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Evaluation of the *in vitro* antiplasmodial and toxicity profiles of novel drug delivery formulations for combination therapies

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Submitted in partial fulfilment of the degree:

Magister Scientiae

Biochemistry

Faculty of Natural and Agricultural Sciences
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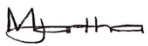
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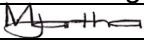
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“Great is thy faithfulness, O God, my Father;
There is no shadow of turning with thee.
Thou changest not, thy compassions, they fail not;
As thou hast been, thou forever wilt be.
Great is thy faithfulness,
Great is thy faithfulness,
Morning by morning new mercies I see.
All I have needed thy hand hast provided;
Great is thy faithfulness,
Lord unto me.”
- Thomas O. Chisholm, *Great Is Thy Faithfulness*

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Dedicated to God, mom, dad, Mimi, Joey, Queeny, Kobe, and Michael. We did it.

Summary

While considerable progress has been made towards malaria elimination over the past several decades, it continues to inflict a heavy burden on socio-economically challenged countries. Artemisinin-based combination therapies, including a fast-acting artemisinin component such as dihydroartemisinin (DHA), have been highly effective in slowing down the rate of drug resistance development and remain the mainstay of control against the disease. However, the complexity of the *P. falciparum* life cycle combined with antimalarial resistance development has necessitated the development of alternative therapeutic options. As malaria control interventions move toward disease elimination, the focus has shifted towards developing compounds targeting transmissible, gametocyte stages, which remain in the human host long after the asexual stages are cleared. While the clinical candidate MMV390048 exhibits multistage antimalarial activity, inefficient pharmacokinetic and pharmacodynamic properties, poor patient compliance, and the requirement for long-term drug administration pose a challenge for developing dual-stage therapies incorporating this compound.

Here, the encapsulation of MMV390048 with the known clinically active DHA in a slow release, bio-functional 3D polymer drug delivery system using *P. falciparum* as a model organism, is interrogated for its ability to deliver novel combinations with therapeutic applications. This drug delivery system uses DHA- and MMV390048-loaded SLNs. SLNs can directly release DHA and MMV390048, ensuring an antimalarial therapeutic effect. The *in vitro* inhibitory activity of a novel, dual active drug combination, DHA and MMV390048, was evaluated against asexual blood stages and transmissible gametocyte stages. Evidence for the combinatorial treatment of asexual and gametocyte stages with DHA and MMV390048 is provided. This revealed that late-stage gametocytes are susceptible to inhibition by the combination, an attractive point for transmission-blocking treatment geared towards elimination. DHA- and MMV390048-loaded SLNs did not retain antimalarial activity compared to the unencapsulated drugs against asexual stages, with excessive haemolytic effects and cytotoxicity. Ultimately, this project presents informative and preliminary evidence of polymer drug delivery systems for administering antimalarials. This strategy may contribute to improved pharmacokinetic and pharmacodynamic properties of transmission-blocking drugs and eventual malaria elimination.

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Abbreviations

ACT	Artemisinin-based combination therapies
ATS	Artesunate
CQ	Chloroquine
DDS	Drug delivery system
DHA	Dihydroartemisinin
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EDC	Extraerythrocytic developmental cycle
EDTA	Ethylene dinitrotetraacetic acid
FIC	Fractional inhibition concentration
FIND	Foundation for Innovative New Diagnostics
GSK	GlaxoSmithKline
GTS	Global Technical Strategy
IDC	Intraerythrocytic developmental cycle
IRS	Indoor Residual Spraying
ITN	Insecticide-Treated Nets
LDH	Lactate dehydrogenase
LLIN	Long-lasting insecticidal nets
MB	Methylene blue
MMV	Medicines for Malaria Venture
MoA	Mode of action
NAG	N-acetylglucosamine
NP	Nanoparticle
NPP	New permeability pathways
PCB	Polycarboxybetaines
PCR	Polymerase Chain Reaction
PD	Pharmacodynamic
<i>Pf</i> CRT	<i>P. falciparum</i> chloroquine-resistance transporter
PK	Pharmacokinetic
PLA	Polylactic acid
PPM	Parasite plasma membrane
PSB	Polysufobetaines
PVM	Parasite vacuolar membrane
QN	Quinine
RDT	Rapid diagnostic test

SLN	Solid lipid nanoparticle
SMA	Styrene-alt-maleic acid
TCP	Target Candidate Profile
WHO	World Health Organisation
WRAIR	Walter Reed Army Institute of Research

1. Introduction

1.1. Global trends in malaria disease burden

In 2022, the World Health Organisation (WHO) estimated 249 million cases and 608 000 deaths in 85 malaria endemic countries [1]. Globally, the WHO African region accounted for about 94 % of cases and 95 % deaths [1, 2]. Malaria exerts a severe socioeconomical impact on these countries, exacerbating poverty and impacting investment, healthcare, labour productivity and trade [2, 3]. Despite this, decreases in malaria case incidence and deaths were reported between 2000 and 2019. The global incidence of malaria decreased by 36 % while deaths due to malaria decreased by 63 % with the most pronounced decreases experienced in the WHO African region [2, 4, 5]. However, a 10 % increase in mortality was reported in 2020 compared to 2019, consequent to resistance towards antimalarial drugs. Additionally, several disruptions to essential malaria services because of the COVID-19 pandemic and overburdened health services in malaria endemic countries contributed to the observed increase in mortality [1]. Disease misdiagnosis and increased observation of antimalarial drug resistance contributed to an increase in malaria incidence and deaths in the 2019-2020 period, further compounding the severe socioeconomic impact of this disease [6].

1.1.1. Causative agent

Malaria is a vector-borne disease caused by protozoan parasites of the genus *Plasmodium* transmitted to humans by female *Anopheles* mosquitoes [7-9]. There are over 200 known *Plasmodium* species, but only six can cause malaria in humans: *P. falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium knowlesi* and *Plasmodium cynomolgi*. *P. knowlesi* and *P. cynomolgi* primarily cause malaria in monkeys, opportunistically infecting humans, while the other four are specific to humans [10]. Out of all the species, *P. falciparum* poses the greatest threat to public health globally, is associated with severe illness and accounts for over 90 % of malaria deaths [1, 11].

The WHO African region carries a more significant global malaria burden than any other malaria endemic region, and *P. falciparum* almost exclusively accounts for this burden (Figure 1.1) [1, 12]. In the countries with high transmission in central, east, and southern Africa, nearly 100 % of the burden is caused by *P. falciparum*. Thus, it is necessary to understand the intricate biology governing *P. falciparum* pathogenesis and transmission.

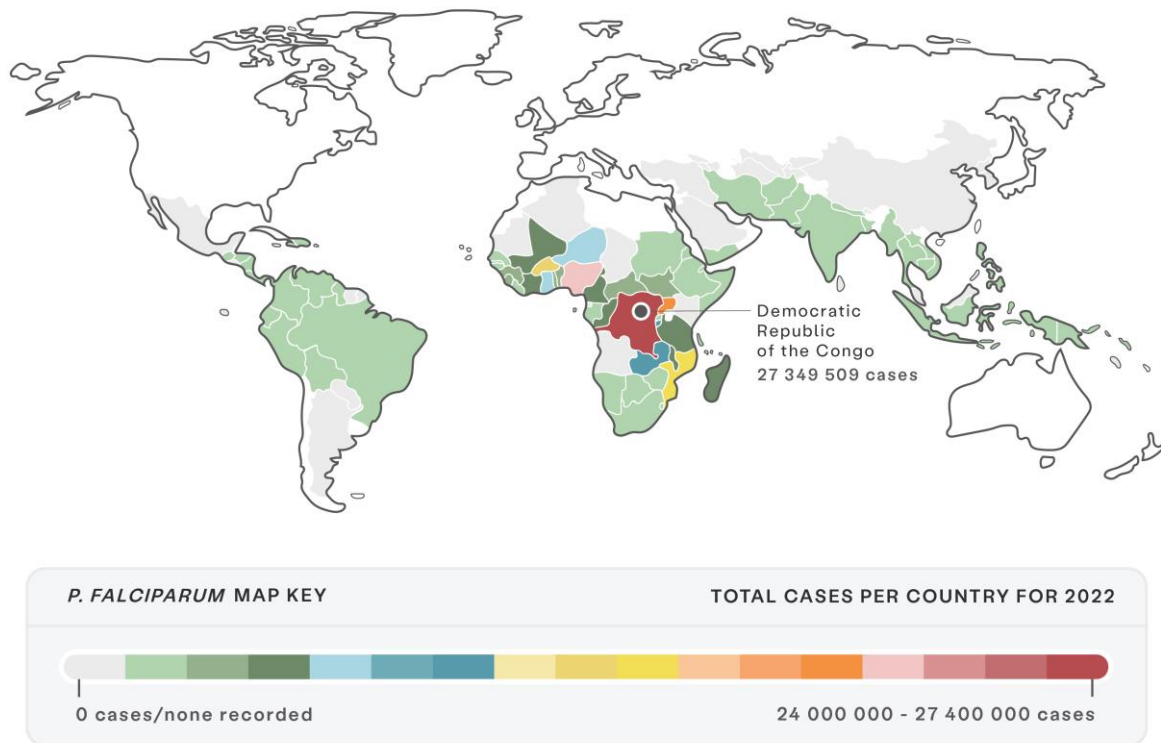


Figure 1.1. *P. falciparum* case incidence in high transmission countries as in 2022. The number of *P. falciparum* cases is indicated on the scale bar, where colour intensity, as shown on the map, is proportional to the increase in cases. The Democratic Republic of the Congo reported the highest case incidences (shown in red), with the WHO African region being the most severely affected. The figure was re-created and adapted from the WHO World Malaria Report 2023 using Adobe Illustrator version 28.5.

1.2. *Plasmodium falciparum* life cycle

Malaria pathogenesis can present as asymptomatic, uncomplicated, or severe malaria. Patients affected with asymptomatic malaria exhibit no symptoms; patients with uncomplicated malaria exhibit symptoms ranging from common fever to anaemia for 7 to 10 days after initial contact with the mosquito [13, 14]. When uncomplicated malaria is left untreated it can develop into severe malaria, which is characterised as cerebral malaria. Cerebral malaria causes the blockage of the small blood vessels within the brain with the remnants of burst erythrocytes, due to erythrocyte lysis after asexual reproduction. Although it can be caused by all *Plasmodium* species, this severe form of malaria is most frequently caused by *P. falciparum* and includes symptoms such as organ failure and severe anaemia [15].

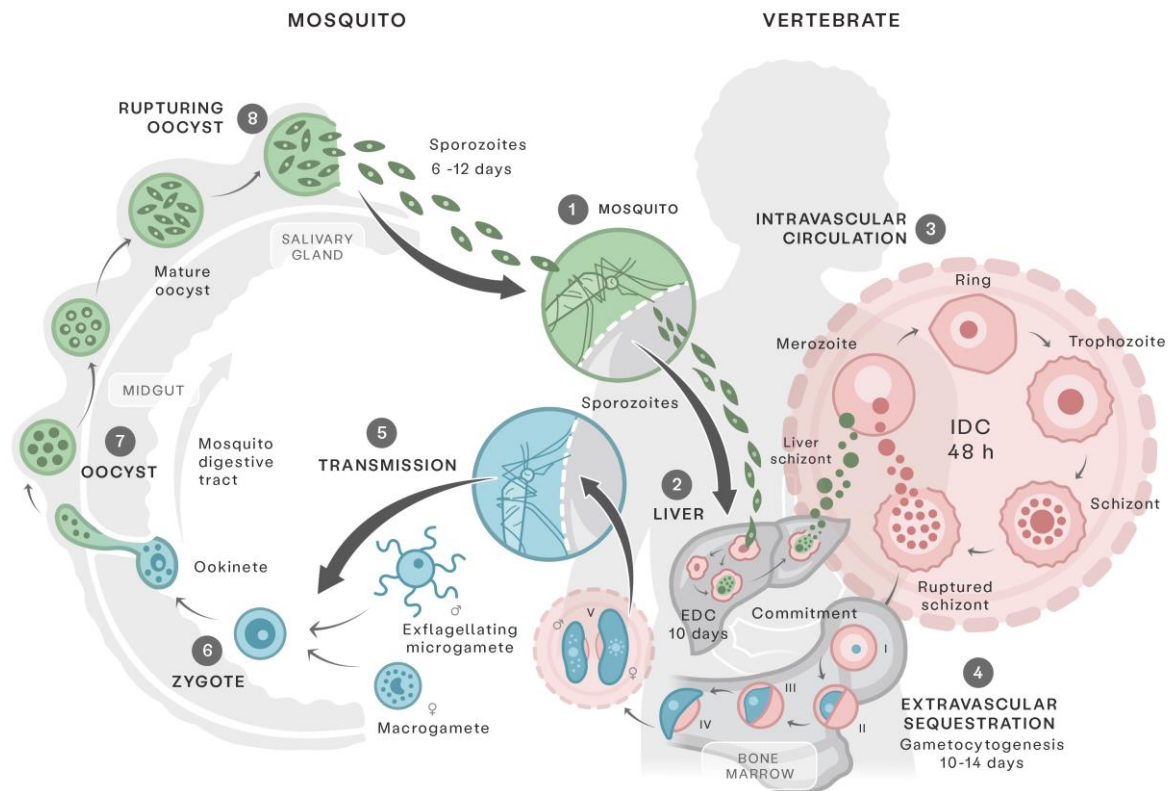


Figure 1.2. The life cycle of *P. falciparum*. The progression of the life cycle is mediated between the sexual developmental stages in an *Anopheles* mosquito and the asexual replicative stages in a vertebrate host. (1) The life cycle begins when sporozoites from the mosquito infect the host during a blood meal. (2) The sporozoites invade hepatocytes and develop into hepatic schizonts, establishing the extraerythrocytic developmental cycle (EDC) that lasts for 10 days. The hepatocytes rupture releasing merozoites into circulation to infect erythrocytes, thus initiating the intraerythrocytic developmental cycle (IDC). (3) This cycle is characterised by the development of ring-stage parasites into trophozoite and schizonts stages over a 48 h period. Upon schizont rupture, subsequent merozoites are released and can invade new erythrocytes effectively reinitiating the IDC. (4) A proportion of merozoites are sexually committed and undergo gametocytogenesis in the bone marrow for 10-14 days to produce mature stage V male and female gametocytes for (5) transmission to the mosquito during a blood meal. Within the digestive tract of the mosquito, gametocytes differentiate into gametes. (6) Following this, the gametes fuse to form a zygote, which becomes a motile ookinete that penetrates the midgut and (7) develops into an oocyst, which in turn (8) ruptures to release sporozoites for over 6-12 days. The sporozoites migrate to the salivary glands of the mosquito for transmission to the host, thereby reinitiating the complete *P. falciparum* life cycle. The figure was created using Adobe Illustrator version 28.5.

The life cycle of *P. falciparum* parasites (Figure 1.2) is a complex process composed of multiple stages, transmitted between the human host and the *Anopheles* vector [16]. There are over 20 recognised *Anopheles* species, many of which are vectors. The major vectors implicated in malaria transmission in the WHO African region are *Anopheles arabiensis*, *Anopheles funestus*, and *Anopheles gambiae* [1]. In South Africa and neighbouring countries, *A. funestus* is the major vector implicated in malaria transmission [17].

The life cycle of the *P. falciparum* parasite begins with a female *Anopheles* mosquito releasing sporozoites from the salivary glands into the host upon taking a blood meal. The sporozoites travel to the liver where they invade the human host's hepatocytes [18]. Here, they undergo hepatic schizogony of over 6 days to produce daughter cells, termed merozoites. This process does not cause clinical symptoms and is known as the pre-erythrocytic stage [19].

Hepatic schizonts rupture to release merozoites into circulation and invade erythrocytes, initiating the asexual intraerythrocytic stage [16, 18, 19]. During this stage, the merozoites develop into ring-stage parasites, followed by trophozoites, which then mature into multinucleated schizonts. These multinucleated schizonts consist of 8-24 daughter merozoites that invade new erythrocytes following schizont rupture. This asexual replication process leads to the clinical symptoms of malaria [20].

A proportion of asexual parasites differentiate into female (macrogametocytes) and male gametocytes (microgametocytes). The gametocytes progress through five intraerythrocytic developmental stages sequestering in the host bone marrow, forming mature female and male gametocytes [18]. These are taken up by an *Anopheles* mosquito taking a blood meal. In the mosquito gut, mature gametocytes are induced to undergo gametogenesis, forming male microgametes and female macrogametes. This initiates the sporogonic stage, initiating fertilisation in the mosquito midgut [18].

Fertilisation produces zygotes that mature into ookinetes and penetrate the gut wall of the mosquito midgut and develop into a cyst-like body called the oocyst, where the production and maturation of multiple sporozoites occurs [16, 20]. After rupture of the oocyst, the sporozoites migrate to the mosquito's salivary glands to initiate infection to a new host during a subsequent blood meal by the mosquito [20].

To date, only the replicative asexual stages of *Plasmodium* parasites have been successfully targeted using antimalarial drugs. Due to the complex life cycle, conventional antimalarial drugs only target pathogenesis and no effective tools for transmission-blocking are known. Further, the development of drugs that target both these stages is also pharmacokinetically challenging. This highlights the unmet medical need associated with antimalarial chemotherapies for malaria elimination strategies.

1.3. Malaria elimination and eradication strategies

The WHO Global Technical Strategy (GTS) for malaria was established to help malaria endemic countries reduce malaria case incidence and deaths by at least 90 % by 2030 [2]. Some countries in the WHO African region, such as Cabo Verde met the GTS 2020 target, but the majority of countries including South Africa are set to meet the GTS 2025 target [1]. However, in 2022, the GTS targets were not achieved globally and the WHO African region was off track for malaria morbidity and mortality by 52 % and 50 %, respectively [1]. Thus, malaria elimination relies on the application of various control measures that target the vector and the parasite.

Vector control aims to prevent parasite transmission by reducing contact between the human host and infected *Anopheles* mosquitoes. Two main vector control interventions are recommended by the WHO: insecticide-treated nets (ITNs) and indoor residual spraying (IRS) [21]. Parasite control aims to prevent or treat malaria by targeting different stages of the malaria parasite, either in the asexual or sexual

stages. Antimalarial drugs have been the mainstay of parasite control; however, vaccine development is gaining traction as a complementary intervention strategy [1, 22].

1.3.1. Vector control

ITNs are impregnated with pyrethroids, and their usage reduced the incidence of malaria cases in Africa by over 50 %. Pyrethroid-treated long-lasting insecticidal nets (LLINs) are the most widely used vector control and provide a physical barrier between mosquitoes and humans [5, 23]. However, insecticide resistance, operational and behavioural constraints, cost, and durability are listed as factors that prevent the effectiveness and life span of ITNs [1, 24, 25].

IRS operates by killing the infected *Anopheles* resting within the habitation or by repelling them before they feed on the human [26]. However, insecticide resistance, timing of spraying, quality of spray, costs and acceptance within communities are factors that impair the effectiveness of IRS, especially in the WHO African region [27, 28].

Although vector control has historically been the most effective measure against malaria transmission and has contributed to the decline in malaria transmission, reduction in the number of cases has stalled and the limitations threaten the effectiveness of vector control as a strategy for eliminating malaria [29].

