposed to be the site of drug action after the observation of accumulated CQ therein, now known to proceed through pH trapping.¹⁵¹ Macomber et al.¹⁵² were the earliest proponents of ferrihaem being the CQ receptor in *P. berghei* in 1967, later corroborated in 1980 by Fitch and co-workers¹⁵³; the latter proposed Fe(III)PPIX to be the specific target of several quinoline compounds, and reported association constants for these compounds and Fe(III)PPIX.¹⁵³ Although these hypotheses were initially met with significant scepticism, the capacity of the quinoline antimalarials to inhibit β -haematin formation provided strong evidence for the proposed drug target: Slater and Cerami⁸⁸ reported the inhibition of parasite extract-induced β -haematin formation by CQ, QD and QN, hypothesising the inhibition of a putative haem polymerase enzyme by the quinoline drugs. Subsequently, the inhibition of abiotic (*in vitro*) β -haematin formation by quinoline-based drugs was reported by multiple research papers.^{97, 99, 154-156} Key to these was the development of efficient β -haematin inhibition assays which appropriately mimic physiological conditions. In a landmark paper by Egan et al.,⁹⁷ the first qualitative β -haematin inhibition assay was reported in acetate medium, utilising infrared spectroscopy to determine the presence of β -haematin through characteristic peaks. Later, a high-throughput and quantitative assay was developed by Kurosawa et al.¹⁵⁷, exploiting ¹³C-labelled haematin. However, despite the suggested robustness of the latter assay, it was not ideal owing to its costliness and prerequisite of special apparatus and skills.

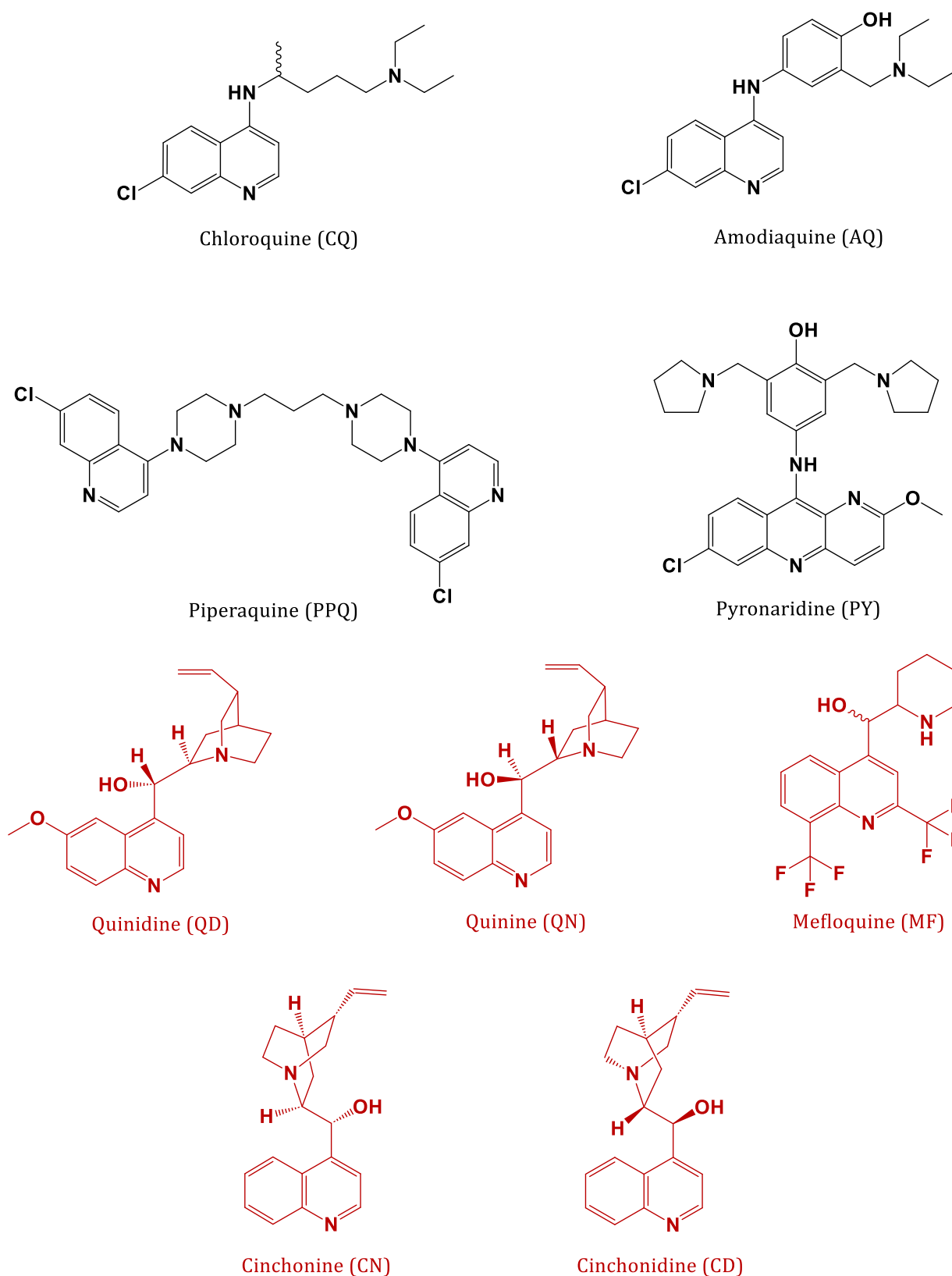


Figure 1.13. The molecular structures of a selection of compounds from the quinoline class of antimalarial drugs, with the 4-aminoquinolines coloured in black and the quinoline methanol compounds in red.

In 2005, the aforementioned acetate-based assay was refined by Ncokazi and Egan¹⁵⁸ by conversion into a pyridine ferrihemochrome assay. The fundament of this assay is the selective reaction of pyridine with free Fe(III)PPIX — be it monomeric, dimeric, or further aggregated free haem — to form a spectroscopically distinct, low-spin bis-pyridyl complex (**Figure 1.14**). This pyridyl complex does not form with β -haematin or haemozoin. Furthermore, upon titration with pyridine, the Fe(III)PPIX Soret band demonstrates a red shift from 389 nm to 404 nm. It also increases significantly in sharpness and intensity, indicative of Fe(III)PPIX monomerization which presumably explains the adherence of the observed absorbance to the Beer–Lambert law.¹⁵⁸ Based on kinetic considerations, a 60-minute incubation period of samples was deemed optimal by Ncokazi and Egan¹⁵⁸ to allow for efficient β -haematin inhibition. An earlier study by Chong and Sullivan¹⁵⁹ reported the assessment of the kinetics behind β -haematin inhibition by a diverse set of antimalarials in which initial inhibition was followed by the eventual formation of β -haematin over time: The authors proposed that the β -haematin inhibition process may either be reversible, or occurs *via* lowering of the rate of β -haematin formation instead of blocking it altogether, thus emphasising the direct impact of incubation period on such assays. Strikingly, as opposed to the findings of Chong and Sullivan,¹⁵⁹ Egan and Ncokazi¹⁶⁰ reported the 50% inhibitory concentration (IC₅₀) of the 4-aminoquinoline antimalarials (CQ and AQ) for β -haematin inhibition to increase substantially with incubation time, as compared to much slower increases in the case of the quinoline methanols QN and QD, suggesting a partial reversal in the hierarchy of inhibition capacity with increasing incubation time.

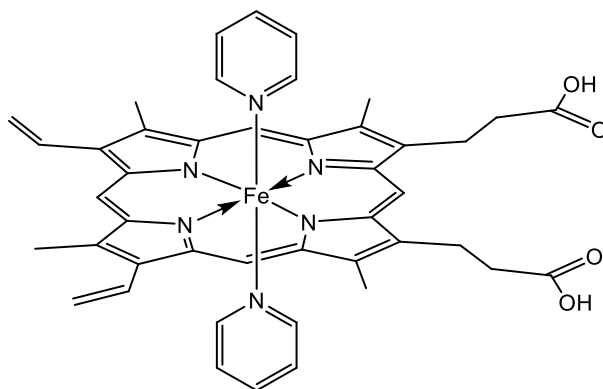


Figure 1.14. The low-spin bis-pyridyl complex formed between unreacted Fe(III)PPIX and pyridine.

Compared to the exhaustive literature on β -haematin inhibition by quinoline antimalarials, direct intracellular evidence of haemozoin inhibition has been scarce. Also, while 4-

aminoquinoline drugs such as CQ and AQ are broadly believed to act through the inhibition of haemozoin biocrystallisation, the mechanism of action of the quinoline methanol compounds (e.g., QD, QN and MF) is much disputed. Despite the extensively reported β -haematin inhibition activity of drugs from both classes, they were observed to trigger different morphological effects on *P. falciparum*: CQ has been reported to provoke an intraparasitic accumulation of haemoglobin transport vesicles and interfere with haemoglobin digestion, whereas QN and MF disrupt haemoglobin ingestion.¹⁶¹⁻¹⁶³ QN and QD have been shown to inhibit β -haematin formation, while their respective stereoisomers, 9-epiquinine^{88, 97} and 9-epiquinidine,¹⁶⁴ do not. This corresponds to the observation that the former two drugs are active against the parasite, whereas the latter two are not.¹⁶⁵ However, compared to CQ, the quinoline methanol compounds are significantly weaker bases since they have a single appreciably basic nitrogen atom.¹⁶⁶ They exist as monoprotonated species under physiological conditions, thus are less efficiently accumulated in the parasitic DV *via* pH trapping. This indicates that their effectiveness as haemozoin formation inhibitors should be notably worse than that of CQ — all the more so since they are weaker inhibitors of β -haematin formation than CQ.^{156, 167} These observations evidently blur the picture of the mode of action of the quinoline methanol antimalarials.

Nevertheless, a definitive paper by Combrinck et al.¹⁶⁸ allayed part of the lingering doubts: The authors employed whole-cell fractionation coupled with a pyridine-ferrihaemochrome assay for ferrihaem quantification, and observed a dose-dependent increase in toxic free ferrihaem and concurrent decrease in parasite survival in cultured *P. falciparum* upon CQ treatment; indeed, ferrihaem detoxification was shown to be the target of CQ, evidenced by the intersection of the two curves representing the free ferrihaem concentration and parasite survival, respectively, near the measured IC₅₀ of the drug (**Figures 1.15A** and **1.15C**). A concomitant decrease in haemozoin was also observed (**Figure 1.15B**). Additionally, the authors demonstrated an increase in free ferrihaem and a simultaneous decrease in haemozoin levels upon single dose administration of each of quinine, artesunate, amodiaquine, mefloquine and lumefantrine at 2.5 times their respective IC₅₀ values. By contrast, no significant changes in ferrihaem or haemozoin levels were caused by the drugs pyrimethamine and sulfadoxine, whose known mode of action against *P. falciparum* is the inhibition of folate biosynthesis. Therefore, this study delivers strong evidence in favour of the haemozoin inhibition pathway as the mode of action for 4-aminoquinolines such as CQ *and* quinoline methanols such as QN and QD. In a separate study, fluorescence microscopic imaging of *P. falciparum* cultured parasites revealed the co-localisation of QN with haemozoin in the DV, providing further evidence for the drug acting *via* the haemozoin inhibition pathway.¹⁶⁹⁻¹⁷⁰

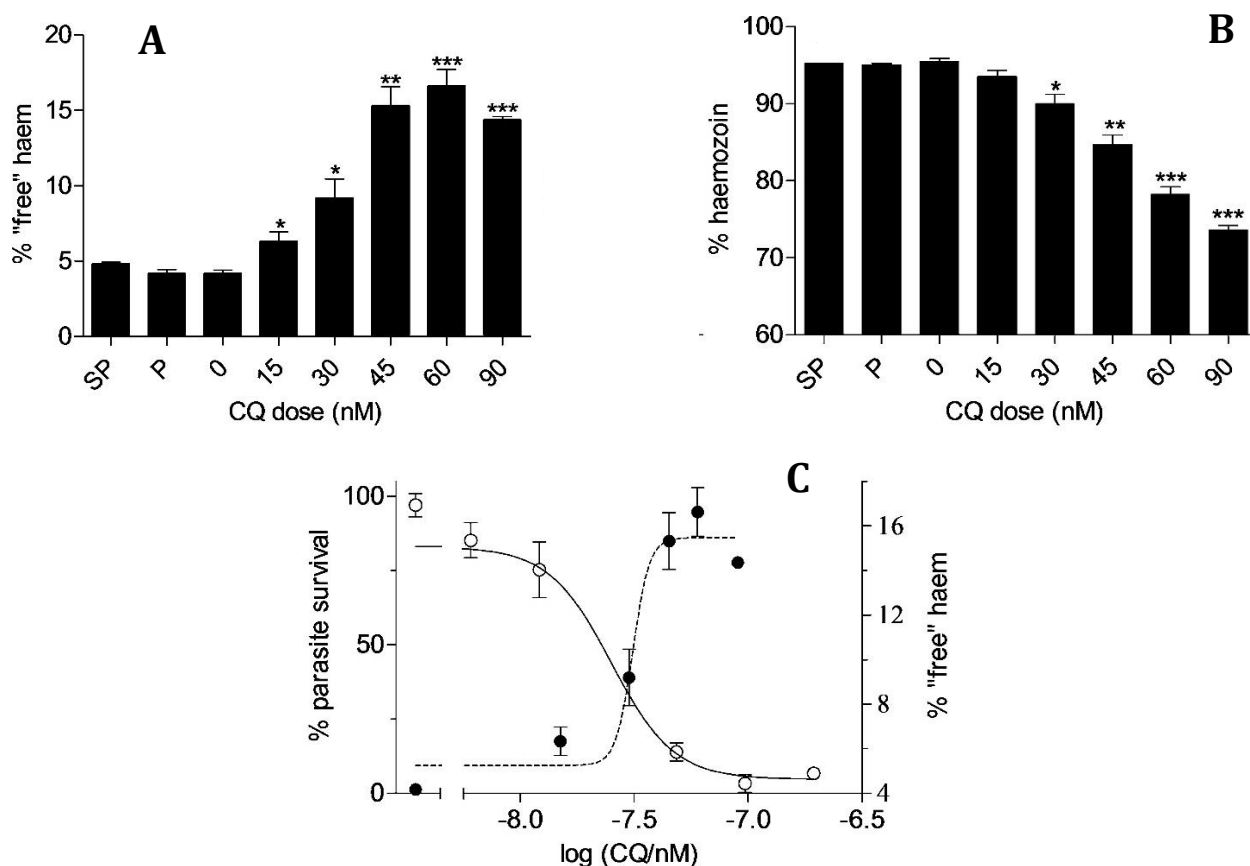


Figure 1.15. Fraction of total haem in *P. falciparum* present as (A) free ferrihaem and (B) haemozoin. Statistical significance relative to the control is denoted by the asterisks: * for $P < 0.05$; ** for $P < 0.01$; *** for $P < 0.001$, $n = 3$. "P" and "SP" stand for pyrimethamine and sulfadoxine/pyrimethamine, respectively. (C) Parasite survival (open circles, left axis) and the concentration of free ferrihaem (filled circles, right axis) as a function of CQ dose. Figures adapted from Combrinck et al., *ACS Chem. Biol.*, **2013**, *8*, 134 with permission from ACS Publications.

Subsequently, a paper by Vanaerschot et al.¹⁷¹ was published in which the latter performed similar experiments to those conducted by Combrinck et al.¹⁶⁸ Analysis of the haem fractionation profiles of the quinoline methanol antimalarials mefloquine and lumefantrine revealed a decrease in haemozoin levels, however, with no significant increase in free ferrihaem levels. The authors hypothesised that in addition to interacting with ferrihaem to some extent, quinoline methanol antimalarials may act outside of the DV, suggesting their possible interference with haemoglobin ingestion and degradation processes by the parasite.¹⁷²⁻¹⁷³ It has been commented that in such cases, the observed decrease in haemozoin

levels is probably an incidental effect, highlighting the markers for “true” haemozoin inhibitors to be *both* a decrease in haemozoin and an increase in free ferrihaem.¹⁷⁴ However, pending conclusive research, the haemozoin inhibition pathway in the activity of quinoline methanol compounds is still of paramount importance.

Having established the relevance of the haemozoin inhibition pathway to quinoline antimalarials, another question emerges: How exactly do these compounds interfere with haemozoin formation? Three theories currently prevail, with the first stating that quinoline antimalarials interact directly with the haemozoin crystal by adsorbing onto its fastest-growing face — which is necessarily the smallest face — and inhibiting crystal growth by “capping” it. This hypothesis was first made by Pagola et al.,¹⁷⁵ followed by the structure of the adsorbed CQ–haemozoin crystal proposed by Leiserowitz and co-workers.¹⁰⁷ Through computational methods, the latter highlighted four key stabilising interactions responsible for strong docking between CQ and haemozoin. More recently, Gildenhuis et al.¹⁷⁶ published a thorough analysis of the kinetics behind the adsorption of CQ and QD onto β -haematin. By way of a pyridine–haemochrome assay, a decrease in the rate of β -haematin formation was observed at low drug concentrations, which the authors ascribed to the inhibition of β -haematin crystal formation *via* drug adsorption. However, higher drug concentrations were conducive to reduced β -hematin yields which was attributed to a possibly irreversible precipitation of Fe(III)PPIX–drug complexes.¹⁷⁶ Subsequently, the inhibition of β -haematin crystal growth by adsorbed CQ was visualised by Olafson et al. through atomic force microscopy.¹⁷⁷

The second hypothesis is the formation of complexes between drugs and free ferrihaem in solution to deter the incorporation of ferrihaem into haemozoin. (Section 1.8.3). It has been proposed, albeit without direct evidence, that the complexes have increased solubilities compared to ferrihaem itself, thus suppressing its biomineralisation into haemozoin.¹⁷⁸ The third possibility is, in essence, an amalgamation of the first two hypotheses, first proposed Sullivan et al. who postulated the “capping” of haemozoin crystal growth by the quinoline–ferrihaem complex.¹⁷⁹

Regardless of the unresolved mechanism of haemozoin inhibition by quinoline antimalarials, the question of how exactly does this process kill *P. falciparum* is controversial. Several schools of thought are prevalent, the majority of which ultimately come down to an increase in toxic levels of free ferrihaem or quinoline–ferrihaem complex: (i) The lysis of malaria parasites by ferrihaem¹⁸⁰ and the quinoline–ferrihaem complex,¹⁸¹ leading to the damage of erythrocytic membranes and thus causing haemolysis; (ii) The pro-oxidant activity of free ferrihaem — exacerbated by formation of the complex — leading to the generation of reactive oxygen

species, in turn causing the peroxidation of lipids¹⁸²; (iii) The inhibition of key parasite enzymes by free ferrihaem¹⁸³; and (iv) Degradation of the DV by quinoline antimalarials *via* a colloid-osmotic mechanism.¹⁸⁴

1.8.3. Investigations of the quinoline–ferrihaem complexes

Complex formation between CQ and ferrihaem in aqueous solution was first reported by Cohen et al.¹⁸⁵ in 1964. The analysis of the absorption spectra upon addition of CQ revealed marked changes in the Soret band shape with insignificant change in its intensity. In the longer wavelength range, however, a concurrent increase in the absorbance values was noted. These observations present a useful artefact for complexation analysis.

However, upon titration of ferrihaem with CQ, QN or other related antimalarials in mixed aqueous solutions (DMSO/water), the Soret band shows an appreciable hypochromic effect (i.e., a decrease in absorbance), as is also observed, albeit to a lesser extent, in organic solvents.^{154, 186} On the other hand, this hypochromic effect appears to be concealed in exclusively aqueous medium due to ferrihaem dimerisation under these conditions, itself effecting a large hypochromic effect.¹¹⁵ Additionally, in aqueous, non-aqueous and mixed solutions, notable spectroscopic changes are observed in the visible region of the spectra such as the apparent disappearance of the *Q* band and a bathochromic shift (i.e., a shift to longer wavelength) in the charge-transfer bands; a pronounced hyperchromism (i.e., a rise in absorbance) is also observed at about 600 nm.^{154, 186-187} Indeed, these changes present an effective marker to detect and monitor ferrihaem complexation with CQ, QN or other related antimalarials.

As mentioned in section 1.6, Fe(III)PPIX has a strong tendency to aggregate in aqueous media which complicates the determination of association constants between ferrihaem and antimalarial drugs in such media, resulting in disagreement over reported data among several publishings.¹¹³⁻¹¹⁵ In particular, titration calorimetric studies often led to extraordinary CQ:ferrihaem binding stoichiometries (e.g., a ratio of 1:9 reported by Vipagunta et al.¹⁸⁸), which was attributed to ferrihaem aggregation. Based on studies with other porphyrins, it seemed as if the determination of association constants in aqueous medium would only be feasible with the porphyrin in its monomeric state.¹⁸⁹⁻¹⁹⁰ Consequently, aqueous DMSO (40% v/v) has proved to be a convenient medium to probe into drug–ferrihaem interactions since ferrihaem has been observed to be strictly monomeric under these conditions.¹⁹¹⁻¹⁹² Also, a major proportion of the medium is water (approximately 86% by mole fraction), making it a

suitable analogue of pure aqueous medium. The strong quenching of the Soret band from titration of ferrihaem with quinoline antimalarials under these conditions facilitates the determination of association constants. For CQ and QD, the ferrihaem association constants computed in aqueous DMSO (40% v/v) assuming a 1:1 binding model were reported as 5.52 ± 0.03^{154} and 5.02 ± 0.03^{99} respectively (reported as log K values).

Thermodynamic studies have emphasised the importance of solvent properties on the quinoline–ferrihaem strength of association. Stronger interactions were noted in pure organic media compared to equally polar mixed aqueous/organic ones, probably due to competitive hydrogen bonding of the water with the ferrihaem or quinoline molecules.¹⁸⁶ Furthermore, association constants increase markedly with increasing solvent polarity, underlining the central role of the hydrophobic effect in complex formation.¹⁸⁶ However, the association strengths in aqueous DMSO (40% v/v) are largely unaltered by varying salt concentrations, indicative of the trifling role of electrostatic interactions in the strength of complexation under these conditions.¹⁸⁶

Despite structural congruencies, 4-aminoquinolines and quinoline methanols differ strikingly in their modes of complexation with ferrihaem. Spectrophotometric titration data of QN and ferrihaem in aqueous DMSO (40% v/v) performed by Egan et al.¹⁵⁴ adhered to a 1:1 binding model. Furthermore, *via* NMR spectroscopy and magnetic susceptibility measurements, Casabianca et al. demonstrated that QN and CQ promote the formation of the monomeric and μ -oxo dimeric forms of ferrihaem, respectively.¹⁹³

The drug–ferrihaem complexes of the quinoline methanol antimalarials halofantrine (HF), QN and QD have been crystallised.¹⁹⁴⁻¹⁹⁵ Crucially, each crystal structure revealed three key interactions between the drug and ferrihaem: coordination of the drug alkoxide (i.e., the deprotonated hydroxyl group) to the Fe(III) centre of the ferrihaem moiety; a π – π stacking interaction between the porphyrin ring system of ferrihaem and the aromatic moiety of the drug; and formation of an intramolecular salt bridge between the drug and propionate groups on the same ferrihaem unit in the case of QN and QD. In the case of HF, besides the aforementioned interactions, intermolecular hydrogen bonding between adjacent propionate groups from neighbouring complexes are also proposed.¹⁹⁴⁻¹⁹⁵ Moreover, an intriguing observation was made based on molecular mechanics modelling of the above drug–ferrihaem complexes: The antimalarial alkaloids quinine and quinidine promptly adopt favourable conformations for an intramolecular salt bridge to form between the drug and its coordinated ferrihaem unit. However, their respective antimalarially inactive stereoisomers, 9-epiquinine and 9-epiquinidine, have to adopt high energy — thus unstable — conformations to sustain such salt bridge formation. As a result, the authors hypothesised the ease of formation of such

salt bridges between the quinoline methanol drugs and ferrihaem to be crucial for complex formation, and thus important for deterring the incorporation of ferrihaem into haemozoin.¹⁹⁴

In a subsequent study, Gildenhuis et al.¹⁹⁶ performed a mass spectrometric analysis of the QN-ferrihaem and QD-ferrihaem complexes prepared in acetonitrile. They successfully identified the alkoxide coordination between the drug and ferrihaem, and confirmed the binding stoichiometry of 1:1 in the complexes. Indeed, the evidence presented for the binding stoichiometries, crystal structures and key interactions within the drug-ferrihaem complexes for the quinoline alcohol antimalarials is undeniable. However, crystal formation was induced in mixed or organic solvent systems; hence it is contested as to whether the same structures arise in aqueous solution. Besides, the proposed crystal structures may not resemble their respective aqueous complex structures due to possibilities of unexpected solvent-dependent interactions.

