

Chapter | 8

This chapter is the conclusion as concluded from the all research done during this study as well as possible further investigation that can also be conducted is mentioned.

Before a product can reach the market for use in treatment or prophylaxis of a disease, certain tests, as described by governing bodies should be conducted. A research strategy is followed to ensure a complete and thorough evaluation of the drug and dosage form. The research strategy usually comprises of a preformulation assessment, pharmacokinetic and pharmacodynamic property evaluation, as well as toxicity and efficacy studies. Acceptable findings leads to clinical trials, after which final evaluation is done before the product is registered (Allen *et al.*, 2005; Allen, 2008; Devalapally *et al.*, 2007; Di *et al.*, 2003).

Malaria, a parasitic infectious disease is a global health problem with limited research that doesn't decrease the burden of malaria (WHO, 2010). Increase in resistance to existing antimalarial drugs and limited amount of new strategies to decrease resistance, will lead to no current antimalarial compound being effective in the treatment of malaria (Bloland, 2010; WHO, 2010). Synthesis of new moieties, derivatives and prodrugs as well as formulation into dosage forms to improve certain characteristics is research currently being conducted (Date *et al.*, 2007). The antimalarial drug, mefloquine, is listed on the World Health Organization list for treatment of malaria and is a blood schizonticidal drug especially used against chloroquine resistant strains (WHO, 2010). The poor solubility, unwanted toxicity profile and high efficacy make it a prime candidate for formulation into drug delivery systems (Barrett *et al.*, 1996; Jacqueroz *et al.*, 2009; Rosenthal, 2004).

Drug delivery systems consist of a combination of various excipients to improve delivery, increase bioavailability, efficacy and toxicity profile of the drug (Allen, 2008; Barich *et al.*, 2005; Gardner, 1987). Lipid excipients are mostly used to improve solubility and consist of long or medium chain fatty acids (Pang *et al.*, 2007). The most extensively researched lipid drug delivery system is liposomes. It consists of lipid bilayers able to entrap both hydrophilic and lipophilic compounds. Spherical in shape, it can be manipulated in size and ingredients for specific functions (New, 1990). Only a few antimalarial drugs have been included into liposomes with promising results (Peeters *et al.*, 1989; Qiu *et al.*, 2008; Stensrud *et al.*, 2000). Incorporation of mefloquine in liposomes is a novel approach in the treatment of malaria. Pheroid™ Technology, similar in shape and size to liposomes, consists of essential natural fatty acids. The ability to entrap both hydrophilic and lipophilic compounds and that Pheroid™ can be modified in shape and function makes this a very versatile drug delivery system. Various antimalarial compounds have been entrapped and

evaluated *in vitro* with increased efficacy. Mefloquine entrapped in Pheroid™ showed promising results but further investigation is needed (Grobler *et al.*, 2007; Grobler, 2009; Langley, 2007; Odendaal, 2009; Van Huyssteen, 2010; Van Niekerk, 2010).

Malaria has a high death toll with increasing resistance to current antimalarial regimes. A currently effective compound, mefloquine has unwanted properties including poor solubility and unwanted adverse reactions including neurotoxicity. The ability of lipid drug delivery systems to increase solubility, bioavailability and the possibility to decrease toxicity makes drug delivery systems prime targets to investigate in combination with mefloquine.

In this study, a specific research strategy was followed to reach certain aims. These aims included:

- The formulation of Pheroid™ vesicles and liposomes.
- The incorporation of mefloquine hydrochloride into Pheroid™ vesicles and liposomes.
- Optimization of methods to determine physicochemical properties.
- Determination of size, pH, entrapment efficacy and stability of the formulations.
- Optimization of methods to determine the efficacy and toxicity of the formulations.
- *In vitro* efficacy evaluation of the formulations on a multidrug resistant *P. Falciparum* strain.
- *In vitro* evaluation of cellular toxicity.

In the subsequent paragraphs, the aims will be discussed with results as obtained during the study, as well as possible further investigation.

Pheroid™ vesicles were successfully formulated as describe previously (Du Plessis *et al.*, 2010). Pheroid™ vesicles could be formulated on a small scale as well as on bigger scales of 200 ml without difference in properties. Incorporation of mefloquine into the oil phase before addition to the nitrous oxide saturated water phase, showed little difference in size and shape. The pH of formulations with mefloquine was more acidic compared to the formulation without mefloquine. The pH was more comparable between the formulations when nitrous oxide saturated phosphate buffer solution (pH 7.4) instead of water was used.

