Chapter 2

This chapter contains the literature study focusing on mefloquine hydrochloride and lipid drug delivery systems, Pheroid™ vesicles and liposomes. Biopharmaceutical evaluation including physiochemical properties and biological considerations to evaluate the lipid drug delivery system is discussed in short. Reference style used in this chapter is a modified version of the Harvard style (as defined in Refworks by the North-West University)

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CO_2	Carbon dioxide
$CO_3^{\cdot}$	Carbonate
DELI	Double-site enzyme-linked lactate dehydrogenase immunodetection
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FP	Ferri/ferroprotoporphyrin
GSH	Glutathione
H_2O	Water
H_2O_2	Hydrogen peroxide
LDH	Lactate dehydrogenase
LUV	Large unilaminer vesicles
MLV	Multilaminer vesicles
N_2O	Nitrous oxide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
$NO^{\cdot}$	Nitric oxide
$NO_2^{\cdot}$	Nitrogen dioxide
NOS	Nitrogen oxygen species

O_2	Oxygen
$O_2^{\cdot}$	Superoxide
$OH^{\cdot}$	Hydroxyl
$ONOO^{\cdot}$	Peroxynitrate
$ONOOCOO^{\cdot}$	Peroxomonocarbonate
ONOOH	Peroxynitrous acid
pLDH	*Plasmodium* lactate dehydrogenase
ROS	Reactive oxygen species
SUV	Small unilaminer vesicles
ULV	Unilaminer vesicle

1. Malaria

Malaria, an infectous disease mainly caused by the parasite, *Plasmodium falciparum,* was responsible for an estimated 863 000 deaths in 2008 (WHO, 2010b). Two distinct phases in the parasitic lifecycle are present, the liver and blood stage, with the latter responsible for the clinical manifestations. The symptoms include fever, headache, anaemia, organ failure and death. Drugs used to treat malaria are stage specific with most drugs eliminating the parasite during the erythrocytic stage known as blood schizonticidal drugs (Daily, 2006). Resistance to the compounds increase the burden of malaria especially on third world countries (Bloland, 2010; WHO, 2010a). Increase in infections, resistance, cost and limited drugs available hinders effective treatment of malaria (Date *et al.*, 2007).

2. Mefloquine

Mefloquine, a blood schizonticidal drug, is used in the treatment and chemoprophylaxis of malaria especially against chloroquine resistant strains (Foley *et al.*, 1998; Rosenthal, 2004; Toovey, 2009). This 4-quinidine methanol compound is a white crystalline powder with a molecular weight of 414.78 g/mol (DrugBank, 2010). It exhibits 98% protein binding, is highly lipophilic with extensive distribution in tissue and is eliminated slowly with a half life of 20 to 30 days (Rosenthal, 2004). Mefloquine has been associated with undesirable adverse effects ranging from moderate to severe that are dependent on the

dosage. The more common adverse reactions include gastrointestinal disturbances like nausea, vomiting and abdominal pains, as well as rash and dizziness. Cardiovascular symptoms are less common but have been reported (Barrett *et al.*, 1996; Rosenthal, 2004; Tin *et al.*, 1982). Neurotoxicity in humans has been widely reported (Jacquerioz *et al.*, 2009; Meier *et al.*, 2004; Thapa *et al.*, 2009; Tran *et al.*, 2006; Van Riemsdijk *et al.*, 2005). Mefloquine showed to have a higher incident of neurotoxic effects compared to atovaqoune-proguanil and doxycycline (Jacquerioz *et al.*, 2009) with only a few paediatric cases reported (Thapa *et al.*, 2009). Neurological symptoms including seizures, psychosis, depression and confusion are serious symptoms associate with mefloquine (Barrett *et al.*, 1996; Rosenthal, 2004; Tin *et al.*, 1982). Dow *et al*. (2006) conducted a study concluding dose and concentration dependent neurological effects in rats.

P. falciparum resistance to mefloquine was first reported in 1982 and has been illustrated *in vitro, in vivo* and in humans (Mockenhaupt, 1995). The biomedical basis of resistance to mefloquine is unknown but is associated with different gene encoding including *Pfmdr1* (Jeffress *et al.*, 2005; Na-Bangchang *et al.*, 2007). Mefloquine resistance has been described in several malaria-endemic regions including Tanzania and can be contributed to the long half life of mefloquine and inadequate blood levels (Mockenhaupt, 1995; Wichmann *et al.*, 2003). Increased sensitivity to *P. falciparum* resistant strains *in vitro* was observed when mefloquine was given in combination with penfluridol (Oduola *et al.*, 1993). To decrease the possibility of mefloquine resistance, combination therapy with artemisinins is presently available (Atequin™, Co-Artem® and Massax™). Combination with artemether illustrated a 97% cure rate with a decrease in recrudescence (Bunnag *et al.*, 1995; Karbwang *et al.*, 1995). Artesunate combination illustrated to be effective against chloroquine resistant strains (Chawira *et al.*, 1987) and is widely used in South East Asia (Olliaro *et al.*, 2004). Current mefloquine prophylaxis regime includes weekly doses with an increase in dosage frequency during treatment and the use of combination therapy with artesunate against chloroquine resistant strains. Mefloquine prophylaxis can be used during the second and third trimester of pregnancy (Nosten *et al.*, 2000) and has no higher risk during the first trimester compared to other malaria drugs (Phillips-Howard *et al.*, 1998).