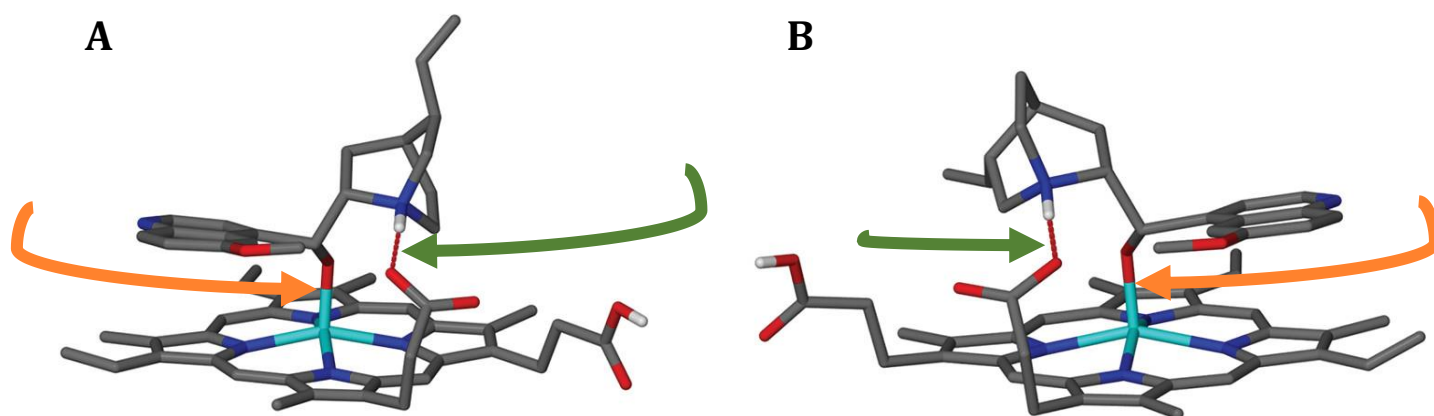


Figure 1.16. The crystal structures of (A) the QN-ferrihaem complex and (B) the QD-ferrihaem complex. Solvent molecules and spectator hydrogen atoms omitted for clarity. Atom colours: O (red), Fe (cyan), N (blue), C (grey) and H (white). For legibility, the orange arrows highlight the alkoxide coordination and the green arrows show the intramolecular salt bridges (dashed red lines). Adapted from de Villiers et al., *ACS Chem. Biol.*, **2012**, 7, 667 with permission from ACS Publications.

Meanwhile, the crystal structure of a 4-aminoquinoline-ferrihaem complex has not yet been elucidated. Solid-state NMR studies on precipitated CQ-ferrihaem by de Dios et al.¹⁹⁷ indicated a 1:1 complex in which CQ, through its quinolinium nitrogen, coordinates to the Fe(III) centre on ferrihaem. The authors proposed an existing equilibrium between the monomeric and the μ -oxo dimeric forms of the CQ-ferrihaem complex. These findings were disputed by Adams et al.,¹⁹⁸ who were proponents of CQ:ferrihaem binding stoichiometry of 1:2 involving the μ -oxo

dimeric form ferrihaem, based on solid-state Mössbauer data. However, from infrared and Raman spectroscopic data of the solid complex obtained by Asghari-Khiavi et al.,¹⁹⁹ the latter rejected the idea of the μ -oxo dimeric complex due to the absence of the characteristic Fe–O–Fe absorption. Indeed, such conflicting reports on solid-state studies of 4-aminoquinoline–ferrihaem complexes present a conundrum.

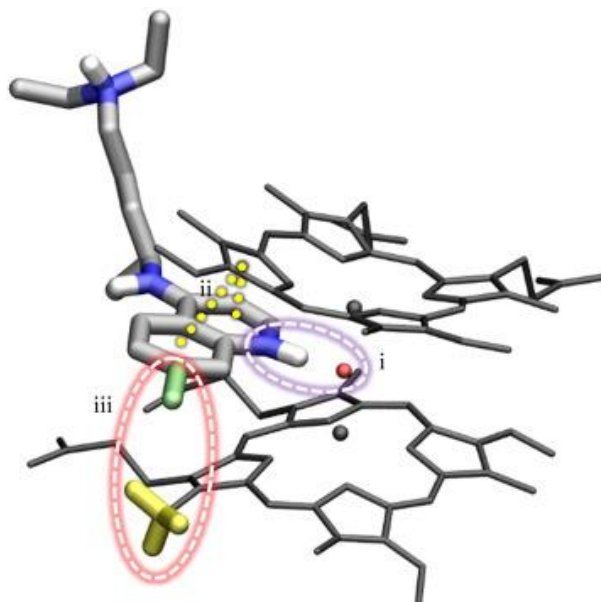


Figure 1.17. Representation of the docked model of the CQ–ferrihaem μ -oxo dimeric complex, highlighting the key predicted interactions: (i) a hydrogen bond between the quinolinium nitrogen of CQ and the bridged oxide of the ferrihaem dimer; (ii) π – π stacking interactions between the quinoline and porphyrin ring systems; and (iii) the 7-chloro substituent on CQ and the methyl hydrogens from ferrihaem in close proximity. Reproduced from Kuter et al., *J. Inorg. Biochem.*, **2016**, 154, 124 with permission from Elsevier.

However, by way of UV–vis spectroscopy, magnetic circular dichroism (MCD) and magnetic susceptibility measurements, Kuter et al.²⁰⁰ conclusively demonstrated the induced formation of the μ -oxo dimeric form of ferrihaem by CQ in aqueous solution. A Job plot indicated a CQ:ferrihaem binding stoichiometry of 1:2 and diffusion measurements suggested a dimeric aggregation state (CQ:ferrihaem = 2:4) with formula $(\text{CQ}-\mu\text{-}[\text{Fe}(\text{III})\text{PPIX}]_2\text{O})_2$. Strong CQ–ferrihaem complexation was observed with a reported association constant of 13.3 ± 0.2 at pH 7.4 (reported a $\log K$ value).²⁰⁰ In a subsequent study,²⁰¹ two proposed models of the CQ–ferrihaem complex in aqueous solution were generated by molecular dynamics simulations: The first comprised CQ π -stacked onto an unligated face of the ferrihaem μ -oxo dimer, while the second involved CQ positioned between the two ferrihaem units making up the dimer. Both

models were fitted to the observed extended X-ray absorption fine structure (EXAFS) spectrum of the frozen solution of the μ -oxo dimeric CQ–ferrihaem complex. The solvated docked model proved to be more consistent with experimental data, indicating the greater likelihood of it representing the true structure in aqueous solution (**Figure 1.17**).²⁰¹

1.9. Research rationale

In a report by Kapishnikov et al. from 2019, the distinct X-ray fluorescence signal of bromine was exploited to determine the intracellular mechanism of action of bromoquine — a bromine analogue of CQ — in rapidly frozen *P. falciparum*-infected erythrocytes.²⁰² Through non-destructive X-ray microscopy, overwhelming overlap between the bromine distributions of the drug and the iron concentrations of haemozoin was observed, from which the authors concluded the “capping” of the haemozoin crystals by bromoquine to inhibit haemozoin formation. In addition, the authors observed high bromoquine concentrations close to the membranes of the DV: They hypothesised the formation of a bromoquine–ferrihaem complex which eventually punctures the DV membrane and spills into the parasitic cytosol, possibly thus killing the parasite.²⁰²

Eventually, the first direct evidence of the intracellular formation of an inhibitor–ferrihaem complex was provided by Openshaw et al.²⁰³ Initially investigating the activity of a diverse set of haemozoin inhibitors including quinolines, the authors surprisingly observed higher levels of ferrihaem with compounds displaying lower antiparasmodial activity compared with more active ones. Employing the inoculum effect, concentrations of intracellular drug accumulation were determined and were observed to vary in linear fashion with exchangeable haem levels, supporting the formation of a drug–ferrihaem complex in the *P. falciparum* CQ-sensitive strain (NF54).²⁰³ Thus, exploiting the low natural abundance of bromine in cells, electron energy loss spectroscopic imaging of a bromo-substituted benzimidazole drug was conducted in the parasite, revealing strong colocalisation between the drug and iron with the Fe signal attributed to both ferrihaem and haemozoin.²⁰³

Subsequently, the authors utilised confocal Raman microscopy to demonstrate direct evidence of drug–ferrihaem complex formation: Principal component analysis exhibited the observed Raman peak positions to be distinct from those of oxy- and deoxyhaemoglobin, free ferrihaem and the drug, but identical to those from the drug–ferrihaem mixture.²⁰³ Interestingly, increasing ferrihaem and inhibitor concentrations were accompanied by lower rates of killing, indicating that slow-acting compounds have more time to accumulate drug–ferrihaem

complexes. Furthermore, not all tested drugs were shown to exhibit pro-oxidant activity, suggesting that each drug–ferrihaem complex has a unique level and mode of parasite killing, either directly, by damaging key enzymes or transporters in the DV, or indirectly, by releasing toxic levels of free ferrihaem in the cytoplasm and effecting impairment through the production of reactive oxygen species.

Consequently, Openshaw et al.²⁰³ proposed a model to incorporate their findings, illustrated in **Figure 1.18**: Following accumulation of the drug in the DV *via* pH trapping, the compound inhibits the formation of haemozoin (most probably by adsorption onto the haemozoin crystal surface, thus “capping” the crystal), which leads to increased levels of free ferrihaem. Precipitation of ferrihaem is likely to occur due to the reported low solubility of haematin of $0.17\ \mu\text{M}$ ²⁰⁴ at the mean DV pH of 5.0.⁷¹⁻⁷² The remaining aqueous ferrihaem forms a drug–ferrihaem complex, itself likely to be highly insoluble.

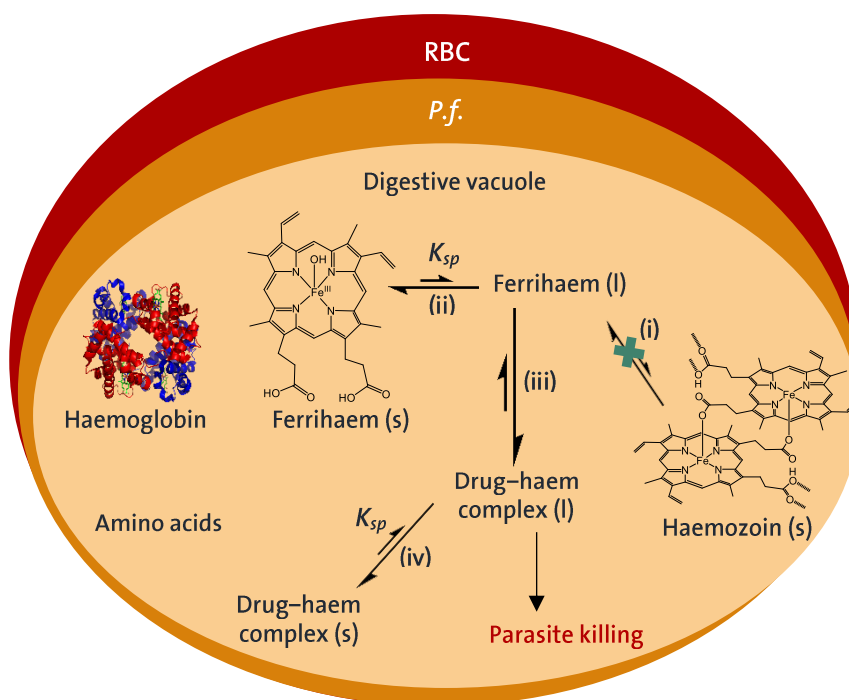


Figure 1.18. Schematic diagram to represent the key factors comprising the proposed model: (i) The inhibition of haemozoin crystal growth by the drug; (ii) precipitation of ferrihaem, followed by (iii) the formation of a drug–ferrihaem complex which leads to parasite killing; and (iv) precipitation of the drug–ferrihaem complex.

Indeed, the drug–ferrihaem complex is probably a key player towards parasite death. Important factors governing the concentrations of the drug–ferrihaem complexes are likely to be the solubility product constant (K_{sp}) of ferrihaem and those of the complexes. The

aforementioned solubility of haematin of 0.174 μM at pH 5.0 was reported by Morrison and Williams based on spectrophotometric titration experiments in 1941,²⁰⁴ with no further attempts of verification reported. With access to more advanced spectroscopic techniques, a verification of the data findings by Morrison and Williams is needed. Moreover, data on the K_{sp} of drug–ferrihaem complexes are largely absent in the literature, and therefore, experimental determination of the solubilities of the complexes is a priority toward modelling the inhibition of haemozoin formation and formation of drug-ferrihaem complexes.

1.10. Aims and Objectives

The aims of this dissertation were twofold:

- i. To determine the solubility of ferrihaem in an exclusively aqueous medium at the physiologically-relevant conditions of the *P. falciparum* digestive vacuole, i.e., at a pH of 5.0 and a temperature of 37°C.
- ii. To determine the solubilities of drug–ferrihaem complexes in exclusively aqueous medium under the same conditions.

To achieve these aims, the following overarching objectives were pursued:

- i. To fully optimise the spectrophotometric base titration methodology employed by Morrison and Williams.²⁰⁴
- ii. To determine the solubility product constant (K_{sp}) of ferrihaem by utilising the optimised UV–vis spectrophotometric titration methodology.
- iii. To compute thermodynamic parameters for ferrihaem solubility from the spectrophotometric titration data.
- iv. To employ a spectrophotometric acid titration methodology in order to determine the solubility of ferrihaem at a pH 5.0 and a temperature of 37°C.
- v. To utilise a spectrophotometric acid titration methodology in order to determine the solubilities of the drug–ferrihaem complexes for the two quinoline antimalarials, chloroquine and quinidine, at a pH of 5.0 and a temperature of 37°C.

CHAPTER 2

Experimental Methodologies

2.1. General methods

Materials used in this research and their commercial sources are listed in **Table 2.1** below. Compounds were of analytical research grade or higher and were used without any further purification, except as otherwise indicated. Unless stated otherwise, double-distilled deionised water (Milli-Q water) for sample preparation and cuvette rinsing was procured from a Millipore Direct-Q3 water purification system.

Table 2.1. Compounds or reagents and their commercial source.

COMPOUND/REAGENT	COMMERCIAL SOURCE
Solids	
Haematin porcine	Sigma-Aldrich
Sodium hydroxide pellets (NaOH)	Sigma-Aldrich
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
Chloroquine diphosphate (CQ)	Sigma-Aldrich
Quinidine sulfate dihydrate (QN)	Sigma-Aldrich
Liquids and solutions	
Nitric acid (HNO ₃) (55%)	Science World
Perchloric acid (HClO ₄) (60%)	Merck
Pyridine (99.0%)	Sigma-Aldrich
Acetone	Sigma-Aldrich

Adsorption of haem onto plastic surfaces — a phenomenon believed to arise from the hydrophobic character of the porphyrin macrocycle — may result in consequential changes in haem concentrations, even more so in dilute solutions.²⁰⁵ In addition, it may cause instability in such solutions with time.^{113, 206} Therefore, plasticware was avidly avoided unless absolutely required. Glass Hamilton microsyringes were employed to dispense haem solutions instead of Gilson or Eppendorf micropipettes. To prevent the accumulation of adsorbed haem, all glassware, including cuvettes and Hamilton microsyringes, were washed thoroughly as previously detailed: scrupulous rinsing with 0.2 M NaOH followed by rinsing with Milli-Q water, then the addition of 1 M HNO₃ and a final water wash.¹²² In case of prolonged use with haem solutions, glassware or cuvettes were soaked in 1 M NaOH for 1 hour and rinsed with copious amounts of water. They were then placed in 1 M HNO₃ and left to rest overnight, after which they were washed with water and carefully soaked in boiling water to remove any excess acid. Glassware were oven-dried overnight at 393 K and cuvettes were blow-dried. During sample or solution preparation, small volumes (100 – 5000 µL) were dispensed using Gilson or Eppendorf micropipettes.

A Sonorex RK 100 H ultrasonic bath and a BV1000 vortex mixer (Benchmark Scientific) were used for the sonication and vortexing of samples respectively, to ensure proper mixing and homogenisation of samples. Centrifugation was performed either on a Prism C2500-R refrigerated microcentrifuge, or an Eppendorf 5810 R centrifuge fitted with adapters to hold 2 mL Eppendorf tubes. Samples were incubated in a GFL shaking water bath equipped with a thermostat.

Baseline-corrected UV-vis spectra were recorded on a Shimadzu UV-1800 spectrophotometer fitted with a TCC-100 temperature control unit set at 25.0°C, and viewed on the UVProbe software. Quartz cuvettes (SUPRASIL®, Hellma) with path length 10 mm and 100 mm were used for analysis. All pH measurements were obtained using a glass micro-electrode on a Jenway Model 3510 pH meter and standard buffer solutions (XS Green Line standard solutions, obtained from Lasec) at pH 4.01 ± 0.01, 7.00 ± 0.01 and 9.21 ± 0.02 (25°C) were used to calibrate the electrode before use.

After preparation, all experimental samples were vortexed and sonicated to ensure adequate mixing before being incubated. Furthermore, prior to pH measurement and UV-vis analysis, all samples were centrifuged and the supernatants were carefully filtered using 0.22 µm syringe filters. UV-vis spectra were obtained in the range 300 – 750 nm.

GraphPad Prism (Version 9.5.0)⁷⁷ was used for analysing and graphing scientific data.

2.2. General preparation of solutions

NaOH solutions were made up by dissolving weighed out masses of NaOH pellets in Milli-Q water using 100 mL volumetric flasks. HClO₄ solutions were prepared through dilution of the 55% stock solution using 100 mL volumetric flasks.

A 1.00×10^{-3} M stock solution of Fe(III)PPIX was prepared by dissolving 0.03168 g hematin in 30.0 mL of 0.1 M NaOH, and the volume made up to 50.0 mL in a volumetric flask. When required, other Fe(III)PPIX solutions were prepared *via* dilution of the 1.00 mM stock solution with Milli-Q water. A Snijders Magnetic Stirrer 34521 was employed to ensure complete dissolution and care was taken to prevent direct exposure of Fe(III)PPIX solutions to light by wrapping the Schott bottles with aluminium foil. In addition, fresh Fe(III)PPIX solutions were prepared before each experiment and were used within 2 hours of preparation.

Solutions of CQ and QD were prepared by dissolving chloroquine diphosphate and quinidine sulfate dihydrate salts, respectively, in Milli-Q water, using 100 mL volumetric flasks. These were portioned into 10 mL aliquots in separate vials and refrigerated.

2.3. Preparation of 2 M HEPES buffer, pH 7.4

47.66 g of HEPES acid were dissolved in 80 mL Milli-Q water and the pH was adjusted to 7.4 by judicious addition of 5 M NaOH, following which the volume was made up to 100.0 mL in a volumetric flask.

2.4. Preparation of 50% pyridine solution

For quantification of haematin concentrations, 50.0 mL of a 50% pyridine solution were prepared as previously reported¹⁵⁸ as follows: 25.0 mL of 99.0% pyridine, 10.0 mL Milli-Q water, 10.0 mL acetone and 5.0 mL of 2 M HEPES buffer. Care was taken to handle pyridine solutions and samples containing pyridine in a fume hood.

2.5. Spectrophotometric titration of haematin with NaOH

As briefly mentioned in section 1.9 of Chapter 1, spectrophotometric titration experiments were performed by Morrison and Williams²⁰⁴ in which 0.350 g samples of crystalline haematin were placed into 30 mL screw-cap bottles followed by the addition of distilled water and 0.12 M NaOH to make up a total volume of 20 mL in each bottle, with the final concentration of added NaOH ranging from 0.6 mM to 90 mM. The bottles were sealed and placed in a rotating incubator set at 30°C for from 3 to 8 days after which they were centrifuged at high speed to pellet any undissolved haematin, and the pH of the supernatants were recorded. After appropriate dilution of the supernatant with 0.036 M NaOH, the absorbance at 545 nm was recorded to determine the concentration of dissolved haematin.

To evaluate the effects of pH and temperature on haematin solubility and to determine its solubility at a pH of 5.0, spectrophotometric titration experiments were performed using a similar methodology as the aforementioned researchers. The volumes and masses, however, were downscaled while keeping the solution concentrations constant: Samples of haematin as close to 0.035 g as possible were weighed out in 2 mL Eppendorf tubes, after which Milli-Q water and 0.12 M NaOH were added to make up a total volume of 2000 μ L in each tube (**Table 2.2**).

Prior to titration studies, experiments were carried out to determine the optimal incubation time to allow for complete sample equilibration at each of five temperatures — 10°C, 20°C, 25°C, 37°C and 50°C. To begin, samples 3 and 8 from the sample set in **Table 2.2** were prepared in triplicate and incubated at 37°C, and analysed after 1, 2, 4, 8 and 16 days of incubation as follows: Following centrifugation of each sample, the pH of the supernatant was recorded and UV-vis spectra were obtained after a hundred-fold dilution of the supernatant with 0.036 M NaOH, whereby 10 μ L of supernatant were carefully taken up without disturbing the pellet at the bottom of the tube and dispensed into a size 0 (2.5 mL) glass vial, to which 990 μ L of 0.036 M NaOH were added. The vial was shaken and the diluted solution was analysed using a 10 mm quartz cuvette, with the spectral *Q* band peak (598 nm) analysed to work out haematin solubility. The data were corrected for dilution. To determine the minimum number of days required for sample equilibration, pH measurements and dilution-corrected absorbance values obtained on successive days of analysis were assessed for significant difference using multiple unpaired *t*-tests with Welch correction and a threshold of 0.05 for *p*-value comparisons. The above experiment was repeated at each of the other four chosen temperatures to establish the optimal incubation time period at each temperature.

