Chapter 7

Chapter 7 contains an article to be submitted to the Indian Journal of Pharmaceutical Science. The article contains the background, aims, experimental methods, results and discussion of the cellular evaluation of mefloquine loaded lipid based drug delivery systems. The article is prepared according to the author guidelines (http://www.ijpsonline.com/contributors.asp). Some formatting have been changed to help with ease of reading. Language and grammer is consitent with US English. Reference style is the Harvard style.

Table of contents

Keywords 100

List of Abbreviations 100

Abstract 101

1. Introduction 101

2. Methods 103

2.1 Formulation and characterization of lipid drug delivery systems 103

2.1.1 Formulation of liposomes 103

2.1.2 Formulation of Pheroid™ vesicles 104

2.1.3 Size determination 104

2.1.4 Entrapment efficacy 105

2.2 *In vitro* cellular evaluation 105

2.2.1 Evaluation of hemolysis 105

2.2.2 Cultivation of *Plasmodium falciparum* cultures 106

2.2.3 ROS analysis of infected and uninfected erythrocytes 106

2.2.4 *In vitro* efficacy of MQ 106

2.2.5 Neurotoxicity 107

2.3 Statistical analysis 107

3. Results 108

3.1 Characterization of lipid drug delivery systems 108

3.2 Cellular evaluation 109

3.2.1 Hemolysis 109

3.2.2 ROS analysis 112

3.2.3 Efficacy 113

3.2.4 Neurotoxicity 113

4. Discussion 114

5. Conclusion 116

6. References 116

List of Figures

Figure 1	Size and entrapment efficacy of Pheroid™ vesicles (left) and liposomes (right) formulated with different MQ concentrations. Size is seen in bars and percentage entrapment efficacy as a line. Note the difference in values of the left y-axis (size) of A and B.	108
Figure 2	Comparison of log concentration-hemolysis curves by spectrophotometric analysis of Pheroid™ vesicles (A) and liposomes (B) over 7 days. Measurements under 10% are non-hemolytic and above 25% are hemolytic. Each point represents the mean ± SEM of triplicate measurements. Data was transformed and normalized before sigmoidal dose response curve was fitted.	110
Figure 3	Curves of the log concentration-hemolysis of MQ as control (A) and entrapped in liposomes (B) over 144 hours. Comparison between MQ as control and in combination with a 0.05% Pheroid™ vesicles or liposome concentration after 48 hours incubation (C). Measurements under 10% are non-hemolytic and above 25% are hemolytic. Each point represents the mean ± SEM of triplicate measurements. Data was transformed and normalized before sigmoidal dose response curve was fitted.	111
Figure 4	Comparison curves of log concentration plotted against the ratio between the geometric mean of the fluorescent intensity and the amount of cells by flow cytometric analysis of Pheroid™ vesicles and liposomes on infected and uninfected erythrocytes (A). ROS analysis of MQ control and entrapped in liposomes and Pheroid™ vesicles after an 24 hour incubation period with infected and uninfected erythrocytes (B). Each point represents the mean ± SEM of triplicate measurements. Data was transformed and normalized before sigmoidal dose response curve was fitted.	112

List of Tables

Table 1	Percentage cell death as determined by flow cytometry after a 48 hour incubation period with MQ. A 0.05% Pheroid™ vesicle and liposome concentration was used. Data is shown as mean ± SEM of duplicate readings.	113

The *in vitro* efficacy and toxicity evaluation of mefloquine loaded lipid based drug delivery systems for treatment of malaria

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Keywords:

- Pheroid™ Technology
- Liposomes
- Neurotoxicity
- ROS analysis
- Hemolysis

List of Abbreviations

%EE	Percentage entrapment efficacy
DCF	2',7' dichlorofluorescein
DCFH	non-fluorescent 2',7'-dichlorfluorescein
DCFH-DA	2',7'-dichlorodihydrofluoresceindiacetate
iRBC	Infected erythrocytes
MQ	Mefloquine
Nb2A	Mouse neuroblastoma cells
PBS	Phosphate buffer solution
RBC	Uninfected erythrocytes
ROS	Reactive oxygen species
W2	*Plasmodium falciparum* multidrug resistant strain