To address the setbacks caused by insecticide resistance, alternative methods for vector control are required and are under investigation, such as the destruction of breeding areas, which is associated with significant environmental impacts. Endectocides, compounds that can be ingested by the human host to target infected *Anopheles* mosquitoes during a blood meal, are another recourse under investigation [26, 29]. However, integration of vector and parasite control is required.

1.3.2. Diagnostic tools

To develop treatment regimens, researchers need to detect parasites using accurate, sensitive, and practical diagnostics. The three main techniques used in malaria diagnostic tests are: microscopic examination, rapid diagnostic tests (RDTs) and polymerase chain reaction (PCR) tests [30].

Microscopic examination entails laboratory confirmation of malaria whereby a blood specimen is collected and examined to detect the presence of parasites infecting the erythrocytes [31] when stained with a nuclear stain such as Giemsa. This tool is relatively simple and allows for the identification of parasite species, development stages, and the number of infected erythrocytes. However, it is time-consuming, requires specific skills that are not readily available in areas with severe malaria, and ensuring accurate diagnosis can be challenging [32].

An alternative to microscopic examination is the use of RDTs that detect specific parasitic antigens found in the patient's blood. This method captures the malaria antigen by using monoclonal antibodies containing selenium dye. The sample containing the malaria antigen migrates across a nitrocellulose membrane to bind a second immobilised monoclonal antibody [33]. This tool resolves the long delay associated with microscopic examination and rapidly determines whether the patient is infected with malaria. However, RDTs cannot provide additional information such as the species and developmental stages of the parasite. RDTs were reported to provide false-positive or false-negative results [31, 34]. PCR-based tests are laboratory bound tests used to identify individual species through nucleic acid amplification, but this tool does not identify the developmental stages of parasites. PCR-based tests are more sensitive than microscopic examinations and RDTs and are used to confirm the parasite species. Despite the advantages, PCR tests are more expensive and difficult to perform in field-based settings, hence requiring trained staff [31, 35].

Early disease detection and accurate disease diagnosis is an important intervention linked with disease surveillance to enable rapid disease management.

1.3.3. Vaccines

Malaria vaccines aim to prevent malaria onset/infection completely by providing host immunity [36]. They are classified into three types depending on what stage of the life cycle is targeted: pre-erythrocytic, blood stage, and transmission-blocking vaccines. Their mode of action (MoA) is to elicit antibodies that can neutralise merozoites by blocking their invasion of erythrocytes and eventually minimise transmission in populations [37].

Despite significant investment, the development of effective malaria vaccines has proven challenging. The most promising vaccine candidate is RTS, S/AS01, sold as Mosquirix, a pre-erythrocytic vaccine created in 1987 through a collaboration between GlaxoSmithKline (GSK) and the Walter Reed Army Institute of Research (WRAIR). This vaccine reached phase III clinical trials where it showed only 36.3 % efficacy with no long-term prevention [22]. RTS, S/AS01 proved most beneficial in areas with high malaria transmission, including a reduction in malaria cases, overall hospital admissions, and the need for blood transfusions. A new R21/Matrix-M (R21) malaria vaccine has entered clinical trials in 2024 and promises more efficacy.

There is a growing interest in finding vaccines that can combine multiple targets in the life cycle of the parasite [36, 37]. However, due to the complexity of the life cycle, genetic diversity, and technical obstacles, it is challenging to develop vaccines as a control strategy [38]. Antimalarial drugs remain the most effective means of parasite control.

1.3.4. Chemoprophylaxis and chemotherapy

Chemoprophylaxis refers to the use of drugs to prevent the development of the disease, while chemotherapy refers to the treatment of a developed disease for complete elimination. Ideally, chemoprophylaxis should prevent all cases of malaria while chemotherapy prevents deaths due to malaria. However, factors such as side effects, toxicity, gene mutation, broad range of parasite species and their life cycle, contribute to the difficulties of finding the ideal drug that can either prevent the development of the disease or treat the disease completely [39, 40].

The factors mentioned above make it difficult to prevent infections or to prevent development by killing all sporozoites and make it more difficult to find a drug that is effective against all stages of the life cycle [41]. Hence, the WHO has recommended the use of more than one drug to target the complete elimination of the developed parasite by chemotherapy [2, 42].

The lack of antimalarial therapeutics that effectively target different stages is attributed to the complex life cycle of the parasite. Current drugs only target pathogenesis and no effective tool for transmission-blocking is known. It is pharmacokinetically challenging to develop drugs that target both stages. The three major drug classes in use for malaria treatment include quinolines, antifolates and artemisinins. However, their use depends on parasite susceptibility.

The first widely used antimalarial drug was quinine (QN) from the Andean *Cinchona* tree. Several synthetic QN derivatives such as mefloquine, primaquine and chloroquine (CQ) make up the quinoline class of antimalarial drugs. Their structures are characterised by heterocyclic aromatic nitrogen and their acidic nature (Figure 1.3). Due to their cost-effectiveness, quinolines were the most commonly used drug worldwide in the 20th century [16, 43].

For example, CQ is a hemozoin inhibitor that accumulates and binds to hemozoin biocrystals in the digestive vacuole to prevent detoxification of the free haem. This leads to swelling of the digestive vacuole and eventual death of the malaria parasite [44, 45] within the erythrocytic stage of the parasite life cycle [46].

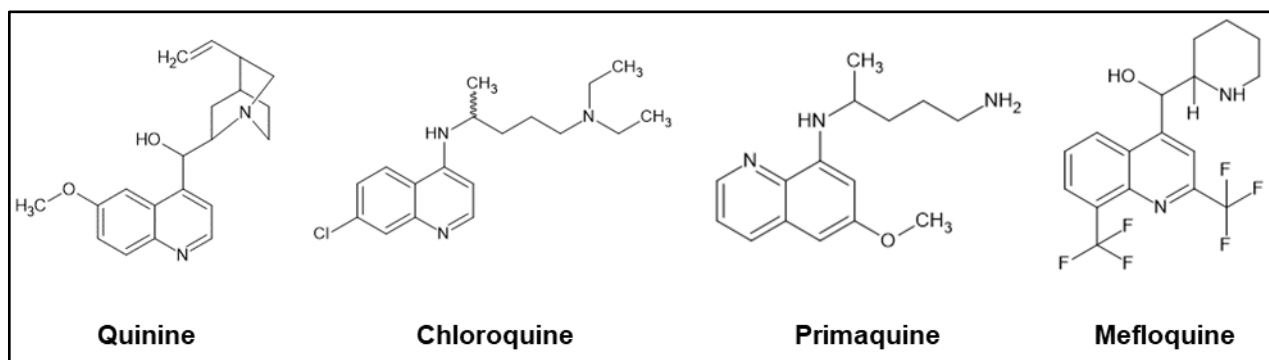


Figure 1.3. Chemical structures of quinine (QN) and its derivatives.

However, using CQ in clinical settings has decreased due to resistance driven by the *P. falciparum* chloroquine-resistance transporter (*PfCRT*), which is responsible for the movement of CQ in and out of the digestive vacuoles. Resistance is due to mutations in *PfCRT* that lead to the active transportation of CQ out of the digestive vacuole to prevent the drug from effectively executing its function [47, 48].

Antifolates are an important class of antimalarial drugs that interfere with folate metabolism important for parasite survival. These drugs inhibit enzymes responsible for the synthesis and use of folate cofactors, namely dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) [49].

Sulfadruugs, such as pyrimethamine and cycloguanil, make up the antifolate class of antimalarial drugs. Most antifolate drugs target the asexual stage of the life cycle [50]. However, resistance to antifolate drugs has been observed and widespread, caused by mutations in the antifolate target enzymes in the malaria parasites [51], effectively negating the use of this drug class.

Artemisinin is a compound isolated from *Artemisia annua*, a herb widely used in Chinese medicine [52]. Artemisinin was derivatised into three clinically approved compounds: dihydroartemisinin (DHA), artesunate (ATS) and artemether (Figure 1.4) [53]. These derivatives are more potent and have more favourable toxicity profiles than artemisinin [54, 55]. Due to their mortality benefits over QN, artemisinin derivatives, especially ATS, are preferred for the treatment of severe malaria in adults [55].

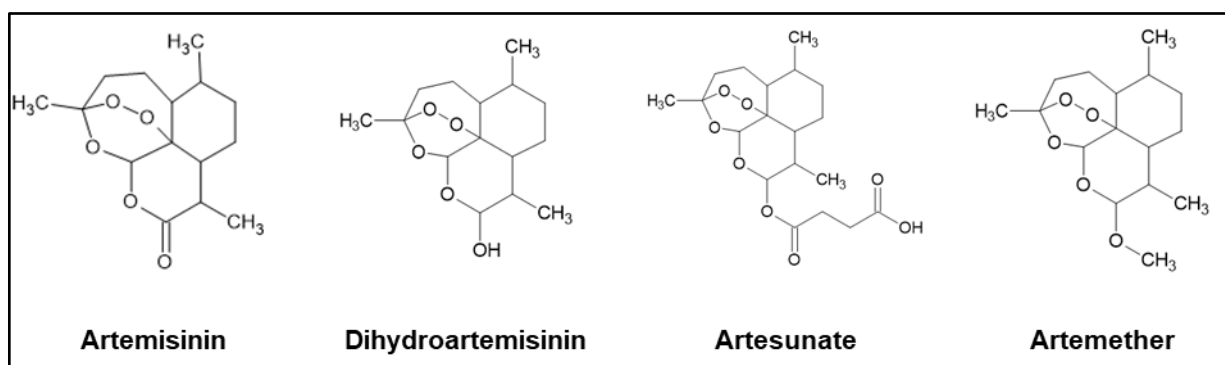


Figure 1.4. Chemical structures of artemisinin and its derivatives.

The MoA of artemisinins is predicted to be multifactorial. However, there is evidence that the chemically labile endoperoxide bridge present in the structure may be responsible for the antimalarial activity [56]. The endoperoxide bridge undergoes activation in the presence of haem in the digestive vacuole. This results in the production of reactive oxygen species that may kill the parasite by covalently modifying some essential parasite proteins including transporters, proteases responsible for haemoglobin degradation and other cellular enzymes [57, 58]. An alternative explanation is that the MoA may also include the inhibition of the enzyme *P. falciparum* ATPase 6 (*PfATP6*), which is essential for oxidative metabolism in the parasite [59].

Unlike other antimalarial drugs, DHA and ATS have demonstrated short half-lives, resulting in less exposure to the malaria parasite [55, 60]. This is beneficial as it minimises rapid development of

resistance, however, it is associated with rapid degradation of the drugs and resistance can still be reached through mutation of target proteins as in the case of antifolate drugs. DHA and ATS are highly efficacious against the asexual stages, especially, ring-stage parasites [61]. Recent studies have shown that resistance to artemisinins is increasing in various parts of the world, especially in several African countries and around the Thai-Cambodian border [57, 62]. However, without negating the issue of resistance, artemisinins remain the most effective antimalarial drug class in malaria treatment.

In response to the increasing burden of artemisinin resistance, the WHO recommends that artemisinins should not be used as monotherapies but used in combination therapies with other drugs known as artemisinin-based combination therapies (ACTs). ACTs are a combination of artemisinin with another class of antimalarials with a different MoA and different properties [63]. ACTs are the preferred, gold-standard treatment for uncomplicated malaria [42].

Due to their fast action combined with a short half-life, artemisinin derivatives are used in combination with drugs with a slower elimination rate and longer half-life to eliminate the residual parasites after artemisinin action. ACTs currently in use are: ATS-mefloquine, ATS-pyronaridine, DHA-piperaquine, and ATS-amodiaquine. However, resistance to these combinations has been noted in several countries, particularly mutations in the *P. falciparum* Kelch 13 (*PfK13*) protein, resulting in the failure of some of these combinations [64]. Using these combination therapies has been instrumental in the remarkable decrease of malaria incidence globally by being fast-acting, with a shorter half-life, having fewer side effects, and providing a reduced risk of resistance [16, 43, 65].

Due to drug-related side effects and the parasite's ability to continuously develop resistance, much investment has been targeted towards finding new combination therapies and developing new antimalarial drugs. Current antimalarial therapies only target the asexual stages. Hence, it is important to find novel targets and strategies that can act as transmission-blocking drugs with new favourable pharmacokinetic (PK) and delivery profiles.

1.4. Novel antimalarial drugs

The challenges observed in the use of current antimalarial drugs include rapid renal clearance and rapid degradation. The latter is of concern because sufficient concentrations may not reach the target. These limitations mean that multiple doses of the drug should be administered, which exposes patients to higher drug concentrations. As a result, patients will develop clinical side effects, potentially leading to non-compliance and increased chances of treatment failure [66].

To overcome these limitations, new strategies need to be developed. Drugs with different targets and MoA should be combined, leading to a lower probability that resistance will develop. Additionally, the development of drug derivatives with improved PK and pharmacodynamic (PD) properties is crucial

[67]. Nanoparticle (NP) drug delivery systems (DDSs) have also been developed as an approach to improve upon these limitations [68].

Finding effective antimalarial drugs is fundamental to parasite control, and prevention of resistance is essential for the long-term goal of malaria elimination. The development of novel compounds and effective DDSs are viewed as possible approaches. The criteria for novel antimalarial drugs should involve targeting more than one stage of the parasitic life cycle, with special focus on blocking transmission of the parasite.

1.4.1. Novel transmission-blocking compounds

Organisations such as Sanofi, the Foundation for Innovative New Diagnostics (FIND), the Medicines for Malaria Venture (MMV), and others are partnering in researching to find lead compounds that will contribute towards eliminating malaria worldwide. MMV have proposed goals in the development of antimalarial drugs that fit certain Target Candidate Profiles (TCPs). Briefly, TCP-1 molecules target asexual stages to treat symptoms. Most antimalarial drugs such as the quinolines (QN), antifolates and artemisinins (DHA) possess this activity [69]. However, it is vital to identify compounds with TCP-5 activity that target transmission. Therefore, these molecules seek to prevent human-to-vector transmission [70].

The current transmission-blocking combination is ATS plus low-dose primaquine, a combination in development for prequalification by the WHO [1]. Primaquine is a prodrug in the quinoline class of antimalarial drugs that blocks transmission. It acts by killing the intrahepatic parasites, mostly *P. vivax* and *P. ovale* in the liver stage and prevents the development of the erythrocytic stage. However, primaquine does not target asexual stages of the life cycle, hence it cannot be used alone [71]. As a prodrug, primaquine must be metabolised to become active, which can influence drug activity based on polymorphisms of the enzyme responsible for the activation of primaquine and genetic variations in the human population i.e. glucose-6-phosphate dehydrogenase (G6PD) deficiency [71, 72].

Several novel compounds have been identified with novel MoA and have reached phase I and II clinical trials [73, 74], where they are being tested as monotherapies and/or combined with other antimalarial drugs [37]. Most of these compounds only target the asexual stages, however, MMV3900048 is a compound from the aminopyridine class and is part of only a few compounds that are active against the asexual and gametocyte stages [37, 75]. MMV390048 has shown efficacy against all stages of the parasite except the latent hypnozoites stage. MMV390048 acts as an inhibitor of the parasite phosphatidylinositol 4-kinase (PI4K) and lacks cross-resistance with current antimalarial drugs [73, 75]. As chemotherapy focuses on the treatment of *P. falciparum* infections and involves a combination of TCP-1 and TCP-5 molecules, MMV390048 can be used in combination therapy to target the asexual

and gametocyte stages through its TCP-1 and TCP-5 activities [76, 77]. Activity against these sexual stages, with suitable PK and PD profiles, would be ideal for the development of combination therapies.

1.4.2. Pharmacokinetic and pharmacodynamic profiles

PK and PD properties of compounds are extraordinarily important for therapeutic efficacy. In antimalarials, PD properties (as defined by what the drug does to the body related to the concentration of the drug at the site of action) are important given the need for oral bioavailability in the target populations of children. PK properties refer to the influence of the body on the drug efficacy through absorption, distribution, metabolism and excretion and are all important considerations in the administration of any drug. PK are associated with the chemical properties and composition of the drug, thus, the concentration profile of the antimalarial drugs in the blood [78]. However, the PK profile of antimalarial drugs to treat the sexual stages would be different to those acting on asexual blood stages. In both scenarios, the drug would have to be quick-acting but in targeting gametocytes, these components of combination therapies would have to be extraordinarily long-lasting ($T_{1/2} > 192$ hours, or 8 days) to target mature gametocytes.

Current combination therapies include compounds with different MoA and speed of action but with matched PK profiles. However, for combinations to be used in elimination strategies and to block transmission, these should involve compounds such as DHA that effectively clear asexual stages and are quick-acting, partnered with slower-acting drugs such as MMV390048 that have shown efficacy against other stages including gametocytes [55, 73]. The challenge remains to match PK profiles for long-lasting compounds to attain transmission-blocking. One-way this can be achieved is through novel drug delivery mechanisms.

1.5. Routes of administration and drug delivery systems

Most drugs are administered orally, intramuscularly, transdermally, subcutaneously, intraocularly, intranasally, or through intrauterine and transmucosal routes (Figure 1.5). However, challenges around bioavailability and side effects are observed for these traditional routes of administration [79]. The investigation of alternative DDSs is supported. Drugs can be encapsulated in NPs and delivered through DDSs via traditional routes of administration.

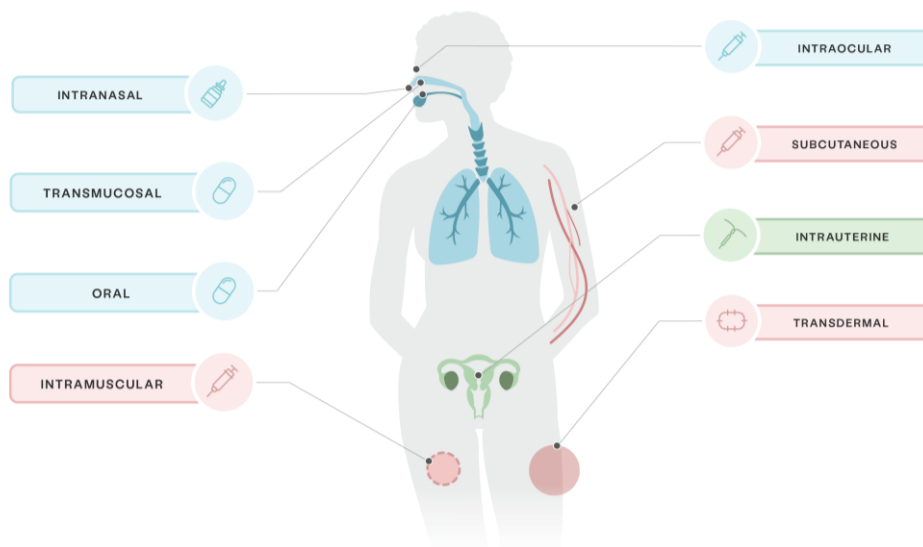


Figure 1.5. Traditional drug delivery system (DDS) examples. Examples of traditional DDSs include intranasal, transmucosal, oral, intramuscular, intraocular, subcutaneous, intrauterine and transdermal routes of administration. The figure was re-created and adapted from Baryakova *et al.* (2023) using Adobe Illustrator version 28.5.

NP-based DDSs are promising technologies, formulations or systems that can alleviate the factors negatively affecting drug administration via traditional routes. These systems are used in drug delivery to modulate drug release to a specific target inside the body [80]. Therefore, NP-based DDSs are associated with various characteristics, such as the route of administration, the device type, the drug profile, and PK properties.