Furthermore, experiments were carried out to assess the necessity for purification of haematin purchased from Sigma-Aldrich *via* recrystallisation. 1.00 g of haematin from Sigma-Aldrich was dissolved in 45 mL of 0.12 M NaOH and diluted to 1700 mL with Milli-Q water. Precipitation of haematin was induced by the slow addition of 50 mL of 0.118 M HCl with constant magnetic stirring to achieve a solution pH of less than 4, ensuring liberation of all haematin. After standing for 12 hours, the clear supernatant was carefully pipetted off and the precipitate was copiously washed with water by centrifugation until the haematin appeared to be colloidally dispersed. Two rounds of washing with each of 65% ethanol and diethyl ether followed. The precipitate was then oven-dried at 40°C for 36 hours. Two batches of samples 1, 3, 5 and 8 from the sample set in **Table 2.2** — the first using haematin without any purification and the second using recrystallised haematin — were prepared in triplicate and incubated for 8 days at 37°C. Following centrifugation, the pH of the supernatants were recorded and UV-vis spectra were obtained as described above. Multiple unpaired *t*-tests with Welch correction and a threshold of 0.05 for *p*-value comparisons were used to test for significant differences between the data obtained using haematin without any purification and those obtained using recrystallised haematin.

Subsequently, spectrophotometric titration experiments were carried out in triplicate at each of the five temperatures, employing their respective determined optimal incubation time, and the samples were analysed as described above.

Table 2.2. Volumes of 0.12 M NaOH and Milli-Q water, and the concentrations of added NaOH in prepared samples for spectrophotometric titration.

Sample number	Vol. of 0.12 M NaOH (μL)	Vol. of MQ water (μL)	[NaOH] added (mM)
1	90	1910	5.4
2	125	1875	7.5
3	170	1830	10.2
4	210	1790	12.6
5	250	1750	15.0
6	295	1705	17.7
7	335	1665	20.1
8	375	1625	22.5
9	415	1585	24.9
10	1335	665	80.1
11	1500	500	90.0

The Beer–Lambert law highlights the direct proportionality between the absorption of light by a solution and the concentration of the solution.²⁰⁷ It is expressed by the equation $A = \epsilon cl$ where A is the absorbance, c represents the concentration of solution under analysis, l is the optical path length and ϵ is the molar absorption coefficient. At each temperature, the absorbances of samples 10 and 11 — where sufficient NaOH was added to ensure complete dissolution — were substituted in the equation to compute a mean ϵ value which was used to determine haematin solubility in the other samples.

2.6. Spectrophotometric titration of haematin with HClO₄

To 2 mL Eppendorf tubes, 1500 μL of 30 μM ferrihaem solution were added, followed by 130 μL of 0.025 M HClO₄. Increasing volumes of 0.0025 M HClO₄ (from 0 to 350 μL) were added to each sample and the volume was made up to 2 mL by the addition of Milli-Q water. The tubes were incubated at 37°C for 48 hours. The pH of the supernatants were measured and UV–vis spectra were obtained without any dilution, using 10 mm quartz cuvettes.

2.7. Pyridine–ferrihaemochrome assay to determine haematin solubility

For this assay, 1500 μL of a 30 μM ferrihaem stock solution were added to 2 mL Eppendorf tubes, to which 130 μL of 0.025 M HClO_4 were added. To each tube, increasing volumes of 0.0025 M HClO_4 and concurrently decreasing volumes of Milli-Q water were added to make up a total volume of 2000 μL (**Table 2.3**).

Prior to spectrophotometric titrations, experiments were performed to determine the optimal incubation time period to allow for sample equilibration. Triplicates of samples 2, 6 and 10 from **Table 2.3** were prepared in 2 mL Eppendorf tubes and incubated at 37°C, and their pH values were recorded after 1, 2, 4 and 6 days. Thus, to deduce the optimal incubation time period, pH measurements obtained on successive days of analysis were assessed for significant difference using multiple unpaired *t*-tests with Welch correction and a threshold of 0.05 for *p*-value comparisons.

Furthermore, solutions of known ferrihaem concentrations in the range of 1 – 15 μM were prepared (in duplicate) in 2 mL Eppendorf tubes by dilution of a 30 μM ferrihaem stock solution with Milli-Q water, and were incubated for 48 hours at 37°C. From each prepared solution, 500 μL of supernatant were carefully dispensed into a size 0 (2.5 mL) glass vial, to which 100 μL of 50% pyridine solution were added, implying 8.33% (v/v) of pyridine content per vial. The vials were vortexed and incubated at 37°C for 15 minutes to allow for equilibration, after which UV–vis spectra of the solutions were recorded using a 10 mm quartz cuvette, with the peak of the distinct Soret band (A_{405}) analysed to determine haematin solubility. The resulting data were used to make up a standard curve to enable the quantification of ferrihaem solubility in unknown samples.

Subsequently, spectrophotometric titration experiments were performed in quintuplicate with the tubes incubated at 37°C for the predetermined optimal number of days, following which the samples were centrifuged. The pH values of the supernatants were then recorded. Without disturbing the pellet, 500 μL of supernatant were carefully dispensed into a size 0 glass vial to which 100 μL of 50% pyridine solution were added, implying 8.33% (v/v) of pyridine content per vial. The vials were vortexed and incubated at 37°C for 15 minutes, and UV–vis spectra of the solutions were recorded using a 10 mm quartz cuvette. The Soret peak (A_{405}) were analysed in conjunction with the standard curve to determine the solubility of haematin.

Table 2.3. Volumes of 0.0025 M HClO₄ and Milli-Q water, and the concentrations of added HClO₄ in prepared samples for spectrophotometric titration.

Sample number	Vol. of 0.0025 M HClO ₄ (μL)	Vol. of MQ water (μL)	[HClO ₄] added (mM)
1	220	150	1.90
2	240	130	1.92
3	260	110	1.95
4	280	90	1.98
5	290	80	1.99
6	300	70	2.00
7	310	60	2.01
8	320	50	2.02
9	330	40	2.04
10	340	30	2.05
11	350	20	2.06

2.8. Second pyridine–ferrihaemochrome assay to determine haematin solubility

To obtain more reliable data points in the low pH region (close to pH 5.0), a second pyridine-based assay was performed employing a similar methodology for spectrophotometric titration as described in section 2.7, differing as follows: 800 μL of 56.25 μM ferrihaem solution were added to 2 mL Eppendorf tubes, followed by 150 μL of 0.0267 M HClO₄. To each tube, increasing volumes of 0.000125 M HClO₄ and concurrently decreasing volumes of Milli-Q water were added to make up a total volume of 2000 μL (**Table 2.4**). Spectrophotometric titration experiments were performed in triplicate.

Table 2.4. Volumes of 0.000125 M HClO₄ and Milli-Q water, and the concentrations of added HClO₄ in prepared samples of ferrihaem for spectrophotometric titration.

Sample number	Vol. of 0.000125 M HClO ₄ (μL)	Vol. of MQ water (μL)	[HClO ₄] added (mM)
1	0	1050	2.002
2	200	850	2.015
3	400	650	2.028
4	500	550	2.034
5	600	450	2.040
6	700	350	2.046
7	800	250	2.052
8	900	150	2.059
9	1000	50	2.065

2.9. Pyridine–ferrihaemochrome assay to determine the solubility of the CQ–ferrihaem complex

103.172 mg of chloroquine diphosphate salt were dissolved in Milli-Q water to make up a 100 mL CQ solution. To 2 mL Eppendorf tubes, 500 μL of 90 μM ferrihaem solution were added, to which 300 μL of the prepared CQ solution were dispensed followed by 40 μL of 0.0795 M HClO₄. Different volumes of 0.025 M HClO₄, 0.000125 M HClO₄ and Milli-Q water were added as specified in **Table 2.5**.

Prior to spectrophotometric titrations, experiments were performed to determine the optimal incubation time period to allow for sample equilibration. Samples 1, 5 and 11 from **Table 2.5** were prepared in 2 mL Eppendorf tubes and incubated at 37°C, and their pH values were recorded in triplicate after 1, 2, 4 and 6 days. Thus, to deduce the optimal incubation time period, pH measurements obtained on successive days of analysis were assessed for significant difference using multiple unpaired *t*-tests with Welch correction and a threshold of 0.05 for *p*-value comparisons.

Subsequently, spectrophotometric titration experiments were performed in quadruplicate with the samples incubated at 37°C for 48 hours, following which they were centrifuged. The pH values of the supernatants were then recorded. Without disturbing the pellet, 500 µL of supernatant were carefully dispensed into a size 0 glass vial to which 100 µL of 50% pyridine solution were added, implying 8.33% (v/v) of pyridine content per vial. The vials were vortexed and incubated at 37°C for 15 minutes to allow for equilibration, after which UV-vis spectra of the solutions were recorded using a 10 mm quartz cuvette; the Soret peak (A_{405}) was analysed in conjunction with the standard curve generated in section 2.7 to determine the solubility of the CQ-ferrihaem complex.

Table 2.5. Volumes of 0.025 M HClO_4 , 0.000125 M HClO_4 and Milli-Q water, and the concentrations of added HClO_4 in prepared samples of the CQ-ferrihaem complex for spectrophotometric titration.

Sample number	Vol. of 0.025 M HClO_4 (µL)	Vol. of 0.000125 M HClO_4 (µL)	Vol. of MQ water (µL)	[HClO_4] added (mM)
1	10	0	1150	1.715
2	15	0	1145	1.778
3	20	0	1140	1.840
4	25	0	1135	1.902
5	30	0	1130	1.965
6	30	100	1030	1.971
7	30	200	930	1.978
8	30	300	830	1.984
9	30	400	730	1.990
10	30	600	530	2.002
11	30	800	330	2.015
12	30	1000	130	2.028

An additional experiment was performed with the CQ–ferrihaem where sample 12 from **Table 2.5** was prepared in sextuplicate, however, with the volumes of the solutions to be added upscaled by a factor of twenty; that is, to 50 mL Falcon tubes, 10 mL of 90 μ M ferrihaem solution were added, to which 6.0 mL of the prepared CQ solution were dispensed followed by 0.8 mL of 0.0795 M HClO_4 . This was followed by the addition of 0.6 mL of 0.025 M HClO_4 , 20 mL of 0.000125 M HClO_4 and 2.6 mL of Milli-Q water, making up a total volume of 40 mL. The samples were incubated at 37°C for 48 hours following which they were centrifuged. Subsequently, the pH values of the supernatants were recorded. Without disturbing the pellet, 25 mL of supernatant were carefully dispensed into a 50 mL glass vial to which 5.0 mL of 50% pyridine solution were added, implying 8.33% (v/v) of pyridine content per vial. The vials were vortexed and incubated 37°C for 15 minutes to allow for equilibration, after which UV–vis spectra of the solutions were recorded using a 100 mm quartz cuvette. The Soret peak (A_{405}) was analysed in conjunction with the standard curve to determine the solubility of the CQ–ferrihaem complex, making use of the Beer–Lambert law to account for the longer optical pathlength of the cuvette.

2.10. Second pyridine–ferrihaemochrome assay to determine the solubility of the CQ–ferrihaem complex

111.598 mg of chloroquine diphosphate salt were dissolved in Milli-Q water to make up a 100 mL CQ solution. To 2 mL Eppendorf tubes, 500 μ L of 90 μ M ferrihaem solution were added, to which 300 μ L of the prepared CQ solution were dispensed followed by 40 μ L of 0.0795 M HClO_4 . Different volumes of 0.025 M HClO_4 , 0.000125 M HClO_4 and Milli-Q water were added as specified in **Table 2.5**. Spectrophotometric titration experiments were performed in quadruplicate with the samples incubated at 37°C for 48 hours, and the solubility of the CQ–ferrihaem complex was obtained as described in section **2.9**.

2.11. Pyridine–ferrihaemochrome assay to determine the solubility of the QD–ferrihaem complex

A similar methodology to that employed to determine the solubility of the CQ–ferrihaem complex was applied here. 156.588 mg of quinidine sulfate (dihydrate) salt were dissolved in Milli-Q water to make up a 100 mL QD solution. To 2 mL Eppendorf tubes, 500 μ L of 90 μ M ferrihaem solution were added, to which 300 μ L of the prepared QD solution were dispensed followed by 50 μ L of 0.0795 M HClO₄. Different volumes of 0.000318 M HClO₄ and Milli-Q water were added as specified in **Table 2.6**.

Prior to spectrophotometric titrations, experiments were performed to determine the optimal incubation time period to allow for sample equilibration. Samples 1, 5 and 11 from **Table 2.6** were prepared in 2 mL Eppendorf tubes and incubated at 37°C, and their pH values were recorded in triplicate after 1, 2, 4 and 6 days. Thus, to deduce the optimal incubation time period, pH measurements obtained on successive days of analysis were assessed for significant difference using multiple unpaired *t*-tests with Welch correction and a threshold of 0.05 for *p*-value comparisons.

Subsequently, spectrophotometric titration experiments were performed in quadruplicate with the samples incubated at 37°C for 48 hours, following which they were centrifuged. The pH values of the supernatants were recorded. Without disturbing the pellet, 500 μ L of supernatant were carefully dispensed into a size 0 glass vial to which 100 μ L of 50% pyridine solution were added, implying 8.33% (v/v) of pyridine content per vial. The vials were vortexed and incubated at 37°C for 15 minutes to allow for equilibration, following which UV–vis spectra of the solutions were recorded using a 10 mm quartz cuvette; the Soret peak (A_{405}) was analysed in conjunction with the standard curve generated in section 2.7 to determine the solubility of the QD–ferrihaem complex.

Table 2.6. Volumes of 0.000318 M HClO₄ and Milli-Q water, and the concentrations of added HClO₄ in prepared samples of the QD–ferrihaem complex for spectrophotometric titration.

Sample number	Vol. of 0.000318 M HClO ₄ (μL)	Vol. of MQ water (μL)	[HClO ₄] added (mM)
1	0	1150	1.99
2	100	1050	2.00
3	200	950	2.02
4	300	850	2.04
5	400	750	2.05
6	500	650	2.07
7	600	550	2.08
8	700	450	2.10
9	800	350	2.11
10	900	250	2.13
11	1000	150	2.15
12	1100	50	2.16

As performed in section 2.9 with the CQ–ferrihaem complex, an additional experiment was performed with the QD–ferrihaem complex where sample 12 from **Table 2.6** was prepared in sextuplicate, however, with the volumes of the solutions to be added upscaled by a factor of twenty; that is, to 50 mL Falcon tubes, 10 mL of 90 μM ferrihaem solution were added, to which 6.0 mL of the prepared QD solution were dispensed followed by 1.0 mL of 0.0795 M HClO₄. This was followed by the addition of 22 mL of 0.000318 M HClO₄ and 1.0 mL of Milli-Q water, making up a total volume of 40 mL. The samples were incubated at 37°C for 48 hours following which they were centrifuged. The pH values of the supernatants were then recorded. Without disturbing the pellet, 25 mL of supernatant were carefully dispensed into a 50 mL glass vial to which 5.0 mL of 50% pyridine solution were added, implying 8.33% (v/v) of pyridine content per vial. The vials were vortexed and incubated at 37°C for 15 minutes to allow for equilibration, and UV–vis spectra of the solutions were recorded using a 100 mm quartz cuvette. The Soret peak (A_{405}) was analysed in conjunction with the standard curve to

determine the solubility of the QD–ferrihaem complex, making use of the Beer–Lambert law to account for the longer optical pathlength of the cuvette.

2.12. Second pyridine–ferrihaemochrome assay to determine the solubility of the QD–ferrihaem complex

510.516 mg of quinidine sulfate (dihydrate) salt were dissolved in Milli-Q water to make up a 100 mL QD solution. To 2 mL Eppendorf tubes, 500 μL of 90 μM ferrihaem solution were added, to which 300 μL of the prepared QD solution were dispensed followed by 50 μL of 0.0795 M HClO_4 . Different volumes of 0.000318 M HClO_4 and Milli-Q water were added as specified in **Table 2.6**. Spectrophotometric titration experiments were performed in quadruplicate with the samples incubated at 37°C for 48 hours and the solubility of the QD–ferrihaem complex was obtained as described in section 2.11.

CHAPTER 3

Base Titration of Haematin

3.1. Introduction

As described in section 2.5 of Chapter 2, the first course of action in pursuit of the aim of investigating ferrihaem solubility at the mean parasitic digestive vacuole (DV) pH of 5.0⁷¹⁻⁷² was to repeat the spectrophotometric titration experiments reported by Morrison and Williams.²⁰⁴ While the spectrophotometric titration data supplied by the latter authors (**Figure 3.1**) are convincing, questions arise on their method of extracting haematin solubility values from the data, especially in view of more recent research findings.

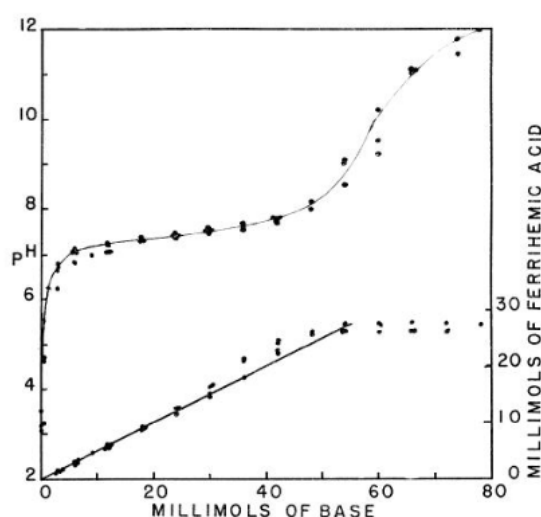
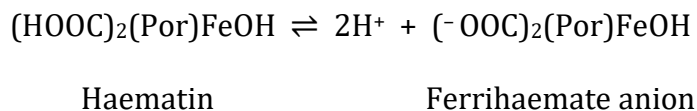


Figure 3.1. Titration and solubility data for haematin, referred to as 'ferrihemic acid' by the authors, obtained at 30°C. Reproduced from Morrison and Williams, *J. Biol. Chem.*, **1941**, 137, 468 with permission from Elsevier.

Importantly, the researchers presumed ionisation of the axial H₂O/OH⁻ ligand to be negligible. At the upper end of the straight line on the solubility curve, they assumed complete solution at a pH of approximately 9.0 at 27.6 mM of haematin after the addition of 55.2 mM of NaOH or approximately two equivalents of base per mole of haematin, due to which they assumed simultaneous deprotonation of the two propionic acid groups on haematin, represented as follows:



Hence,

$$K_{\text{ionisation}} = \frac{[(-\text{OOC})_2(\text{Por})\text{FeOH}] \times [\text{H}^+]^2}{[(\text{HOOC})_2(\text{Por})\text{FeOH}]}$$

Assuming complete ionisation in the saturated solution,

$$\text{Solubility product, } K_{sp} = [\text{H}^+]^2 \times [(-\text{OOC})_2(\text{Por})\text{FeOH}] \quad (3.1)$$

Taking the common logarithm on both sides of the equation and rearranging leads to a simplified equation where C is the concentration of ferrihaemate salt,

$$\text{Log } C = 2\text{pH} + \text{log } K_{sp} \quad (3.2)$$

By fitting titration and solubility data from the more effectively buffered sections of their curves to the above equation, Morrison and Williams obtained $\text{log } K_{sp} = -16.76 \pm 0.04$. Using this equation, the solubility of haematin at a pH of 5.0 is computed to be 0.174 μM . The authors, however, did not publish any $\text{log } C$ vs. pH graph; thus, a graph of $\text{log } C$ against pH was plotted (**Figure 3.2**) to verify the validity of equation 3.2 in describing the data in **Figure 3.1**. To depict the simultaneous deprotonation of the two carboxyl groups as presumed by Morrison and Williams and conform to equation 3.2, a linear regression model fitted to data points over the more effectively buffered portion of the curve would be expected to yield a slope of two. However, a slope of approximately one is observed indicating a single deprotonation process, and the predicted solubility at pH 5.0 using the generated equation in **Figure 3.2B** is $27 \pm 13 \mu\text{M}$ (Standard error of the mean (SEM) computed using a propagation of error formula:²⁰⁸⁻²⁰⁹ If $y = \text{log } C$, then $\text{SEM } (C) = \text{SEM } (y) \times C \times \ln(10)$. From the graph analytics of **Figure 3.2B**, $\text{SEM } (y) = 0.2163$ when pH = 5.0).

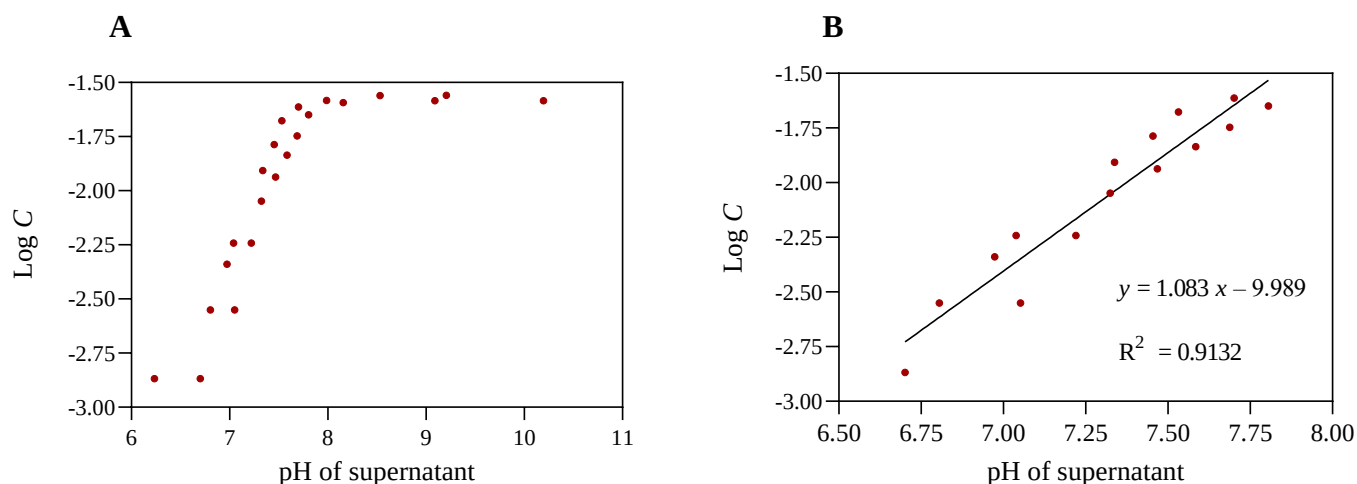


Figure 3.2. (A) Log C vs. pH graph extracted from the full titration and solubility dataset obtained by Morrison and Williams, and (B) excluding data points above pH 8 and a linear regression model fitted to the remaining data points.