The exact mode of action of mefloquine is unknown (Dow *et al.*, 2003) but it interacts with a variety of biological systems. The long half life of mefloquine may be due to the binding

to infected and uninfected erythrocytes and cell membranes with high affinity (Chevli *et al.*, 1982; Desneves *et al.*, 1996; San George *et al.*, 1984; Schwartz *et al.*, 1982). Mefloquine has an influence on neuronal calcium homeostasis (Dow *et al.*, 2003), the calcium pump (Go *et al.*, 1995), blood brain barrier glycoproteins (Lu *et al.*, 2001; Pham *et al.*, 2000), potassium channels (Gribble *et al.*, 2000), endoplastic reticulum (Dow *et al.*, 2003) and acetylcholinesterase (Lee *et al.*, 1988). Unlike chloroquine, no accumulation of mefloquine in the food vacuole, inhibiting haem polymerization of the parasite, is seen (Slater, 1993). Mefloquine is still widely used in both treatment and prophylaxis because of the potent activity and absence of effective alternatives (Foley *et al.*, 1998).

3. Drug delivery systems

The pure chemical form of drugs are seldom administered alone. Various excipients are added when preparing the final dosage form for administration to a patient (York, 2002). It is therefore important when choosing a delivery method and formulation, to take all drug and excipients properties and preferred administration route into account to improve the pharmaceutical active compound (Allen, 2008; Barich *et al.*, 2005). Excipients have unique characteristics to solubilise, preserve, modify dissolution and flavour to improve drug delivery (York, 2002). Drug delivery is thus a method used to administer drugs to reach a therapeutic concentration by the addition of various excipients with specialised pharmaceutical functions. Drug delivery is used to improve the bioavailability, patient compliance and reduce side effects of an active compound (Gardner, 1987; Speiser, 1998). Development is based on convenience, safety and specific targeting (Han *et al.*, 2005). The main objective of drug formulation is the delivery of the drug into circulation and site of action and maintaining therapeutic levels for a specific time period (Barich *et al.*, 2005; Han *et al.*, 2005). A drug can only be therapeutically active when dissolved in either the dosage form or bodily fluids with high physical and chemical stability (Barich *et al.*, 2005).

Various excipients are used during the formulation of dosage forms (Barich *et al.*, 2005) to improve the properties of poorly soluble drugs (Speiser, 1998). Lipid excipients, most commonly used for this purpose, consists of long- or medium-chain fatty acids, phospholipids and steroids. Lipid excipients can modulate the activity of drug efflux transporters, serve as fuel and help with important biological functions (Pang *et al.*, 2007)

and have the unique ability to solubilise hydrophobic drugs and thus improve drug absorption (Jeong *et al.*, 2007; Pang *et al.*, 2007). Lipid excipients are adaptable and can be formulated in emulsions, suspensions, self emulsifying systems and micro emulsions. Disadvantages of lipid formulation include low stability, high cost of manufacturing and potential side effects. Novel properties of lipid formulation are imperative in drug delivery (Jeong *et al.*, 2007). Lipid based drug delivery systems are when a drug in combination with lipid excipients with unique pharmaceutical functions are manufactured to achieve a therapeutic dose after administration. The ability of lipid drug delivery systems to improve solubility and drug absorption makes these formulations important research targets. Two lipid based drug delivery systems, liposomes and Pheroid™ Technology, will be discussed in the subsequent section.

3.1 Liposomes

The model membrane drug delivery system, liposomes, are one of the most comprehensively researched carrier systems (Müller *et al.*, 2007; New, 1990). Spherical in shape, it consists of single or multiple lipid bilayers enclosing aqueous compartments (Jeong *et al.*, 2007; New, 1990; Roerdink *et al.*, 1987). A variety of artificial or natural phospholipids can be used to formulate liposomes. The most common of them is phosphatidyl choline, usually in combination with cholesterol (New, 1990; Roerdink *et al.*, 1987). The lipid molecule consists of a hydrophilic head group and a hydrophobic chain (New, 1990; Ranade *et al.*, 2004; Roerdink *et al.*, 1987). These amphipatic molecules arrange themselves by exposing the polar head to the water phase and the hydrophobic chains adhere together to form the lipid bilayer (Figure 1) (Ranade *et al.*, 2004; Roerdink *et al.*, 1987). Phosphatidyl choline is tubular in shape and is more suitable for aggregation in planar sheets to form closed sealed vesicles. The low cost, neutral charge, chemical inertness and the fact that phosphatidyl choline can be derived from both natural and synthetic sources, makes it ideal to use in liposomes. Changes in the properties of the bilayer of liposomes are seen with incorporation of cholesterol (New, 1990). Cholesterol, a main component of natural membranes gives rigidity to the liposome vesicle altering the permeability and fluidity characteristics of the vesicles (New, 1990; Roerdink *et al.*, 1987). Lipophilic compounds can be entrapped in the lipid bilayer and hydrophilic drugs in the aqueous compartment (Allen, 2008; New, 1990; Ranade *et al.*, 2004; Roerdink *et al.*,

1987). The amount of lipophilic compound that can be entrapped in the liposomes is directly proportional to the lipid contents of the liposome and not the size (New, 1990).