Abstract

Malaria is a major health problem affecting millions of people especially in Africa. A limited amount of new drugs are being developed to help alleviate this problem. Incorporation of existing drugs into drug delivery systems to improve bioavailability and decrease toxicity can be used in the fight against malaria. Mefloquine was chosen for further investigation due to the advantageous high efficacy and limited resistance, but as disadvantage toxicity concerns and poor solubility. This study investigates the use of lipid drug delivery systems in combination with mefloquine. Incorporation of mefloquine in liposomes and Pheroid™ vesicles was successful with 62.96% and 63.03% entrapment efficacy respectively. Cellular evaluation of Pheroid™ vesicles showed concentration dependent hemolysis and increase in ROS levels. Liposomes in contrast, showed little hemolysis and ROS production. A decrease of 186% and 207% in parasitemia levels were seen after a 48 hour incubation period. Pheroid™ vesicles illustrated the possibility of neuroprotection during *in vitro* studies on Nb2A cells. Pheroid™ vesicles with mefloquine showed high efficacy and neuroprotection with an increase in ROS levels. The current study illustrates the potential of Pheroid™ vesicles to increase efficacy and decrease toxicity of mefloquine.

1. Introduction

Malaria, caused by *Plasmodium* spp., is responsible for millions of deaths annually and has a high economic burden mainly affecting third world countries (1). An increase in resistance and limited amounts of drugs available (2) contribute to the lack of effective disease control. Research to help alleviate this burden includes evaluation of combination therapy, including a 3 day mefloquine artesunate regime (3-5), synthesis of new moieties (6) and incorporation of existing antimalarial compounds in drug delivery systems to improve bioavailability and decrease toxicity (7-10).

Mefloquine (MQ) is a highly effective drug used against multidrug resistant strains of *Plasmodium falciparum* (11). The exact mode of action of MQ is unknown but the drug is known to interact with a variety of biological systems and physiological processes. Some of these include neuronal calcium homeostasis (12), the calcium pump (13), blood brain

barrier glycoproteins (14,15) and potassium channels (16). MQ, unlike chloroquine does not accumulate in the food vacuole inhibiting heme polymerization (17) and is, therefore, considered a more effective drug (18). MQ is known to bind with high affinity to infected and uninfected erythrocytes and cell membranes (19-21) that may be responsible for the long half life of MQ (22).

Shortcomings of MQ include the dose dependent adverse reactions ranging from mild to severe. Mild adverse reactions include headache, nausea, fatigue and depression whereas the more severe reactions include hallucinations and physcosis (23). Other limitations of the drug include the extensive tissue and protein binding, slow clearance, poor solubility and variation in oral absorption. MQ is known for its neurological effects as established by numerous case studies (24-26) as well as *in vitro* and *in vivo* neurotoxicity studies (12,27). The neurological effects have been ascribed to increased reactive oxygen species (ROS) production responsible for neurodegeneration in cortical neurons (28).

ROS are chemical reactive oxygen containing molecules and play an essential role in both cell death and malaria pathology. They have unpaired electrons making them highly reactive leading to damage of DNA, lipids, proteins and carbohydrates. ROS are used as defense mechanism during infections, but when the ROS levels in relation to the antioxidant defense mechanisms of the host is too high, irreversible damage occurs. Increasing of ROS leads to mitochondrial damage followed by DNA damage initiating apoptosis and cell death (29-31). Hemoglobin degradation by malaria parasites, a source of amino acids, releases heme that is oxidized to produce ROS. The parasite has various methods of protection against ROS (32,33) including hemoglobin scavaging of ROS. Parasites are surrounded by hemoglobin when inside erythrocytes and are only susceptible to ROS for a short period of time between erythrocyte rupturing and reinvading of erythrocytes (34). Decreasing the natural defense or overwhelming the antioxidant protection against ROS can be used as a mechanism to decrease parasitemia levels (33).

MQ has been formulated in submicron emulsions. This formulation proved to be stable and good vehicles for this poorly soluble drug. High antimalarial effect was reported as well as the need for further investigation (7,8). Drug delivery systems are utilized to improve bioavailability and reduce side effects (35) by enhancing drug solubility (36)(36). Lipid excipients, mainly long and medium chain fatty acids, phospholipids and steroids are

a popular choice because they modulate the activity of drug efflux, serve as fuel and yield important biological functions (37). Liposomes, a model membrane system consists of either a single or multiple lipid bilayers enclosing an aqueous compartment (38,39). Liposomes have the ability of entrapping both hydrophilic and hydrophobic drugs. Hydrophillic drugs are entrapped in the aqueous compartment and are dependent on the volume of the compartment. Dependent on the lipid concentration, the hydrophobic drug is entrapped in the lipid bilayer (38,40,41). Liposomes are mainly used to reduce toxic side effects and increase efficacy (42). Disadvantages include rapid removal from circulation, as well as chemical and physical instability (39).