The pK_a of the haem propionic acid groups is now believed to lie in the range of 5 – 6,²¹⁰ implying that those groups would likely exist in their carboxylate form even at the lowest pH examined by Morrison and Williams; that is, even in the ferrihaem samples containing no base. This conflicts with their assumption of deprotonation of the propionic acid groups over the studied pH range during spectrophotometric titration and hints towards deprotonation of the axial H_2O/OH^- ligand, strongly corroborated by De Villiers et al.¹²² The latter prepared a series of aqueous haematin solutions — with Fe(III)PPIX in its π - π dimeric form — in 0.050 M HEPES buffer to different pH values, ensuring a final Fe(III)PPIX concentration of 1.00×10^{-4} M in each solution. Absorption data recorded at 610 nm (from the charge transfer bands) were fitted to both a one and two pK_a model, where the pH dependence was observed to be consistent with two consecutive deprotonation steps of the two axial H_2O/OH^- ligands on the dimer with fitted pK_a values of 6.2 ± 0.3 and 8.5 ± 0.3 , respectively. These observations strongly suggest that the mathematical approach employed by Morrison and Williams was incorrect, encouraging further investigation into the methodology and the interpretation of results therein.

3.2. Determination of optimal incubation time period

Optimal incubation time periods to allow for sample equilibration were not elaborated on by Morrison and Williams and were therefore determined as described in section 2.5. The incubation time period required for equilibration was observed to decrease with increasing temperature (**Figures 3.3 and 3.4**). As the pH of the supernatant levelled off after decreasing, the dilution-corrected absorbance followed an increasing trend before reaching a plateau. This may be explained by more and more solute haematin molecules moving into the aqueous phase with time and their ionisable protons neutralising the hydroxide ions present, to effectively buffer the system, thus the decrease in pH with time. Interestingly, as observed in **Figure 3.3**, the separation between the pH curves of samples 3 and 8 seemed to diminish with increasing temperature, suggesting a narrowing of the pH range spanned through from the same sample set with increasing temperature. As mentioned in section 2.5, to determine the minimum number of days required for sample equilibration, multiple unpaired *t*-tests with Welch correction were used to test for significant difference between pH measurements and solubility readings obtained on successive days of analysis.

Table 3.1 summarises the results of the analysis at 37°C in which both the pH of supernatant and dilution-corrected absorbance at λ_{max} (598 nm) after day 8 were tested to be significantly different from those after day 16, implying an optimal incubation time period of 8 days at that temperature. At the other four temperatures, the dilution-corrected absorbances at 598 nm were observed to equilibrate faster than the pH of the supernatants, thus the latter were the preferred marker for determining the optimal incubation times, observed to be 16 days at both 10°C and 20°C, 12 days at 25°C and 8 days at 50°C.

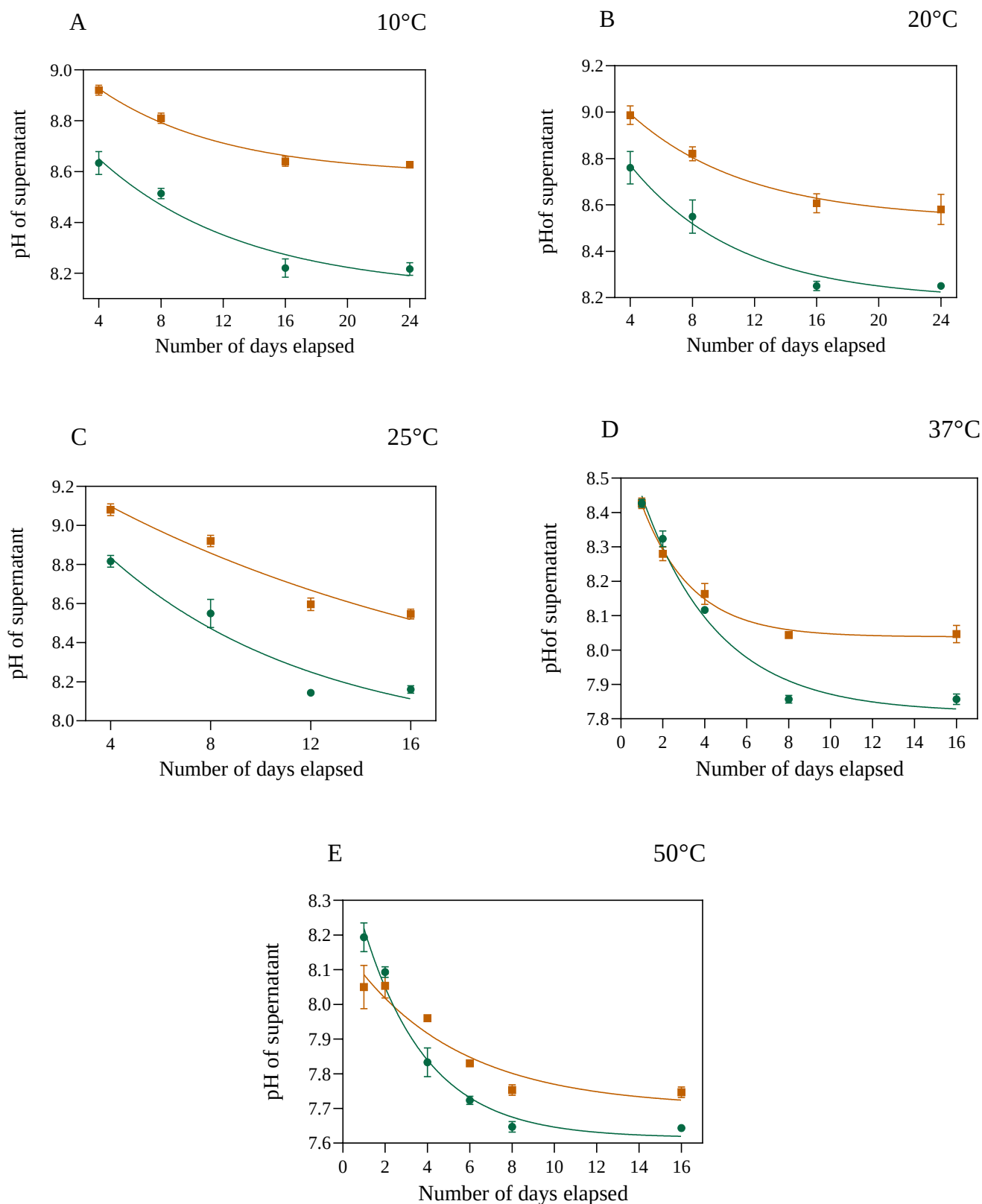


Figure 3.3. The variation of pH with the number of days elapsed for samples 3 (green) and 8 (orange) as defined in **Table 2.2**, at (A) 10°C, (B) 20°C, (C) 25°C, (D) 37°C and (E) 50°C. The error bars represent one standard deviation computed over triplicate measurements.

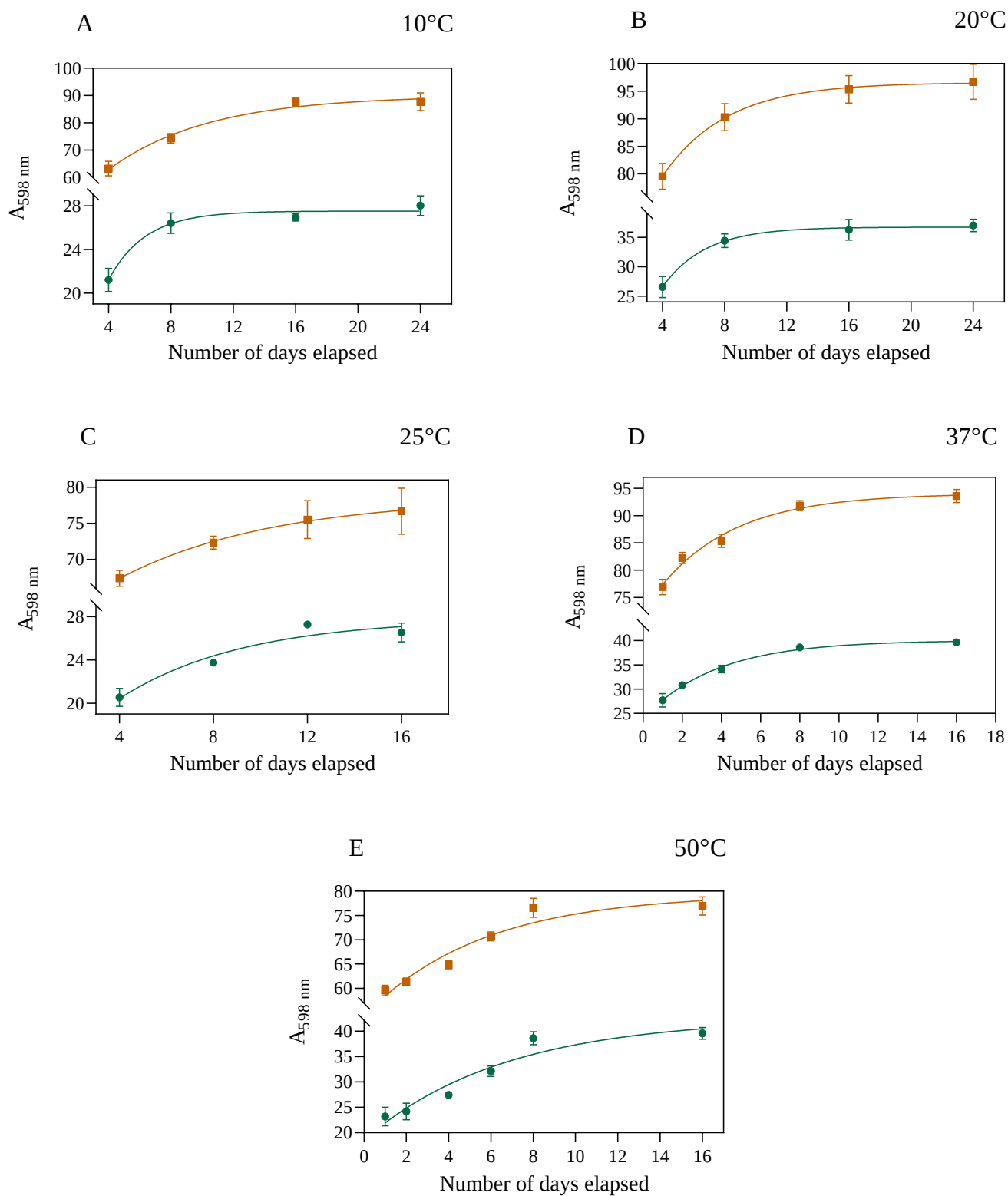


Figure 3.4. The variation of the dilution-corrected absorbance at 598 nm with the number of days elapsed for samples 3 (green) and 8 (orange) as defined in **Table 2.2**, at (A) 10°C, (B) 20°C, (C) 25°C, (D) 37°C and (E) 50°C. The error bars represent one standard deviation computed over triplicate measurements.

Table 3.1. Summary of the results from the multiple unpaired *t*-tests with Welch correction at 37°C for the optimal incubation time studies, carried out as described in section 2.5.

SAMPLE 3				
	pH of supernatant		Dil. corr. absorbance at $\lambda_{\max}$	
	<i>p</i> -value	Below threshold	<i>p</i> -value	Below threshold
After day 1 vs. after day 2	0.006562	Yes	0.049326	Yes
After day 2 vs. after day 4	0.002652	Yes	0.006662	Yes
After day 4 vs. after day 8	0.000061	Yes	0.003179	Yes
After day 8 vs. after day 16	>0.999999	No	0.113452	No
SAMPLE 8				
	pH of supernatant		Dil. corr. absorbance at $\lambda_{\max}$	
	<i>p</i> -value	Below threshold	<i>p</i> -value	Below threshold
After day 1 vs. after day 2	0.000755	Yes	0.008144	Yes
After day 2 vs. after day 4	0.007968	Yes	0.027181	Yes
After day 4 vs. after day 8	0.018088	Yes	0.002426	Yes
After day 8 vs. after day 16	0.842058	No	0.115082	No

3.3. Recrystallisation of haematin

As described in section 2.5, experiments were performed to assess the importance of recrystallisation of haematin prior to sample preparation. In each of the tested samples 1, 3, 5 and 8 (as defined in Table 2.2), the multiple unpaired *t*-tests with Welch correction did not detect any significant difference — both in pH of supernatant and dilution-corrected absorbance at 598 nm — between the data obtained using haematin without any purification and those obtained using recrystallised haematin (Figure 3.5). Therefore, the results justified the use of haematin procured from Sigma-Aldrich without any further purification for spectrophotometric titration.

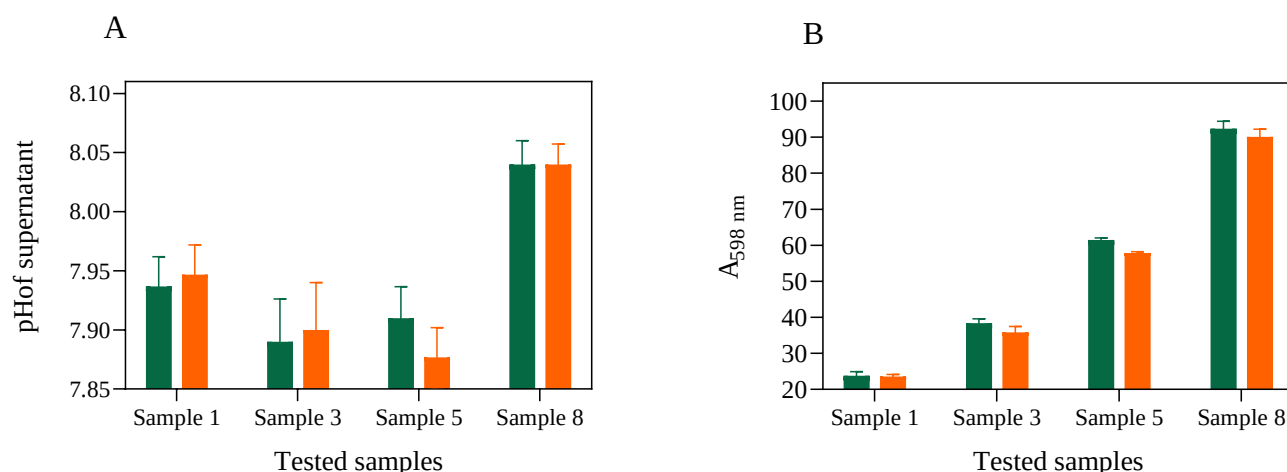


Figure 3.5. (A) The pH of the supernatants and (B) the dilution-corrected absorbance values at 598 nm for the tested samples using haematin without any purification (green bars) and recrystallised haematin (orange bars). The error bars represent one standard deviation computed over triplicate measurements.

3.4. Spectrophotometric titration of haematin with NaOH

UV-vis spectra from preliminary titration experiments of haematin with NaOH at 37°C confirmed the presence of the π - π dimeric form of ferrihaem present in the chosen aqueous environment (**Figure 3.6A**). As detailed by Asher et al.,¹²³ the π - π dimer is identified by the characteristic *Q* band without any shoulder at 598 nm and the broad Soret band which appears to be resolved into two bands. Given that absorbance = $-\log_{10} (I_1 / I_0)$ where I_1 and I_0 denote the incident and emerging intensities of light, respectively, an absorbance higher than one is indicative of more than 90% of the incident radiation being absorbed by the sample at that wavelength. This implies less than 10% of the incident radiation being detected by the instrument, which raises the possibilities of large errors in measurement. Hence, as a rule of thumb, application of the Beer-Lambert law is often restricted to absorbances in the range of 0.1 – 1.0,²¹¹ which is why the *Q* band peak was preferred for quantification of haematin solubility due to measured absorbance values of the Soret peaks largely exceeding the threshold absorbance of 1.0.

Figure 3.6B summarises preliminary titration and solubility data obtained at 37°C. Despite the data by Morrison and Williams (**Figure 3.1**) being obtained at 30°C, several comparisons may be drawn. To repeat, they assumed complete solution at a pH of approximately 9.0 at 27.6 mM of haematin after the addition of 55.2 mM of NaOH or approximately two equivalents of

base per mole of haematin. In contrast to their findings, solution was seemingly complete in this study at a pH of 8.2 at 27.8 mM of haematin after the addition of 35.0 mM of NaOH, corresponding to $1.26 \approx 1$ equivalent of base per mole of haematin as observed in **Figure 3.6B**, consistent with a single deprotonation per haematin molecule. A possible reason for higher NaOH concentrations required by Morrison and Williams for complete solution is the insufficient incubation of samples, given that their samples were incubated for 3 – 8 days. However, the observed discrepancy could also be attributed to the difference in incubation temperatures. Furthermore, the more effectively buffered region over a pH range of 7.0 – 8.0 in **Figure 3.1** is somewhat steeper than that observed in this research over a smaller pH range of 7.7 – 8.2. The smaller range could be attributed to the difference in incubation temperatures while the lower pH could be due to Morrison and Williams making use of haematin obtained *via* the reaction of hemin with NaOH and recrystallised using HCl, suggested by the authors as a potential source of error through contamination of samples with trace amounts of residual HCl.

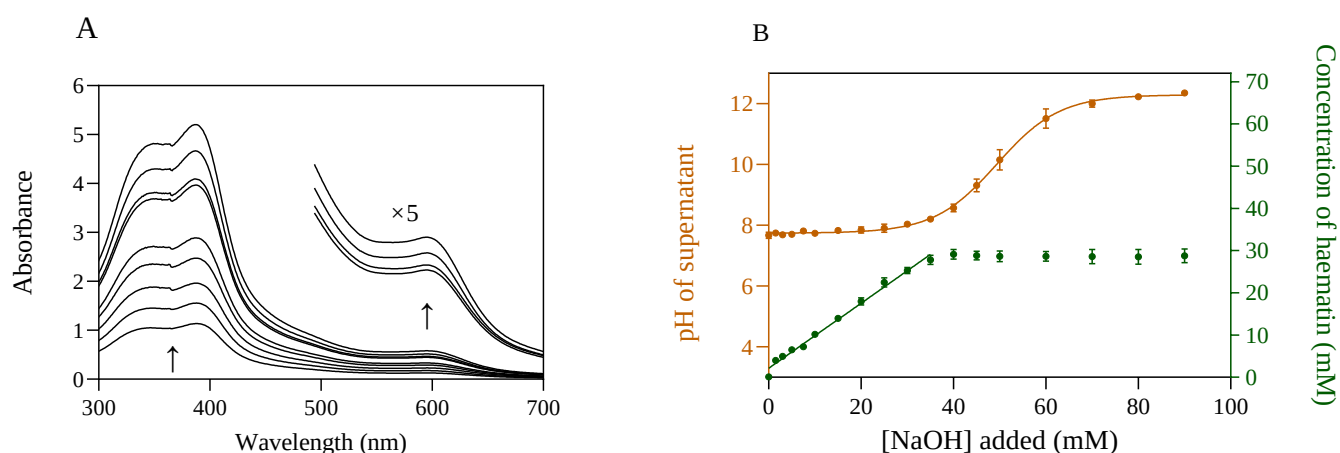


Figure 3.6. (A) UV-vis spectra from the titration of haematin with NaOH in aqueous solution. Arrows show the direction of change in spectra with increasing pH. *Q* band peaks were preferred for quantification of haematin solubility, falling between the absorbance range of 0–1. (B) Titration (orange, pH of supernatant) and solubility (green, concentration of dissolved haematin) data vs [NaOH] added, obtained at 37°C.

Subsequently, spectrophotometric titration experiments were conducted to determine the solubility of haematin at as close to the mean parasitic digestive vacuole (DV) pH of 5.0⁷¹⁻⁷² as possible, using the refined sample set in **Table 2.2** as described in section 2.5. At each of the five incubation temperatures, the log *C* vs. pH data from all three replicates, where *C* denotes the concentration of dissolved haematin, are displayed in **Figure 3.7**.

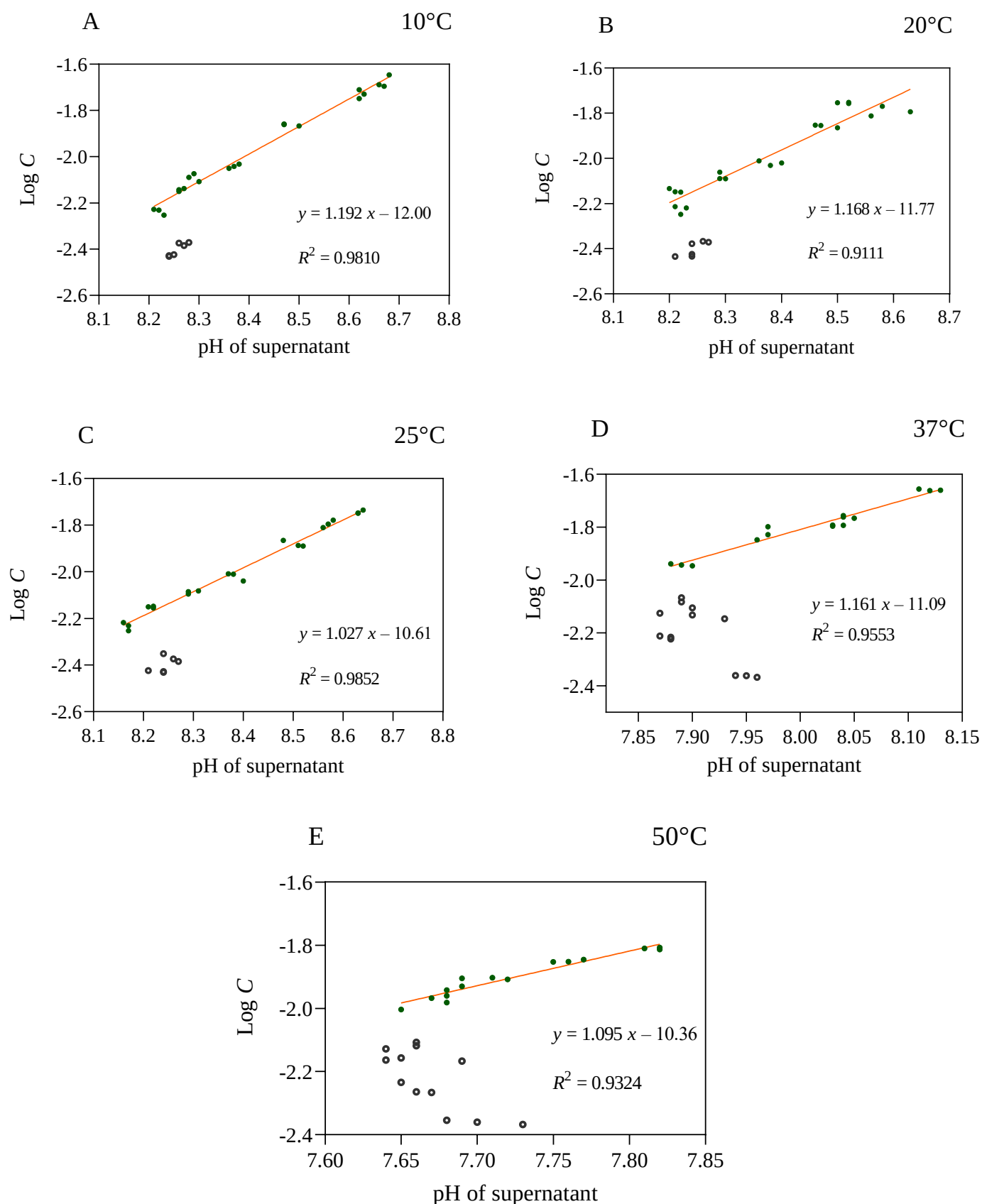


Figure 3.7. $\text{Log } (C \text{ (mol} \cdot \text{dm}^{-3}))$ vs. pH graphs obtained at (A) 10°C, (B) 20°C, (C) 25°C, (D) 37°C and (E) 50°C, with linear regression models fitted to each dataset, represented by solid orange lines. Unfilled grey data points were excluded from regression analysis.