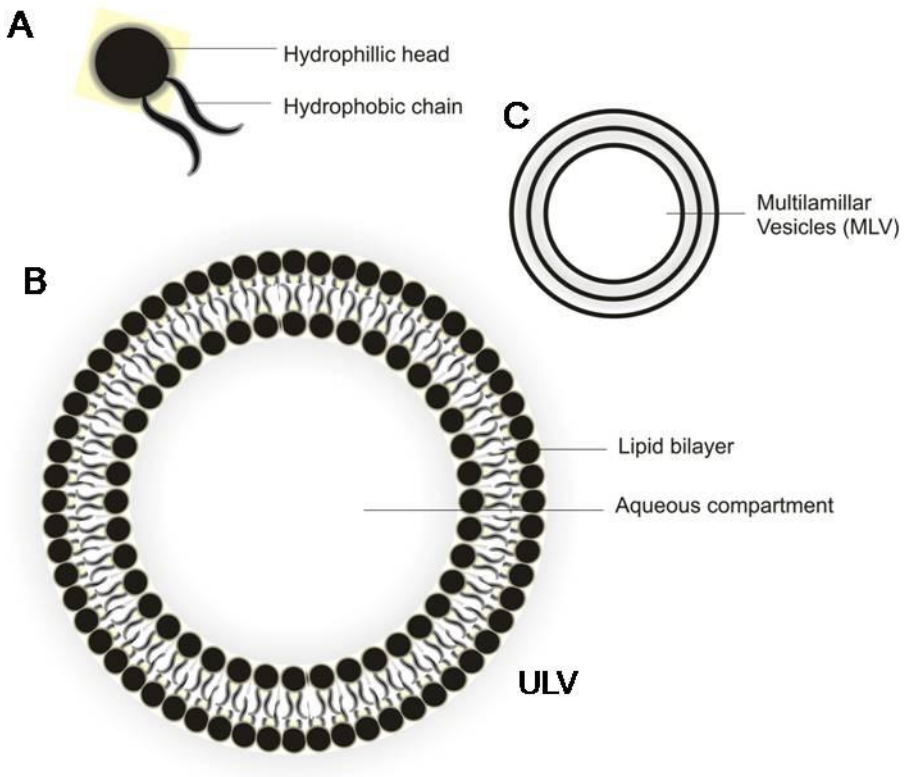

Figure 1 Liposomes consist of amphipatic molecules (A) and groups together exposing the hydrophilic head to the water phase and the hydrophobic chain, adhering together to form the lipid bilayer of the unilaminer vesicle (ULV) (B). Multilamillar liposome vesicles containing more than one lipid bilayer can also be formed (Adapted from New, 1990; Roerdink *et al.*, 1987).

Properties of liposomes can be altered by using different lipid combinations and/or methods of manufacturing (Torchilin, 2007) depending on the intended use. Different lipid excipients are used to alter properties such as membrane fluidity, charge density and permeability of liposomes (New, 1990). Size and shape of liposomes also plays an important role in the intended use. The size of liposomes can range from 25 nm to 10 µm and greater (Allen, 2008; New, 1990; Ranade *et al.*, 2004). Different types, according to size can be used as drug delivery systems, as seen in Figure 1. Multilaminer vesicles (MLV) are heterogeneous in size distribution and consist of more than one bilayer. They consist of neutral lipids forming tightly packed multilayers with adjacent bilayers stacked

very closely with little aqueous volume between the bilayers. MLV are prepared by gentle manual agitation during hydration with the aqueous medium after formation of a thin film on the side of the flask. This is the simplest and most widely used method. Small unilaminer vesicles (SUV) are 25 nm in size with a homogeneous size distribution. This is obtained by exposing MLV to high levels of energy like sonication. Increase in temperature leads to the risk of degradation of the lipids, which can be reduced by cooling the liposomes during sonication. Sonication is a very good method for reducing the size of liposomes. Large unilaminer vesicles (LUV) have a size distribution in the micron range, a homogeneous population and is mostly formulated using the reverse phase evaporation method. Large liposomes can entrap more hydrophilic compound due to a greater aqueous compartment (New, 1990; Roerdink *et al.*, 1987). It is therefore important to determine which ingredients and type of liposomes will be used to best reach the desired effect.

Liposomes have been distinguished as a promising drug delivery systems since they were first formulated in the 1960's (Müller *et al.*, 2007; New, 1990). Controlling the rate of release, rapid removal from circulation and chemical and physical stability, the use of artificial lipids foreign to the body and high cost of manufacturing are some of the disadvantages of liposomes (Jeong *et al.*, 2007). Advantages of this lipid drug delivery system are the reduction in toxic side effects of the drug, increased efficacy of treatment (Müller *et al.*, 2007) and site specific liposomes and can be formulated for targeted delivery (Roerdink *et al.*, 1987). Liposomes can be administrated parentarally, topically and orally. Products on the market include antifungal (AmbiSone), antineoplastic and immunomodulators (Roerdink *et al.*, 1987; Torchilin, 2007). Antimalarial drugs incorporated into liposomes include chloroquine (Qiu *et al.*, 2008), primaquine (Stensrud *et al.*, 2000) and artemisinins (Joshi *et al.*, 2008a; Joshi *et al.*, 2008b). Chloroquine has successfully been entrapped in liposomes and showed increased antifungal efficacy (Khan *et al.*, 2005). For cancer therapy, the pH-gradient method was successfully employed resulting in high entrapment efficacy of chloroquine (Qiu *et al.*, 2008). For the use in the treatment of malaria, both gel state and fluid state liposomes illustrated that chloroquine could be administered in higher doses leading to successful treatment of *P. berghei* infections *in vivo* (Peeters *et al.*, 1989a; Peeters *et al.*, 1989b). The pH-gradient method was used to entrap primaquine in liposomes for IV administration. The entrapment efficacy was dependent on the lipid composition, buffer, charge of liposomes and drug to

lipid ratio (Stensrud *et al.*, 2000). Oral administration of artemether liposomes illustrated high bioavailability with faster and increased absorption compared to the aqueous solution (Bayoni *et al.*, 1998). Other studies conducted on artemether incorporated into lipid carriers also showed increased efficacy (Joshi *et al.*, 2008a; Joshi *et al.*, 2008b). Incorporation of mefloquine, a highly lipophilic drug like artemether, into liposomes can possible lead to increased bioavailability and efficacy for the treatment of malaria.