Liposomes was successfully formulated by the film hydration method (Mozafari, 2005; New, 1990; Yamabe *et al.*, 2003). Differences were seen in formulations with addition of phosphatidyl glycerol. The method followed during formulation also produced liposomes with different properties (will be discussed in later paragraphs). Longer sonication using a rod lead to a decrease in size. The film that was formed on the side of the flask also had an influence on the characteristics of the liposomes. The thicker the film, the more irregular in size and shape the liposomes were. It is therefore a necessity to have a thin film before hydration with a buffer. Up scaling of liposomes was difficult because of the necessity of formation of a thin film after removal of the organic solvent (Crommelin *et al.*, 2010). Addition of mefloquine to the lipid formulation gave liposomes vesicles, but oil droplets were present. Up scaling was very difficult with formulation of oil droplets instead of vesicles. This could be due to the high affinity of mefloquine for phosphatidyl choline (Chevli *et al.*, 1982). The possibility to entrap mefloquine in liposomes consisting of cholesterol and other fatty acids like phosphatidyl ethanolamine, phosphatidyl glycerol or phosphatidyl serine should be investigated (New, 1990).

Different characteristics can be determined by different methods when evaluating drug delivery systems. It is necessary to determine whether the method used to determine the characteristics is efficient and accurate for the specific drug delivery system. Size can be determined by a variety of methods including light scattering, light defraction and microscopically. Light scattering is based on the equivalent spherical principle (Gaumet *et al.*, 2008) and includes the determination of particle size and distribution by a Malvern Mastersizer. Size determination of Pheroid™ vesicles and Pheroid™ microsponges were determined with accuracy due to uniform shape as visible with confocal laser scanning microscopy. Size ranged between 0.365-12.619 µm and 0.159-12.619 µm for Pheroid™ vesicles and Pheroid™ microsponges respectively. Repeated samples gave similar size distributions. Liposomes evaluation by dynamic light scattering was less accurate because of the irregular shapes of liposome vesicles (as seen with confocal laser scanning microscopy). Repeated measurements lead to different size distribution between the same sample. Malvern Mastersizer was less accurate due to the fact that the method is base on the equivalent spherical principle and not all liposome vesicles were spherical in shape. Another disadvantage was the big sample volume needed for measurement of size. A more accurate method with smaller sample size was needed.

Evaluation of size by flow cytometric evaluation is based on light defraction (Vorauer-Uhl *et al.*, 2000). The properties of each particle were measured as it flows through a beam of light, defracting the light. The size of the particles is represented by the forward light scatter parameter and the exact size of each particle was calculated by evaluation of beads with exact sizes. The evaluation of the different bead sizes lead to distinct peaks on a forward scatter histogram. The geometric mean of each bead was plotted against the size and fitted with linear regression yielding an equation of $y=1.607x+0.4496$ with a correlation of $r^2=0.9781$. This equation was successfully used to determine the size of each particle. The size of Pheroid™ vesicles and microsponges showed similar values compared to the Malvern data. No difference was observed between Pheroid™ vesicles and Pheroid™ microsponges. Liposomes showed more accurate size distributions with better comparison with confocal laser scanning microscopy. As mentioned earlier, addition of phosphatidyl glycerol as well as modification of the method of manufacturing lead to formulation of particles with different size distributions. Size distributions similar to Pheroid™ vesicles was found with the addition of phosphatidyl glycerol and when longer sonication was used. Liposomes consisting of cholesterol and phosphatidyl choline as prepared by the film hydration method with increase sonication was used for further experiments and evaluation. Because of the similar size profile of Pheroid™ vesicles and Pheroid™ microsponges, Pheroid™ vesicles was chosen for further investigation due to the similarity in structure to liposomes. Size determination during stability testing and cellular evaluation was done by flow cytometry. Small sample volume and accurate size determination of each particle is some of the advantages of this method of analysis.