Interestingly, data points corresponding to less than 7.5 mM of added NaOH seemed to record unexpectedly high and erratic pH values, causing appreciable deviation from the apparent trend observed in the other data points. These erratic data points were excluded from linear regression analysis using successive ROUT tests (False Discovery Rate = 5%) for outlier detection on GraphPad Prism⁷⁷ with the data points flagged after one run excluded before the next run, until the detection of no outliers. It is possible that such low NaOH concentrations did not suffice to adequately dissolve the solid haematin, leading to unbuffered solutions with higher pH values. This phenomenon was more pronounced at the higher temperatures of 37°C and 50°C, with deviation arising from data points which correspond to less than 13 mM of added NaOH. Therefore, it seems likely that higher concentrations of added NaOH were required to trigger the dissolution of haematin at increasing temperatures. Additionally, from **Figure 3.8A**, the observed pH range during titration did not vary significantly going from 10°C to 25°C. At 37°C and 50°C, lower pH values were observed with smaller range spans, indicated by the narrowing of the boxes in the box plot. Furthermore, in view of the fitted pK_a values of 6.2 ± 0.3 and 8.5 ± 0.3 reported by De Villiers et al.¹²² to describe the consecutive deprotonation equilibria in dimeric Fe(III)PPIX, it is highly probable that the more acidic of the two axial H₂O/OH⁻ ligands existed in its deprotonated state, with only the less acidic one involved in deprotonation equilibria over the pH ranges observed in this study. Concurrently, as observed in **Figure 3.8B**, there was no marked variation in the observed ferrihaem solubility range from 10 to 25°C but higher solubilities with smaller range spans recorded at 37°C and 50°C. With higher ferrihaem solubilities at increasing temperature, a larger proportion of haematin molecules are in the aqueous phase with their ionisable protons available to neutralise hydroxide ions present, thus buffering the system more effectively and constituting a likely explanation for the lower pH values observed at the higher temperatures of 37°C and 50°C. The reduced range spans in pH values and ferrihaem solubility likely occurred as a result of faster equilibration at increasing temperatures.

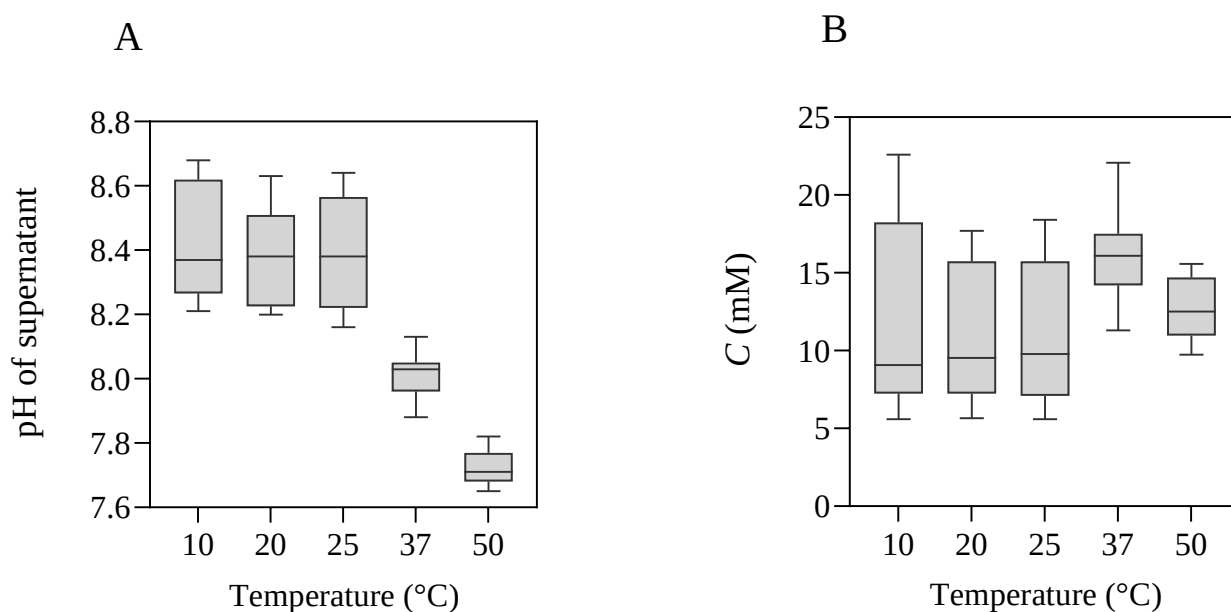


Figure 3.8. Box-and-whisker plots showing (A) the variation of pH values of the supernatants with temperature, and (B) the variation of ferrihaem solubility with temperature.

It is important to note that in the above spectrophotometric titration data, caution must be exercised when excluding data points as small changes in the slopes and y -intercepts may cause significant changes in the predicted haematin solubility values. Interestingly, at each of the five temperatures, linear regression analysis on the spectrophotometric titration data yielded a slope of approximately one, similar to the data from Morrison and Williams. This observation corroborates the deprotonation of the axial $\text{H}_2\text{O}/\text{OH}^-$ ligand and refutes the simultaneous deprotonation of the two haematin propionic acid groups. Thus, the solubility of haematin at a pH of 5.0 may be derived using the equations resulting from the linear regression analyses in **Figure 3.7** (method 1). Alternatively, the equation $\log C = \text{pH} + \log K_{sp}$ is derived from equation 3.2 by assuming single deprotonation equilibria and may be used to determine the solubility at pH 5.0 by employing the $\log K_{sp}$ values computed in **Figure 3.7** (method 2). The results are summarised in **Table 3.2** and **Figure 3.9** with the SEMs computed using the propagation of error formula described in section 3.1. At each temperature, predicted values from method 1 were observed to be higher than those predicted from method 2 due to the regression line slopes being slightly higher than one. Also, relatively large standard error values were observed, presumably due to back-transforming from the logarithmic to the normal scale when computing SEMs. While a general ascending trend with temperature was observed in the solubility values computed using method 1, the predicted solubility value at 37°C from method 2 was inexplicably smaller than that at 25°C. It may be argued that the

regression line equations provide an unbiased representation of the spectrophotometric titration data whereas using the equation $\log C = \text{pH} + \log K_{sp}$ introduces some bias by restricting the slope to one, possibly the reason behind the seemingly anomalous reading at 25°C.

Table 3.2. Summary of the solubility data at a pH of 5.0.

Temperature (°C)	Solubility of haematin at pH 5.0 obtained using method 1 (μM)	Solubility of haematin at pH 5.0 computed using method 2 (μM)
10	0.9 ± 0.3	0.10 ± 0.07
20	1.2 ± 0.8	0.2 ± 0.3
25	3.4 ± 0.8	2 ± 1
37	5 ± 2	1 ± 1
50	13 ± 7	4 ± 6

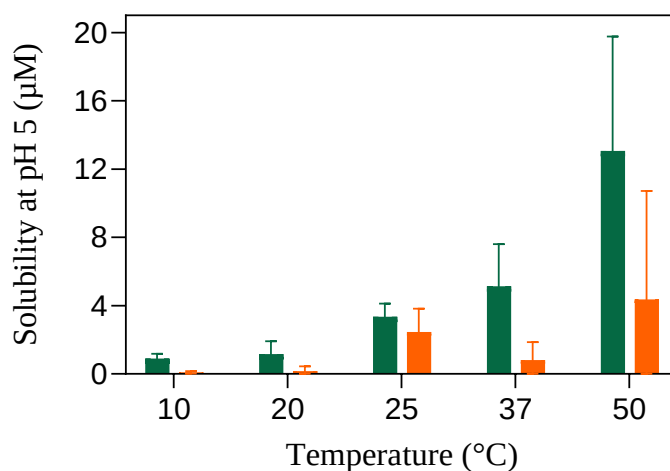


Figure 3.9. Graphical representation of the data in **Table 3.4** showing the solubility at pH 5.0 from extrapolation of regression analysis (green bars) and from the equation $\log C = \text{pH} + \log K_{sp}$ (orange bars). The error bars represent the computed standard error of the means.

3.5. Determination of thermodynamic constants for ferrihaem solubility

From the thermodynamic description of chemical equilibrium of a reaction, the standard change in Gibbs's free energy, ΔG^0 , is defined as follows²¹²:

$$\Delta G^0 = -RT \ln (K^0)$$

where R is the molar gas constant ($8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$); T is the temperature in kelvin and K^0 is the thermodynamic equilibrium constant.

The Gibb's free energy change may also be defined by:

$$\Delta G^0 = \Delta H^0 - T \Delta S^0$$

where ΔH^0 and ΔS^0 are the standard changes in enthalpy and entropy, respectively.

Combining the above two equations and rearranging yields the linear form of the Van 't Hoff equation:

$$\ln K^0 = - \left(\frac{\Delta H^0}{R} \right) \cdot \left(\frac{1}{T} \right) + \left(\frac{\Delta S^0}{R} \right) \quad (3.3)$$

This equation constitutes a convenient non-calorimetric method of determining reaction enthalpies. In practice, ΔH^0 is weakly temperature-dependant in most cases.²¹² Therefore, in the interests of simplicity, it may be assumed that the changes in enthalpy and entropy do not vary remarkably across the temperature range spanned in this study. The $\log K_{sp}$ values obtained from spectrophotometric titration data in section 3.4 were transformed to $\ln K_{sp}$ with the associated standard errors computed using the propagation of error formula described in section 3.1, followed by the plotting of a graph of $\ln K_{sp}$ against $1/T$, shown in **Figure 3.10A**. Employing equation 3.3, the linear regression fitted to the data yielded ΔH and ΔS values of $(67 \pm 26) \text{ kJ} \cdot \text{mol}^{-1}$ and $(9 \pm 85) \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, respectively. The standard error of the means (SEMs) were computed using a propagation of error formula:²⁰⁸⁻²⁰⁹

$$\text{If } L = - \left(\frac{\Delta H}{R} \right), \text{ then SEM } (\Delta H) = \text{SEM } (L) \times R$$

$$\text{If } N = \left(\frac{\Delta S}{R} \right), \text{ then SEM } (\Delta S) = \text{SEM } (N) \times R$$

where L and N denote the regression slope and y -intercept, respectively, with $\text{SEM } (L)$ and $\text{SEM } (N)$ obtained from the graph analytics.

Even though no outlier was flagged by the ROUT outlier removal method (False Discovery Rate = 5%) during regression analysis, the data point at 25°C was excluded in an *ad hoc* attempt of assessing the impact on the goodness-of-fit, leading to a remarkable improvement in the R^2 value (**Figure 3.10B**) which somewhat rationalised the exclusion, although caution must be exercised when making such simplistic removal of data points. Nevertheless, employing equation 3.3, the linear regression fitted to the data in **Figure 3.10B** yielded ΔH and ΔS values of $(72 \pm 9) \text{ kJ} \cdot \text{mol}^{-1}$ and $(21 \pm 31) \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, respectively. These results indicate that haematin solubility in aqueous medium is predominantly entropically driven and spontaneous only at very high temperatures. The endothermic enthalpy of solution conforms to the observed rise in haematin solubility with increasing temperature and highlights weaker interactions between the Fe(III)PPIX dimer and the aqueous solvent as opposed to stronger interactive forces within the reported multicrystalline structures comprising distinctly regular units in haematin crystals.²¹³

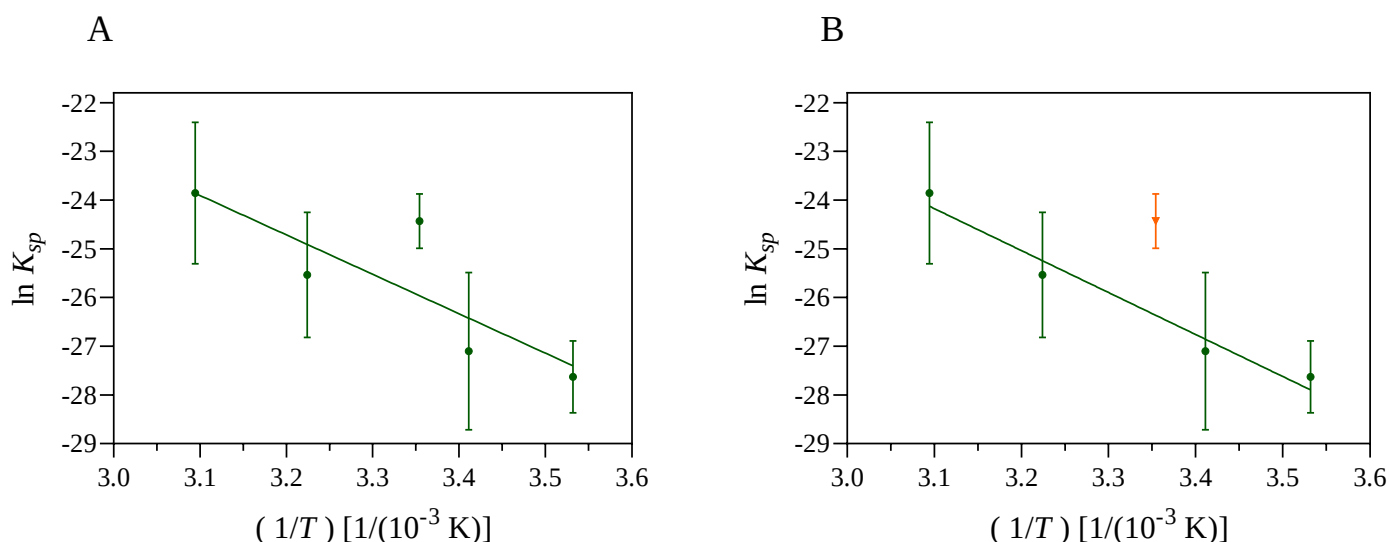


Figure 3.10. Van 't Hoff plot describing the solubility of haematin with (A) a linear regression fitted to the entire data set, described by the equation $y = -8.097x + 1.196$, $R^2 = 0.70$, and (B) the data point at 25°C (orange) excluded with the resulting linear regression described by the equation $y = -8.619x + 2.546$, $R^2 = 0.97$. The error bars represent the computed standard error of the means.

3.6. Conclusions

The spectrophotometric titration methodology from Morrison and Williams was refined by comprehensive optimisation of parameters such as the incubation time period. The observed titration and solubility data refuted their assumption of deprotonation of the ferrihaem propionic acid groups and emphasised single deprotonation equilibria, presumably corresponding to deprotonation of the less acidic axial $\text{H}_2\text{O}/\text{OH}^-$ ligand in the $\text{Fe(III)PPIX } \pi\text{-}\pi$ dimer. A haematin solubility of $(5.1 \pm 2.5) \mu\text{M}$ was observed at 37°C , but ferrihaem dimerisation was not accounted for during molarity calculations. It is therefore more appropriate to report the solubility of the Fe(III)PPIX dimer as $(2.6 \pm 1.3) \mu\text{M}$ in view of the predominance of the $\pi\text{-}\pi$ dimeric form in aqueous solution. Notably, the observed ferrihaem solubility value is larger than that predicted by Morrison and Williams by approximately thirty-fold.²⁰⁴ However, erratic pH values and solubilities were encountered in samples with low concentrations of added base, presumably due to solid haematin not dissolving, and these data points were excluded from regression analysis. Therefore, despite the statistical significance of the linear regression fitted to the remaining data points, uncertainties remain with regard to the arbitrary selection of outlier removal method and its aggressiveness, ergo calling into question the accuracy of extrapolation to determine the solubility of haematin at a pH of 5.0 and the computed values of K_{sp} . The latter values were used to determine parameters for ferrihaem solubility through a Van 't Hoff plot which yielded ΔH and ΔS values of $(72 \pm 9) \text{ kJ}\cdot\text{mol}^{-1}$ and $(21 \pm 31) \text{ J}\cdot\text{mol}^{-1} \cdot \text{K}^{-1}$ respectively, underlining the endothermic nature of entropically-driven ferrihaem solubility. The relatively large standard errors presumably resulted from carrying forward of the uncertainties associated with the K_{sp} values, or from a lack of robustness in the linear regression fitted in the Van 't Hoff plot due to experimentation at a relatively small set of temperatures. Therefore, while the base titration experiments supplied useful ferrihaem solubility data and thermodynamic data, further investigation is required to improve the reliability of the methodology and reproducibility of the results.

CHAPTER 4

Acid Titration of Haematin

4.1. Introduction

The key disadvantage of the base titration methodology was that the lowest solution pH achieved during spectrophotometric titration across the five temperatures was approximately 7.6 which is appreciably far from the mean parasitic digestive vacuole (DV) pH of 5.0.⁷¹⁻⁷² Thus, in order to probe a more relevant pH range, an acid titration methodology was proposed. However, as described in Chapter 1 (Section 1.6), mineral acid-based solvents are probably inducers of β -haematin formation, although not as potently as acetate-based solvents, with aggregation probably inevitable in both cases. Nevertheless, in view of the aim of experimenting in an exclusively aqueous and inorganic medium, an acid titration methodology employing a mineral acid was deemed the most suitable way of investigating the solubility of haematin at as close to a pH of 5.0 as possible. Furthermore, all experiments described in this chapter were performed at 37°C to ensure experimental parameters as relevant to human physiological conditions as possible.

4.2. Spectrophotometric titration of haematin with HClO₄

Spectrophotometric titration experiments were conducted as described in section 2.6, with the results summarised in **Figure 4.1**. At high pH values, the characteristic *Q* band without any shoulder at 608 nm and the broad Soret band which appears to be resolved into two bands indicated the presence of the π - π dimeric form of Fe(III)PPIX.¹²³ The use of HClO₄ was

preferred over HCl to avoid any chloride ligand coordination to Fe(III)PPIX resulting from the latter acid.

Similar phenomena to those reported by Wood et al.¹⁰⁸ were observed with decreasing pH: The collapsing of the Soret band into a single broad band, the apparent collapsing of the *Q* band at 608 nm and the appearance of the charge transfer band at 633 nm were all indicative of Fe(III)PPIX aggregation. Also, it is impossible to discount the formation of the amorphous intermediate phase (B-haematin) to be responsible for the observed spectroscopic changes, as described in section 1.6 of Chapter 1. As a result, these spectra were not helpful in determining the solubility of haematin at pH 5.0, requiring further refinement of the methodology.

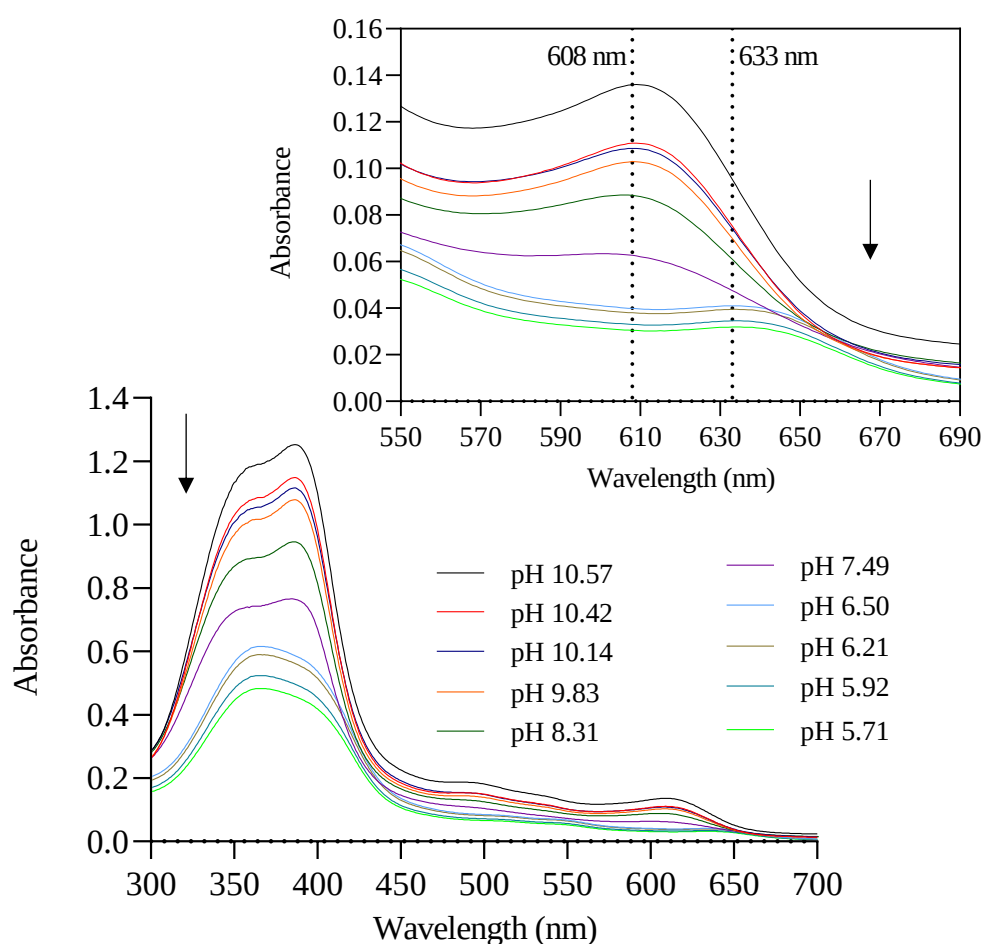


Figure 4.1. UV-vis spectra recorded during the acidification of an alkaline haematin solution using HClO₄. The inset highlights the changes in the *Q* band region with decreasing pH. For clarity, the arrows show the direction of change in spectra with decreasing pH.

4.3. Pyridine–ferrihaemochrome assay to quantify haematin solubility

As described in section 2.7, a modification of the fully validated pyridine–ferrihaemochrome assay developed by Ncokazi and Egan¹⁵⁸ was used to determine the solubility of haematin. As detailed in Chapter 1 (section 1.8.2), key to this assay is the selective reaction of pyridine with free haem — be it monomeric, dimeric, or further aggregated free haem — to form a low-spin bis-pyridyl complex (**Figure 1.14**), but not with β -haematin. Pyridine with ferrihaem in strongly basic conditions has previously been observed to induce the formation of the μ -oxo dimer of Fe(III)PPIX¹²¹; this was avoided by buffering the pyridine solution at pH 7.4 with 2 M HEPES.

Prior to spectrophotometric titrations, experiments were performed to determine the optimal incubation time period to allow for sample equilibration as described in section 2.7. Multiple unpaired *t*-tests with Welch correction did not reveal any statistical difference between the pH readings after day one compared to those after day two (**Figure 4.2**). Nonetheless, an optimal incubation time of two days was chosen as a measure of precaution. In contrast to the base titration experiments described in the previous chapter where the samples were prepared using solid haematin as starting reagent, faster equilibration was observed, possibly due to much smaller initial ferrihaem concentrations per Eppendorf tube.