Pheroid™ technology is a novel, colloidal system of lipid bilayers constructed of natural essential fatty acids necessary for normal cell function. Submicron and micro sized structures are formed when the oil phase is dispersed in a nitrous oxide saturated water phase. Pheroid™ can also entrap both hydrophilic and hydrophobic drugs and can be administered orally, transdermally and nasally. Applications include nasal delivery of calcitonin and peptide drugs (43-45).

Because the main focus of drug delivery systems is to increase efficacy and decrease toxicity it is, therefore, necessary to determine the efficacy and toxicity of the drug delivery systems (46). The aim of this study was to characterize two lipid drug delivery systems, Pheroid™ vesicles and liposomes loaded with MQ and to determine their *in vitro* biological effects utilizing methods to determine efficacy, neurotoxicity, ROS analysis and hemolysis.

2. Methods

2.1 Formulation and characterization of lipid drug delivery systems

2.1.1 Formulation of liposomes

Liposomes were prepared by the film hydration method. L-α-phosphatidylcholine and cholesterol (Sigma-Aldrich®, St. Louis, MO, USA) were dissolved in chloroform:methanol (2:1 v/v). The organic solvent was removed under reduced pressure to obtain a thin film on the side of the flask. Hydration with phosphate buffer solution (PBS) (pH 7.4) to form the lipid suspension was obtained with gentle swirling with glass beads. Suspensions

were submitted to sonication to reduce the size of particles for 5 minutes at 4°C (38,47,48). Mefloquine hydrochloride (MQ) (Sifavito S.p.A. Mairano, Italy) was added to the organic solvent phase before evaporation to obtain loaded liposomes. The pH of each formulation was measured before analysis.

2.1.2 Formulation of Pheroid™ vesicles

Vitamin F ethyl ester (CLR, Berlin, Germany) and Cremophor® EL (BASF, Germany) was heated to 75°C and left to cool after which DL-α-tocopherol (DSM, Basel, Switzerland) was added and heated to 55°C. The oil phase was added to the nitrous oxide saturated PBS, preheated to 75°C and homogenized at 13500 rpm until a temperature of below 40°C was reached. The suspension was shaken until room temperature was attained (44). Loading of Pheroid™ vesicles with MQ was obtained by addition of MQ to the oil phase. The pH of each formulation was measured before analysis.

2.1.3 Size determination of Pheroid™ vesicles and liposomes

Size determination was done as previously described by Vorauer-Uhl *et al.* (2000). Briefly, FACSCalibur™ (Becton and Dickson, Mountain View, CA, USA) benchtop flow cytometer equipped with a 488 nm Argon ion laser was used to determine the size distribution. Size calibration beads (Molecular Probes, Invitrogen™, Breda, The Netherlands) were analyzed and the geometric mean of each bead size was used to plot a graph yielding a equation of y=mx+c. Diluted samples, to give a flow rate of less than 2000 events per second were analyzed, yielding size distribution (49). Span was determined by the following equation:

$$\text{Span } (\mu m) = \frac{(S_{95\%} - S_{5\%})}{S_{50\%}}$$

where $S_{95\%}$ and $S_{5\%}$ is the size where 95% and 5% of particles are smaller and $S_{50\%}$ is the median.

2.1.4 Entrapment efficacy

The amount of unentrapped MQ in Pheroid™ vesicles and liposomes was determined by UV-Spectrophotometry as described by Rao and Murthy (2002). The unentrapped MQ was separated from the lipid drug delivery system using Sephadex G50 centrifuge columns (50). A comparative study to determine the maximum entrapment efficacy was done. The percentage entrapment efficacy (%EE) was determined by the following equation:

$$\%EE = \frac{\text{(Initial drug load – unentrapped drug)}}{\text{Initial drug load}} \times 100$$