3.2 Pheroid™ Technology

This novel lipid based colloidal system contains vesicular submicron and micron sized structures. Pheroid™ technology comprises of a lipid bilayer constructed mainly of ethylated and pegylated poly unsaturated fatty acids. This drug delivery system has three phases, an oil, water and gas phase. Essential and plant fatty acids necessary for normal cell function, are dispersed in a water phase saturated with nitrous oxide (N_2O) to spontaneously form lipid structures. Pheroid™ mainly consist of Vitamin F ethyl ester, Cremophor® EL, DL-α-tocopherol and nitrous oxide (Grobler, 2009). Vitamin F ethyl ester is an essential fatty acid and is vital for humans but cannot be synthesized in the body (Das, 2006; Dobryniewski *et al.*, 2007). DL-α-tocopherol is an antioxidant and emulsion stabilizer and can be used as solvent for lipophilic drugs (Rossi *et al.*, 2007). It can possibly promote the growth of parasites *in vitro* but has limited influence *in vivo* (Skinner-Adams *et al.*, 1998). Cremophor® EL is an excipient used to prevail over the problem of poor water solubility but is toxic (Gelderblom *et al.*, 2001; Jeong *et al.*, 2007). N_2O is fairly soluble in a selection of solvents including water and oils (Mattson *et al.*, 2010). Pheroid™ can be manipulated in size and morphology to optimize therapeutic effect. The size is determined by the type, ratio and saturated state of the fatty acids as well as the manufacturing procedure. Lipophillic and hydrophilic drugs can be entrapped and is influenced by the size of the vesicle, concentration and character of the fatty acids and the hydration medium. Pheroid™ can transport the active compound across the different membranes and is possibly metabolized in either the mitochondria or peroxisomes releasing the compounds (Grobler *et al.*, 2007; Grobler, 2009)

Other advantages include the vast range of routes of administration including oral, transdermal and nasal. Pheroid™ can be used as pro-delivery system and targeted delivery. High stability in shelf life and body fluid is observed. Applications include the

transdermal delivery of anti-infective agents as well as cosmetic products (Grobler *et al.*, 2007; Grobler, 2009). Increased nasal delivery of calcitonin (Du Plessis *et al.*, 2010) and other peptide drugs (Steyn *et al.*, 2010) were observed *in vivo*. Entrapment of tuberculosis drugs increased drug plasma levels and bioavailability with a decrease in side effects. Agriculturally, Pheroid™ can be utilized to deliver pesticides, micro nutrients and growth regulator factor making Pheroid™ a very versatile drug delivery system (Grobler *et al.*, 2007; Grobler, 2009). Antimalarial drugs, including azithromycin, erythromycin, mefloquine, chloroquine, artemether and artesunate were incorporated into Pheroid™ technology and showed higher efficacy compared to the aqueous solutions (Langley, 2007; Odendaal, 2009; Van Huyssteen, 2010; Van Niekerk, 2010). Comparison between the aqueous and Pheroid™ vesicles mefloquine solutions, lower parasitemia levels was observed with Pheroid™ vesicles (Langley, 2007). A decrease in the $IC_{50\%}$ values of 36% and 51% after 24 and 72 hours respectively was observed *in vitro* (Van Huyssteen, 2010). Other studies conducted showed a 46.86% and 58.91% reduction in $IC_{50\%}$ values (Odendaal, 2009). This illustrates the potential of Pheroid™ vesicles to improve the efficacy of mefloquine *in vitro.*

4. Biopharmaceutical evaluation

The relationship between the physical, chemical and biological effect of drugs and dosage forms is known as biopharmaceutics. Biopharmaceutical studies utilises *in vitro* and *in vivo* methods of evaluation to determine, among other things, the stability of the drug and dosage form, properties of the dosage form and biological response (Allen *et al.*, 2005; Shargel *et al.*, 2005). Different methods can be used to determine one property of the drug delivery system. Evaluation of the lipid drug delivery systems with regard to physicochemical properties and biological effect will be discussed.

4.1 Physiochemical properties

Characterization of drug delivery systems is essential and can be done by determining a selection of properties by a variety of methods. These include particle size, entrapment efficacy, morphology and stability (Gou *et al.*, 2009; Ishii *et al.*, 2001; Shanmuganathan *et al.*, 2008; Vicentini *et al.*, 2010). Stability and solubility are important physiochemical properties and form a crucial part of the pharmaceutical process and is based on certain guidelines (Han *et al.*, 2005; ICH. 2006; Vicentini *et al.*, 2010).

4.1.1 Stability testing

Stability is dependent on the physical and chemical properties and environmental factors. Physical and chemical properties include entrapment efficacy, appearance, consistence, colour, odour, taste, pH and particle shape and size (Matthews, 1999). Environmental factors for instance temperature, humidity and light and their effect are documented and must be submitted for marketing authorization. The aim of stability testing is to determine the quality of a drug or substance and if it varies over time under different environmental factors. Data documented should give a satisfactory product summary to determine storage conditions, shelf life and re-test intervals (ICH. 2006; Matthews, 1999; WHO. 2009). Accelerated storage conditions are used to determine the chemical and physical change at exaggerated storage conditions (ICH. 2006). These temperatures and relative humidity, as summarized in Table 1, is depended on the intended storage conditions and period over which stability testing is done.

Table 1 The temperature and relative humidity of the different storage conditions for products intended for storage at room temperature or in a refrigerator or freezer (Adapted from ICH. 2006; Matthews, 1999; WHO. 2009).

Room temperature		
Long term	25°C ± 2°C	60% ± 5%
	30°C ± 2°C	65% ± 5%
Intermediate	30°C ± 2°C	65% ± 5%
Accelerated	40°C ± 2°C	75% ± 5%
Refrigerator		
Long term	5°C ± 3°C	-
Accelerated	25°C ± 2°C	60% ± 5%
Freezer		
Long term	-20°C ± 5°C	-

4.1.2 Size and shape characterization

The size of particles of drug delivery systems influence the degradation, flow properties, clearance, uptake and bio distribution (Champion *et al.*, 2007; Gaumet *et al.*, 2008) and is a key physical stability consideration (Burgess *et al.*, 2004). The intended use, method of manufacturing and route of administration are all factors influencing the size of particles. Particles are seldom spherical, therefore shape and morphology plays an important role in size determination. Size analysis is dependent on various facets including the method of sampling and analysis (Burgess *et al.*, 2004). Different methods can be used to determine size distribution.