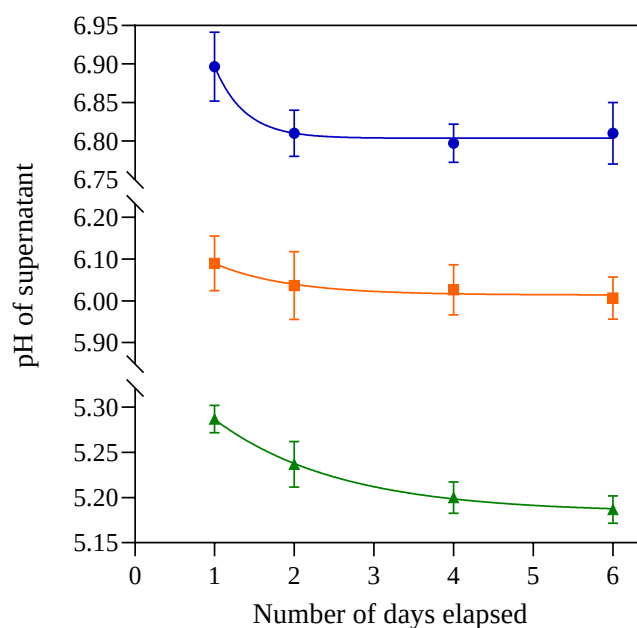


Figure 4.2. The variation of pH with the number of days elapsed for sample 2 (blue), sample 6 (orange) and sample 10 (green) as defined in **Table 2.3**, at 37°C. The error bars represent one standard deviation computed over triplicate measurements.

Furthermore, as detailed in section 2.7, prepared solutions of known ferrihaem concentrations in the range of 1 – 15 μM were analysed to make up a standard curve for the solubility of haematin; the Beer–Lambert law was obeyed across the observed range of concentrations of haematin as displayed in **Figure 4.3A**. The haematin concentrations were corrected for changes in volume from addition of the pyridine solution, and the observed molar extinction coefficient was comparable to literature values.¹²² Also, the titration curve from preliminary experiments (**Figure 4.3B**) exhibited two inflection points, probably indicating the consecutive protonation of the two axial ligands in the Fe(III)PPIX dimer with reported fitted pK_a values of 6.2 ± 0.3 and 8.5 ± 0.3 , respectively, followed by protonation of the propionate groups.¹²² The concentrations of ferrihaemate and haematin in the aqueous phase are identical when the initial number of moles of ferrihaemate—which acts as a base in this case—has been half neutralised by the added HClO_4 . Thus, these terms cancel in the Henderson–Hasselbalch equation ($\text{pH} = pK_a + \log([A^-]/[HA])$ where $[A^-]$ and $[HA]$ denote the equilibrium concentrations of an acid–base conjugate pair). At these ‘half-titration points’, $\text{pH} = pK_a$ and the buffer capacities of the solutions are maximal; the titration curve is flattest, resulting in the smallest changes in pH from the addition of acid or base.²¹⁴ It was, however, not possible to accurately obtain the pK_a values of the protonation sites from **Figure 4.3B** for comparison with the reported values¹²² due to the limited number of data points.

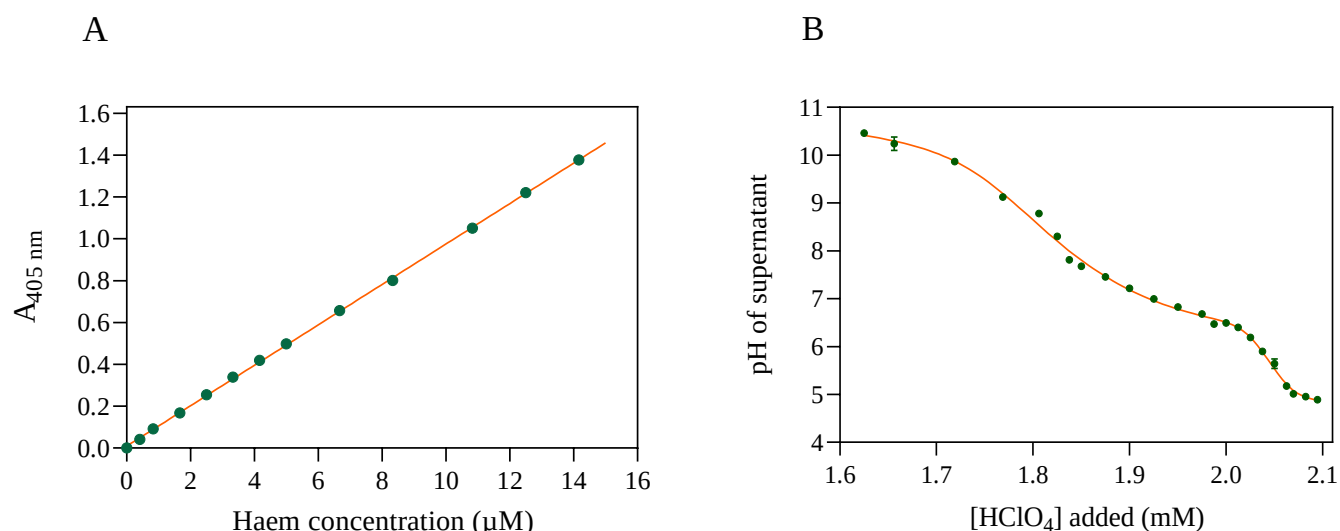


Figure 4.3. (A) Standard curve for the solubility of haematin measured *via* the bis-pyridyl complex which has an absorbance maximum at 405 nm. The linear regression fitted to the data (orange line) is described by the equation $y = 0.09661x + 0.008771$, $R^2 = 0.99$. The molar extinction coefficient, $\epsilon = 96\,607\text{ M}^{-1}\cdot\text{cm}^{-1}$ was obtained from the slope of the linear regression. (B) Titration curve for an alkaline haematin solution with HClO_4 .

Subsequently, spectrophotometric titration experiments were conducted in quintuplicate at 37°C using the refined sample set in **Table 2.3** as described in section 2.7. As observed in **Figure 4.4B**, the linear regression fitted to the data points was statistically significant; however, predictions from such a fit could be misleading due to the data points seemingly grouped near the top and bottom ends of the regression line. Therefore, further honing of the methodology was deemed necessary to obtain more solubility readings at pH close to 5.0 in order to obtain a more robust fit.

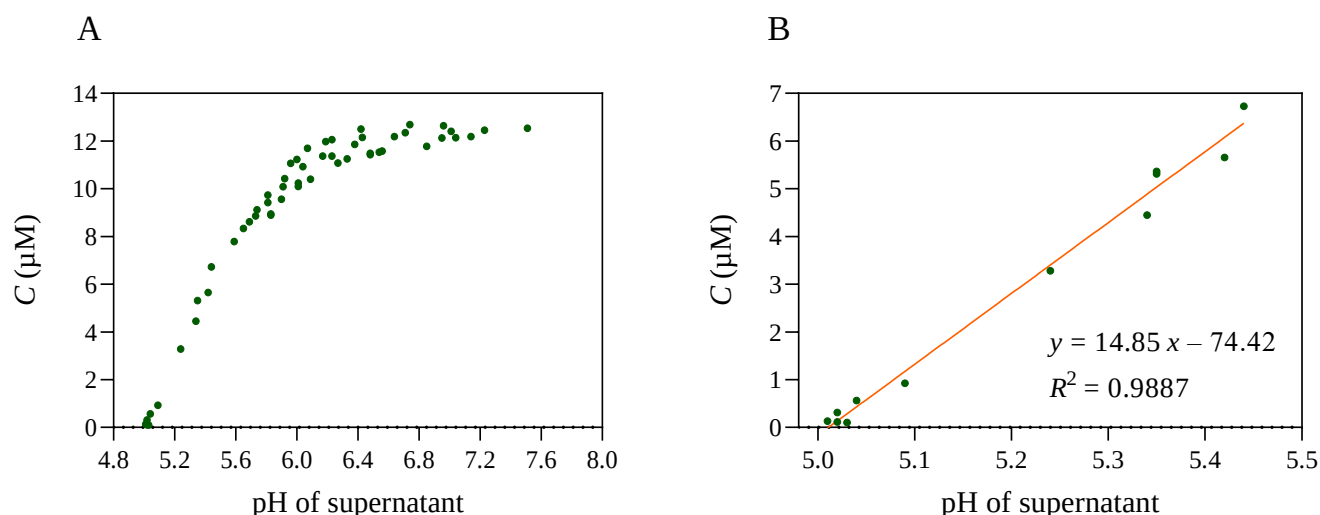


Figure 4.4. (A) The solubility of haematin in perchloric acid solution as a function of pH, with C denoting the concentration of dissolved ferrihaem. (B) The linear regression (orange line) fitted to the data in the lower pH region.

To this end, a second assay was devised as described in section 2.8, employing a combination of more dilute solutions of HClO_4 to observe smaller incremental decreases in pH across the sample set. As shown in **Figure 4.5B**, the linear regression fitted to the spectrophotometric titration data was statistically significant; from the graph analytics, the solubility of haematin at pH 5.0 is predicted to be equal to $0.5 \pm 0.2 \mu\text{M}$ ($0.47 \pm 0.15 \mu\text{M}$), which is lower by one order of magnitude than that predicted from the base titration experiments in Chapter 3 ($5.1 \pm 2.5 \mu\text{M}$). This discrepancy may be explained by the fact that the base titration experiments comprised extrapolation from a relatively high pH region with significantly higher ferrihaem solubility compared to the more relevant and lower pH range inspected in this chapter.

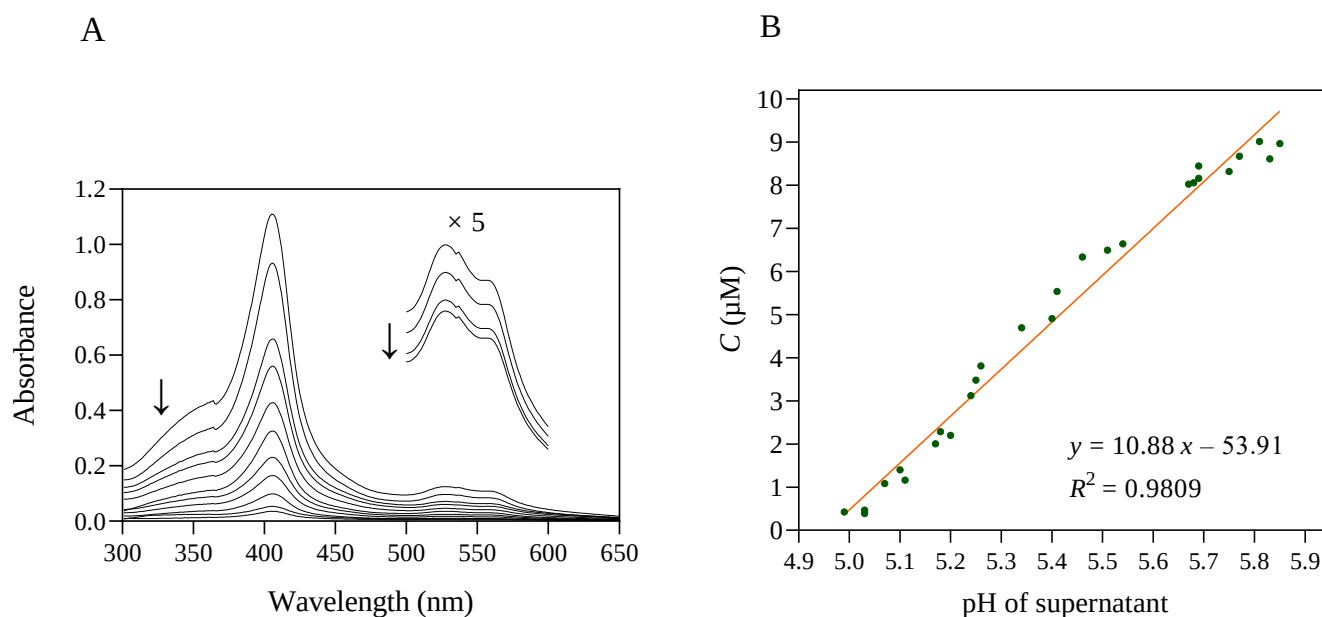


Figure 4.5. (A) UV-vis spectra from the titration of alkaline haematin with HClO_4 combined with aqueous pyridine to quantify the solubility of haematin. The arrow shows the direction of change in the spectra with decreasing pH. The *Q* bands, although not used for measuring Fe(III)PPIX concentration, are magnified for clarity. (B) Graphical representation of the solubility data where *C* represents the concentration of dissolved haematin.

Given the reported pK_a range of the ferrihaem propionic acid groups of 5 – 6,²¹⁰ the assumption of protonation of the two propionates was plausible over the pH range examined in the above assay; thus, the protonation equilibria described in section 3.1 (Chapter 3) was considered. From equation 3.2 ($\text{Log } C = 2\text{pH} + \text{log } K_{sp}$), a graph of $\text{log } C$ against pH would be expected to yield a slope of two, representative of the protonation of the two ferrihaem propionates. However, as shown in **Figure 4.6B**, the linear regression fitted to the set of data points closest to pH 5.0 was statistically significant and resulted in a slope of approximately three, indicative of triple protonation equilibria. This was probably due to continued protonation of the axial OH^- ligand as well, even though this hypothesis does not conform to the reported pK_a value of 6.2 ± 0.3 for the more acidic axial ligand on the ferrihaem dimer.¹²²

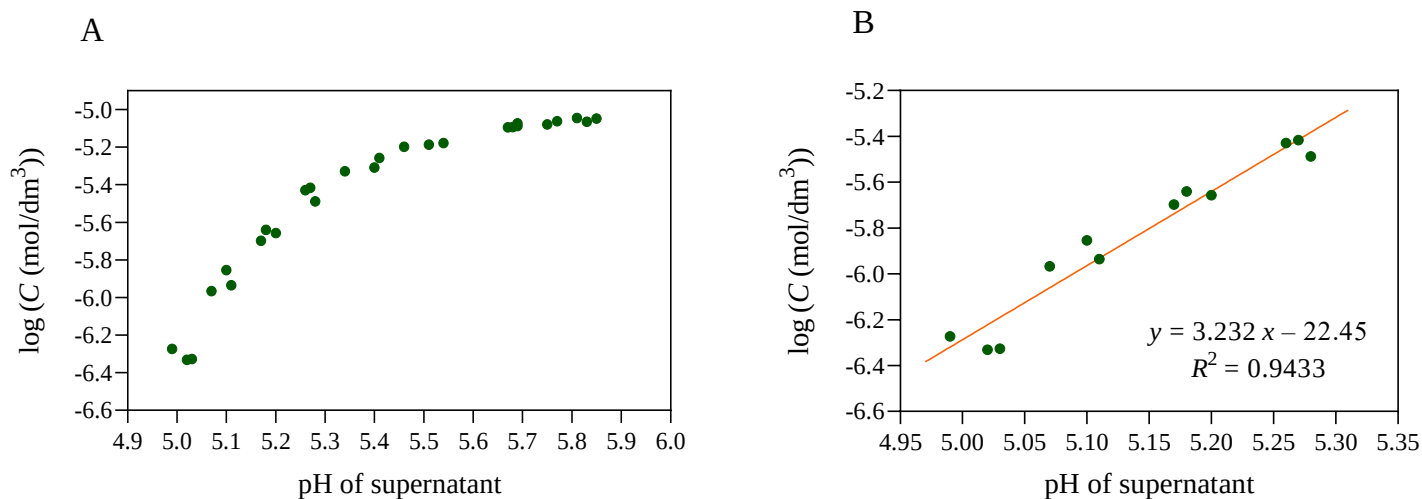
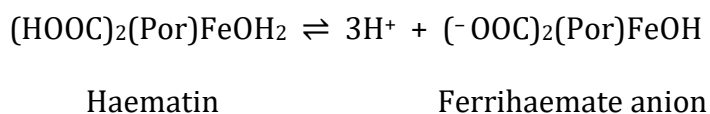


Figure 4.6. (A) Log C vs. pH graph for the solubility data obtained from the second pyridine–ferrihaemochrome assay. (B) The linear regression (orange line) fitted to the data in the lower pH region.

Assuming triple protonation equilibria, the solubility product constant for ferrihaem at pH 5.0 may be computed as follows:



Hence,

$$K_{\text{ionisation}} = \frac{[(-\text{OOC})_2(\text{Por})\text{FeOH}] \times [\text{H}^+]^3}{[(\text{HOOC})_2(\text{Por})\text{FeOH}_2]}$$

where $[(-\text{OOC})_2(\text{Por})\text{FeOH}]$ represents the concentration of dissolved haematin at pH 5.0, equal to $0.47 \pm 0.15 \mu\text{M}$.

Assuming complete ionisation in the saturated solution and substituting $[(-\text{OOC})_2(\text{Por})\text{FeOH}]$ with [Monomeric ferrihaem] for easier equation reading,

$$\text{Solubility product, } K_{sp} = [\text{H}^+]^3 \times [\text{Monomeric ferrihaem}]$$

and

$$[\text{H}^+] = 3 \times [\text{Monomeric ferrihaem}]$$

$$= 3 \times (0.47 \times 10^{-6})$$

$$= 1.41 \times 10^{-6} \text{ M}$$

Combining the above two equations,

$$\text{Solubility product, } K_{sp} = [\text{H}^+]^3 \times [\text{Monomeric ferrihaem}]$$

$$= (1.41 \times 10^{-6})^3 \times (0.47 \times 10^{-6})$$

$$= (1.3 \pm 0.3) \times 10^{-24}$$

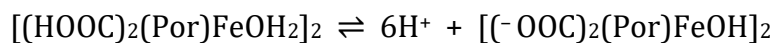
with the SEM computed using the following propagation of error formulae in succession:²⁰⁸⁻²⁰⁹

- If $[\text{H}^+] = 3 \times [\text{Monomeric ferrihaem}]$, then $\text{SEM} ([\text{H}^+]) = 3 \times \text{SEM} ([\text{Monomeric ferrihaem}])$ with $\text{SEM} ([\text{Monomeric ferrihaem}])$ known to be equal to (0.1503×10^{-6}) from the graph analytics of **Figure 4.5B**.
- If $y = [\text{H}^+]^3$, then $\frac{\text{SEM} (y)}{y} = 3 \times \frac{\text{SEM} ([\text{H}^+])}{[\text{H}^+]}$, using which $\text{SEM} (y)$ was computed.
- If $K_{sp} = [\text{H}^+]^3 \times [\text{Monomeric ferrihaem}]$, then

$$\frac{\text{SEM} (K_{sp})}{K_{sp}} = \sqrt{\left(\frac{\text{SEM} ([\text{H}^+]^3)}{[\text{H}^+]^3}\right)^2 + \left(\frac{\text{SEM} ([\text{Monomeric ferrihaem}])}{[\text{Monomeric ferrihaem}]}\right)^2}$$

from which $\text{SEM} (K_{sp})$ was computed.

To account for ferrihaem dimerisation with triple protonation equilibria per haematin molecule,



Dimeric haematin

Dimeric ferrihaemate anion

with the concentration of the dimeric ferrihaemate anion, denoted by [Dimeric ferrihaem], being equal to half of the concentration of the monomeric anion:

$$[\text{Dimeric ferrihaem}] = \frac{[\text{Monomeric ferrihaem}]}{2} = \frac{0.47 \times 10^{-6}}{2} = 0.24 \times 10^{-6} \mu\text{M}.$$

Hence,

$$\begin{aligned} \text{Solubility product for the Fe(III)PPIX dimer, } K_{sp} &= [\text{H}^+]^6 \times [\text{Dimeric ferrihaem}] \\ &= (1.41 \times 10^{-6})^6 \times (0.24 \times 10^{-6}) \\ &= (1.9 \pm 0.4) \times 10^{-42} \end{aligned}$$

with the SEM computed using the propagation of error formulae²⁰⁸⁻²⁰⁹ as described above.

4.4. Conclusions

While the acetate-mediated assays have been reported to produce β -haematin in high yields, the use of mineral acids instead of organic ones probably leads to β -haematin formation in low amounts. Distinct changes were observed in the UV-vis spectra with decreasing pH, caused by Fe(III)PPIX aggregation and probably B-haematin or β -haematin formation; these changes were induced by the mineral acid HClO_4 , concurring with the observations by Wood et al.¹⁰⁸ Hence, the acid titration methodology was not useful to obtain any solubility data at low pH values. Subsequently, the procedure was refined by coupling with a pyridine-ferrihaemochrome assay to quantify the solubility of haematin at 37°C, observed to be equal to $0.5 \pm 0.2 \mu\text{M}$ ($0.47 \pm 0.15 \mu\text{M}$) at a pH of 5.0 using which the solubility product constant for haematin was computed to be $(1.3 \pm 0.3) \times 10^{-24}$. To account for the predominance of the π - π dimeric form in aqueous solution, it is more appropriate to report the solubility of the ferrihaem dimer as $0.2 \pm 0.1 \mu\text{M}$ ($0.24 \pm 0.08 \mu\text{M}$), with the solubility product constant for the dimer computed to be $(1.9 \pm 0.4) \times 10^{-42}$.

In essence, the pyridine-ferrihaemochrome assay supplied reliable and reproducible ferrihaem solubility data at the mean parasitic DV pH of 5.0.⁷¹⁻⁷² However, despite the absence

of significant scattering in the solubility data at low pH values, the recorded absorbance values of the Soret peak below a pH of 5.2 were less than 0.1, with concentration determinations below this limit previously reported to be prone to high error.²¹⁵ Hence, further investigation is required into more accurate methods of ferrihaem quantification in the micro- and nanomolar concentration ranges.

CHAPTER 5

Solubility of the CQ–ferrihaem and QD–ferrihaem Complexes

5.1. Introduction

As detailed in Chapter 1 (section 1.9), the intracellular modes of action of quinoline antimalarials such as chloroquine (CQ) and quinidine (QD) were observed to involve adsorption onto the fastest-growing face of haemozoin to inhibit crystal growth by “capping” it.²⁰² Subsequently, the formation of an intracellular drug–ferrihaem complex was demonstrated by Openshaw et al.²⁰³ The mechanism of parasite killing, however, is yet to be fully elucidated. Nonetheless, as hypothesised by Openshaw et al.,²⁰³ the dissolved complex may kill the parasite by inhibiting key enzymes or transporters in the digestive vacuole (DV), or by diffusing out of the parasitic DV and dissociating to release free haem in the cytoplasm, responsible for the generation of toxic reactive oxygen species (ROS). However, a majority of studies have hitherto made use of organic solvents — such as DMSO or acetic acid — to prepare drug–ferrihaem complexes.^{154, 200} Therefore, in line with the aim of experimenting in an exclusively aqueous inorganic medium, the methodology employed in Chapter 4 was combined with the addition of aqueous drug solution to obtain the solubilities of the drug–ferrihaem complexes at as close to a pH of 5.0 as possible. Furthermore, similar to Chapter 4, all experiments described in this chapter were performed at 37°C to ensure experimental parameters as relevant to human physiological conditions as possible.