A study by Zech *et al.* (2021) showed that dermal NP-based DDSs circumvent complications observed with oral or parenteral routes of administration alone. The same study showed effective delivery of antimalarial drugs using a preclinical mouse model where successful recovery was observed in mice previously infected with the malaria parasite [81]. NP-based DDSs have several advantages over traditional routes, however, some challenges persist (Table 1.1). NP-based DDSs can improve the PK of a drug by enabling different routes and potentially reducing the initial dose, thereby resulting in reduced costs and mitigating the chance of overdosing [80, 82]. These benefits play a role in increasing patient acceptability and compliance.

Table 1.1. Advantages and disadvantages of traditional vs. nanoparticle (NP)-based drug delivery systems (DDSs).

	Traditional DDSs	NP-based DDSs
Advantages	Simple ^a	Avoids first-pass metabolism ^a
	Cost-effective ^b	Improved bioavailability ^a
	Self-administered ^c	Controlled and prolonged drug release ^a
	Most convenient ^c	Less side effects ^{ab}
	No sterility needed ^{bc}	Drug dose reduction ^b
Disadvantages	Gastrointestinal absorption ^a	Not compatible with all medications ^b
	Gastrointestinal side effects ^a	
	First-pass metabolism ^a	
	Random distribution ^a	Unknown side effects ^b
	Poor bioavailability ^b	
	Poor solubility ^c	

Data are from Shen *et al.* (2015)^a, Baryakova *et al.* (2023)^b and Zech *et al.* (2021)^c.

Traditional formulations administered via traditional routes alone result in rapid drug clearance from the blood, whereas some NP encapsulated drugs have prolonged duration where the concentration remains at therapeutic levels during the treatment window [83]. NP-based DDSs administered via the intradermal route have shown a potential to decrease the initial dose concentration and rapidly reach the target. Studies have reported intradermal delivery to be more suitable compared to other traditional routes [83, 84].

Some caveats associated with the use of intradermal DDSs are largely based on the devices used to administer the drugs. Intradermal injections using needles may result in poor patient compliance, needle injuries, needle phobia, and the production of dangerous biological waste [85]. It is, therefore, necessary to use devices that promote safer, more reliable and easier intradermal delivery; such as disposable needle-free syringe jets, microneedle patches and prefilled syringes [86].

1.5.1. Nanoparticles in delivery systems

NPs are sub-micron sized particles used to carry drugs to specific targets. They improve penetration across barriers, accumulation at targeted sites, and decrease toxicity and side effects [87, 88]. In some cases, NPs can also reduce or overcome drug resistance and help improve the efficacy of the drug [88,

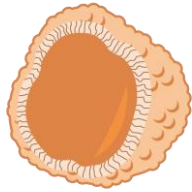
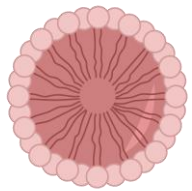
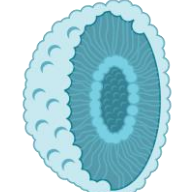
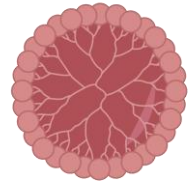
89]. Ideal NPs should be temperature, pH and salt-responsive to provide a triple stimulus response in one material and help with the controlled release of the drugs [90]. NPs can enhance the concentration of drugs in the target cells with less cytotoxicity through passive and active targeting techniques.

Passive transport depends on size and shape and relies on structural changes through the phenomenon known as the enhanced permeation and retention effect [91, 92]. This is where the permeability features of the drugs are enhanced through structural changes, allowing the drugs or NPs to be more available to diseased cells than healthy cells. Passive transport is mostly observed in cancerous cells, where there is higher permeability compared to healthy cells [77]. In erythrocytes infected with *P. falciparum* parasites, the structural changes involve the deposition of new permeability pathways (NPPs) to increase the permeability of the infected cells as the parasite moves through different stages of the life cycle [93]. However, there are some limitations associated with passive transport such as the movement of small concentrations of solutes across the membranes of healthy cells. Hence, the development of NPs that can enhance transport in infected cells and reduce distribution into healthy cells is necessary.

1.5.2. The structure of nanoparticles

There are several types of NPs used in DDSs, such as polymers, micelles, liposomes, and dendrimers (Table 1.2). It is, therefore, important to select the most appropriate NP based on the desired outcome. This takes into consideration the nanoscale, the end goal and the rate of degradation required [94].

Table 1.2. Types of nanoparticles (NPs) in drug delivery systems (DDSs).

NPs	Size (nm)	Structure	Description	Carried agent	Advantages
Polymers	10 – 1000 ^a		Conjugation between drugs and the side chain of the polymer ^a .	DNA ^a	Assist with the localisation of drugs for weeks ^a
				Proteins ^a	
				Small compounds ^a	
Solid lipid	15 – 1000 ^b		Made of natural plant wax or a synthetic analogue with a matrix and surfactants ^b .	Small compounds ^b	High melting point ^b
					High lipophilicity ^b
					Low dissolution profile ^b
Micelles	< 100 ^a		Formation of a micelle between amphiphilic block, hydrophobic and hydrophilic core/shell ^a .	DNA ^a	Suitable carriers for water-insoluble drugs ^a
				Proteins ^a	
				Anticancer agents ^a	
Liposomes	50 – 100 ^a		Colloidal structures composed of lipid bilayers ^a .	DNA ^a	Efficient reduction of system toxicity ^a
				Proteins ^a	
				Anticancer agents ^a	
Dendrimers	< 10 ^a		Hyperbranched polymer with regular and repeated patterns ^a .	DNA ^a	Possible modifications to carry hydrophilic or hydrophobic drugs ^a .
				Anticancer agents ^a	
				Antiviral agents ^a	
				Large compounds ^a	

Data are from Yoh *et al.* (2006)^a and Tzachev (2021)^b. The images were created using Adobe Illustrator version 28.5.

1.5.3. Solid lipid nanoparticles

Due to their properties, lipids are used as vehicles in drug delivery for all routes of administration, allowing adjustments to physical state, drug loading, and release kinetics [95]. Solid lipid nanoparticles

(SLNs) are composed of lipids and stabilisers such as surfactants, co-surfactants, and coating materials.

SLNs are small spherical particles made from plant wax or a synthetic analogue, surrounded by surfactants while encapsulating the drug, and act as lipid-based carriers for the intracellular release of drugs [96]. Their small/nanoscale dimensions and high membrane affinity make them ideal for entering cells easily and preventing macrophage uptake. This process allows for the transportation and release of the encapsulated drug, making SLNs suitable for systemic delivery [97, 98].

Since most SLNs are composed of lipids found in food sources, they have high safety profiles and high LD₅₀ (half the maximal lethal dose) values with well-known degradation profiles. Several studies have investigated and confirmed the safety of SLNs and have also reported lower toxicity for SLN formulations compared to other NPs such as polylactide (PLA) and polylactide/glycolide (PLA/GA) [99-102].

Compared to other polymeric NPs, SLNs have several advantages as DDSs. In addition to protecting the drug in difficult environments, SLNs can be produced on a large scale using high-pressure homogenisation. Additionally, SLNs are biocompatible, have lower toxicity, are biodegradable, and improve drug stability, delivery, and absorption [102]. SLN DDSs are characterised by high melting points, high lipophilicity, high dissolution profiles and lipase resistance, which allows them to freely penetrate through the cell membrane of target cells and release the drugs while being digested intracellularly [90, 96].

1.6. Background to the project

There is an urgent need for novel antimalarial combination strategies to target asexual and gametocyte stages through innovative administration routes of antimalarial combination therapies. This project proposes the encapsulation of SLNs into an injectable or dermal patch hydrogel to allow the subcutaneous release of drugs from a drug depot. Hydrogels are three-dimensional network structures that can be used for the delivery of antimalarial drugs of different PK properties, with prolonged release properties [103].

In the current scenario, it is postulated that two antimalarials can be combined, to allow 1) quick release of the quick-acting component to target asexual blood stage parasites and have therapeutic benefit, and 2) slow release of the partner drug to target gametocytes and have a transmission-blocking effect (Figure 1.6).

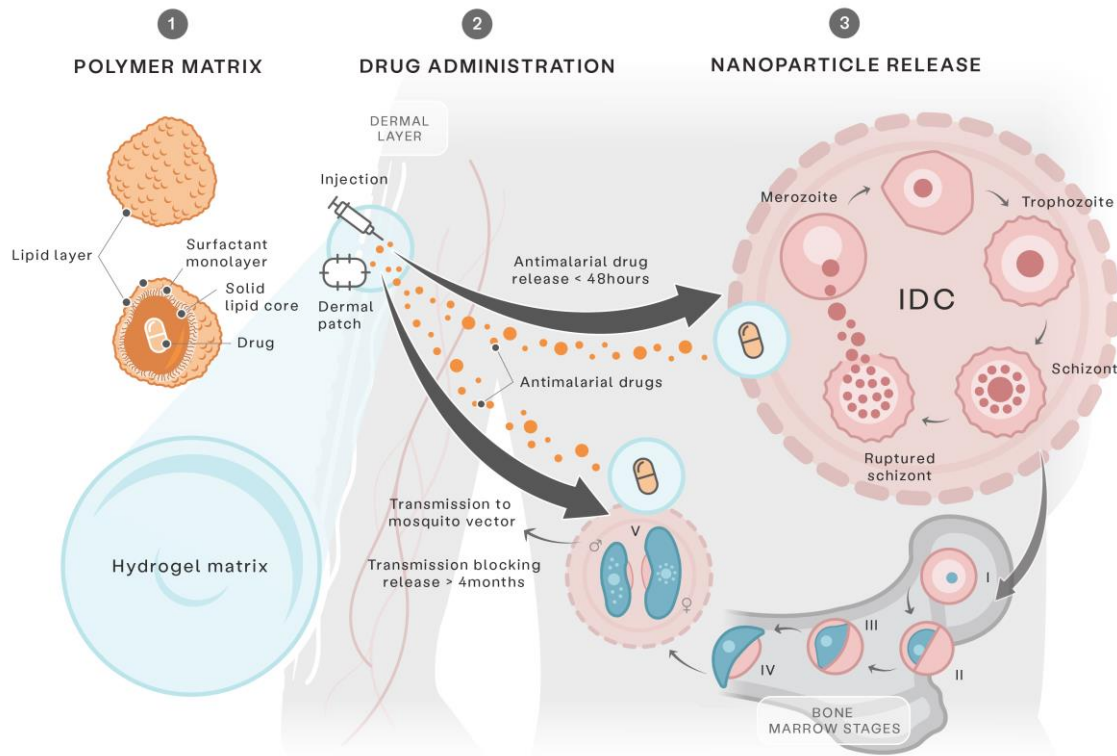


Figure 1.6. The proposed design and action of a novel nanoparticle (NP)-based drug delivery system (DDS) for simultaneous treatment against *P. falciparum* parasites. The solid lipid nanoparticles (SLNs) were synthesised with lipids and surfactants encapsulating the antimalarial drug. The encapsulated drug would be administered via a dermal/injectable hydrogel. The system will ensure the sustained release of the drug within 48 h to disrupt the intraerythrocytic developmental cycle (IDC) and maintain transmission-blocking activity for over 4 months. The figure was created using Adobe Illustrator version 28.5.

To promote the controlled release of the drugs and enhance the properties of the drugs and polymers, different adjustments to the polymer drug systems need to be evaluated. NPs can be developed as intradermal hydrogels to facilitate their application, and this can promote the controlled release of encapsulated drugs to target the whole malaria cycle using a single application. Here, we will evaluate the use of this novel SLN DDS with DHA and MMV390048 as proof-of-principle drugs that can be directly released from the SLN and administered via an injectable or dermal patch hydrogel.

This system can help circumvent the factors that contribute to poor patient compliance including poor drug PK and PD properties combined with the extensive therapeutic times required, which is challenging in an African setting. This remains a challenge for the development of such combination therapies.

Hypothesis

SLNs will allow the controlled release of DHA and MMV390048 to kill asexual and gametocyte stages of *P. falciparum* with minimal haemolytic and cytotoxic effects on erythrocytes and mammalian cells, respectively.

Aim

Evaluate the multistage antimalarial efficacy of antiplasmodial compounds DHA and MMV390048 encapsulated into a novel SLN DDS.

Objectives:

- I. Evaluate the multistage antiplasmodial activity of DHA and MMV390048, independently and in combination, against asexual and gametocyte stages of *P. falciparum* parasites.
- II. Evaluate the antiplasmodial efficacy of DHA and MMV390048 incorporated into a SLN delivery system.
- III. Evaluate cytotoxic effects of SLNs, encapsulated and unencapsulated compound formulations on erythrocytes and mammalian hepatocellular (HepG2) cells.
- IV. Evaluate the uptake and subcellular distribution of SLN delivery systems to gauge the potential effect of encapsulation on DHA and MMV390048 distribution using fluorescent tagging.

Outputs

- Muruya, M., Coertzen, D and Birkholtz, L. Evaluation of a novel nanodelivery system for long-lasting release of antimalarial drugs. 7th Southern African Malaria Research Conference. Poster presentation. Virtual. 2-4 August 2022.
- Muruya, M., Coertzen, D and Birkholtz, L. Evaluation of novel nanodelivery systems for long-lasting antimalarial drug release. 8th Southern African Malaria Research Conference. Oral presentation. Pretoria, South Africa. 1-3 August 2023.

2. Materials and Methods

2.1. Ethics statement

All *in vitro* experiments were carried out in a biosafety level 2 (BSL2) certified facility (registration certificate 39.2-University of Pretoria-19/160) in the Malaria Parasite Molecular Laboratory (M²PL) at the University of Pretoria. All experiments involving human blood donors hold ethics approval from the Faculty of Health Sciences Research Ethics Committee (NAS040/2023) and for work with human malaria parasites, umbrella ethics approval from the Faculty of Natural and Agricultural Sciences Research Ethics Committee (180000094) for the SARCHI programme under Prof. Lyn-Marié Birkholtz.

2.2. Materials

The SLN samples were derived from a research collaboration with Prof. Christo Tzachev at Sofia University “St. Kliment Ohridski” (Sofia, Bulgaria) (Table 2.1). The samples were prepared from stock solutions in 0.9 % (w/v) NaCl and stored at 4 °C for long-term storage and when regularly used.

Table 2.1. List of solid lipid nanoparticles (SLNs) with and without surfactants.

Sample ID	Sample name
CHPL	Placebo SLNs
CHPLW	Placebo SLNs free of surfactants
CHQN	SLNs loaded with QN
CHQNW	SLNs loaded with QN free of surfactants
CHDHA	SLNs loaded with DHA
CHDHAW	SLNs loaded with DHA free of surfactants
CHMMV	SLNs loaded with MMV390048
CHFL	SLNs fluorescently labelled with fluoresceine
CHSI	SLNs fluorescently labelled with sudan I

CHPL and CHPLW working solutions were prepared from 100 % polymer dispersion stock solutions in supplemented RPMI-1640 culture medium or 1x PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄). CHQN and CHQNW working solutions were prepared from 10 mM QN or 29.4 % polymer dispersion stock solutions, diluted in supplemented RPMI-1640 culture medium or 1x PBS. CHDHA and CHDHAW working solutions were prepared from 5 mM DHA or 100 % polymer dispersion stock solutions in supplemented RPMI-1640 culture medium or 1x PBS. CHMMV working solutions were prepared from 23.13 mM MMV390048 or 100 % polymer dispersion stock solutions in

supplemented RPMI-1640 culture medium or 1x PBS. CHFL working solutions were prepared from a 150 μ M fluorescein stock solution, and CHSI working solutions were prepared from a 201 μ M Sudan I stock solution or 100 % polymer dispersion stock solutions, in supplemented RPMI-1640 culture medium or 1x PBS.

2.3. *In vitro* cultivation of intraerythrocytic asexual *P. falciparum* parasites

Cryopreserved, drug sensitive P₁NF54 parasites were obtained from the Malaria Research and Reference Reagent Centre, MR4 BEI Resources (Manassas, USA). Cryopreserved parasites were subsequently thawed and cultivated *in vitro* following a method adapted from Trager and Jansen [104]. Parasites were cultured at 5 % (v/v) haematocrit (Type O/A⁺) in complete RPMI-1640 culture medium (Sigma-Aldrich). The complete medium was supplemented with 25 mM HEPES (Sigma-Aldrich), pH 7.4 and 24 mM NaHCO₃ (Sigma-Aldrich), both of which were added to buffer the lactic acid produced by proliferating asexual *P. falciparum* parasites. Additionally, 11 mM glucose (Sigma-Aldrich), 200 μ M hypoxanthine (Sigma-Aldrich) and 0.024 mg/mL gentamycin (HyClone, USA) were added to the medium. The medium was completed by adding 5 g/L Albumax II (Life Technologies, USA), a lipid-rich substitute for human serum. Parasites were cultivated at 37 °C under hypoxic conditions using a special gas mixture comprising 90 % N₂, 5 % CO₂ and 5 % O₂ (Afrox, South Africa) with shaking at 60 rpm. The parasitaemia (proportion of *P. falciparum* infected erythrocytes) was maintained at between 2-6 %. Parasite proliferation and morphology were evaluated daily on methanol fixed, RapiDiff-stained thin blood smears at 1000x oil magnification using a light microscope (Nikon, Japan). Briefly, parasites were fixed and stained with an eosin dye that stains cytoplasmic components red and a thiazine dye that serves as a nucleic acid stain.

2.4. Sorbitol synchronisation of intraerythrocytic asexual *P. falciparum* parasites

While *in vivo* parasites maintain synchrony throughout the IDC, *in vitro*, parasites become asynchronous after several cycles of the IDC [105]. Therefore, *in vitro* *P. falciparum* cultures must be routinely synchronised for stage-specific studies. To this end, 5 % (w/v) D-sorbitol (Sigma-Aldrich) was used to ensure a > 95 % synchrony. Synchronisation of asexual parasites relies on the impermeability of non-infected erythrocytes to D-sorbitol. However, as parasites progress through the IDC, NPPs are expressed increasing erythrocyte membrane fluidity and permitting sorbitol uptake. As such, mature asexual parasites (trophozoites) are selectively eliminated through osmolysis, resulting in a culture that consists of non-infected erythrocytes and ring-stage infected erythrocytes. Parasites were synchronised twice over a 96 h period during the ring developmental stages prior to use in stage-specific assays.