Light scattering is a rapid method based on the equivalent sphere principle where each particle is viewed as a sphere (Gaumet *et al.*, 2008). This non-invasive approach determines the mean size and distribution (Gaumet *et al.*, 2008; Villari *et al.*, 2008) of a large number of particles smaller than 1 µm (Gaumet *et al.*, 2008). Laser light defraction, used for particles larger than 1 µm (Gaumet *et al.*, 2008) measures the forward diffraction light where the angle of diffraction is an indication of the particle size (Allen, 1981). A large sample volume and over estimation of small particles are some disadvantages. Scanning electron microscopy provides information regarding the size and shape but has difficulty in observation of particles smaller than 100 nm. Other methods include transmission electron microscopy, atomic force microscopy, analytical ultracentrifugation, field flow fractionation and capillary electrophoresis (Gaumet *et al.*, 2008).

4.1.3 Entrapment efficacy

It is important to determine the amount of drug entrapped before biological studies can be done because the effect is dose related (New, 1990). Entrapment of an adequate amount of drug is a sought after property (Sharma *et al.*, 1997). Drugs are entrapped to enhance delivery and decrease toxicity resulting in an increased therapeutic index (De Jong *et al.*, 2008). The efficacy of a drug is dependent on the amount absorbed as well as the absorption rate (Karalis *et al.*, 2010). Drug delivery systems are used to increase the solubility of the drugs (Gardner, 1987). Different methods are utilized to determine entrapment efficacy and are dependent on the drug entrapped (New, 1990).

4.2 Biological considerations

Drug delivery systems are used to improve the bioavailability and efficacy and decrease the toxicity of drugs. It is therefore important to prove this by evaluation of drug delivery systems, loaded and non-drug loaded (De Jong *et al.*, 2008). Biological data on the efficacy and toxicity of drug delivery systems have been documented for *in vitro* and *in vivo* studies. A variety of methods, animals and cell lines can be used to determine efficacy and toxicity (Atobe *et al.*, 2007; Gorle *et al.*, 2009; Pollock *et al.*, 2010; Pulford *et al.*, 2010; Rodrigues *et al.*, 1995; Rodriguez *et al.*, 2009) to give adequate data to proceed to clinical trials (Devalapally *et al.*, 2007).

4.2.1 Efficacy studies

In vitro assays to determine the sensitivity of the *P. falciparum* to drugs are a valuable tool for drug development and monitoring resistance. No single standardised method of *in vitro* evaluation is available, but four major types of assays can be used. These include morphological analysis, radioisotope, ELISA based and fluorometric assays and can be used for field applications. The metabolic activity of the parasites is measured by incorporation of radio labelled precursors when utilising radio isotope assays (Basco, 2007). [^{3}H]hypoxanthine assays allow rapid, sensitive and accurate determination (Basco, 2007; Wein *et al.*, 2010) and is known as the golden standard (Basco, 2007). Fluorometric analysis is based on DNA labelling with fluorochromes (Basco, 2007) including Hoechst 3358, ethidium bromide, Pico Green and SYBR® Green (Basco, 2007; Smeijsters *et al.*, 1996; Wein *et al.*, 2010). LDH, a non-ELISA based colorimetric assay, is a rapid and reliable method but has low sensitivity (Basco, 2007; Martin *et al.*, 2009; Ramazani *et al.*, 2010; Wong *et al.*, 2010). ELISA based assays, used in rapid tests utilise relatively cheap reagents and equipment, is non-radioactive but numerous washing steps are needed (Basco, 2007). DELI, double site enzyme linked pLDH immunodetection (Barends *et al.*, 2007; Mayxay *et al.*, 2007) and HRP II (Kurth *et al.*, 2009; Martin *et al.*, 2009; Wallach *et al.*, 1983) are some of the assays used. Flow cytometry, a sophisticated method, inappropriate for field applications is a rapid, sensitive method able to determine various properties of the cells (Basco, 2007). DNA binding dyes including Hoechst 33342, SYBR®

Green, propidium iodide, arcidine orange, YoYo-1, DAPI and thiazole orange have successfully been used to determine parasite susceptibility to drugs (Barkan *et al.*, 2000; Bei *et al.*, 2010; Contreras *et al.*, 2004; Deitch *et al.*, 1982; Grimberg *et al.*, 2009; Jimenez-Diaz *et al.*, 2005; Nyakeriga *et al.*, 2006; Theron *et al.*, 2010). Flow cytometry is costly but is highly DNA specific, objective, automated and non-radioactive (Basco, 2007). Efficacy of mefloquine was successfully determinded by flow cytometry using different DNA dyes. These dyces include SYBR® Green (Karl *et al.*, 2009), propidium iodide (Grimberg *et al.*, 2009) and YoYo-1 (Li *et al.*, 1997).

4.2.2 Cellular toxicity

Determination of cellular toxicity plays an important role in the evaluation of drug delivery systems. Different toxicity analysis is needed to portray the overall influence and safety of the drug delivery system. Toxicity cannot only be analysed *in vitro* on different cell lines, but various methods can be used to analyse the toxicity. Toxicity on a neuronal cell line and erythrocytes will be discussed specifically for the method of analysis used.

4.2.2.1 Neurotoxicity

Chemical, biological and certain physical agents have adverse effects on the nervous system (Harry *et al.*, 1998). Neurotoxicity evaluation is required by regulatory bodies (Coecke *et al.*, 2010) and can be determined by *in vitro* and *in vivo* studies (Harry *et al.*, 1998). Elimination or reduction of neurotoxicity is a key aim when formulating these substance in drug delivery systems. Mefloquine showed neurotoxicity during *in vitro* and *in vivo* studies (Dow *et al.*, 2006; Dow *et al.*, 2003). A variety of cell lines can be used to determine neurotoxicity and includes brain, spinal cord and ganglion cell cultures. Human and mouse neuroblastoma cells have been established as good candidates to evaluate neurotoxicity (Harry *et al.*, 1998). Mouse neuroblastoma cells have successfully been used to determine neurotoxicity of antimalarial compounds (Smith *et al.*, 2001; Wesche *et al.*, 1994). Different assays can be used to determine the effects of compounds on cell cultures. A basic effective method is the simultaneous specific staining of either the live or dead cells with two fluorescent probes and analysing it, among others, by flow cytometry (Molecular Probes. 2005). *In vitro* evaluation is used to give a better understanding of the

effects of the compounds on the nervous system before expensive *in vivo* evaluation is done (Coecke *et al.*, 2010).