5.2. Determination of optimal incubation time periods

Prior to spectrophotometric titrations, experiments were performed to determine the optimal incubation time period to allow for sample equilibration, as described in sections 2.9 and 2.11 for the CQ–ferrihaem and QD–ferrihaem complexes, respectively. Multiple unpaired *t*-tests with Welch correction revealed an optimal period of 48 hours to ensure sample equilibration of either complex (**Figures 5.1A** and **5.1B**).

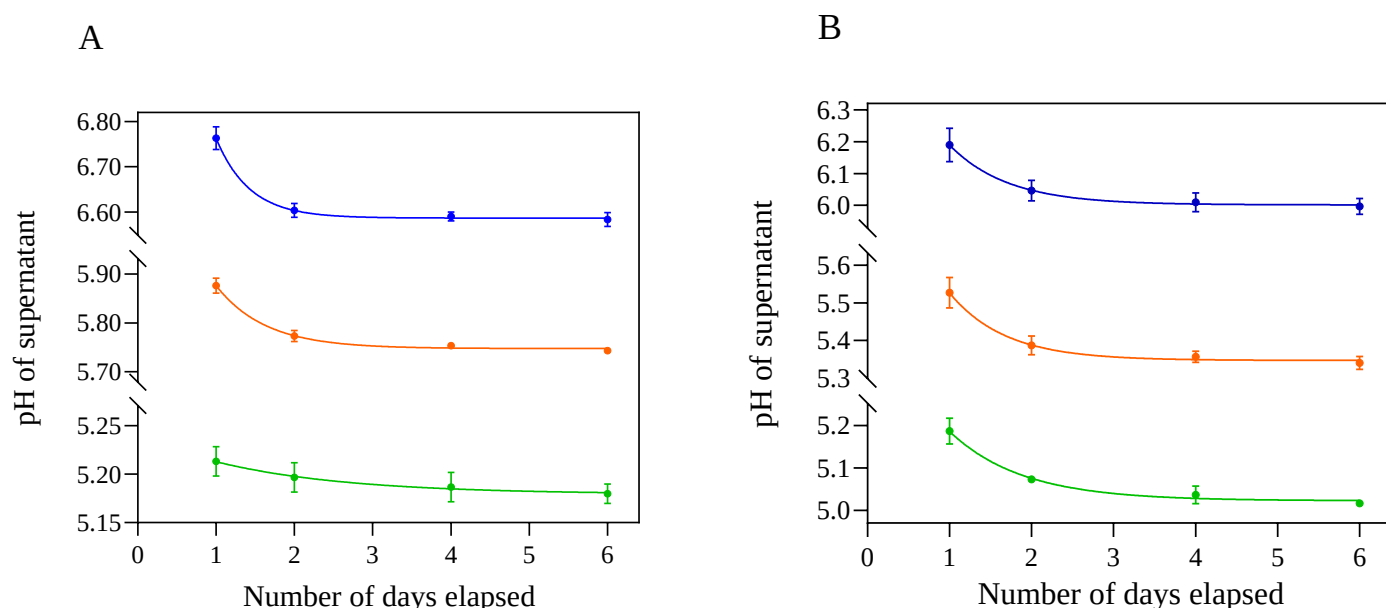


Figure 5.1. (A) For the CQ–ferrihaem complex, the variation of pH with the number of days elapsed for sample 1 (blue), sample 5 (orange) and sample 11 (green) as defined in **Table 2.5**, at 37°C. (B) For the QD–ferrihaem complex, the variation of pH with the number of days elapsed for sample 1 (blue), sample 5 (orange) and sample 11 (green) as defined in **Table 2.6**. The error bars represent one standard deviation computed over triplicate measurements.

5.3. Spectrophotometric titration experiments

For the CQ–ferrihaem complex, the titration curve from preliminary experiments (**Figure 5.2A**) exhibited two inflection points, probably indicating the consecutive protonation of the side chain tertiary nitrogen and quinolyl nitrogen of CQ with reported pK_a values of 10.18 and 8.38,¹⁶⁴ respectively, followed by protonation of the ferrihaem propionate groups. Meanwhile, for the QD–ferrihaem complex, preliminary titration curves (**Figure 5.2B**) showed a single inflection point, probably indicating the protonation of the side chain tertiary nitrogen of QD with a reported pK_a value of 8.58,¹⁶⁴ followed by protonation of the quinolyl nitrogen of QD having a reported pK_a value of 4.42¹⁶⁴ and the ferrihaem propionate groups. However, for either complex, it was impossible to discount the possible protonation of the Fe(III)PPIX axial H_2O/OH^- ligands as well.

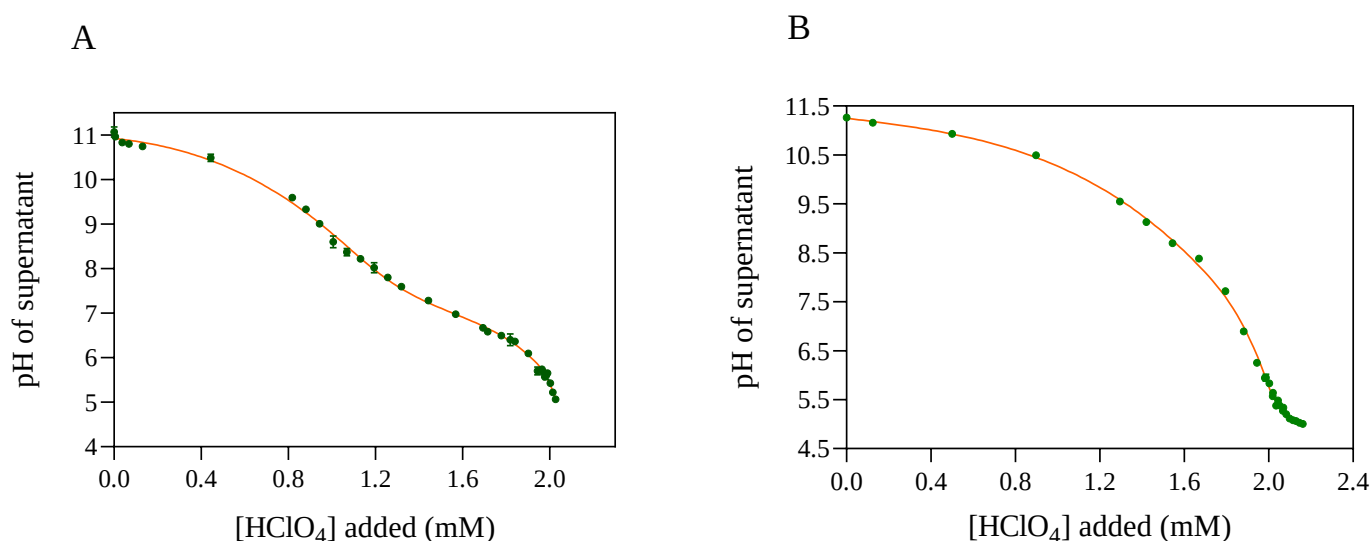


Figure 5.2. Titration curves for (A) the CQ–ferrihaem complex with $HClO_4$, and (B) the QD–ferrihaem complex with $HClO_4$.

Subsequently, spectrophotometric titration experiments were conducted in quadruplicate at 37°C using the refined sample set in **Table 2.5** as described in section 2.9 for the CQ–ferrihaem complex, and that in **Table 2.6** as detailed in section 2.11 for the QD–ferrihaem complex. Given the reported binding stoichiometries of 1:2 CQ:ferrihaem²⁰⁰ and 1:1 QD:ferrihaem,²¹⁶ a drug concentration exceeding that of haematin by more than tenfold was chosen to ensure the complexation of all the ferrihaem present in solution. Thus, for either complex, the

concentrations of haematin and drug in each sample were 22.5 μM and 300 μM , respectively. Each of CQ or QD alone is known to be UV inactive in the haem Soret band region,²¹⁷⁻²¹⁸ hence negating any potential effects on the spectra due to excess unreacted drug. Also, there were no significant differences (no red or blue shifts in the Soret or *Q* band positions) between the absorption spectra from the acid titration of haematin (**Figure 4.5A**) and those from the acid titration of either complex (**Figures 5.3** and **5.4**), implying formation of the same low-spin bis-pyridyl complex (**Figure 1.14**) in each case. The latter observations were not surprising since the pyridine-ferrihaemochrome assay has previously been reported to successfully detect protein-bound ferrihaem,²¹⁹ making it likely to displace coordinating substances such as drugs as well.

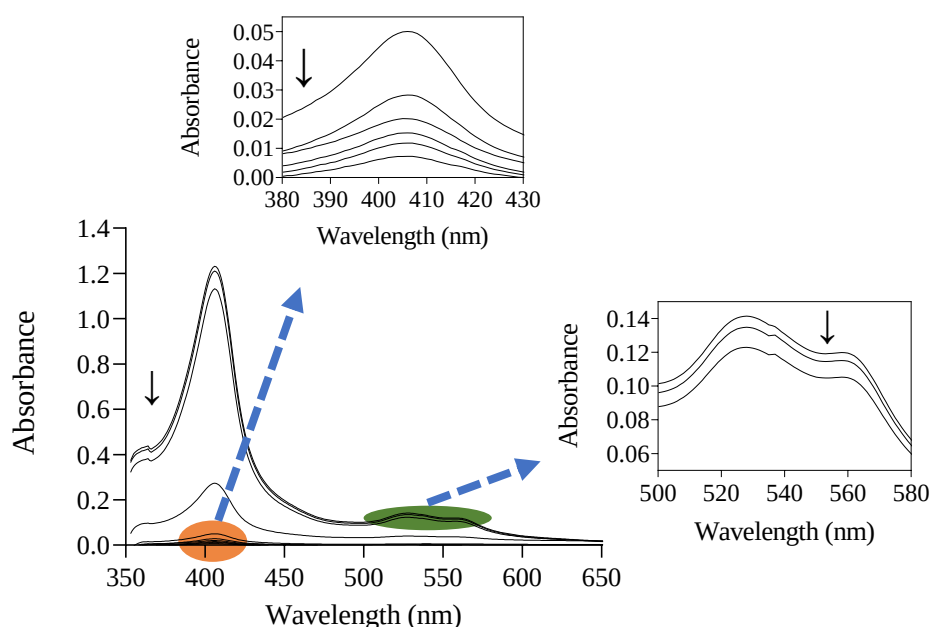


Figure 5.3. UV-vis spectra from the titration of the CQ-ferrihaem complex with HClO_4 combined with aqueous pyridine to quantify the solubility of the complex. The arrows show the direction of change in spectra with decreasing pH.

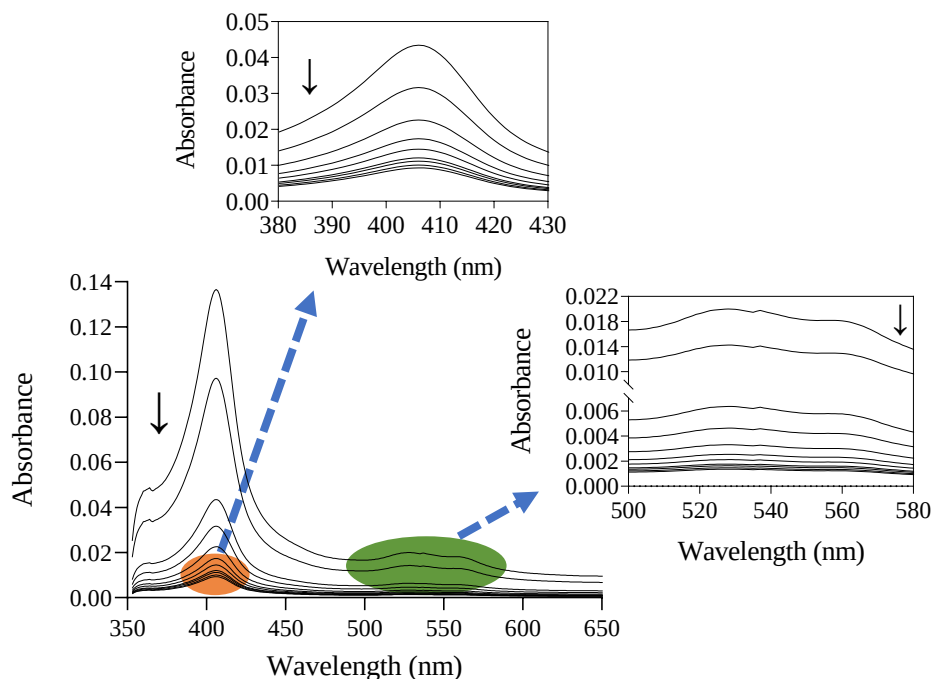


Figure 5.4. UV-vis spectra from the titration of the QD-ferrihaem complex with HClO_4 combined with aqueous pyridine to quantify the solubility of the complex. The arrows show the direction of change in spectra with decreasing pH.

Interestingly, an appreciable decrease in the concentrations of the complexes was observed as the pH was lowered from 6.4 to 5.8 for the CQ-ferrihaem complex (**Figure 5.5A**), and from 6.0 to 5.2 for the QD-ferrihaem complex (**Figure 5.5C**). A closer look at the data points close to pH 5.0 (**Figures 5.5B** and **5.5D**, green-filled circles) revealed a significant degree of scatter in the nanomolar range, hence the use of a linear regression to determine the solubility of either complex at pH 5.0 was not possible. Thus, as described in sections 2.9 and 2.11 for the CQ-ferrihaem and QD-ferrihaem complexes, respectively, an additional single point experiment was performed in an attempt to accurately determine the solubility of the complex at as close to a pH of 5.0 as possible, employing a 100-mm quartz cuvette for increased pathlength (10×) and therefore sensitivity in the recorded absorbances. However, it still proved difficult to accurately obtain a mean solubility value for the complex at pH 5.0 due to the pH readings across the samples varying from 5.01 to 5.08 (**Figures 5.5B** and **5.5D**, orange-filled triangles).

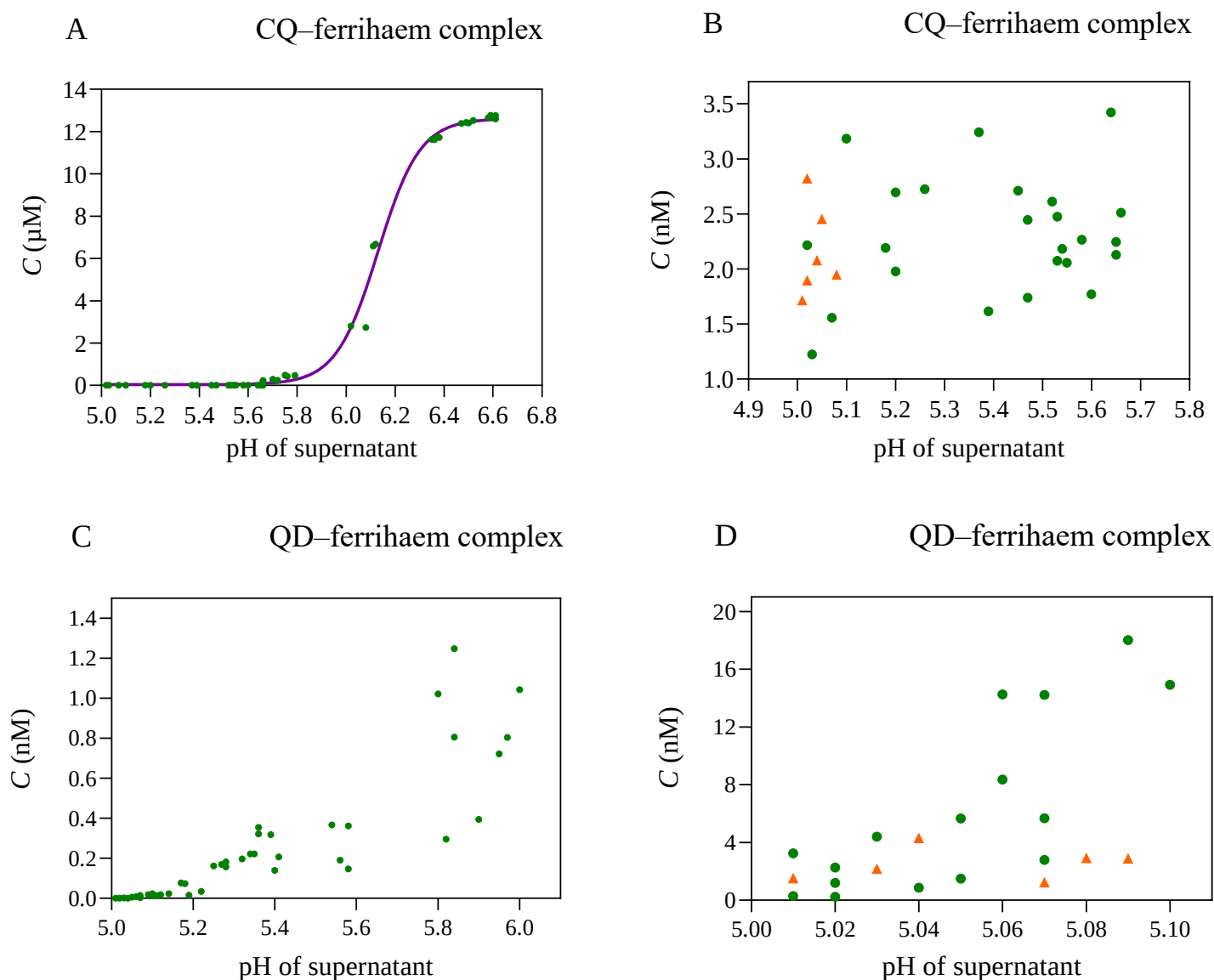


Figure 5.5. (A) The solubility of the CQ–ferrihaem complex in perchloric acid solution as a function of pH. (B) Emphasis on the lower pH region: Green-filled circles represent the data points from **Figure 5.5A** and orange-filled triangles represent the data points from the single point experiment performed in a cuvette with 10× pathlength. (C) The solubility of the QD–ferrihaem complex in perchloric acid solution as a function of pH. (D) Emphasis on the lower pH region: Green-filled circles represent the data points from **Figure 5.5C** and orange-filled triangles represent the data points from the single point experiment performed in a cuvette with 10× pathlength. Ferrihaem concentration is denoted by C .

A probable cause for the scatter in the observed data was posited to be the presence of significant amounts of unbound ferrihaem. Therefore, a mathematical analysis was performed to verify whether the concentration of CQ solution was sufficient to ensure maximum shifting

of the equilibrium towards formation of the CQ–ferrihaem complex with minimum unbound haematin. With haematin denoted by H and the binding constant represented by K ,

$$\begin{aligned} \text{CQ} + \text{H} &\rightleftharpoons \text{CQ-H} \\ K &= \frac{[\text{CQ-H}]}{[\text{CQ}] \cdot [\text{H}]} \\ \therefore [\text{CQ}]_{\text{free}} &= \frac{[\text{CQ-H}]}{[\text{H}]} \times \frac{1}{K} \end{aligned} \quad (5.1)$$

The reported value of $\log K$ for the complexation of CQ with Fe(III)PPIX is 5.52.¹⁵⁴ Assuming that 100% of the haem forms a complex with CQ, $[\text{CQ-H}] = 100$ and $[\text{H}] = 1$. Thus,

$$\frac{[\text{CQ-H}]}{[\text{H}]} = \frac{100}{1}$$

and substituting into equation 5.1 leads to

$$[\text{CQ}]_{\text{free}} = \frac{100}{1} \times \frac{1}{10^{5.52}} = 302.0 \mu\text{M}$$

A mass balance equation may be defined as follows:

$$[\text{CQ}]_{\text{total}} = [\text{CQ}]_{\text{free}} + [\text{CQ-H}]$$

Assuming that 100% of the haematin is bound, the following simplification may be made:

$$[\text{H}]_{\text{total}} = [\text{CQ-H}]$$

Substituting in the mass balance equation leads to $[\text{CQ}]_{\text{total}} = [\text{CQ}]_{\text{free}} + [\text{H}]_{\text{total}}$ where $[\text{H}]_{\text{total}}$ in each prepared sample was known to be equal to 22.5 μM from the experimental set-up. Hence, the concentration of CQ theoretically required to ensure complexation of all the haematin present in solution was $[\text{CQ}]_{\text{total}} = 302.0 + 22.5 = 324.5 \mu\text{M}$.

A similar mathematical analysis was performed to verify whether the concentration of QD solution was sufficient to ensure maximum shifting of the equilibrium towards formation of the QD–ferrihaem complex with minimum unbound haematin. Employing the reported value for $\log K$ of 5.02²²⁰ for the QD–ferrihaem complex, the concentration of QD theoretically required to ensure complexation of all the haematin present in solution was equal to 977.5 μM .

Consequently, as described in section 2.10, spectrophotometric titration experiments were performed again, differing only in the CQ concentration per sample of 324.5 μM compared to 300 μM in the previous experiments described in section 2.9. Also, as detailed in section 2.12,

the titration experiments with QD were repeated, differing only in the QD concentration per sample of 977.5 μM compared to 300 μM in the previous experiments described in section 2.11. Interestingly, as observed in **Figures 5.6A, 5.6B, 5.6C and 5.6D**, the increased drug concentration per sample did not seem to have any significant effect on the observed scatter in the spectrophotometric titration data for either drug-ferrihaem complex; it may therefore be deduced that the initial approach of using a CQ or QD concentration exceeding that of haematin by more than tenfold was sufficient to ensure maximum CQ-ferrihaem or QD-ferrihaem complex formation.