The entrapment efficacy is generally determined when evaluating liposomes. The entrapment efficacy can be determined by a variety of methods. The ability of Pheroid™ vesicles and liposomes to entrap both hydrophilic and lipophilic compounds was evaluated quantitatively and qualitatively. Fluorescent labelled proteins were entrapped in both liposomes and Pheroid™ vesicles and evaluated with confocal laser scanning microscopy and flow cytometry. Entrapment of the protein in the drug delivery systems was clearly visible on the micrographs. The ability of Pheroid™ vesicles to passively entrap compounds after formulation was determined by addition of labelled proteins to the Pheroid™ vesicles over time. An increase in the entrapped proteins were seen with both confocal laser scanning microscopy and flow cytometry.

The evaluation of the entrapment efficacy of the highly lipophilic drug, mefloquine was evaluated by a twofold method. The first was the separation of the untrapped drug from the entrapped. This involved the separation of the drug delivery system and water utilizing a Sephadex G50 mini column (Fry *et al.*, 1978). Successful separation was found with little dilution of the sample. Untrapped mefloquine remained in the column and was dissolved by methanol before analysis by UV-spectrophotometry. A standard calibration curve was prepared by measuring the absorbance of different concentration for this second part of the evaluation. Plotting the concentration against the absorbance values at 283 nm yielded a linear equation of $y=0.01493x-0.1086$ with $r^2=0.9975$. Using this equation it was possible to determine the amount of untrapped drug (Rao *et al.*, 2002). It was possible to combine the separation and spectrophotometric evaluation of mefloquine successfully and was used for further evaluation. Entrapment efficacy for both Pheroid™ and liposomes were above 60% when mefloquine was added to the oil phase during formulation. Mefloquine was also added to already formulated Pheroid™ vesicles to determine the ability to entrap lipophilic compounds after formulation. Entrapment increased over time to above 60% after 14 days. It is possible to entrap mefloquine before and after formulation in Pheroid™ vesicles with high efficacy. Similar results were observed with the labelled proteins. Pheroid™ vesicles and liposomes can entrap both hydrophilic and lipophilic compounds, but similar studies should be conducted for specific compounds when entrapped in either Pheroid™ vesicles or liposomes.

During a three month accelerated stability testing samples of both Pheroid™ vesicles and liposomes were kept at $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$, $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}/60\%\text{ RH}$ and $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}/65\%\text{ RH}$. The size, morphology, pH and entrapment efficacy were measured at regular intervals. Pheroid™ vesicles was stable with a slight increase in size, no morphological changes and an increase in entrapment efficacy was observed. A decrease in pH was observed during the three month stability testing at all temperatures. Liposomes on the other hand showed a significant higher size distribution after three months at $30\text{ }^{\circ}\text{C}$. No morphological changes were observed but a significant decrease in pH was seen. The addition of mefloquine to the formulation, lead to the formation of large aggregates with a significant decrease in pH at months two and three. The amount of untrapped mefloquine decreased over time, but this could be due to the high affinity of mefloquine for

phosphatidyl choline (Chevli *et al.*, 1982). The instability of liposomes could be resolved by lyophilisation (New, 1990), but should be investigated to confirm.

After formulation studies of Pheroid™ vesicles and liposomes loaded with mefloquine it was apparent that the size and entrapment efficacy could be successfully and accurately determined. Characterization of the lipid drug delivery systems during a three month accelerated stability testing illustrated instability of liposomes and high stability of Pheroid™ vesicles with exclusion of pH. Pheroid™ vesicles and liposomes formulation loaded with mefloquine with the above characteristics was used during the cellular evaluation of the drug delivery systems.

Biocompatibility of drug delivery systems and cells as well as the assay was investigated. To determine the haemolytic activity of Pheroid™ vesicles and liposomes with and without mefloquine, it was first necessary to determine the influence of the drug delivery systems on the assay. During haemolysis, haemoglobin is released after lysis of erythrocytes, and the amount of haemoglobin released is directly proportional to the percentage hemolysis as determined spectrophotometrically at an absorbance of 540 nm (Reed *et al.*, 1985). The milky white appearance of both Pheroid™ vesicles and liposomes has an influence on the absorbance measurement. The absorbance of a concentration range for Pheroid™ vesicles and liposomes was evaluated. It was possible to subtract the absorbance values of each concentration from the sample, resulting in absorbance value only representing haemolysis. Pheroid™ vesicles illustrated a concentration dependent increase in haemolytic activity with a Pheroid™ vesicles concentration of 0.05% defined as non-haemolytic. Liposomes showed dose and time dependent increase in haemolytic activity but to a lesser extent than Pheroid™ vesicles. Mefloquine showed no haemolysis, but when entrapped in Pheroid™ vesicles illustrated a dose related increase in haemolysis resulting in significant increase in haemolysis at concentrations above 0.75 µg/ml. Liposomes only showed significant higher haemolytic activity for concentration above 10 µg/ml after a 7 days incubation period. More accurate *in vitro* haemolysis assay can be conducted to confirm these results as well as *in vivo* studies.