4.2.2.2 Haemolytic activity

Erythrocytes circulate in the blood stream for approximately 120 days but certain effects can shorten the lifespan of the erythrocytes (Beutler, 1969). Haemolysis is an alteration or destruction of the erythrocyte membrane resulting in the release of haemoglobin. Evaluation of haemolytic potential is used to assess the safety and utility of the pharmaceutical additives and drugs (Amin *et al.*, 2006; Krzyzaniak *et al.*, 1997). Haemolysis below 10% is non-haemolytic and above 25% is judged to be haemolytic (Amin *et al.*, 2006). Morphological changes in the membrane of erythrocytes occur, and haemolysis is a valuable method of studying these changes (Dourmashkin *et al.*, 1966).

4.2.2.3 ROS analysis

Oxidative stress is when reactive species in relation to available antioxidants is too high. This causes damage to macromolecules including DNA, lipids, proteins and carbohydrates (Halliwell, 2006; Halliwell, 2007; Stahl *et al.*, 2010). ROS, reactive oxygen species and NOS, reactive nitrogen species, are terms used to describe radical as well as nonradical derivatives of oxygen and nitrogen respectively. ROS is used in defence against infections and signalling but in high amounts can cause irreversible effects. Mild oxidative stress leads to an increase in intracellular calcium and protein phosphorylation. With an increase in ROS, mitochondrial damage, DNA damage initiating apoptosis and cell death leading to inflammation occurs (Halliwell, 2006; Halliwell, 2007).

ROS can be formed by means of different reactions as seen in Figure 2. Oxygen (O_2) essential to survival undergoes electron reduction to form superoxide ($O_2^{\cdot}$) by among others NADPH oxidase or xanthine oxidase. This reactive radical is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. H_2O_2 is a nonradical and is less potent than other ROS types. H_2O_2 is converted through a metal catalyzing process convertor to the highly reactive hydroxyl ($OH^{\cdot}$). $OH^{\cdot}$, because of its free electron pair attacks immediately at the site it was generated. H_2O_2 can be removed by different enzymes including glutathione peroxidase and peroxiredoxins forming water (H_2O), thus

reducing the amount of $OH^{\cdot}$ production. H_2O however, can be reduced to $OH^{\cdot}$ by haemolytic cleavage. Nitric oxide ($NO^{\cdot}$), a NOS molecule, forms a covalent bond with $O_2^{\cdot}$ to form peroxynitrate ($ONOO^{\cdot}$) a non-radical that rapidly protonates at physiological pH to peroxynitrous acid (ONOOH). ONOOH is an oxidizing and nitrating agent that can directly damage proteins, lipids and DNA. ONOOH is converted to nitrogen dioxide ($NO_2^{\cdot}$) and $NO^{\cdot}$. $ONOO^{\cdot}$ in the presence of carbon dioxide (CO_2) change to the nonradical peroxomonocarbonate ($ONOOCOO^{\cdot}$) leading to the formation of $NO_2^{\cdot}$ and carbonate ($CO_3^{\cdot}$) (Halliwell, 2006; Halliwell, 2007; Stahl *et al.*, 2010).