2.5. Evaluation of the antiplasmodial activity of compounds and SLNs against intraerythrocytic asexual *P. falciparum* parasites

To determine the activity of selected compounds and SLNs against asexual intraerythrocytic *P. falciparum* parasites, a SYBR Green I-based proliferation assay was used. The assay is based on the

amplification of SYBR Green I fluorescence a thousand-fold upon intercalation into double stranded parasitic DNA since human erythrocytes are anucleate [106]. Therefore, a higher SYBR Green I fluorescence is expected where a higher proportion of DNA is present, giving a proxy for parasite proliferation. The selected compounds or NPs were added to 96-well plates and serially diluted. A synchronised *Pf*NF54 ring-stage parasite suspension at 1 % parasitaemia and 1 % (v/v) haematocrit was added to each well. Compound working solutions were prepared from a 10 mM stock concentration in 100 % (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich) in complete RPMI-1640 culture medium with a final DMSO concentration of < 0.1 % (v/v), previously found to be non-toxic to intraerythrocytic asexual parasites and gametocytes [107]. Untreated parasites were used as a negative control and 1 μ M CQ was used as a positive control for inhibition. The drug treated parasite suspensions were incubated in a stationary incubator at 37 °C for 96 h under hypoxic conditions. Following this, 100 μ L of the parasite suspension was incubated with 100 μ L of SYBR Green I lysis buffer (1x SYBR Green I dye (Invitrogen, USA), 20 mM Tris pH 7.5, 0.08 % (v/v) Triton X-100 (Sigma-Aldrich), 5 mM ethylene dinitrotetraacetic acid (EDTA) (Merck) and 0.0008 % (w/v) saponin (Merck)) for 1 h in the dark at room temperature to allow complete cell lysis. SYBR Green I fluorescence was detected at an excitation wavelength of 490 nm and an emission wavelength of 520 nm using a Fluoroskan Ascent FL microplate fluorometer (Thermo Fisher, USA). The fluorescence from drug treated parasites was expressed as a percentage of the untreated control. Data were analysed using GraphPad Prism v 8.02 using non-linear regression curves from which the IC₅₀ (half maximal inhibitory concentration) was determined. Compounds and SLNs were screened in technical triplicate for three biological repeats (n=3).

2.6. *In vitro* cultivation of *P. falciparum* gametocytes

P. falciparum gametocytes are induced from asexual parasite cultures under stress conditions. As such, asexual drug sensitive, *Pf*NF54-*pfs16*-GFP-Luc, *Pf*NF54-*mal8p1.16*-GFP-Luc [108] or *Pf*3D7*elo1-pfs16*-CBG99 [109] parasites were exposed to two stress factors, nutrient deprivation, and a decrease in the haematocrit. Asexual parasites were synchronised twice as in section 2.4 and the parasitaemia was adjusted to 0.5 % at a 6 % (v/v) haematocrit in gametocyte induction medium. Gametocyte induction medium is comprised of complete RPMI-1640 culture medium without the additional 11 mM glucose to provide the initial stress factor. These cultures were maintained for 72 h under hypoxic conditions. Thereafter, the haematocrit was reduced to 4 % (v/v) in complete culture medium supplemented with 50 mM N-acetylglucosamine (NAG). The NAG was added for the selective elimination of asexual parasites to produce a homogenous gametocyte culture. Gametocyte cultures were maintained in NAG-supplemented medium until all asexual parasites had been eliminated, after which gametocytes were returned to complete culture medium and maintained at 37 °C without agitation under hypoxic conditions. Gametocyte cultures were kept stationary for up to 12 days post gametocyte induction and monitored daily on methanol fixed, RapiDiff-stained thin blood smears at 1000x magnification using a light microscope (Nikon, Japan).

2.7. Evaluation of the gametocytocidal activity of selected compounds and SLNs against *P. falciparum* gametocytes

To determine the activity of the selected compounds and SLNs against *P. falciparum* gametocytes, a luciferase assay was used. Parasite lines expressing GFP-firefly luciferase were used. These lines express firefly luciferase under the control of the gametocyte-specific promoter *pfs16* [110]. The luciferase catalyses the oxidation of D-luciferin to oxyluciferin with the concomitant production of light. Hence, the amount of bioluminescence can be used as an indicator of gametocyte viability. Selected compounds or NPs were serially diluted in 96-well plates and incubated with a gametocyte suspension at > 1 % gametocytaemia and 2 % (v/v) haematocrit under hypoxic conditions. Untreated gametocytes were used as a negative control for inhibition and 1 mM methylene blue (MB) treatment was used as a positive control for inhibition. The 96-well plates were incubated for 48 h at 37 °C under hypoxic conditions. The bioluminescence was detected by mixing equal volumes of the gametocyte suspension and a luciferase reagent (Promega, USA) or by the addition of D-luciferin in citrate buffer (0.1 M citric acid (Merck) and 0.1 M tri-sodium citrate dihydrate (Merck) adjusted to pH 5.5). Luminescence was measured using a GloMax Explorer Detection System (Promega). The luminescence values were normalised to the untreated control and data were expressed as a percentage of the untreated gametocytes. Data were analysed on GraphPad Prism as in section 2.5. Compounds and SLNs were screened in technical triplicate for three biological repeats (n=3).

2.8. Fixed ratio isobole analysis

The *in vitro* interaction of DHA and MMV390048 was evaluated using fixed ratio isobole analyses for asexual *PfNF54* parasites using a SYBR Green I-based fluorescence assay and for the sexual stages, *Pf3D7elo1-pfs16*-CBG99 parasites using a luciferase assay. The drugs were added to 96-well plates alone and in fixed drug combination ratios (DHA: MMV390048) of 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100. These were two-fold serially diluted and incubated with an *PfNF54* parasite suspension at 1 % parasitaemia and 1 % (v/v) haematocrit for asexual assays and a *Pf3D7elo1-pfs16*-CBG99 gametocyte suspension at > 1 % gametocytaemia and 2 % (v/v) haematocrit for the sexual stages. The plates were incubated for 96 h for asexual parasites (SYBR Green I) and 48 h for gametocyte stages (luciferase) at 37 °C under hypoxic conditions. Dose-response curves were generated for each drug alone and in the fixed ratio as described above. Thereafter, the fractional inhibition concentration (FIC) values for each drug alone and in combination were determined as:

$$\text{FIC (DHA)} = \frac{\text{IC}_{50} \text{ of DHA in combination with MMV390048}}{\text{IC}_{50} \text{ of DHA alone}}$$

The same formula was applied in determining the FIC for MMV390048 alone and combined with DHA. The paired FIC values were linearly plotted in GraphPad Prism v 8.02 producing an isobologram. The mean FIC ($\sum \text{FIC}$) of FIC (DHA) combined with FIC (MMV390048) was determined and a representative FIC value for the combination of DHA and MMV390048 was obtained for asexual parasites and

gametocytes. A $\Sigma\text{FIC} < 0.8$ is indicative of a synergistic interaction, a $\Sigma\text{FIC} > 1.4$ represents an antagonistic interaction and an ΣFIC between 0.8 and 1.4 show an additive or indifferent interaction.

2.9. *In vitro* haemolytic toxicity

Intraerythrocytic *P. falciparum* parasites reside within human erythrocytes; therefore, any proposed drug or delivery system should show specificity to *P. falciparum* parasites and not towards the host-cell. Thus, to determine whether the observed activity was due to specific parasite interactions or host erythrocyte toxicity, an *in vitro* haemolysis assay was performed. Human erythrocytes at 2 % haematocrit in 1x PBS adjusted to pH 7.5 were incubated with serially diluted compounds or SLNs in 96-well plates. Untreated erythrocytes were used as a negative control for haemolysis and 1 % (v/v) Triton X-100 (Sigma-Aldrich) was used as a positive control for haemolysis. The plates were incubated at 37 °C for 48 h. Following this, the erythrocyte suspensions were centrifuged at 2500 $\times g$ for 3 min and 30 μL of the supernatant was diluted 3:7 in sterile water. The haemoglobin content in the diluted suspensions was determined by measuring the absorbance at 540 nm using a SpectraMax Paradigm microplate reader (Molecular Devices). Data was normalised to the untreated control and expressed as a percentage of the untreated erythrocytes. Data were analysed using GraphPad Prism v 8.02 where non-linear regression curves from which the HC_{50} (half the maximal haemolytic activity) were determined.

2.10. *In vitro* cultivation of human hepatocellular carcinoma (HepG2) cells

Adherent human hepatocellular carcinoma liver cells were used for cytotoxicity screening. These cells were thawed at 37 °C with the addition of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum (GE Healthcare) and were kept at 37 °C under 5 % CO_2 and 90 % humidity in a Heracell 150i incubator (Thermo Fisher Scientific, USA) and the medium was replaced every 3 days. Cell confluency was evaluated at 40 x magnification using a phase contrast microscope until a > 70 % monolayer confluency was observed. After this, the medium was aspirated, and the cells were washed three times in 1x PBS. Thereafter, the cells were incubated in trypsin-EDTA for 5 min at 37 °C to detach them from the flask. The cells were pelleted through centrifugation at 1000 $\times g$ for 2 min and washed once in DMEM. The cells were split and maintained further or prepared for downstream experiments.

2.11. *In vitro* cytotoxicity

The cytotoxic effect of the selected compounds and SLNs was evaluated against HepG2 cells by proxy of measuring the lactate dehydrogenase (LDH) levels released from cells. The cells were seeded into a clear 96-well plate at 3.33×10^5 cells/mL, with 150 μL added per well and incubated for 24 h at 37 °C in a 5 % CO_2 and 90 % humidity. Following the 24 h incubation, the cells were treated with the selected compounds and SLNs for a further 48 h at 37 °C. As a negative control for background LDH levels, 1 % Triton X-100 solution (Cell Biolabs) was added to cells for 15 min at room temperature. The LDH

levels within each well as a proxy for cytotoxicity were determined using the CytoSelect LDH Cytotoxicity assay kit (Cell Biolabs) as per the manufacturer's instructions using the SkyHigh spectrometer (Thermo Fisher) to determine the absorbance of each sample at 450 nm. The negative control was subtracted from the data and the data were normalised to the viable mammalian cell population. Data from three independent experiments, in technical duplicate, were analysed using Microsoft Excel. Data were visually represented using GraphPad Prism v 8.02 and statistical significance between data sets was determined.

2.12. Live-cell confocal fluorescence microscopy

Fluorescent markers are potentially useful for the localisation of the drug-loaded SLNs in the body. SLNs have shown promise as bioimaging agents for *in vitro* investigations of DDSs [111, 112] and have been used as carriers of fluorogenic dyes designed to bind nucleic acids and to visualise cellular organelles [97]. The charge of SLNs allows binding to fluorescent dyes via electrostatic interactions. This contact ion pair creates SLN-dye particles with a fluorescent marker that emits a fluorescent signal once SLN-dye particles interact with DNA [98]. This shows that fluorescently labelled SLNs can be used as a potential delivery system for the imaging agent.

The fluorophores investigated to this end were fluorescein and sudan I. Fluorescein is a xanthene dye used in forensics to detect traces of blood. Sudan I is a nitrogen (azide) dye (Figure 2.1) [113].

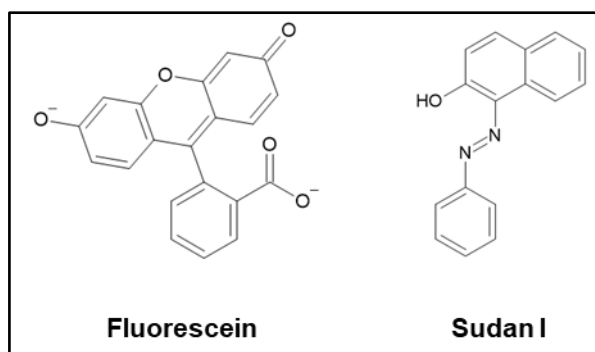


Figure 2.1. Chemical structures of fluorescein and sudan I.

Live-cell confocal fluorescence microscopy was used to visualise erythrocytes and *P. falciparum* parasites interrogated with SLN covalently linked to fluorogenic dyes (Table 2.2) and co-stained with Hoechst 33342 or DRAQ5 nuclear stains. Uninfected and *P. falciparum* infected human erythrocytes (2.5 % haematocrit) were interrogated in complete RPMI-1640 culture medium for 1 h at 37 °C with the SLNs under the below conditions.

Table 2.2. Excitation lasers and emission filter settings used for confocal imaging of uninfected erythrocytes and *P. falciparum* infected erythrocytes.

SLN	Fluorophore	Excitation laser (nm)	Emission filter (nm)
CHFL	Fluorescein	488	525-550
CHSI	Sudan I	647	665-800

The parasite suspensions were each co-stained with a nucleic acid stain; both CHFL with 1 µg/mL of Hoechst 33342 (Thermo Fisher Scientific) for 10 min, and CHSI with 5 µM of DRAQ5 (Thermo Fisher Scientific) for 30 min, at 37 °C, to visualise the nuclei. Following incubation, the suspensions were washed three times and re-suspended in Ringer's solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 11 mM D-glucose, 25 mM HEPES and 1 mM NaH₂PO₄) by centrifugation at 500 xg for 30 s. The stained samples (8 µL) were placed on a glass slide, covered with a 1.0 borosilicate cover glass, and the edges sealed with nail polish. Images were captured with a Zeiss Inverted Confocal Laser Scanning Microscope (LSM) 880, with an Airyscan detector (Zeiss, Germany) using the alpha Plan-Apochromat 100x/1.46 oil immersion DIC M27 Elyra objective. Images were then processed with Zeiss ZEN lite 3.8 (blue edition) software (Zeiss, Germany) and analysed with ImageJ (NIH, USA).

3. Results

3.1. Activities of DHA and MMV390048 against *P. falciparum* parasites

3.1.1. Drug activities against the asexual stages of *P. falciparum* parasites

Prior to SLN encapsulation studies, the antiplasmodial activities of DHA (clinically used) and MMV390048 (investigative) were determined against asexual parasites, and early- and late-stage gametocytes of *P. falciparum*. The IC_{50} of the drugs was determined against *PfNF54* asexual parasites using the SYBR Green I-based proliferation assay. The inhibitory activity of both DHA and MMV390048 in asexual parasites was confirmed as potent with low nanomolar IC_{50} values of 4.28 ± 0.13 nM and 6.44 ± 0.22 nM, respectively (Figure 3.1), similar to previous reports of 2.51 nM and 28 nM using the same assay platform [73, 114].

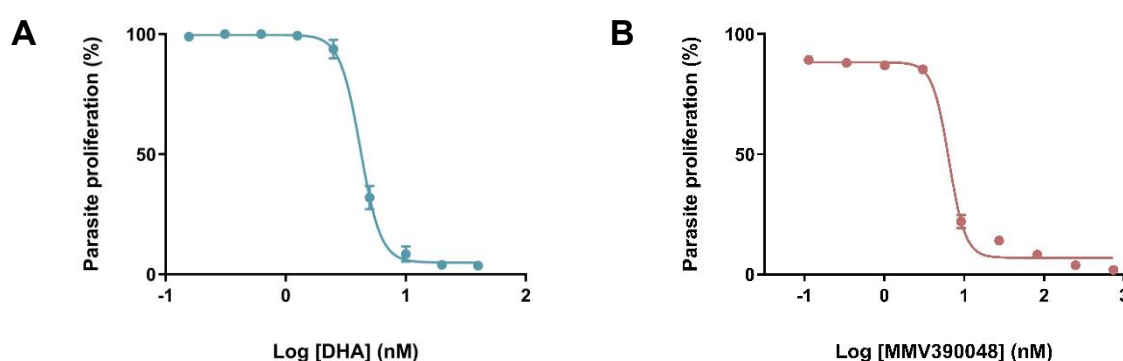


Figure 3.1. *In vitro* activity of DHA and MMV390048 against asexual *P. falciparum* parasite proliferation. Dose-response evaluation of (A) DHA and (B) MMV390048 against asexual parasites using the SYBR Green I-based fluorescence assay. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

3.1.2. Drug activities against the gametocyte stages of *P. falciparum* parasites

The drugs were tested for their activity against both gametocyte stages of *PfNF54-pfs16*-GFP-Luc or *Pf3D7elo1-pfs16*-CBG99 *P. falciparum* parasites using a luciferase assay. In early-stage gametocytes, DHA and MMV390048 demonstrated IC_{50} values of 49.5 ± 6.3 nM and 435.9 ± 8.6 nM, respectively (Figure 3.2 A). In late-stage gametocytes, both drugs showed a decrease in activity to 66.7 ± 8.1 nM and 669.7 ± 19.7 nM, respectively (Figure 3.2 B). These results confirmed DHA and MMV390048 as potent inhibitors of gametocyte viability correlating to previous reports of 34 nM and 214 nM in early-stage gametocytes and 88 nM and 140 nM in late-stage gametocytes, respectively using the same assay platform [73, 114].

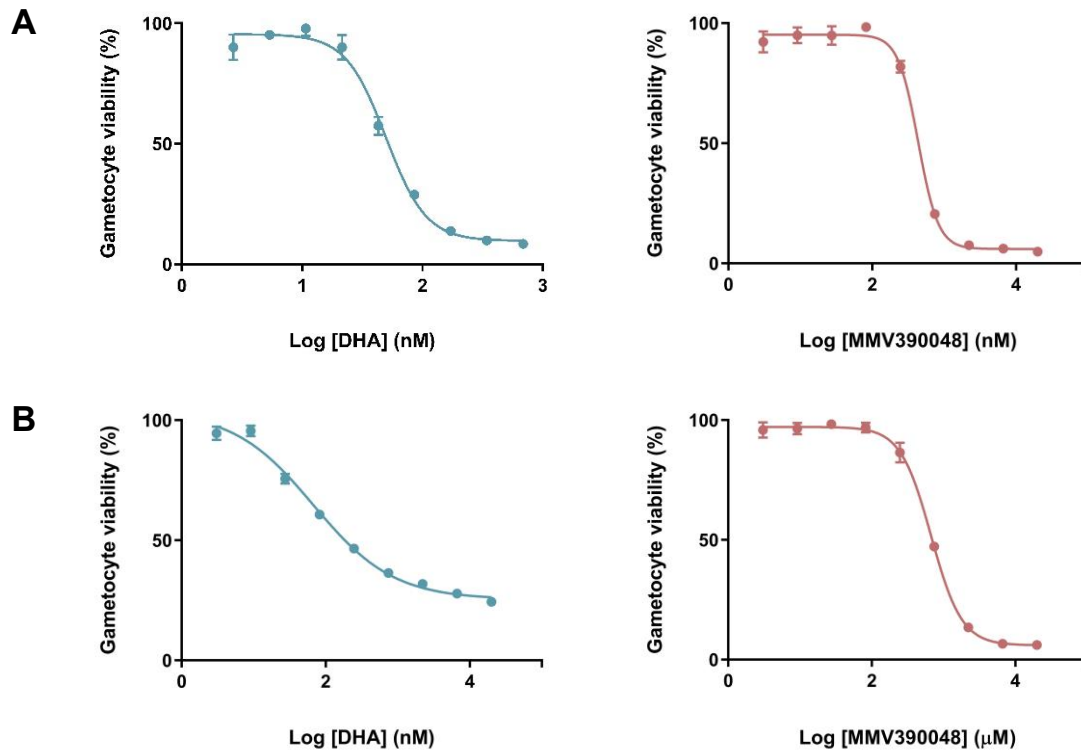


Figure 3.2. *In vitro* activity of DHA and MMV390048 against *P. falciparum* gametocyte viability. Dose-response evaluation of DHA and MMV390048 against (A) early- and (B) late-stage gametocytes determined using a luciferase assay. Data are from three biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

3.1.3. Isobologram analysis of the combination of DHA and MMV390048 against asexual and gametocyte stages of *P. falciparum* parasites

The proposed use of a dual active drug combination, DHA and MMV390048, was evaluated by constructing fixed ratio isobolograms and measuring the mean Σ FIC values against a combination index to determine whether their combined activity was representative of a synergistic, antagonistic, or additive interaction. A drug combination is considered additive or indifferent if the cutoff Σ FIC value ranges between 0.8-1.4 and a linear regression line is observed. Σ FIC values < 0.8 with curves displaced to the bottom left are regarded as synergistic interactions. Finally, combinations that are consistent with Σ FIC values > 1.4 and curves displaced to the top right are considered antagonistic [115, 116].