2.2 *In vitro* cellular evaluation

2.2.1 Evaluation of hemolysis

Hemolytic activity assays were performed with and without MQ at different concentrations. Pheroid™ vesicles and liposomes, without MQ were added at different concentrations to erythrocytes in 96 well plates. Human erythrocytes were isolated from whole blood and after washing, resuspended in RPMI1640 medium. Plates were incubated at 37°C for 7 days with daily analysis. Plates were centrifuged at 2000 rpm for 8 minutes where after 100 µl supernatant was removed and added to a new plate. Lysis of erythrocytes was quantified by spectrophotometrically measuring the release of hemoglobin in the supernatants at 540 nm. Control of 100% hemolysis was obtained by addition of water (51). Results were expressed as absorbance and background subtraction was done before determining the percentage hemolysis by the following equation:

$$\%\ \text{Hemolysis} = \frac{\text{Abs of sample}}{\text{Abs of 100\% hemolysis}} \times 100$$

The optimal drug delivery system concentration where no hemolysis was observed was used to determine the hemolytic activity of MQ. MQ at different concentrations was analyzed as described above. MQ dissolved in ethanol at a final concentration of less than 0.1% was used as control.

2.2.2 Cultivation of *Plasmodium falciparum*

The method as described by Trager and Jensen was used (1976). In short, W2 strain (kindly supplied by Prof P. Smith, University of Cape Town, Department Pharmacology) was maintained in continuous culture supplemented with RPMI 1640 medium (Sigma-Aldrich®, St. Louis, MO, USA) and Albumax II (Gibco®, Invitrogen™, Breda, The Netherlands). Cultures were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and the balance nitrogen. Cultures were used in analysis of ROS and efficacy studies.

2.2.3 ROS analysis of infected and uninfected erythrocytes

ROS analysis was performed by flow cytometry using a FACSCalibur™ bench top flow cytometer linked to Cell Quest Prof Software (2002, Becton and Dickson, Mountain View, CA, USA). DCFH-DA (2',7'-dichlorodihydrofluoresceindiacetate) (Molecular Probes, Invitrogen™, Breda, The Netherlands) is converted to DCFH (non-fluorescent 2',7'-dichlorfluorescein) by intracellular esterase and in the presence of ROS is converted to the highly fluorescent DCF (2',7'dichlorofluorescein). DCF is excited at 488 nm and emits green fluorescence proportional to the intracellular levels of ROS. DCFH-DA was added to each sample at a final concentration of 10 µM after a 24 hour incubation period of Pheroid™ vesicles and liposomes at different concentrations. Histograms with markers representing the negative control (M1) and positive control (M2) were set to each sample. A ratio between the geometric mean of each sample and the percentage of events in marker M2 was determined. ROS analysis of a MQ concentration range was determined at the optimal Pheroid™ vesicle and liposome concentration and compared to MQ in ethanol (52,53).

2.2.4 *In vitro* efficacy of MQ

MQ, incorporated into the drug delivery system or alone was added to infected erythrocytes (iRBC) in a 1:1 ratio. After 48 hours incubation at 37°C, samples were

analyzed by flow cytometry. Samples were fixated with 0.025% gluteraldehyde, perforated with 0.005% saponin (Sigma-Aldrich®, St. Louis, MO, USA) followed by incubation with propidium iodide (PI) (Molecular Probes, Invitrogen™, Breda, The Netherlands) for 50 minutes (54). Nile Red (Sigma-Aldrich®, St. Louis, MO, USA) at a final concentration of 0.8 µg/ml was added and left for an incubation period of 10 minutes (44,55). Samples were gated to exclude noise and analyzed at a flow rate of less than 2000 events per second as previously described (Technical Note: Chapter 6).

2.2.5 Neurotoxicity

Mouse neuroblastoma cells (Nb2a) (ATCC, Manassas, Virginia) were cultivated in modified DMEM medium supplemented with 10% fetal bovine serum (Hyclone®, Thermo Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% carbon dioxide. Various concentrations of free or loaded MQ were added to cells in duplicate with the inclusion of appropriate controls. Controls included 100% viable cells and 100% dead cells obtained by addition of hydrogen peroxide (10 mM). After a 24 hours incubation period, the endpoint of cell viability was determined with the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen™, Breda, The Netherlands). Calcein AM and ethidium homodimer-1 were added to each sample and left to incubate for 20 minutes. Markers on the histogram representing viable and dead cells were drawn up. The toxicity was expressed as a percentage of the dead cells (56).