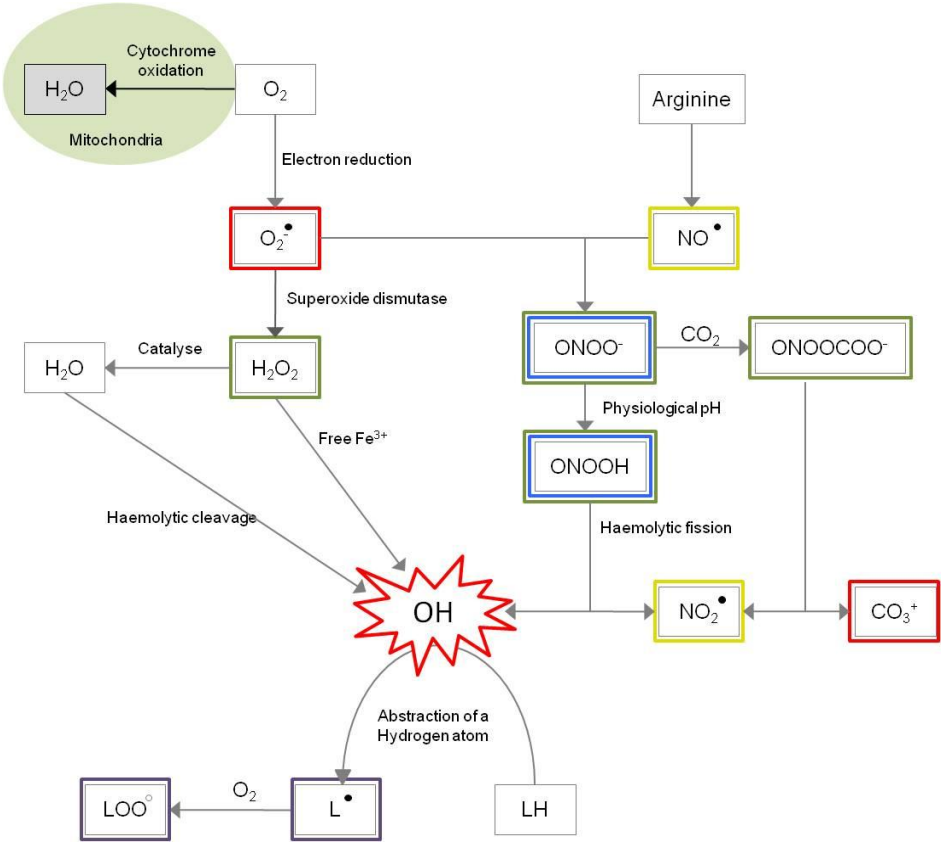

Figure 2 Formation of reactive oxygen species. Free radical ROS, $O_2^{\cdot}$ (superoxide), $OH^{\cdot}$ (hydroxyl) and $CO_3^{\cdot}$ (carbonate) as seen in red and free radical NOS, NO· (nitric oxide) and NO2· (nitrous oxide) in yellow. Non-radicals in green and blue represents ROS and NOS respectively includes H2O2 (hydrogen peroxide), $ONOOCOO^{-}$ (peroxomonocarbonate), $ONOO^{-}$ (peroxynitrate) and ONOOH (peroxynitrous acid). Purple is an indication of lipid peroxidation, LH (polyunsaturated fatty acid) and LOO· (peroxylipid). Other molecules include O2 (oxygen), H2O (water) and CO_2 (carbon dioxide) (Adapted from Halliwell, 2006; Halliwell, 2007; Stahl *et al.*, 2010).

Any system producing $NO^{\cdot}$ and $O_2^{\cdot}$ can result in biological damage. To protect the body against ROS, different defence mechanism are present. Damage due to ROS can be repaired by a variety of enzymes (Halliwell *et al.*, 2007; Kohen *et al.*, 2002). Tocopherol and phospholipids stabilize biological membranes and form part of the physical defence mechanism (Kohen *et al.*, 2002). The antioxidant defence of the human body is to either decrease O_2 to, in effect reduce ROS and scavenging of ROS. The mitochondria is responsible for 95% of O_2 being converted to H_2O by a very complex and effective system, cytochrome oxidase. If these systems fail, damages occur leading to a variety of problems including cancer, neurodegenerative diseases and aging (Halliwell, 2006; Halliwell, 2007). Prevention of ROS by metal chelation leads to the prevention of the Fenton reaction decreasing ROS levels (Halliwell *et al.*, 2007; Kohen *et al.*, 2002). The antioxidant molecules can be grouped into different classes as seen in Table 2. Antioxidant enzymes naturally present in the body reacts with ROS molecules to form less reactive molecules. The three major ROS scavenging enzymes include superoxide dismutase, glutathione reductase and catalase (Crohns, 2010; Halliwell *et al.*, 2007; Kohen *et al.*, 2002). Low molecular weight molecules act directly with radical molecules by donating an electron. The main source of vitamin C, vitamin E and carotenoid is the diet where as GSH, uric acid, albumin and bilirubin is naturally present in the body (Halliwell *et al.*, 2007; Kohen *et al.*, 2002). Vitamin E, also known as α-tocopherol is a strong reducing agent providing protection against ROS (Metzger *et al.*, 2001). In the presence of iron or with a depletion of α-tocopherol levels, this antioxidant may act as a prooxidant that induces ROS (Yamamoto *et al.*, 1988). The other ingredients in Pheroid™ vesicles, vitamin F ethyl ester, a polyunsaturated fatty acid and Cremophor EL also have an influence on ROS production. The unsaturated fatty acid decreases ROS levels in a variety of cells (Ambrozova *et al.*, 2010; Kim *et al.*, 2010; Yu *et al.*, 2009) but ROS production is dependent on the degree of unsaturated fatty acids and presence of the ethyl ester derivative (Bondy *et al.*, 1995). Cremophor EL is cytotoxic for cells (Csoka *et al.*, 1997) and leads to production of ROS by peroxidation of polyunsaturated fatty acids. A decrease in GSH levels are observed with Cremophor EL leading to increased ROS levels (Gutierrez *et al.*, 2006; Iwase *et al.*, 2004). Phosphatidyl choline provides structure to membranes and the degree of saturation determines the amount of damage ROS can cause (Kohen *et al.*, 2002; Shea *et al.*, 2003). Oxidative damage occurs when ROS levels overwhelms the defence mechanism of the body.

Table 2 Different groups of antioxidant molecules used in the defence mechanism of ROS.

Antioxidant enzymes	Low molecular weight molecules
Superoxide dismutase	Vitamin C
Ascorbate peroxidise	Vitamin E (α-tocopherol)
Monoascorbate reductase	Carotenoid
Dehydroascorbate reductase	GSH
Glutathion reductase	Uric acid
Catalase	Billirubin
	Albumin

The rapidly multiplying and growing malaria parasite has a high metabolic rate producing toxic redoxactive by products. As seen in Figure 3, haemoglobin in the food vacuole of the parasite is broken down to free haem (ferri/ferroprotoporphyrin: FP). Detoxifying the toxic FP is achieved by formation of haemozoin through biomineralization or by FP degradation, binding to FP-binding proteins and reactions with glutathione (GSH). Failure to detoxify FP leads to redox damage to the host and parasite. Oxygen and iron leads to ROS formation via the Fenton reaction. ROS is also generated by the host immune response to fight the infection, increasing the burden. The parasite is protected from ROS by antioxidant molecules. These include superoxide dismutase converting $O_2^{\cdot-}$ to the less reactive H_2O_2 and GSH, a redox buffer used to help with detoxification of FP. Oxidative stress may be responsible for many of the clinical signs of malaria, but at the same time can be used as chemotherapy. Chloroquine, primaquine and artemisinins are thought to disrupt the parasite's natural defence against ROS. Chloroquine inhibits the detoxification of haem as well as decreasing GSH levels. This leads to increased haem and ROS. Primaquine and artemisinins interfere with the natural metabolic pathways of the parasite increasing ROS levels. ROS is an important pathobiochemical and clinical factor as well as possible drug target (Becker *et al.*, 2004; Egan *et al.*, 2002; Foley *et al.*, 1998; Harwaldt *et al.*, 2002; Loria *et al.*, 1999; Zhang *et al.*, 1992).