The combination of DHA and MMV390048 in asexual parasites presented a mean Σ FIC value of 1.49, indicative of an indifferent interaction tending toward antagonism (Figure 3.3 A). Combining DHA and MMV390048 in early-stage gametocytes resulted in a mean Σ FIC value of 1.36, which falls within the cutoff range for additivity (Figure 3.3 B). Late-stage gametocytes were more susceptible to dual inhibition by DHA and MMV390048 than asexual parasites and early-stage gametocytes. A strongly synergistic interaction was observed between the combination (mean Σ FIC 0.52) with a concave deviation from the line (Figure 3.3 C).

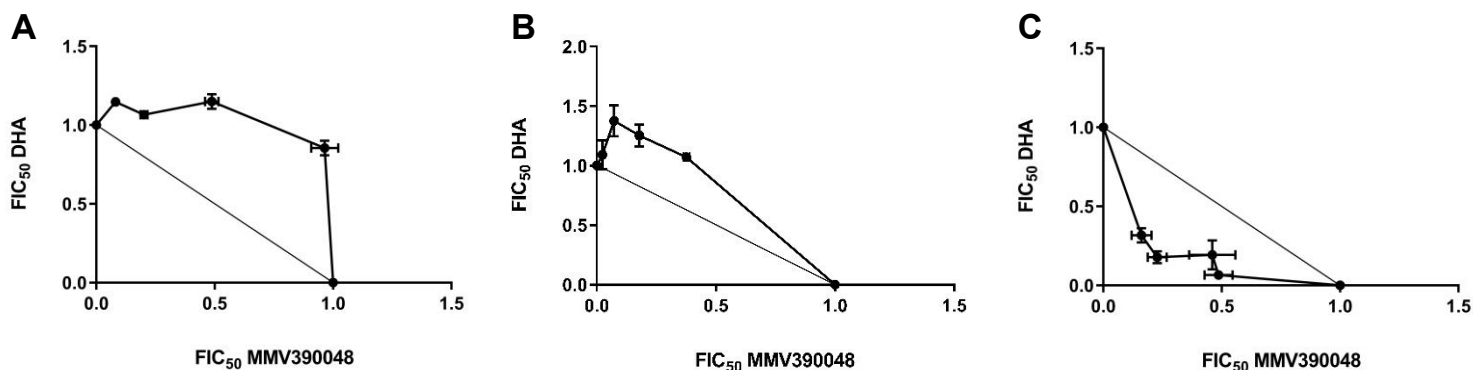


Figure 3.3. Antiplasmodial activity of DHA and MMV390048 in combination in *P. falciparum*. Isobolograms show either additive or indifferent (FIC 0.8-1.4, slight deviation from linear regression line), synergistic (FIC < 0.8, curve to left) or antagonistic (FIC > 1.4, curve to right) interactions between DHA and MMV390048 in (A) asexual parasites and (B) early- and (C) late-stage gametocytes (Table 3.1). Results are from the SYBR Green I-based fluorescence assay against asexual parasites and a luciferase assay against gametocyte stages. The mean Σ FIC data are from three biological repeats ($n=3$) performed in technical triplicates, $\pm$ S.E. Error bars fall within symbols where not shown.

Table 3.1. The average fractional inhibitory concentrations (FIC) of DHA and MMV390048 combinations against *P. falciparum* parasites.

	Mean Σ FIC
Asexual parasites	1.49 $\pm$ 0.28
Early-stage gametocytes	1.36 $\pm$ 0.09
Late-stage gametocytes	0.52 $\pm$ 0.15

The antagonism that extends to the combination in asexual parasites and early-stage gametocytes supports the assumption that no interaction would be expected between DHA and MMV390048 based on their different MoA in *P. falciparum*. In contrast, late-stage gametocytes were more susceptible to dual inhibition by DHA and MMV390048. The pronounced synergistic effect implies that there is a positive interaction between the combination during this stage, which was not expected.

3.2. *In vitro* activity of drugs incorporated into SLNs

Given that *P. falciparum* parasites were susceptible to the dual active drug combination of DHA and MMV390048 and considering the poor PK and PD properties of the compounds alone, it was reasonable to assume that this therapeutic intervention would be advantageous in encapsulation. Therefore, it was necessary to evaluate if SLN formulations of DHA and MMV390048 reflected improved antiplasmodial activity without negating the efficacy of the compounds alone.

Thus, the antiplasmodial activity of DHA and MMV390048-loaded SLNs against *P. falciparum* NF54 asexual parasites was investigated using the SYBR Green I-based proliferation assay. QN, unencapsulated and encapsulated formulations, was evaluated as a comparative/reference control to verify the different polymers being used.

Dose-response curves were observed for CHQN and CHQNW from which an IC_{50} of 14.15 ± 1.71 nM and 48.44 ± 4.96 nM was obtained, respectively (Figure 3.4 A). Therefore, QN-encapsulated SLNs did not present improved activity compared to QN alone, with an IC_{50} of 5.82 ± 0.04 nM (compared to a previous report of 56 nM using the same assay platform [117]). CHQNW demonstrated an eight-fold decrease in activity compared to QN alone, a significant difference (one-way ANOVA, Tukey's multiple comparisons test, $n=3 \pm$ S.E., $p < 0.001$). By contrast, no appreciable difference was observed between CHQN and QN alone (Figure 3.4 B).

Therefore, it was important to determine if the observed antiparasmodial activity was influenced by surfactants. This was achieved by comparing the activities of CHQN and CHQNW at the same concentration range of QN and percentage of polymer, across the dose-response curves, to accurately and simultaneously identify if surfactants influence QN activity. CHQNW demonstrated a three-fold decrease in activity compared to CHQN (Figure 3.4 A), a significant difference (one-way ANOVA, Tukey's multiple comparisons test, $n=3 \pm$ S.E., $p < 0.001$). This suggests that the removal of surfactants from formulations influences the antiparasmodial activity of QN-encapsulated SLNs. Surfactants are stabilisers and their removal possibly promotes NP aggregation, thus reducing the concentration of QN delivered to or taken up by parasites.

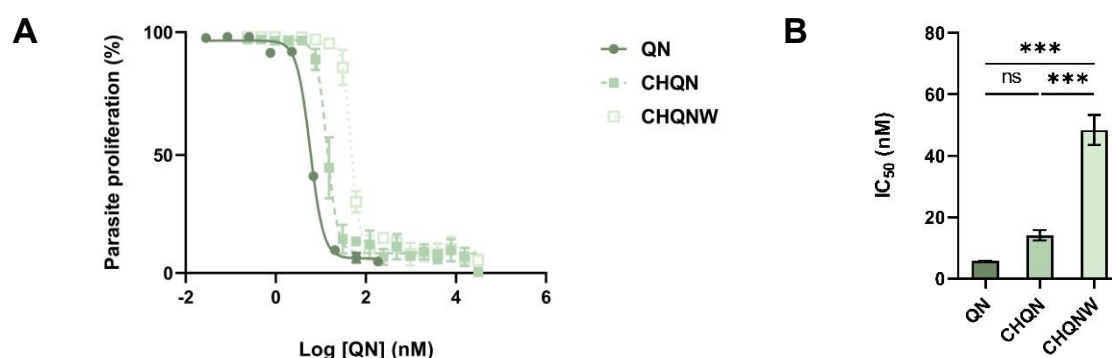


Figure 3.4. Effect of encapsulation on the antiparasmodial activity of QN against asexual *P. falciparum* parasite proliferation. Dose-response evaluation of (A) QN, CHQN and CHQNW against asexual parasites was determined using the SYBR Green I-based fluorescence assay. (B) Comparative histogram of observed IC_{50} activity between QN, CHQN and CHQNW. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

Given the observed activity of QN and its encapsulated formulations against asexual parasites, it was important to investigate if this was a systematic trend observed with DHA, MMV390048 and their encapsulations.

As with QN and its encapsulated polymers, DHA-encapsulated SLNs did not present improved antiparasmodial activity compared to DHA alone. A dose-response curve was observed for CHDHA and CHDHAW from which an IC_{50} of 2.46 ± 0.59 nM and 48.67 ± 5.32 nM was obtained, respectively (Figure 3.5 A). CHDHA did not show an appreciable difference in activity compared to DHA alone where CHDHAW indicated a significant, 11-fold decrease (one-way ANOVA, Tukey's multiple comparisons

test, $n=3 \pm \text{S.E.}$, $p < 0.001$) in activity, compared to DHA alone (Figure 3.5 B). Furthermore, at equivalent concentrations of DHA and percentage polymer, CHDHAW demonstrated a 20-fold decrease in activity compared to CHDHA (Figure 3.5 B), a significant difference (one-way ANOVA, Tukey's multiple comparisons test, $n=3 \pm \text{S.E.}$, $p < 0.001$). The results correspond to what was observed with QN and its encapsulated formulations: overall, CHDHAW showed the most significant decrease in antiparasmodial activity.

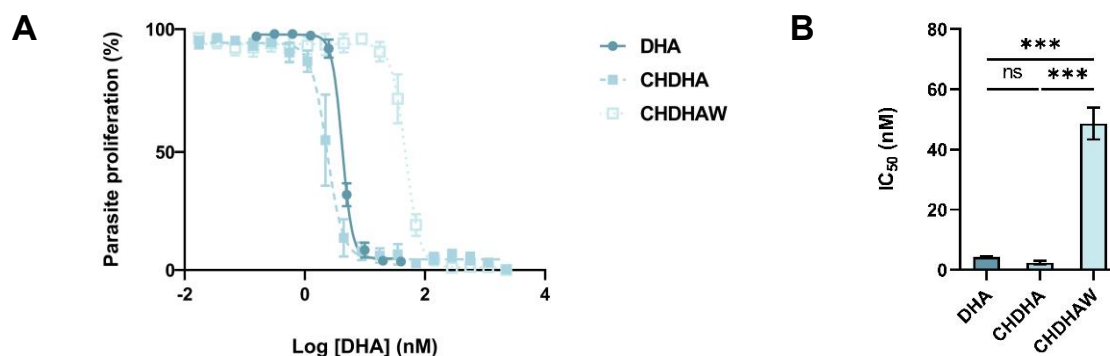


Figure 3.5. Effect of encapsulation on the antiparasmodial activity of DHA against asexual *P. falciparum* parasite proliferation. Dose-response evaluation of (A) DHA, CHDHA and CHDHAW against asexual parasites was determined using the SYBR Green I-based fluorescence assay. (B) Comparative histogram of observed IC₅₀ activity between DHA, CHDHA and CHDHAW. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

For MMV390048-encapsulated SLNs, CHMMV demonstrated a significant decrease (unpaired, two-tailed Student's *t*-test, $n=3 \pm \text{S.E.}$, $p < 0.05$) in activity compared to MMV390048 alone (IC₅₀: 5.76 ± 1.78 μM vs. IC₅₀: 6.44 ± 0.22 nM, respectively), seen in Figure 3.6. Notably, CHMMVW samples were unavailable to determine if surfactants influenced the observed antiparasmodial activity. Thus, it was important to determine if the observed ~ 900-fold decrease in activity was due to the encapsulating SLN formulations presenting some *in vitro* antiparasmodial activity.

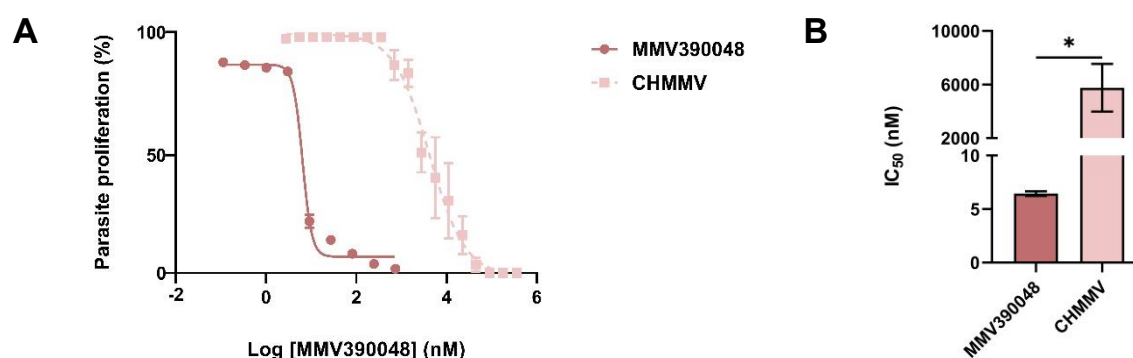


Figure 3.6. Effect of encapsulation on the antiparasmodial activity of MMV390048 against asexual *P. falciparum* parasite proliferation. Dose-response evaluation of (A) MMV390048 and CHMMV against asexual parasites was determined using the SYBR Green I-based fluorescence assay. (B) Comparative histogram of observed IC₅₀ activity between MMV390048 and CHMMV. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

Given the SLN formulations of QN, DHA and MMV390048 did not present improved antiparasmodial activity compared to using the unencapsulated formulations, it was important to determine if the

observed antiparasmodial activity was attributable to the encapsulating formulations as well or if the activity was due to the release of the incorporated drugs only. This was achieved by, first, investigating the antiparasmodial activity of the SLNs alone and then comparing the activities to corresponding SLN formulations.

A dose-response curve was observed for CHPL and CHPLW from which an IC_{50} of $5.78 \times 10^{-3} \pm 0.68 \times 10^{-3} \%$ and $7.26 \times 10^{-3} \pm 1.36 \times 10^{-3} \%$ was obtained, respectively (Figure 3.7 A and B). The SLNs indicate inhibitory activity against asexual parasites. Interestingly, the SLNs alone had an opposite profile to what was observed above, where surfactants did not influence the activity (Figure 3.7 C).

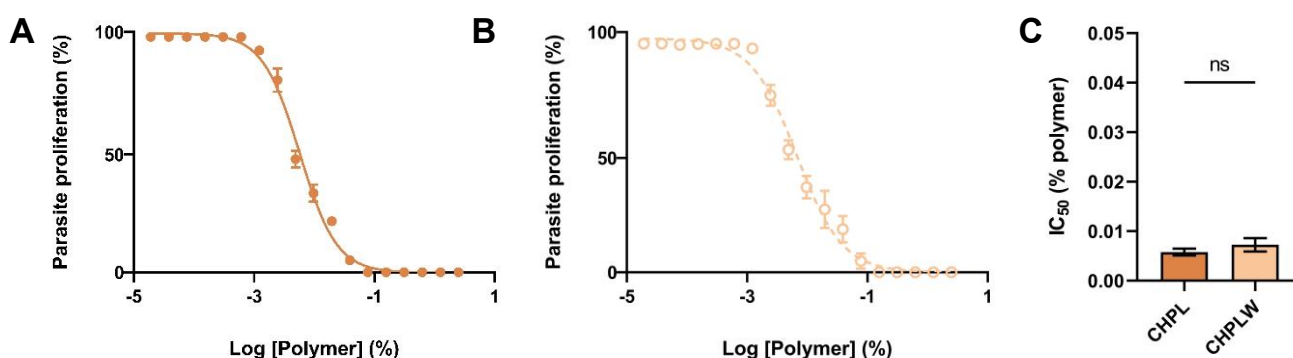


Figure 3.7. Dose-response evaluation of placebo SLNs against asexual *P. falciparum* parasite proliferation. The *in vitro* activity of (A) CHPL and (B) CHPLW against asexual parasites was determined using the SYBR Green I-based fluorescence assay. (C) Comparative histogram of observed IC_{50} activity between CHPL and CHPLW. No significant difference is indicated at $p > 0.05$ (ns) as determined with an unpaired, two-tailed Student's *t*-test. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

As expected, a significant difference (unpaired, two-tailed Student's *t*-test, $n=3 \pm$ S.E., $p < 0.01$) was observed when comparing the activities of CHPL and CHQN (IC_{50} : $5.78 \times 10^{-3} \pm 0.68 \times 10^{-3} \%$ vs. IC_{50} : $4.16 \times 10^{-5} \pm 0.50 \times 10^{-5} \%$, respectively) and CHPLW and CHQNW (IC_{50} : $7.26 \times 10^{-3} \pm 1.36 \times 10^{-3} \%$ vs. IC_{50} : $14.21 \times 10^{-5} \pm 1.45 \times 10^{-5} \%$, respectively), seen in Figure 3.8.

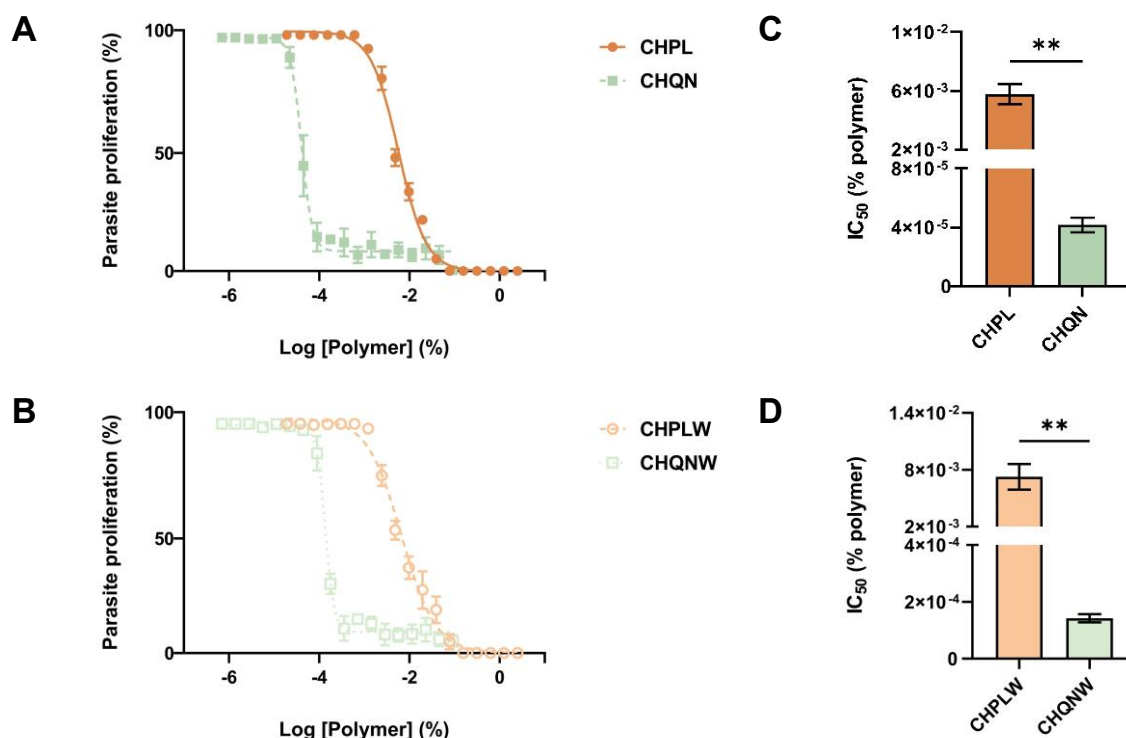


Figure 3.8. Impact of encapsulating formulations on the antiplasmodial activity of QN-encapsulated SLNs against asexual *P. falciparum* parasite proliferation. (A) *In vitro* activity of CHPL and CHQN, and (B) CHPLW and CHQNW against asexual parasites as a measure of percentage polymer. (C) Comparative histograms of observed IC_{50} activity between CHPL and CHQN, and (D) CHPLW and CHQNW. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

As with QN-encapsulated polymers, for the activities between CHPL and CHDHA (IC_{50} : $5.78 \times 10^{-3} \pm 0.68 \times 10^{-3} \%$ vs. IC_{50} : $4.23 \times 10^{-5} \pm 1.01 \times 10^{-5} \%$, respectively) and CHPLW and CHDHA (IC_{50} : $7.26 \times 10^{-3} \pm 1.36 \times 10^{-3} \%$ vs. IC_{50} : $83.73 \times 10^{-5} \pm 9.17 \times 10^{-5} \%$, respectively), a significant difference (unpaired, two-tailed Student's *t*-test, $n=3 \pm S.E.$, $p < 0.01$) was observed (Figure 3.9).