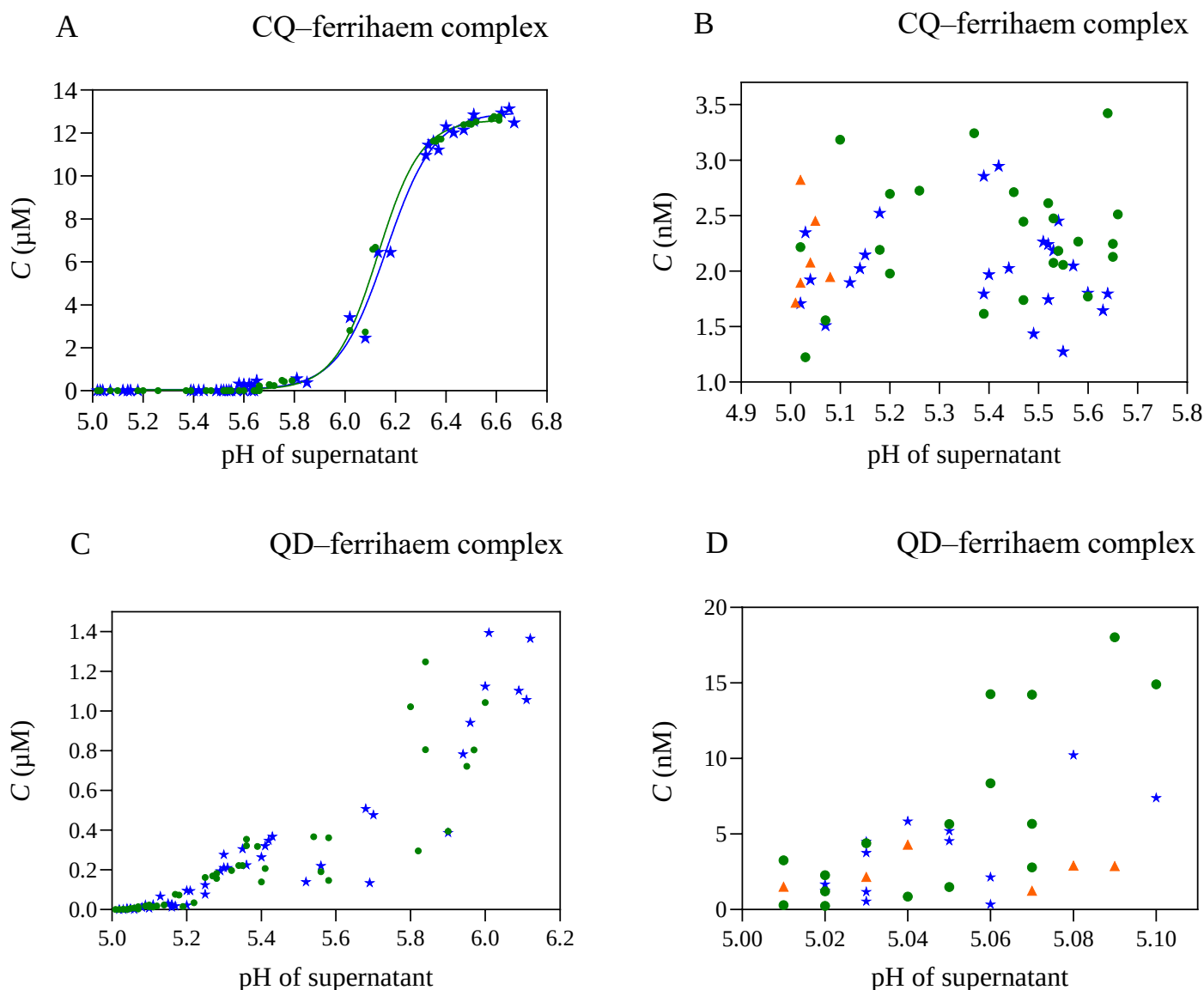


Figure 5.6. (A) Spectrophotometric titration data with the CQ concentration per sample of 300 μM (green-filled circles) and 324.5 μM (blue-filled stars). (B) Spectrophotometric titration data with the CQ concentration per sample of 324.5 μM (blue-filled stars) superimposed onto **Figure 5.5B**. (C) Spectrophotometric titration data with the QD concentration per sample of 300 μM (green-filled circles) and 977.5 μM (blue-filled stars). (D) Spectrophotometric titration data with the QD concentration per sample of 977.5 μM (blue-filled stars) superimposed onto **Figure 5.5D**.

However, as a result of non-success in diminishing scatter in the data points, it was deemed appropriate to report ranges of values that the true solubilities of the complexes at pH 5.0 would most likely fall within. For the CQ–ferrihaem complex, below an arbitrary cut-off pH value of 5.7, the Gaussian function fitted to the frequency distribution of the observed

concentration data (**Figure 5.7A**) yielded a 95% confidence interval of 1.9 – 2.2 nM. Analogously, the Gaussian function fitted to the frequency distribution of the observed concentration data for the QD–ferrihaem complex (**Figure 5.7B**) yielded a 95% confidence interval of 2.0 – 2.9 nM.

Hence, the solubility of the CQ–ferrihaem complex may be reported to lie within 1.0 nM and 3.5 nM (lower and upper bounds, respectively, from **Figure 5.6B**) with well-founded evidence of falling within 1.9 nM and 2.2 nM. However, given the 1:2 CQ:ferrihaem binding ratio in aqueous solution, it is more appropriate to report the solubility of the complex to lie within 0.5 nM and 1.8 nM, with well-founded evidence of falling within 1.0 nM and 1.1 nM. Meanwhile, the solubility of the QD–ferrihaem complex may be reported to lie within 0.2 nM and 18.0 nM (lower and upper bounds, respectively, from **Figure 5.6D**) with dependable evidence of falling within 2.0 nM and 2.9 nM.

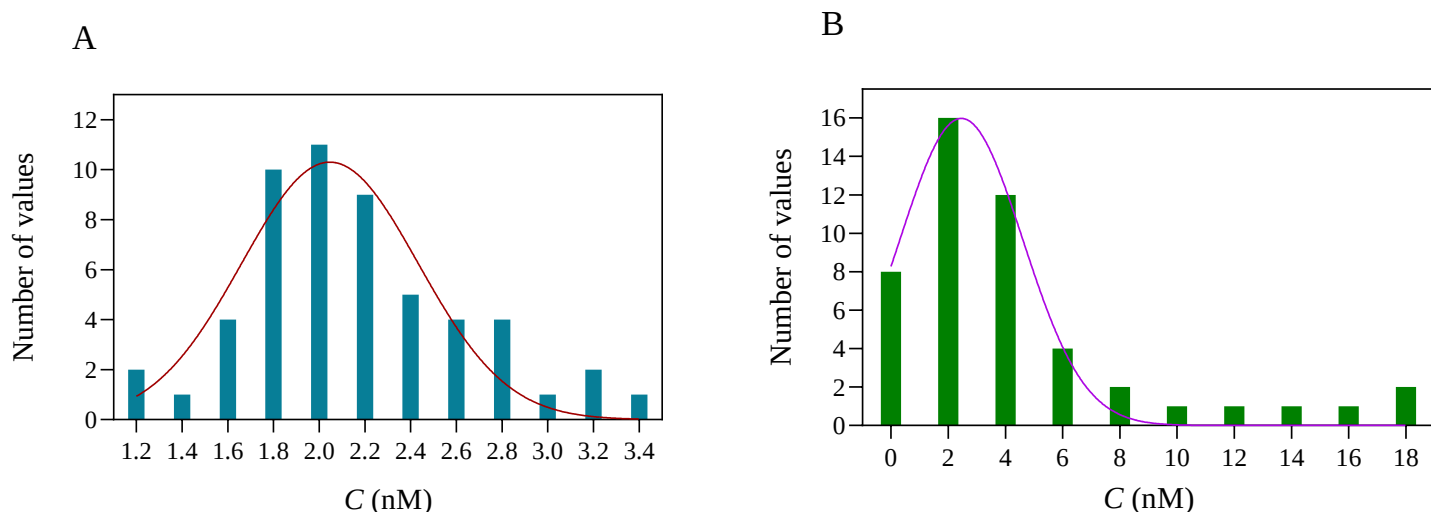


Figure 5.7. (A) Frequency distribution histogram (blue bars) for the solubility data of the CQ–ferrihaem complex from **Figure 5.6B**. The fitted Gaussian function (solid red curve) has mean and standard deviation values of 2.0 nM and 0.4 nM, respectively, with $R^2 = 0.84$. (B) Frequency distribution histogram (green bars) for the solubility data of the QD–ferrihaem complex from **Figure 5.6D**. The fitted Gaussian function (solid purple curve) has mean and standard deviation values of 2.5 nM and 2.1 nM, respectively, with $R^2 = 0.96$.

Unfortunately, for either complex, significant scatter was also observed in the $\log C$ vs. pH graph (**Figures 5.8A** and **5.8B**) due to which protonation equilibria of the CQ–ferrihaem and

QD–ferrihaem complexes with decreasing pH could not be ascertained. As a result, it was not possible to compute the solubility product constants for the complexes.

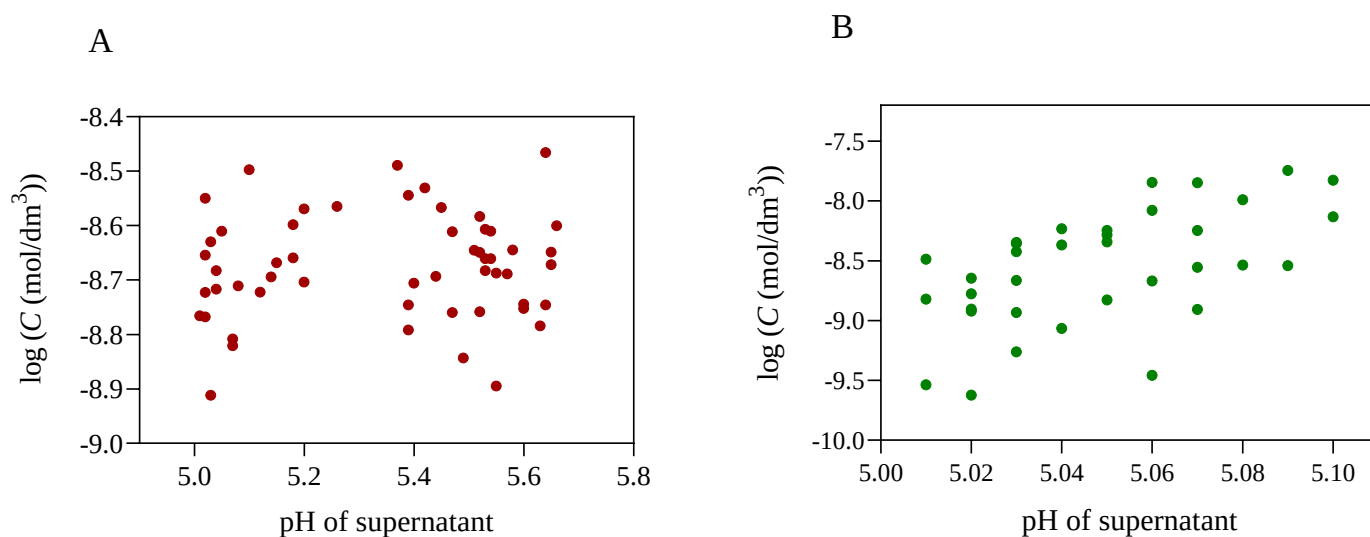


Figure 5.8. Log C vs. pH graph for (A) the spectrophotometric titration data for the CQ–ferrihaem complex from Figure 5.6B, and (B) the spectrophotometric titration data for the QD–ferrihaem complex from Figure 5.6D.

5.4. Conclusions

The pyridine–ferrihaemochrome assay employed in Chapter 4 was adapted in an attempt to determine the solubilities of the CQ–ferrihaem and QD–ferrihaem complexes at 37°C, at as close to the parasitic DV pH of 5.0^{71–72} as possible. Due to the capacity of pyridine to complex with both free ferrihaem and drug-bound ferrihaem, it was crucial to shift the reaction equilibria as far as possible towards complex formation. For both CQ and QD, a drug concentration exceeding that of haematin by more than tenfold per sample was observed to be sufficient for ensuring maximum complexation of the ferrihaem present in solution, thus minimum levels of free ferrihaem. However, accurate solubility data could not be derived due to observed Soret peak absorbances being appreciably lower than 0.1, indicating a high propensity for error; the resulting spectrophotometric titration data exhibited significant scatter due to the extremely low solubilities — down to the nanomolar range — of the complexes at pH values close to 5.0. Therefore, it was deemed appropriate to report ranges of values that the true solubility of the complexes would most likely fall within. For either

complex, a wider range was reported based on the lower and upper bounds of the observed spectrophotometric titration data close to a pH of 5.0, while a narrower range was derived from the 95% confidence interval of the Gaussian distribution fitted to the data: The solubility of the CQ-ferrihaem dimeric complex was reported to lie within 0.5 nM and 1.8 nM with evidence of falling within 1.0 nM and 1.1 nM, while that of the QD-ferrihaem complex was observed to lie within 0.2 nM and 18.0 nM with evidence of falling within 2.0 nM and 2.9 nM. These findings contest the hypothesis made by Ursos et al.¹⁰⁹ that drug-ferrihaem complexes are likely to be more soluble than ferrihaem itself to suppresses its biomineralisation into haemozoin.

As a further consequence of significant scatter in the observed spectrophotometric data, the solubility product constants for the CQ-ferrihaem and QD-ferrihaem complexes could not be computed, warranting further investigation into more sensitive methods for ferrihaem quantification.

CHAPTER 6

Overall Conclusions and Future Work

6.1. Conclusions

Despite obstacles to the treatment of malaria posed by the emergence of resistance, the quinoline-based antimalarials remain of significant interest as therapeutic agents used in combination therapy against *P. falciparum*. Chloroquine (CQ) — the archetypal 4-aminoquinoline drug — is part of the first-line treatment in the region of the Americas, while the *Cinchona* alkaloid quinine (QN) is used to treat severe cases in certain African countries.¹⁹ The inhibition of haemozoin biocrystallisation within the acidic digestive vacuole (DV) of the parasite is the established mode of action of these two classes of antimalarials; however, uncertainties over its mechanistic details have presented a serious stumbling block for many years. Recently, Kapishnikov et al.²⁰² exhibited the haemozoin crystal “capping” action by a CQ analogue intracellularly and hypothesised the formation of a drug–ferrihaem complex, whose existence was conclusively demonstrated intracellularly by Openshaw et al.²⁰³ In addition, formation of the aqueous drug–ferrihaem complex has been hypothesised by the latter authors to be the causative factor of the parasitocidal effects of various haemozoin inhibitors. Thus, the solubilities of ferrihaem itself and of the drug–ferrihaem complexes under conditions as relevant as possible to physiological conditions were identified as the key questions for this dissertation. In this work, CQ and QD were selected as representatives of the 4-aminoquinoline and quinoline methanol classes of antimalarials, respectively.

The solubility of haematin at the mean parasitic DV pH of 5.0⁷¹⁻⁷² was reported by Morrison and Williams²⁰⁴ in 1941, constituting the only known investigation of ferrihaem solubility. In pursuit of the first aim of this project which was to quantify ferrihaem solubility at the physiologically-relevant pH of the digestive vacuole, the primary course of action was to repeat

the experiment performed by these authors — involving the spectrophotometric titration of haematin with base — in order to verify their findings. The observed ferrihaem solubility value differed significantly from that obtained by Morrison and Williams²⁰⁴: 0.087 μM as reported by the latter *versus* $2.6 \pm 1.3 \mu\text{M}$ observed in this work for the ferrihaem dimer. Comparatively speaking, the observed solubility data in this work present a more reliable set of results than that reported by Morrison and Williams. However, despite a seemingly low degree of scatter in the observed spectrophotometric titration data, reliability and reproducibility of the results were reduced by erratic pH values in the lower pH region examined. As a result, the accurate determination of dependable thermodynamic data for ferrihaem solubility was not possible. The principal shortcoming of the methodology was the observation of data points corresponding to pH values far from the mean DV pH of 5.0, emphasising the need for a more suitable assay.

Consequently, an acid spectrophotometric titration methodology was adopted. To avoid difficulties in spectroscopic analysis at low pH values, the pyridine-ferrihaemochrome method was used to quantify the concentrations of free ferrihaem, yielding the solubility for the ferrihaem dimer at 37°C to be equal to $0.24 \pm 0.08 \mu\text{M}$ at pH 5.0, with the solubility product constant for the dimer computed to be $(1.9 \pm 0.4) \times 10^{-42}$. Having probed the relevant pH range as close to a pH of 5.0 as possible, the data suggested that the acid titration coupled with the pyridine-ferrihaemochrome assay yielded more accurate and reliable information on the solubility of ferrihaem than the base titration experiments performed in this work as well as those conducted by Morrison and Williams.²⁰⁴

Subsequently, a similar methodology was employed to determine the solubilities of the CQ-ferrihaem and QD-ferrihaem complexes. A mathematical approach was employed to estimate the minimum initial drug concentrations required for maximum complexation of all ferrihaem present. However, the solubility of either complex was observed to be less than that of ferrihaem determined in this work, probably by multiple orders of magnitude. This was manifested in the form of consistent scattering in the spectrophotometric titration data at pH values close to 5.0, due to which mean solubility values for the complexes could not be computed. Instead, *ranges* of values were reported within which the true solubility of the complexes would most likely lie. 95% confidence intervals were reported for the solubilities of either complex at 37°C: 1.0 – 1.1 nM for the CQ-ferrihaem dimeric complex, and 2.0 – 2.9 nM for the QD-ferrihaem complex. Thus, the sensitivity limitations of the pyridine-based spectrophotometric assay at concentrations of the nanomolar range were accentuated.

The formation of drug-ferrihaem complexes is a key piece of the bigger jigsaw puzzle that is the haemozoin inhibition pathway. The parasite killing activity of an antimalarial drug is likely

to depend on the concentration of its drug–ferrihaem complex in the DV, which in turn hinges on ferrihaem solubility; the concentration of ferrihaem is itself influenced by the potency of the drug to inhibit the formation of haemozoin, and thus, by the rate of drug accumulation in the DV. This accumulation also determines the position of equilibrium for the haem–drug complex formation. Indeed, it is a complex process comprising an interplay of various factors. Taken together, the work presented in this study has supplied the first quantitative insight for physiologically-relevant solubility data which will further equip an *in silico* model towards the investigation of the modes of action of important antimalarials (Chapter 1, **Figure 1.18**).

6.2. Future work

To build on the current study, the determination of ferrihaem concentrations *via* the pyridine-based acid titration methodology could be performed at a set of different temperatures, at which the solubility product constants (K_{sp}) could be determined. By fitting these in the Van ‘t Hoff equation,

$$\ln K^0 = - \left(\frac{\Delta H^0}{R} \right) \cdot \left(\frac{1}{T} \right) + \left(\frac{\Delta S^0}{R} \right),$$

reliable and reproducible thermodynamic parameters for ferrihaem solubility could be obtained which would enhance our understanding of the complex behaviour of ferrihaem in solution, and also constitute important additions to the haemozoin inhibition model. In addition, it also remains to be verified whether the solubility data obtained from cell-free systems remain valid under intracellular conditions. As described in section 1.6 of Chapter 1, recent research has diverted the focus of the site of haemozoin nucleation and its inhibition by quinoline drugs from an aqueous to a non-polar, predominantly neutral lipid microenvironment which reduces the activation energy needed for efficient haemozoin biocrystallisation.⁹³ In 2010, an optimised and validated detergent-based assay was reported in which the neutral and non-denaturing detergent NP-40 was found to be an ideal mimic of the amphiphilic lipids. This assay has since become the cornerstone of various high-throughput screening studies for β -haematin inhibition in order to identify new antimalarial chemotypes.²²¹⁻²²² Therefore, to achieve conditions as relevant to intracellular ones as possible, spectrophotometric titration experiments to determine the solubilities of ferrihaem and drug–ferrihaem complexes could be performed in aqueous NP-40-based and neutral lipid-based media.

Over the course of this study, caution was required while handling pyridine solutions due to their reported toxicity, especially in case of inhalation of emanating volatile fumes.²²³ In 2014, Kuter et al. reported the formation of a spectroscopically distinct, low-spin bis-imidazole complex between ferrihaem and the aromatic heterocycle imidazole, with an intense Soret peak at 411 nm.²⁰⁰ Furthermore, the reported pH-independent imidazole–ferrihaem association constant (2.17 ± 0.05) was higher than that for the pyridine–ferrihaem complex (0.77 ± 0.05).²²⁴ The validity of an intracellular imidazole-based HPLC–UV-Vis assay coupled with haem fractionation to assess the activity of antimalarials is under investigation in ongoing studies, including analysis of the interactions of imidazole with ferrihaem/ β -haematin mixtures. If validated, imidazole would constitute a less toxic alternative to pyridine in such an assay, which would be the preferred route for future studies on the solubilities of ferrihaem and drug–ferrihaem complexes.

The pyridine–ferrihaemochrome assay is the most common method of ferrihaem detection and quantification. However, in 2006, an intriguing enzymatic assay for ferrihaem quantification was developed by Masuda and Takahashi²²⁵ which has been relatively overlooked: The fundament of the assay is the spontaneous reconstituting of horseradish peroxidase apo-enzyme with ferrihaem to form an active holo-enzyme which catalyses the degradation of hydrogen peroxide to form two hydroxyl radicals. The latter, in turn, react with luminol to yield aminophthalic acid in a reaction which exhibits luminescence at 428 nm. Working back to determine ferrihaem concentration, the luminescent activity varies linearly with the rate of catalytic hydrogen peroxide breakdown, which is in turn proportional to the concentration of reconstituted holo-enzyme; in excess HRP apo-enzyme, the concentration of reconstituted enzyme equals that of the initial ferrihaem. The high sensitivity of this assay — demonstrated by its capacity to measure ferrihaem levels as low as 100 pM²²⁶ — emphasises its untapped potential as a stellar technique to complement the established pyridine–ferrihaemochrome assay in the determination of the solubilities of ferrihaem and the drug–ferrihaem complexes.

The notable drawback of the spectrophotometric titration methodology was the reduced quantification sensitivity of the assay at extremely low ferrihaem concentrations. This was particularly conspicuous during the determination of the solubility of the CQ–ferrihaem and QD–ferrihaem complexes, observed to reach as low as the single-digit nanomolar range and causing consistent scatter in the spectrophotometric titration data. Consequently, more sensitive quantification techniques could be explored: Exploiting the presence of iron in ferrihaem, a strong option would be inductively coupled plasma mass spectrometry (ICP-MS), a highly selective and sensitive analytical technique boasting a diverse range of applications, such as the detection of inorganic contaminants in pharmaceuticals and toxicology analysis.²²⁷

The approximate lower detection limit for iron quantification using ICP-MS has been reported to be 0.05 nM.²²⁸ This technique allows the simultaneous detection of multiple elements at ultra-trace levels: Following the nebulisation of liquid samples, the resulting fine aerosol is ionised by high-temperature argon plasma. A quadrupole mass filter separates the ions by their mass-to-charge ratios, which are quantitatively detected through mass spectrometry.²²⁷ Indeed, capitalising on this technique to investigate ferrihaem solubility could alleviate the issue of scattering in the data at pH values close to 5.0, thus yielding accurate and reliable solubility data for ferrihaem and drug–ferrihaem complexes of CQ and QD.

As detailed in section 1.9 of Chapter 1, the drug–ferrihaem complex is likely to be more toxic to *P. falciparum* compared to free ferrihaem by itself. The aforementioned suggestions for future work could be applied to investigate the drug–ferrihaem solubilities for CQ and QD, as well as for a larger set of antimalarials that may include the following: (i) the 4-aminoquinoline drug piperazine which makes up the artemisinin-based combination therapy of dihydroartemisinin–piperazine (DHA–PPQ); (ii) another 4-aminoquinoline drug, pyronaridine, which constitutes the artesunate–pyronaridine (AS–PY) combinatorial therapy; and (iii) the 4-aminoquinoline compound amodiaquine which forms the artesunate–amodiaquine (AS–AQ) therapy, all of which are first-line treatments against *P. falciparum* in Africa.¹⁶ In addition, the solubilities of the drug–ferrihaem complexes of the *Cinchona* alkaloids quinine, cinchonine and cinchonidine could also be determined (See **Figure 1.13** for molecular structures of the antimalarials mentioned). Such a diverse set of solubility data would indeed enrich the proposed haemozoin detoxification model (Chapter 1, **Figure 1.18**) and would allow for better predictions in target deconvolution of novel antimalarials.

In light of the revived recent interest in the activity of drug–ferrihaem complexes,¹⁷⁴ this work has contributed useful knowledge towards explicating their modes of action. A thorough understanding of the haemozoin inhibition pathway remains elusive. However, starting with the premise that relevant solubility data can resolve many mechanistic details, this study has re-emphasised the value of spectrophotometry in such investigations: It contributes a small, albeit useful piece, in hope of bringing big change towards eradication of the deadly disease of malaria.

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