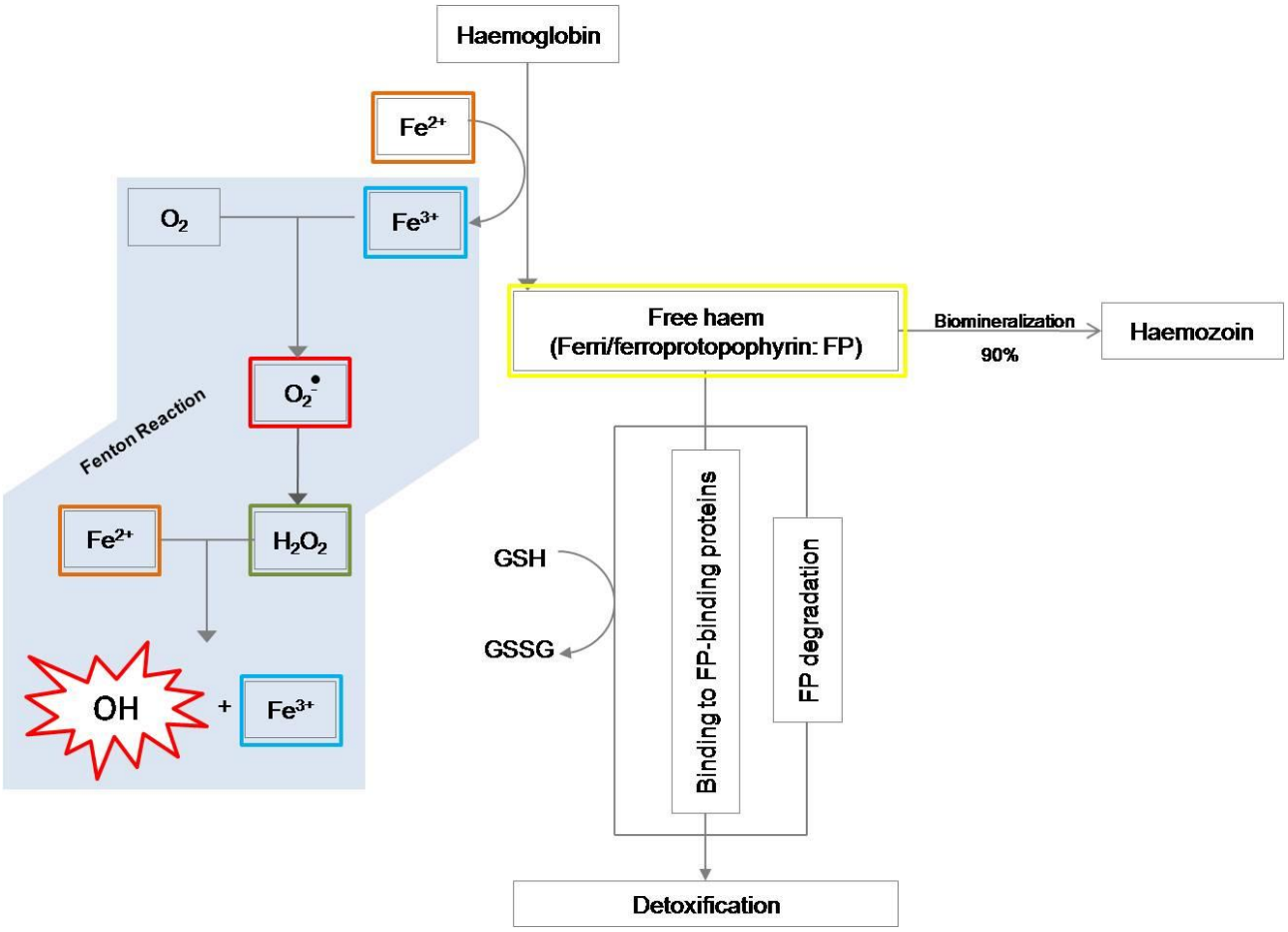

Figure 3 Formation of reactive oxygen species in *Plasmodium falciparum* during the haemoglobin digestion. Free radical ROS, $O_2^{\cdot}$ (superoxide), $OH^{\cdot}$ (hydroxyl) is seen in red. Non-radicals in green represent ROS includes H_2O_2 (hydrogen peroxide). Other molecules include O_2 (oxygen). Iron (Fe^{2+} and Fe^{3+}) is seen in orange and blue. Haem is represented by the yellow block. The blue shaded part is known as the Fenton reaction (Adapted from Becker *et al.*, 2004).

5. Conclusion

Millions of people are affected by malaria on an annual basis, leading to high economic burdens. Due to an increase in resistance and limited new compounds reaching the market, different approaches for treatment of malaria should be followed. One of these is the incorporation of current drugs into drug delivery systems to improve the efficacy and decrease toxicity. Mefloquine, a lipophilic drug with unwanted toxicity has successfully been incorporated into oil-in-water emulsion and Pheroid™ vesicles with an increase in efficacy. Before the efficacy and toxicity of mefloquine in the drug delivery systems can be evaluated, the characteristics, including size, entrapment efficacy and stability should be

evaluated. After the formulation study, cellular assays including efficacy and toxicity should be determined. A research strategy should be followed to give structure and bring to light any possible problems. During this study two distinctive components are present. The first was the formulation and characterisation of the drug delivery systems followed by cellular analysis. To accurately determine the characteristics of the formulation, optimisation of size (Chapter 3) and entrapment efficacy analysis (Chapter 4) was done. This was followed by stability testing (Chapter 5) where these methods were used during the evaluation of the stability of Pheroid™ vesicles and liposomes with and without mefloquine. Out of these studies, optimal formulations was identified and used during the cellular assays. Optimisation of cellular assays (Chapter 6) and evaluation of the efficacy and toxicity, including haemolysis, ROS production and neurotoxicity (Chapter 7), was investigated. By following this strategy, an overall picture of the possibility to use these drug delivery systems in the treatment of malaria, could be evaluated.

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