Notably, CHMMV samples were synthesised with a different batch of polymers with a different lipid fraction compared to the polymers above. Since the polymer on its own was not available, no direct comparison could be made to confirm if the observed antiplasmodial activity was attributable to the encapsulating formulation, and not only due to the release of MMV390048. Taken together, the results substantiate that the encapsulating formulations have antiplasmodial activity. However, the observed death effect was attributed to the encapsulated compounds rather than the encapsulating formulations alone. SLN formulations of the compounds did not present improved efficacy against asexual parasites compared to using the compounds alone. Surfactants are also necessary for therapeutic efficacy, possibly by minimising NP aggregation.

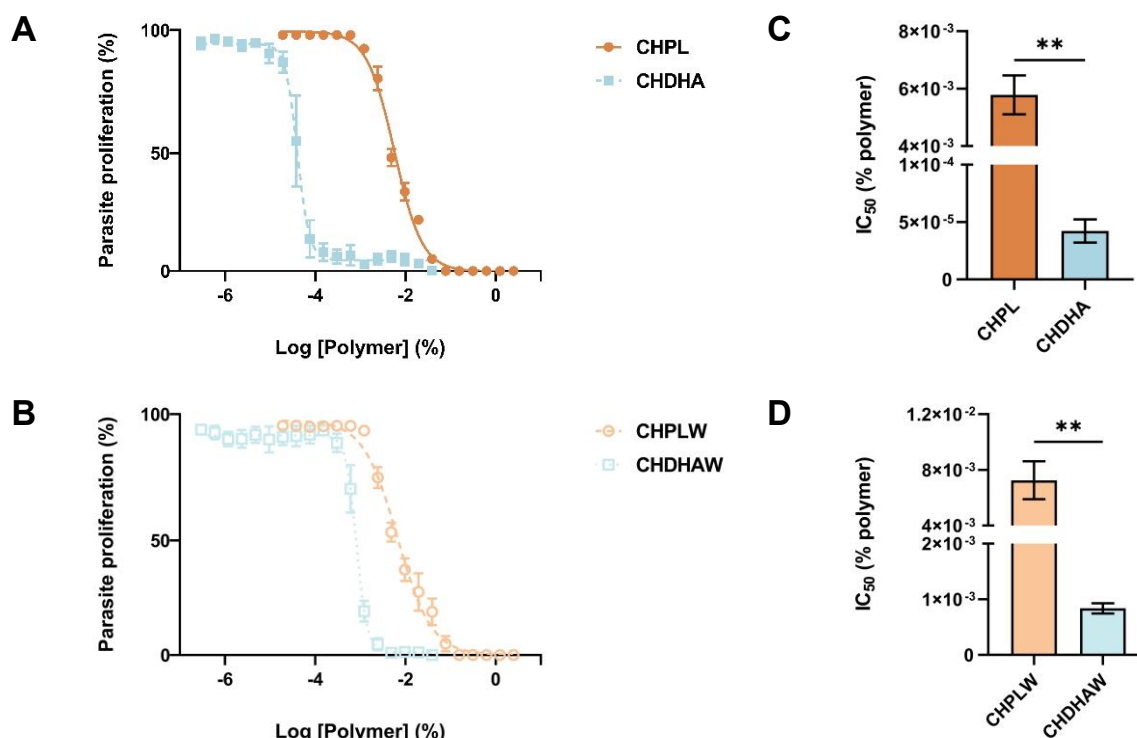


Figure 3.9. Impact of encapsulating formulations on the antiparasmodial activity of DHA-encapsulated SLNs against asexual *P. falciparum* parasite proliferation. (A) *In vitro* activity of CHPL and CHDHA, and (B) CHPLW and CHDHAW against asexual parasites as a measure of percentage polymer. (C) Comparative histograms of observed IC_{50} activity between CHPL and CHDHA, and (D) CHPLW and CHDHAW. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

3.3. Evaluation of the biocompatibility of SLNs

3.3.1. Haemolytic toxicity of compounds incorporated into SLNs

Given antiparasmodial activity was observed by encapsulated formulations, it was important to evaluate to what extent this was due to specific effects of the encapsulated compound rather than host-cell toxicity of the encapsulating SLN formulations. This was achieved by, first, using haemolysis as an indicator of toxicity. QN, unencapsulated and encapsulated formulations, were evaluated as a comparative/reference control to verify the different polymers being used. The SLNs alone indicated haemolysis of erythrocytes, with an HC_{50} of 0.12 ± 0.02 % for CHPL and 0.12 ± 0.01 % for CHPLW (Figure 3.10 A and B). Furthermore, no appreciable difference was observed between CHPL and CHPLW, suggesting that surfactants do not influence the haemolytic activity observed (Figure 3.10 C). Given the SLNs alone were toxic to erythrocytes *in vitro*, it was essential to determine whether this was the case with the drug-loaded SLNs and, if so, whether toxicity was influenced by drug incorporation or only due to the encapsulating formulations.

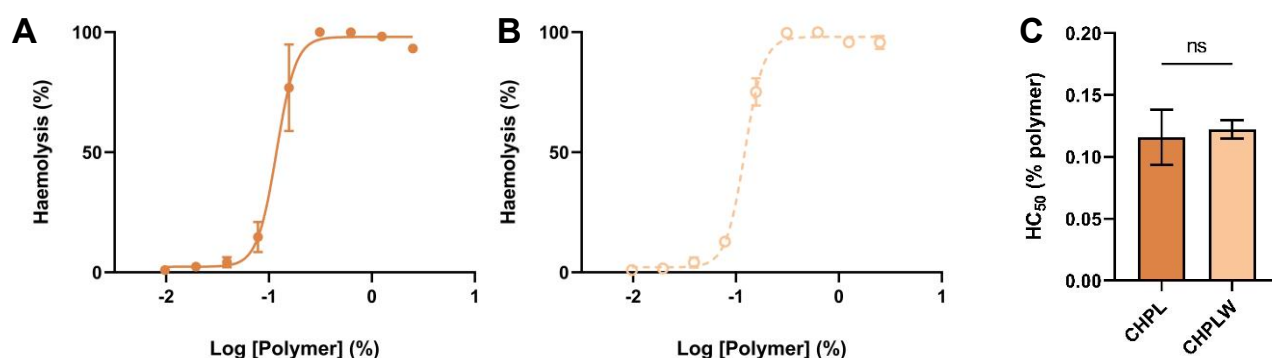


Figure 3.10. Dose-response evaluation of placebo SLNs against human erythrocytes. The haemolytic activity of (A) CHPL and (B) CHPLW was determined using an *in vitro* haemolysis assay. (C) Comparative histogram of observed HC₅₀ activity between CHPL and CHPLW. No significant difference is indicated at $p > 0.05$ (ns) as determined with an unpaired, two-tailed Student's *t*-test. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

At equivalent concentrations of QN, where QN alone was not haemolytic to erythrocytes and in the presence of polymers, CHQN and CHQNW caused haemolysis (HC₅₀: $28.31 \pm 2.86 \mu\text{M}$ and HC₅₀: $39.47 \pm 3.36 \mu\text{M}$, respectively), seen in Figure 3.11. This equates to an HC₅₀ of $0.08 \pm 0.01 \%$ for CHQN and $0.12 \pm 0.01 \%$ for CHQNW as a measure of percentage polymer, and as with the SLNs alone, indicates no appreciable difference between the encapsulated formulations. The results substantiate that surfactants do not influence the haemolytic activity. Furthermore, the observed haemolytic activity of QN-encapsulated SLNs was not due to drug incorporation. It was then important to determine whether the observed toxicity was due to the encapsulating formulations alone. This was achieved by comparing the haemolysis caused by the SLNs alone to CHQN and CHQNW.

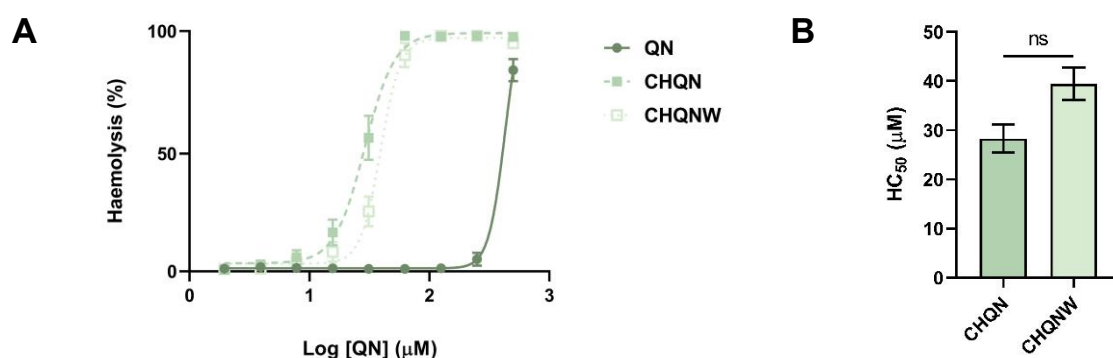


Figure 3.11. Effect of encapsulation on the haemolytic activity of QN against human erythrocytes. Dose-response evaluation of erythrocyte lysis caused by (A) QN, CHQN and CHQNW was determined using an *in vitro* haemolysis assay. (B) Comparative histogram of observed HC₅₀ activity between CHQN and CHQNW as a measure of drug concentration. No significant difference is indicated at $p > 0.05$ (ns) as determined with an unpaired, two-tailed Student's *t*-test. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

At equivalent polymer concentrations, CHPL and CHQN did not show an appreciable difference in HC₅₀ (HC₅₀: $0.12 \pm 0.02 \%$ vs. HC₅₀: $0.08 \pm 0.01 \%$, respectively). Similarly, no appreciable difference was observed between the haemolysis caused by CHPLW and CHQNW (HC₅₀: $0.12 \pm 0.01 \%$ vs. HC₅₀:

0.12 ± 0.01 %, respectively), seen in Figure 3.12. Interestingly, these results suggest that the encapsulating formulations were not solely responsible for the haemolytic activity observed. A possible explanation for the haemolytic activity due to the encapsulated formulations, compared to the compound alone, is that other excipients constituting the SLNs are at play.

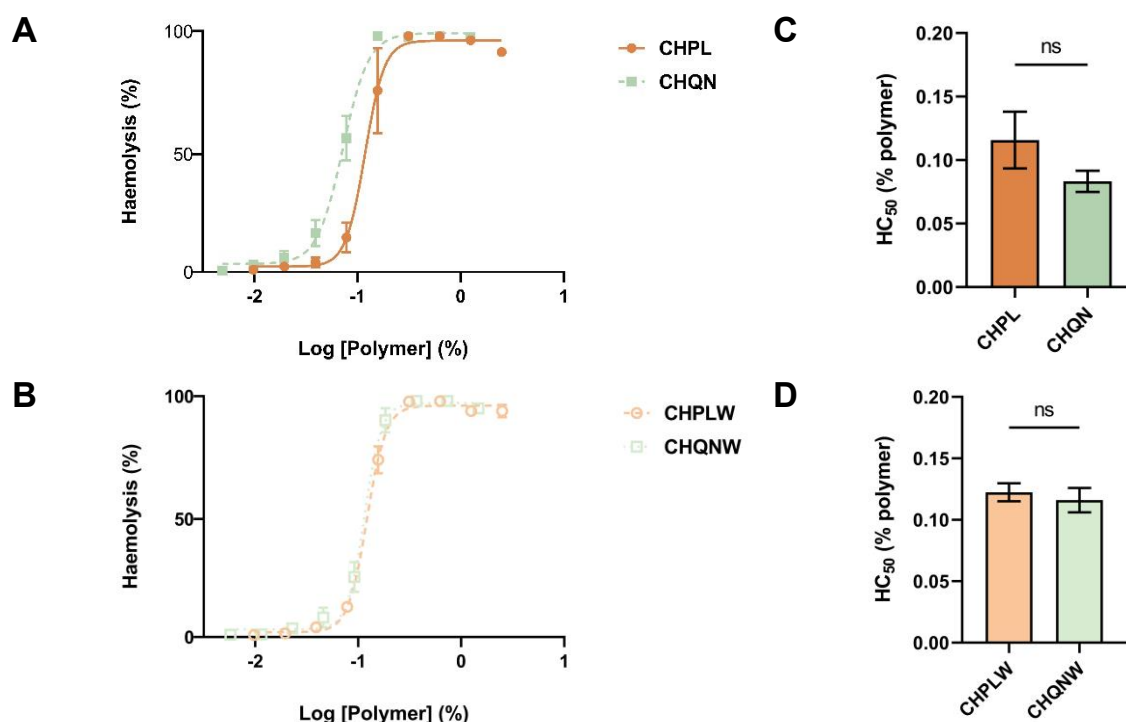


Figure 3.12. Impact of encapsulating formulations on the haemolytic activity of QN-encapsulated SLNs against human erythrocytes. (A) Erythrocyte lysis caused by CHPL and CHQN, and (B) CHPLW and CHQNW as a measure of percentage polymer. (C) Comparative histograms of observed HC₅₀ activity between CHPL and CHQN, and (D) CHPLW and CHQNW. No significant difference is indicated at $p > 0.05$ (ns) as determined with an unpaired, two-tailed Student's t -test. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

At equivalent concentrations of DHA, where DHA alone was haemolytic to erythrocytes, and in the presence of polymers, CHDHA and CHDHAW were five and three-fold less haemolytic ($7.85 \pm 1.56 \mu\text{M}$ and $14.96 \pm 3.46 \mu\text{M}$, respectively) than DHA alone ($38.20 \pm 0.78 \mu\text{M}$), seen in Figure 3.13 A. This equates to HC₅₀ values of 0.14 ± 0.03 % for CHDHA and 0.26 ± 0.06 % for CHDHAW as a measure of percentage polymer. As expected, the results indicate that surfactants do not influence the haemolytic activity. By contrast, CHDHA and CHDHAW had an opposite profile to QN-encapsulated SLNs, where a significant difference (one-way ANOVA, Tukey's multiple comparisons test, $n=3 \pm \text{S.E.}$, $p < 0.001$) was observed between the formulations (Figure 3.13 B). This indicates that drug incorporation was attributable to the haemolytic activity observed and suggests that SLNs significantly improved the safety of DHA towards erythrocytes *in vitro*. It is noteworthy that the haemolytic activity of DHA alone was determined at concentrations more than $>1000\times$ than the antiparasmodial IC₅₀ for DHA. It was, therefore, essential to further evaluate if the observed toxicity was attributable to the encapsulating formulations.

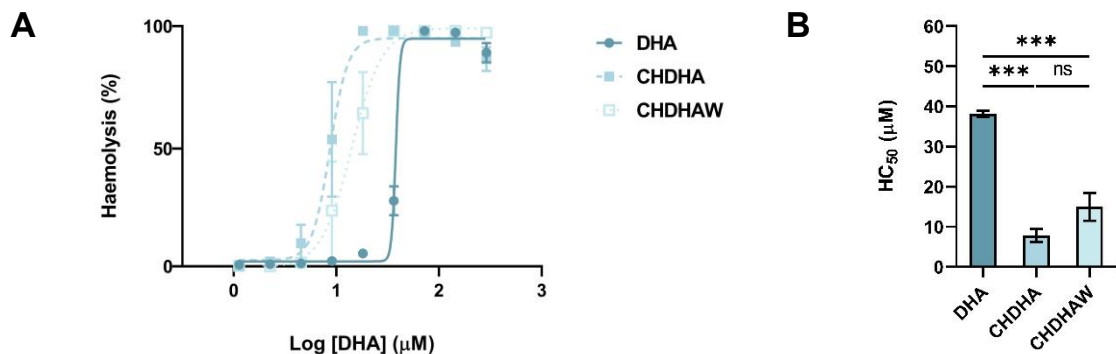


Figure 3.13. Effect of encapsulation on the haemolytic activity of DHA against human erythrocytes. Dose-response evaluation of erythrocyte lysis caused by (A) DHA, CHDHA and CHDHAW was determined using an *in vitro* haemolysis assay. (B) Comparative histogram of observed HC₅₀ activity between DHA, CHDHA and CHDHAW. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

At equivalent polymer concentrations, no appreciable difference in haemolytic activity was observed between CHPL and CHDHA (HC₅₀: 0.12 ± 0.02 % vs. HC₅₀: 0.14 ± 0.03 %, respectively) and CHPLW and CHDHAW (HC₅₀: 0.12 ± 0.01 % vs. HC₅₀: 0.26 ± 0.06 %, respectively), seen in Figure 3.14. These results suggest that the observed toxicity was due to drug incorporation.

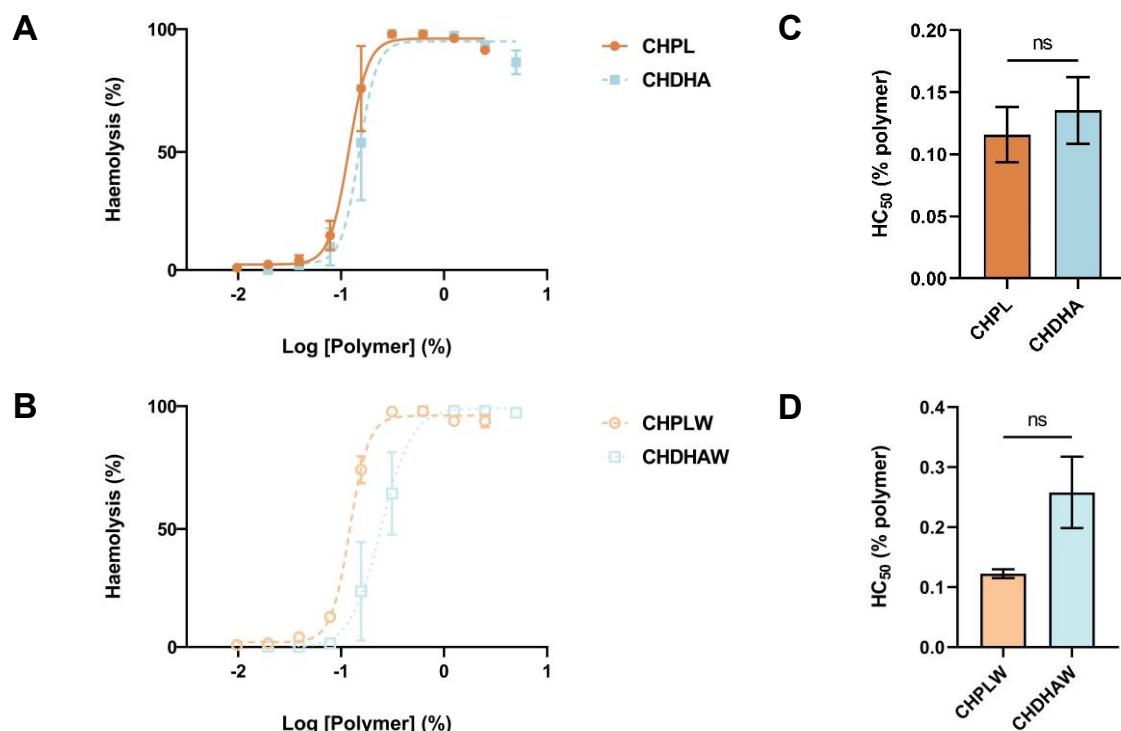


Figure 3.14. Impact of encapsulating formulations on the haemolytic activity of DHA-encapsulated SLNs against human erythrocytes. (A) Erythrocyte lysis caused by CHPL and CHDHA, and CHPLW and (B) CHDHAW as a measure of percentage polymer. (C) Comparative histograms of observed HC₅₀ activity between CHPL and CHDHA, and (D) CHPLW and CHDHAW. No significant difference is indicated at $p > 0.05$ (ns) as determined with an unpaired, two-tailed Student's *t*-test. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates. Error bars fall within symbols where not shown.