Oxidative damage occurs if the natural defence of the body is overwhelmed by ROS levels. This leads to lipid, protein and DNA damage that can cause apoptosis and cell death (Halliwell, 2006). ROS was evaluated by flow cytometry by converting DCFH to the

fluorescent DCF in the presence of ROS. The fluorescent intensity is directly proportional to the ROS levels. Pheroid™ vesicles and liposomes had no influence on the assay. Evaluation on erythrocytes showed a concentration dependent increase in ROS for Pheroid™ vesicles and liposome without MQ. Pheroid™ vesicles had significantly higher ROS production compared to liposomes. Little to no ROS production was seen with MQ but a dose dependent increase in ROS was observed for both liposomes and Pheroid™ vesicles. Infected erythrocytes showed similar results to uninfected erythrocytes, with an overall increase in ROS levels. The higher ROS levels seen with Pheroid™ vesicles could be explained by the presence of Cremophor EL in the formulation (Iwase *et al.*, 2004). The antioxidant effect of DL- α -tocopherol was overwhelmed by the ROS production, thus showing no protection. This can also be explained by DL- α -tocopherol acting as a prooxidant in the presence of metal ions inducing ROS (Yamamoto *et al.*, 1988). Further investigation into ROS production in erythrocytes as well as the antioxidant/prooxidant effect of DL- α -tocopherol is needed.

In vitro efficacy study was an indication to determine whether the drug delivery system increased the efficacy of a compound. Similarity in size and structure between erythrocytes and the drug delivery systems lead to optimization of the assay to accurately determine the efficacy of mefloquine. Cells were stained with the DNA specific dye, propidium iodide and evaluated with flow cytometry. Samples containing only Pheroid™ vesicles and liposomes were run yielding distinctive population on dot plots. Infected and uninfected erythrocytes also showed two distinct populations. Utilizing a gating strategy, a large amount of background noise, liposomes and Pheroid™ vesicles could be eliminated from the events analysed resulting in more accurate readings. No decrease in growth was observed with mefloquine, but when entrapped in both liposomes and Pheroid™ vesicles showed a decrease in parasitemia of 207% and 186% respectively. No dose dependent response was seen. This increase in efficacy could be due to increased solubility of mefloquine. Higher efficacy can also be related to the haemolytic activity and ROS production but further investigation should be done to confirm this theory.

Because of the known neurotoxicity of mefloquine (Dow *et al.*, 2006), it is necessary to determine the neurotoxicity of mefloquine when entrapped in the drug delivery systems. No dose dependent response in cell death was observed. Pheroid™ vesicles illustrated higher cell viability with significant difference seen when compared to mefloquine and

liposomes. Even though Pheroid™ vesicle ROS analysis on erythrocytes lead to increase in ROS levels, Pheroid™ vesicles seemed to have a neuroprotective property during *in vitro* studies on Nb2A cells. ROS analysis *in vitro* on neuronal cell lines should be done to confirm a reduction in ROS leading to increase cell viability as well as *in vivo* studies to confirm the neuroprotection of Pheroid™ vesicles.

It is apparent from the above mentioned results that mefloquine was successfully incorporated into Pheroid™ vesicles with high stability. Incorporation of mefloquine into liposomes was less successful because of trouble during formulation and stability, but these problems may be overcome by not using phosphatidyl choline and to use lyophilisation to increase stability. The size and entrapment efficacy was successfully determined. The influence of the drug delivery systems on the *in vitro* haemolysis and efficacy assays was minimized by optimization leading to accurate results. Liposomes illustrated little haemolysis and ROS production with little difference with incorporation of mefloquine into the drug delivery system. Increase efficacy was seen but no difference in neurotoxicity was observed. Pheroid™ on the other hand showed to be highly haemolytic with high ROS production with similar results when mefloquine was entrapped. An increase in efficacy and possible neuroprotection was observed. Mefloquine entrapped in Pheroid™ vesicles can possibly be used for the treatment of malaria, but further studies are required to confirm this findings.

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