CHMMV indicated haemolysis of erythrocytes at an HC₅₀ of 0.45 ± 0.04 %. Unfortunately, CHMMVW samples were not available to determine whether surfactants influence the observed haemolytic activity.

At equivalent concentrations of MMV390048, where MMV390048 alone was not haemolytic to erythrocytes, and in the presence of polymers, haemolysis was observed by CHMMV (HC_{50} of $0.10 \pm 0.01 \mu\text{M}$), seen in Figure 3.15. These results suggest that the observed haemolytic activity was not attributable to drug incorporation. No evaluations were performed on CHMMV to confirm if surfactants influence the observed haemolytic activity and if the toxicity was caused by the encapsulating formulations alone. The results further point to the toxicity of the SLNs against healthy, non-infected erythrocytes *in vitro*.

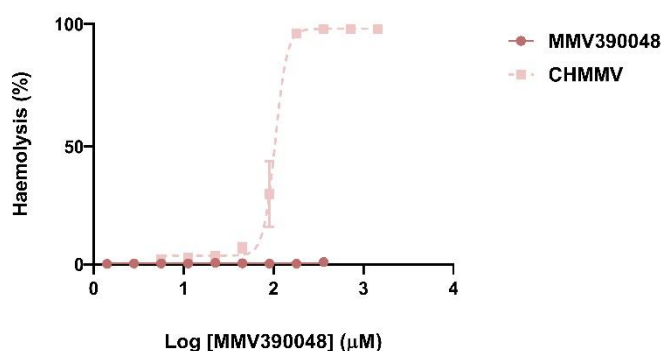


Figure 3.15. Haemolytic activity of MMV390048 against human erythrocytes and the effect of encapsulation thereof. Dose-response evaluation of erythrocyte lysis caused by MMV390048 and CHMMV was determined using an *in vitro* haemolysis assay. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

3.3.2. Cytotoxicity of compounds incorporated into SLNs

Previous reports on the cytotoxicity of SLNs indicate that the polymers present no acute nor chronic toxic effects on rabbit and human nasal mucosa cells. However, no information exists on its effects on human hepatocellular cells. Therefore, here, we evaluated to what extent the observed antiplasmodial activity of the encapsulated formulations was due to host-cell toxicity of the encapsulating SLN formulations using HepG2 viability as an indicator of toxicity. Cytotoxic drug Emetine was used as a control drug for cytotoxicity ($\sim 89\%$) at $50 \mu\text{M}$. The SLNs alone were cytotoxic to HepG2 cells, with no appreciable difference observed between CHPL and CHPLW at 9 % and 90 % polymer exposure, suggesting that surfactants do not influence the cytotoxicity observed (Figure 3.16). Since the SLNs alone were cytotoxic to HepG2 cells *in vitro*, it was important to determine whether this was the case with the drug-loaded SLNs and, if so, whether toxicity was influenced by drug incorporation or only due to the encapsulating formulations.

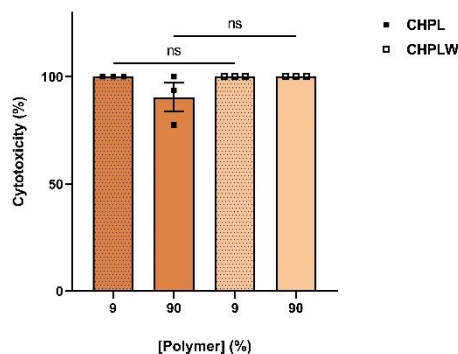


Figure 3.16. *In vitro* cytotoxicity of placebo SLNs against human hepatocellular carcinoma (HepG2) cells. Dual point inhibitory activity of (A) CHPL and CHPLW against HepG2 cells was determined using the lactate dehydrogenase (LDH) cytotoxicity assay. No significant difference is indicated at $p > 0.05$ (ns) as determined with a one-way ANOVA and Tukey's multiple comparisons test. Data are from three independent biological repeats ($n = 3$), performed in technical duplicates, individual data points indicated.

Negligible cytotoxicity of QN alone, CHQN and CHQWN to HepG2 cells was observed at 2 μM of QN and no significant difference was observed at 20 μM (Figure 3.17 A). Thus, the observed cytotoxicity was not attributable to drug incorporation. As with the SLNs alone, at equivalent polymer concentrations, surfactants did not influence the observed cytotoxicity of CHQN and CHQNW (Figure 3.17 B). These results suggest that incorporating QN likely improved the biocompatibility of SLNs to HepG2 cells *in vitro*. However, to make a direct comparison to the SLNs alone, a cytotoxicity dose-response would need to be investigated.

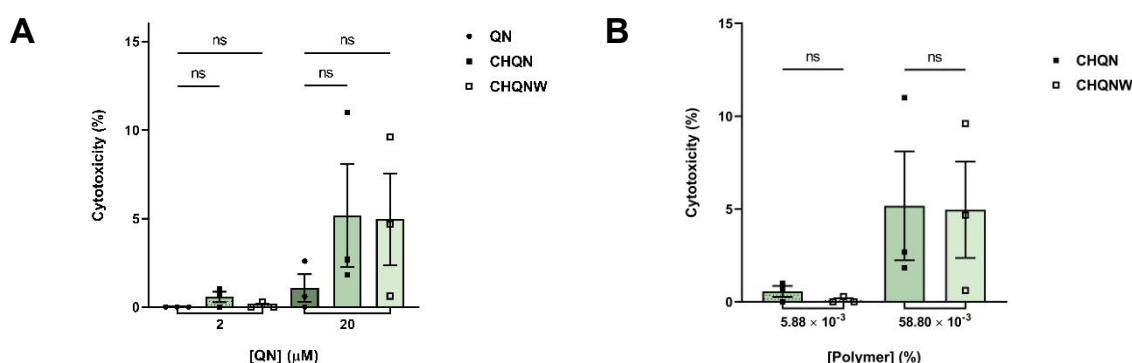


Figure 3.17. Effect of encapsulation on the cytotoxicity of QN against human hepatocellular carcinoma (HepG2) cells. Dual point inhibitory activity of (A) QN, CHQN and CHQNW against HepG2 cells was determined using the lactate dehydrogenase (LDH) cytotoxicity assay. (B) Cytotoxicity of CHQN in comparison to CHQNW as a measure of percentage polymer. No significant difference is indicated at $p > 0.05$ (ns) as determined with a one-way ANOVA and Tukey's multiple comparisons test. Data are from three independent biological repeats ($n = 3$), performed in technical duplicates, individual data points indicated.

Similarly, at 2 μM of DHA, no appreciable difference was observed between DHA alone, CHDHA and CHDHAW (Figure 3.18 A). However, at 20 μM , significant differences were observed between DHA vs. CHDHA (two-way ANOVA, Tukey's multiple comparisons test, $n=3 \pm \text{S.E.}$, $p < 0.001$) and DHA vs. CHDHAW (two-way ANOVA, Tukey's multiple comparisons test, $n=3 \pm \text{S.E.}$, $p < 0.01$). There was also no indication that surfactants influenced the cytotoxicity of CHDHA and CHDHAW to HepG2 cells when evaluated at equivalent polymer concentrations (Figure 3.18 B). This further suggests that drug

incorporation decreases the cytotoxicity of SLNs to HepG2 cells *in vitro*, and that cytotoxicity was likely due to the encapsulating formulations alone.

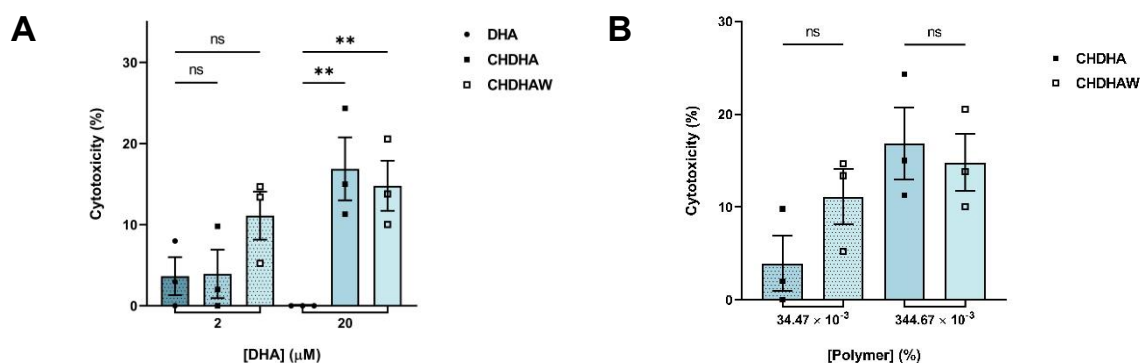


Figure 3.18. Effect of encapsulation on the cytotoxicity of DHA against human hepatocellular carcinoma (HepG2) cells. Dual point inhibitory activity of (A) DHA, CHDHA and CHDHAW against HepG2 cells was determined using the lactate dehydrogenase (LDH) cytotoxicity assay. (B) Cytotoxicity of CHDHA in comparison to CHDHAW as a measure of percentage polymer. Data are from three independent biological repeats ($n = 3$), performed in technical duplicates, individual data points indicated.

At 2 μM and 20 μM of MMV390048, no significant difference was observed between the cytotoxicity of MMV390048 alone and CHMMV to HepG2 cells (Figure 3.19), suggesting that the observed cytotoxicity was not attributable to drug incorporation. Since CHMMV was synthesised with a different batch of polymers, no evaluation could be made that would further substantiate the cytotoxicity of SLNs to human hepatocellular cells *in vitro*.

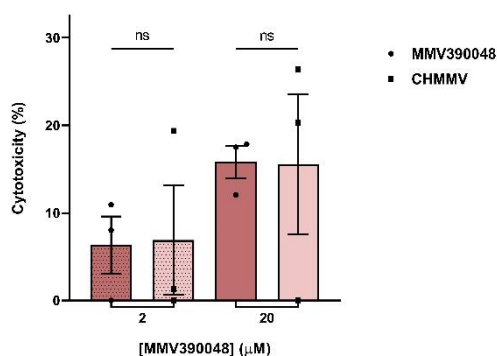


Figure 3.19. Effect of encapsulation on the cytotoxicity of MMV390048 against human hepatocellular carcinoma (HepG2) cells. (A) Dual point inhibitory activity of MMV390048 and CHMMV against HepG2 cells was determined using the lactate dehydrogenase (LDH) cytotoxicity assay. No significant difference is indicated at $p > 0.05$ (ns) as determined with a one-way ANOVA and Tukey's multiple comparisons test. Data are from three independent biological repeats ($n = 3$), performed in technical duplicates, individual data points indicated.

Collectively, the results exemplify that while the encapsulated formulations confer toxicity against mammalian cells and are not biocompatible to non-infected erythrocytes, the observed antiplasmodial activity was likely due to the release of the incorporated drugs and not attributable to host-cell toxicity. As expected, the toxicity caused by the encapsulating formulations, which was not attributable to the encapsulated compounds, was not influenced by surfactants.

3.4. Localisation of fluorescently labelled SLNs

It is possible that encapsulation of DHA and MMV390048 into SLNs would affect the uptake and subcellular distribution of these compounds and thus their activity. To determine whether this was the case, fluorescently labelled encapsulating SLN formulations were used to explore uptake and subcellular distribution using fluorescence microscopy.

Given the overt haemolysis and cytotoxicity observed by the SLNs, it was important to determine at what concentrations the fluorescently labelled SLNs would be suitable to be tested on erythrocytes and parasites to visualise uptake so that the effect observed is parasite-specific and not due to host-cell toxicity. Furthermore, it was important to deduce which fluorophore was most suitable for determining this.

First, using haemolysis as an indicator of toxicity, HC_{50} values of $0.57 \pm 0.03 \mu\text{M}$ for CHFL and $0.76 \pm 0.01 \mu\text{M}$ for CHSI were determined from the dose-response curves observed in Figures 3.20 A and B respectively. No appreciable difference in haemolytic activities between the fluorescently labelled SLNs was observed (Figures 3.20 C and D).

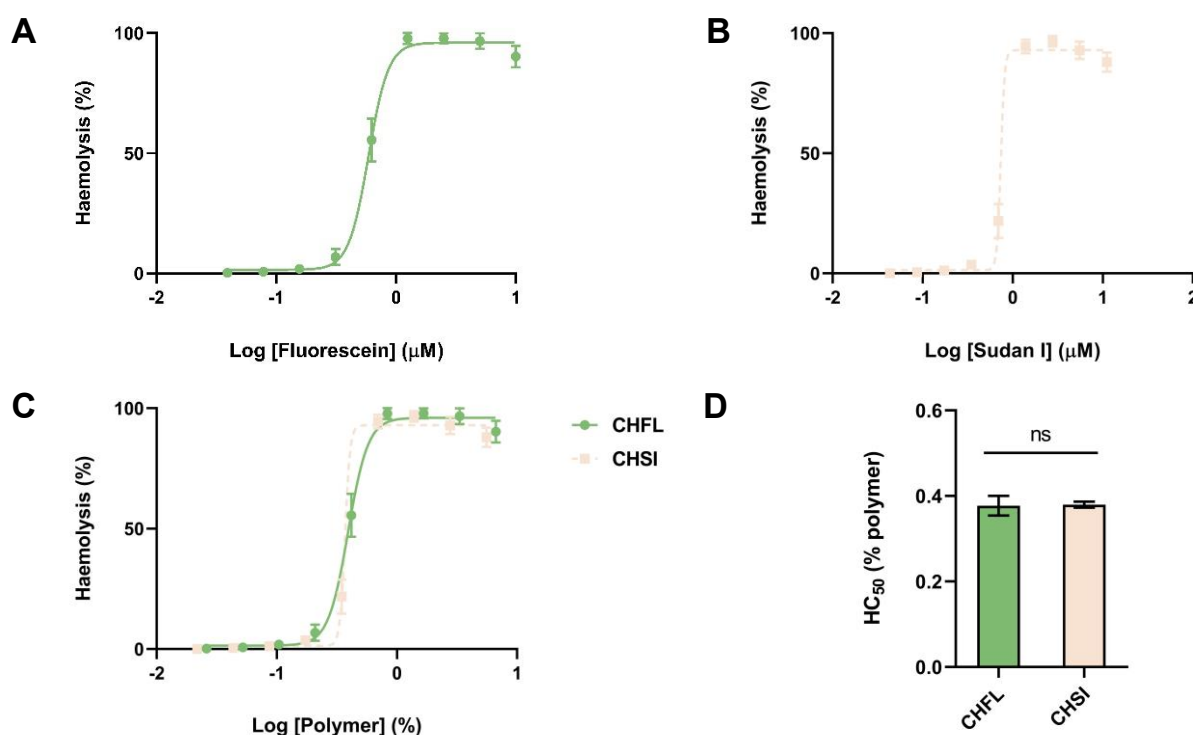


Figure 3.20. Haemolytic activity of fluorescently labelled SLNs against human erythrocytes. Dose-response evaluation of erythrocyte lysis caused by (A) CHFL and (B) CHSI was determined using an *in vitro* haemolysis assay. (C) Comparing the observed HC_{50} activities between CHFL and CHSI as a measure of percentage polymer. (D) No significant difference is indicated at $p > 0.05$ (ns) as determined with an unpaired, two-tailed Student's *t*-test. For all graphs, data are from three independent biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

Secondly, using HepG2 viability as an indicator of toxicity, the concentrations at what the fluorescently labelled SLN formulations would avoid cytotoxicity were investigated. The cytotoxicity criteria in HepG2 cells is set at a cutoff of <50 %, such that compounds that result in at least 50 % cell viability are deemed acceptable [118].

CHFL was significantly (unpaired, two-tailed Student's *t*-test, $n=3 \pm$ S.E., $p < 0.001$) more cytotoxic at 20 μ M (>50 %) than at 2 μ M (Figure 3.21 A), while CHSI showed minimal cytotoxicity (<50 %) for 2 μ M and 20 μ M (Figure 3.21 B). Ideally, concentrations that do not exceed 2 μ M for CHFL and CHSI would be suitable for determining parasite uptake and subcellular distribution.

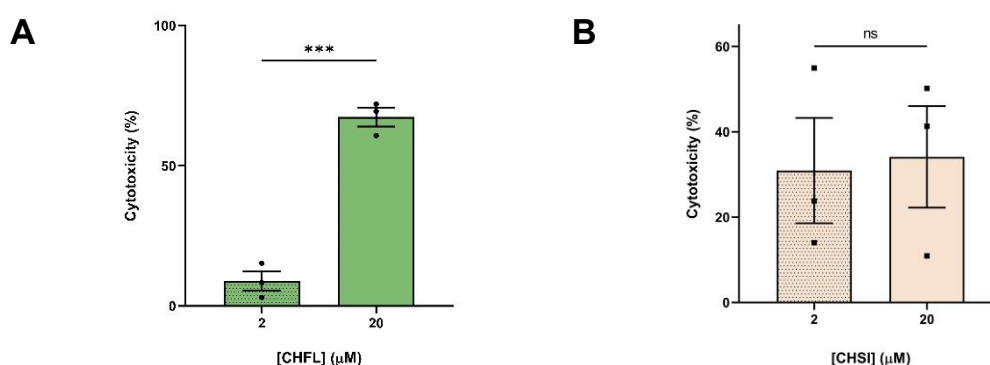


Figure 3.21. *In vitro* cytotoxicity of fluorescently labelled SLNs against human hepatocellular carcinoma (HepG2) cells. Dual point inhibitory activity of (A) CHFL and (B) CHSI against HepG2 cells was determined using the lactate dehydrogenase (LDH) cytotoxicity assay. Data are from three independent biological repeats ($n = 3$), performed in technical duplicates, individual data points indicated.

Since antiplasmodial activity was observed by the SLNs against asexual parasites, a dose-response evaluation was necessary to determine at what concentrations fluorescently labelled SLNs would not result in parasite death. An IC_{50} value of 0.04 ± 0.01 μ M was determined for CHSI (Figure 3.22). However, CHFL showed no activity against parasites, with 100 % parasite proliferation retained even at the highest tested concentration (10 μ M).

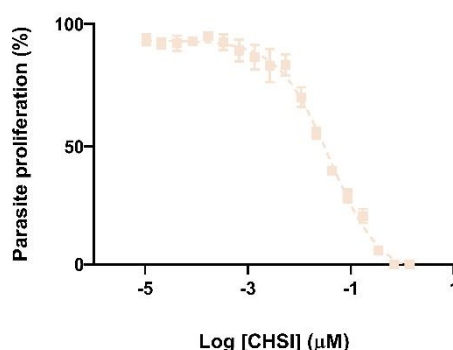


Figure 3.22. Dose-response evaluation of fluorescently labelled SLNs against asexual *P. falciparum* parasite proliferation. The *in vitro* activity of CHSI against asexual parasites was determined using the SYBR Green I-based fluorescence assay. Data are from three independent biological repeats ($n = 3$), performed in technical triplicates, mean $\pm$ S.E. indicated. Error bars fall within symbols where not shown.

Confocal microscopy of uninfected erythrocytes revealed that CHFL does not accumulate in erythrocytes. For CHSI, however, accumulation was observed in erythrocytes with a dispersed appearance. The signal from the Hoechst and DRAQ5 nuclear dyes was produced by autofluorescence as erythrocytes lack nuclei (Figure 3.23). In *P. falciparum* infected erythrocytes, co-staining with CHFL and Hoechst revealed that CHFL accumulates in the parasite with localisation in the cytoplasmic compartment and a dispersed distribution. Similarly, treatment of infected erythrocytes with CHSI and DRAQ5 showed that CHSI accumulates in the cytoplasmic compartment and with a discrete punctate distribution (Figure 3.23).

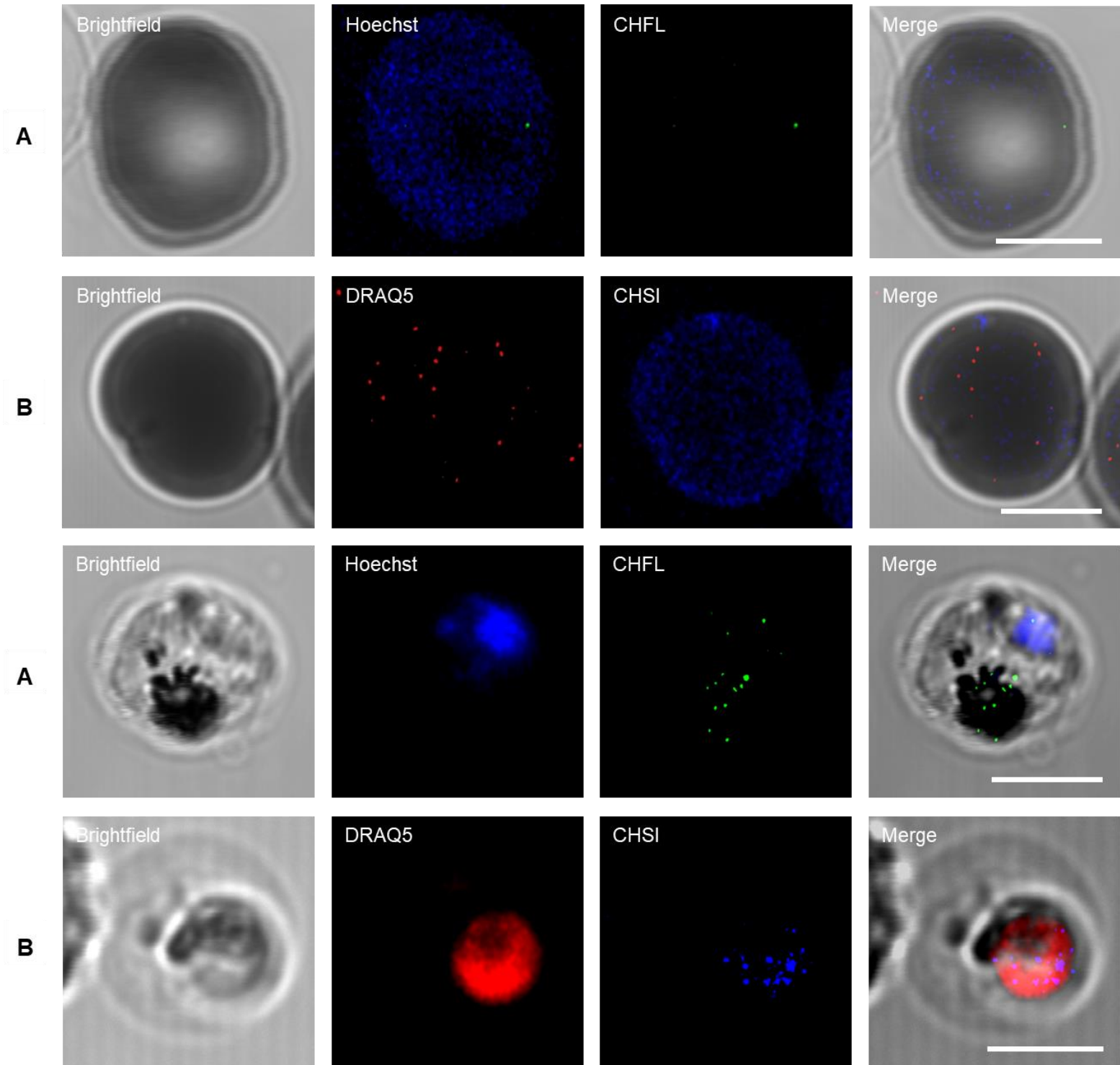


Figure 3.23. Confocal microscopic analysis of the intracellular uptake and distribution of fluorescently labelled SLNs in erythrocytes or *P. falciparum* infected erythrocytes. The top panels indicate uptake into erythrocytes and the bottom panels into infected erythrocytes. (A) shows co-stained with 0.5 μ M CHFL (green) and 1 μ g/mL Hoechst (blue), (B) 0.5 μ M of CHSI (blue) and DRAQ5 (red). Scale bars represent 5 μ M.

4. Discussion

The complex life cycle of the malaria parasite involves a 48 h asexual life cycle characterised by massive population expansion in the bloodstream. These stages cause symptoms in humans and are the main driver for drug discovery programmes. Ten percent of these parasites undergo differentiation to form gametocytes, which takes about 10-14 days in *P. falciparum* [18]. This occurs in the bone marrow in the earlier stages, but the mature stage V male and female gametocytes are found in the peripheral capillaries ensuring uptake by mosquitoes during bloodmeals. Stage V gametocytes are the targets of most drug discovery efforts targeting transmission, as they are the most pharmacologically amenable compartment to target the gametocytes. To impact malarial elimination strategies, this transmission cycle needs to be targeted to prevent onward sexual maturation in mosquitoes. Although most antimalarial drugs only target the asexual blood stages and not gametocytes, despite gametocytes remaining in the human host long after the asexual stages are cleared and where transmission to a mosquito vector can occur.

Drug uptake is suspected to differ between asexual and gametocyte stages of *P. falciparum* because, in asexual parasite-infected erythrocytes, the drug must traverse three membrane systems, namely the erythrocyte membrane, parasite vacuolar membrane (PVM), and parasite plasma membrane (PPM). In stage V gametocytes, the erythrocyte membrane is still present but is non-viable. Additionally, asexual parasites express NPPs, which are porous channels that go from the parasites through all these membranes to the erythrocytes, allowing solutes and compounds to enter the parasite. The NPPs are not active in stage V gametocytes. Therefore, a partial loss in the efficacy of compounds killing both stages is observed [119]. The IC_{50} values of both drugs decreased in early- and late-stage gametocytes compared to asexual parasites.

According to TCP-1 classifications for asexual-targeting drugs, DHA effectively enters the parasite. It diffuses through the erythrocyte membrane, but it also uses NPPs [69]. DHA is an effective and well-characterised artemisinin derivative and well-established in the use of ACTs. It is potent against asexual stages, has rapid action and a short half-life, and is a target indication of protein alkylation as a polypharmacological MoA [55, 60, 61]. However, dynamically, we need to control the lifespan and release of this compound as the compound has an extremely short half-life, rendering it almost completely ineffective against gametocytes that develop over a longer period.

Late-stage DHA activity is only observed when stage IV gametocytes are present in the culture. It is not active against stage V gametocytes because its known MoA is to target haem detoxification in the food vacuole, which is a process that is only active until stage III-IV developmental stages and not mature male and female differentiated stage V gametocytes [120]. The chemical properties that make DHA good for reaching an asexual blood stage parasite are not innately good for gametocyte uptake. This necessitates developing strategies to improve this compound's delivery and longevity.

The second partner drug of interest in this study, MMV390048, has TCP-1 asexual blood stage and TCP-5 transmission-blocking potency and is classified as a dual active compound [76, 77]. It has rapid action but a much longer half-life and a well-characterised MoA as a PI4K inhibitor [73, 75]. Drugs that in addition to targeting the symptomatic asexual stages, block transmission by rapidly killing transmissible forms of the parasite that persist in carriers for months must be long-lasting. Novel classes of compounds that will delay resistance development and have the added benefit of being potent inhibitors of multiple stages of the parasite are attractive therapeutic targets.

It is possible to use drug combinations to target more than one life cycle stage simultaneously. The combination of protein alkylating agents (DHA) and PI4K inhibitors (MMV390048) offers a potential therapeutic effect against *P. falciparum* infections. Compounds that have demonstrated poor activity can be partnered with another compound to enhance their effect in ACTs. No effective combination targeting the transmission stages exists yet. Drug combinations that differ considerably in terms of MoA are desirable to delay resistance development and prevent resistance development and an extended life cycle footprint is achieved. DHA and MMV390048 have not yet been explored in combinations and pose a vast avenue of exploration.

Isobole analyses were conducted where DHA and MMV390048 were combined in a fixed ratio to each other, and FIC values were observed for different life cycle stages. In asexual parasites and early-stage gametocytes, the combination functioned in an almost mutually exclusive manner from one another, recognising the fact that they have different MoA and expect a single compound to be more active than the compounds in treatment together. In asexual parasites, evidence of these combinations is artemisinin derivatives with pyrimethamine, which targets folate biosynthesis and is also clearly antagonistic [121] and artemisinin with thapsigargin, which is an ATP6 transport inhibitor and is additive [116]. This is expected since artemisinins inhibit alkylase ATP6 *in vitro*. Finally, artemisinin with a pro-oxidant redox partner MB and is additive [107]. It is difficult to interpret antagonism in an *in vitro* model because we do not know if these compounds chemically interact with each other or whether there is a metabolic interaction outside the cell.

Late-stage gametocytes were susceptible and sensitive to the drug combination given they lack certain pathways to adapt including NPPs, displaying synergism (Figure 3.3 C). This raises an interesting question as to the particular MoA and even expression profile of DHA, and to decipher its specific role in late-stage gametocytes given that DHA was the least active compound tested in late-stage gametocytes. Evidence of this kind of interaction is sparse but includes artemisinin derivatives with MB and is also synergistic [107]. However, the oxidative stress induced by the artemisinins was sustained and even suspected to be enhanced by the redox cycling action of MB, which is not the case with MMV390048.

Isobolograms are limited in accurately reflecting the MoA, mechanism of action, etc., between the drugs since the readout for this experiment is only cell viability, and isobolograms' graphical composition assumes a constant relative potency [122]. Isobolograms are, however, a useful tool in asserting drug interaction, furthering our understanding of the actions of individual drugs and determining what ratios to combine these in for therapeutic applications. Further studies are required to validate the observed potent killing effect of the combination of DHA and MMV390048 in late-stage gametocytes. This discovery garners the combination of DHA and MMV390048 are of considerable interest for their potential inhibitory effects.

Combining drugs that target asexual proliferation, and sexual differentiation poses a developmental challenge in terms of the PK and PD profiles of these combinations. Here, we hypothesised that SLNs will allow the controlled release of DHA and MMV390048 to kill asexual and gametocyte stages of *P. falciparum* with minimal haemolytic and cytotoxic effects on erythrocytes and mammalian cells, respectively.

Another major challenge with current antimalarials is oral bioavailability, which this project proposes to address. The oral bioavailability challenges associated with current antimalarial treatments, in addition to poor targeting of gametocyte stages due to their prolonged formation period, were challenges this study attempted to overcome. To this end, we integrated the drugs into SLNs, so the particles can be released from the hydrogel matrix (Figure 1.6). An *in vitro* study on hydrogels synthesised from gum acacia provides clear evidence that hydrogels can be used to deliver two or more antimalarial drugs with different PKs. It indicates prolonged and sustained release profiles for curcumin, while 40-aminoquinoline exhibited a short-term release profile at 37 °C [103]. Using an injectable polymer matrix capable of releasing more than one drug presents as an interesting innovation, possibly offering a solution to challenges in treating multiple diseases or diseases with comorbidities. This approach will further propel progress in therapeutics. To date, injectable hydrogels have only been reported for cancer treatment [123].

One ongoing challenge is how to track the release of the particles into the erythrocytes or the parasites. One way to address this was to test parasite viability as a readout to evaluate parasite death. Antiplasmodial activity was observed for SLN formulations of QN, DHA and MMV390048 indicating that SLNs did reach *P. falciparum* infected erythrocytes. However, this is not direct evidence of drug release or drug uptake into the parasite. In the case of intrinsic fluorescence of the SLNs, we traced this inside the parasite through fluorescence microscopy. Analysis of parasite-infected erythrocytes showed that CHFL and CHSI localised to the cytoplasmic compartment, indicating that the SLNs reached the parasites (Figure 3.23). CHSI, however, also accumulates in erythrocytes, which is a likely explanation for the observed toxicity of the SLNs (Figure 3.23). However, a more plausible explanation is that the SLNs associate with erythrocytes and in so doing, disrupt the cell membranes causing haemolysis.

To exert their effects, SLNs need to be taken up by parasites; first by traversing the erythrocyte membrane and then the PVM and PPM. This poses a challenge to uptake unless they can use NPPs to traverse the erythrocyte surface. This can be determined by inhibitors of proteins constituting the channels so we can tell whether the SLNs get in through diffusion, active uptake or through the NPPs [119]. However, one would need to determine whether the particles need to be taken up into the parasites directly or whether they can be released outside of the parasites from the hydrogel matrix and then be taken up by the parasites. DHA and MMV390048 can enter the parasite, albeit SLN encapsulation significantly decreased their antiparasmodial activity, contrary to what was hypothesised. Subsequently, we did not proceed with studies in the early and late sexual developmental stages of the parasite. This loss in activity may be explained by uptake and release. Re-evaluation of the delivery system for the SLNs to stabilise them for some time in the hydrogel matrix and regulation of their release is indicated. Upon release, the SLNs would disintegrate to release the drugs into the system to maintain continuous drug pressure.

Due to the transdermal nature of the delivery system, it is also necessary to ensure that the particles reach the parasites as they must traverse the skin, enter the bloodstream and eventually reach the parasite. It is also necessary to ensure that the SLNs do not affect other cells. It would be easier to use the SLNs as a method to get the drugs from the hydrogel matrix, which will be degraded over time via collagenase treatment [124, 125] then into the bloodstream and then released into the blood. By using a trigger, such as a polymer matrix that responds to stimuli like temperature, pH, and salts such as polysulfobetaines (PSB) and polycarboxybetaines (PCB), and the combination of them (e.g., as interpenetrating polymer networks or copolymers), it is possible to create a material that responds to all three stimuli. This behaviour can be exploited to trigger the release of NPs and the encapsulated antimalarial drugs in response to changes in the local pH of surrounding tissue, this is the case with malaria-induced acidosis.

It was hypothesised that because SLNs are known to be able to directly release drugs into the cell, and that they possess almost 100 % drug release efficacy due to their specific mechanism of intracellular action [96], SLNs will allow the controlled release of DHA and MMV390048 to kill asexual and gametocyte stages of *P. falciparum* with minimal haemolytic and cytotoxic effects on erythrocytes and mammalian cells, respectively. The toxicity counter screens of these compounds were confirmed. These compounds alone do not have toxicity issues, evidenced by the lack of haemolytic toxicity and cytotoxicity against erythrocytes and HepG2 liver cells, respectively. Possibly because passive transport is observed in erythrocytes infected with *P. falciparum* through NPPs, non-specific tissue targeting was observed in healthy, non-infected erythrocytes and mammalian cells by the encapsulating SLN formulations indicating that they were not well tolerated evidenced by the overt toxicity to HepG2 cells and localisation to the erythrocyte membrane. This shows that while the SLNs are active against *P. falciparum* parasites, this is likely not due to drug release but rather to SLN toxicity. Nanoencapsulation further presents and substantiates a promising approach to improved antimalarial

activity, reduced drug toxicity and prolonged therapeutic effects. However, in the case of this study, nanoencapsulation did not improve the activity of DHA and MMV390048 and overt toxicity was observed by the SLNs alone. This indicates a correlation between the low concentration at which SLN-encapsulated compounds enter cell types and this being released into cells that are not the intended target.

5. Concluding Discussion

NP-based dual drug delivery devices posit a solution to the underlying pharmacological issues hindering malaria treatment including low aqueous solubility of antimalarial drugs, which limits their *in vivo* efficacy. The encapsulation approach presented here uses SLNs with a carefully controlled lipophilic/hydrophobic balance to ensure controlled drug release. This device also proposes an effective way to target multidrug-resistant malaria by delivering drugs with different chemical properties and MoA. The long-term goal is to take compounds that have toxicity or uptake issues and be able to use them in combination. Thus, if we can have a formulation that allows the particle to be taken up into the parasite and then released, we can overcome uptake and toxicity issues. However, after the interactions between the novel drug delivery device and the biological host were studied *in vitro*, the SLNs alone would need to be re-evaluated owing to their toxicity.

In cell cultures, we determined whether the particles get taken up by erythrocytes, infected erythrocytes, and other mammalian cells. Future studies include first improving the toxicity profiles of the SLNs to get to a point to look at the gradual degradation of the particles and the release of the drugs associated with that, then qualitatively characterise the system. From there, one can start measuring the kinetics of the processes that are important and define the minimal ideal dose needed to be included in the SLNs and hydrogel matrix.

A proposed solution to particularly solve the polymer toxicity issue observed is to alternatively use polylactic acid (PLA), and poly(lactic acid)-block-poly(styrene-alt-maleic acid) (PLA-*b*-SMA) as they are examples of non-toxic, biodegradable and biocompatible polymers with excellent properties. PLA-*b*-SMA is a novel PLA block copolymer used for the potential stabilisation of polymeric NPs used in the formulation and encapsulation of lipophilic drugs [126]. This novel block copolymer contains styrene-alt-maleic acid (SMA) as a hydrophilic block that has been shown to improve DDSs without obvious side effects. SMA facilitates increased electrostatic stabilisation of NPs, improved loading efficiency and prolonged circulation due to the high affinity of the copolymer [126, 127].

PLA NPs are biodegradable, providing a prolonged release of drugs through their erosion mechanism of action. This allows the drug to remain in the host for a longer circulation time, helping to ensure long-term transmission-blocking activity of the malaria parasite against mosquito vectors.

Using biodegradable polymers will enable gradual degradation of the polymer scaffold in the host ensuring the controlled release of drug-loaded NPs and delivery of almost the entire load of antimalarial drugs over a sustained period.

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