2.3 Statistical analysis

Data was analyzed with repeated measure ANOVA using Statistica 9 (StatSoft, San Diego, California, USA). All data were termed statistically significant with p-values less than 0.05. P-values for the formulation data were determined by Tukey's post hoc test and the Bonferroni post hoc test was performed on ROS, hemolysis, efficacy and neurotoxicity data. Data for cellular assay were transformed, X = log (X), normalized between 0% and 100% and fitted with sigmoidal dose response curves.

3. Results

3.1 Characterization of lipid drug delivery systems

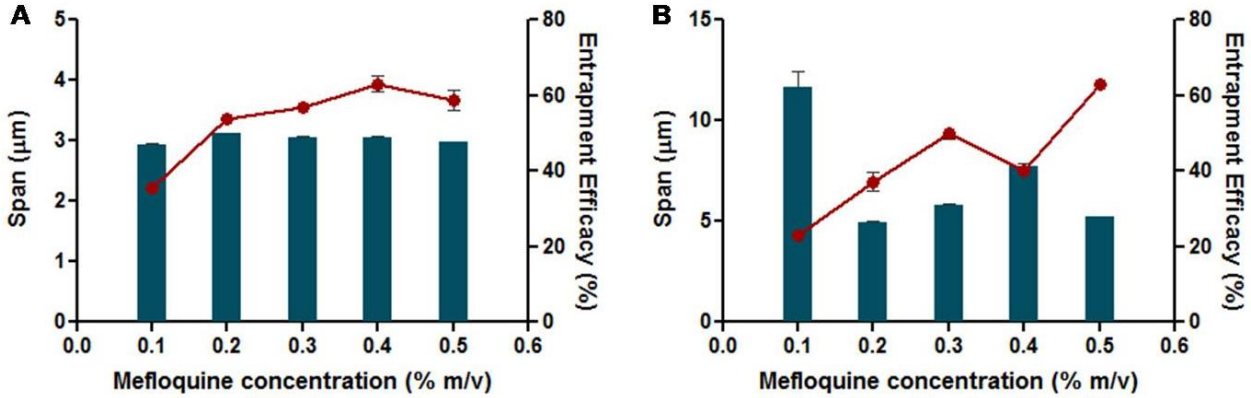

Figure 1 Size and entrapment efficacy of Pheroid™ vesicles (A) and liposomes (B) formulated with different MQ concentrations. Size is seen in bars and percentage entrapment efficacy as a line. Note the difference in values of the left y-axis (size) of A and B.

MQ added in concentrations ranging from 0.1% to 0.5% was formulated in combination with Pheroid™ vesicles and liposomes. Pheroid™ vesicles with MQ showed an average size of approximately 3 µm at all concentrations. No significant difference in size of Pheroid™ vesicles was noted with the different MQ formulations (as seen in Figure 1A). Liposomes formulated with 0.2% - 0.5% MQ showed size distribution of less than 8 µm with 5.22 µm at 0.5% (Figure 1B). The 0.5% formulation differed significantly from Pheroid™ vesicles. Pheroid™ vesicles illustrated an increase in entrapment efficacy with an increase in MQ concentration. No significant difference was seen between 0.2% to 0.5% with the maximum %EE of 63.03% obtained at 0.4%. Liposomes did not show significant lower entrapment efficacy compared to Pheroid™ vesicles with 62.96% at 0.5% MQ concentration. The pH of all formulations was between 7.07 and 7.48. No difference in size or entrapment efficacy was observed for Pheroid™ vesicles between 0.2% and 0.5% whereas the optimal liposome formulation appears to be 0.5%. Therefore, cellular evaluation was done with a initial concentration of 0.5% MQ in both liposomes and Pheroid™ vesicles.

3.2 Cellular evaluation

3.2.1 Hemolysis

The hemolytic activity of Pheroid™ vesicles and liposomes without MQ was determined over a 7 day period. As seen in Figure 2A, Pheroid™ vesicles showed an increase in hemolytic activity at concentrations ranging from 0.01% to 0.5% with hemolysis above 90% from 72 hours for the highest concentration. Hemolysis under 10% wais observed with concentration below 0.05%. Daily readings were significantly higher from the initial reading with p-values < 0.05. No significant difference was observed between measurements from 24 hours to 144 hours (data not shown). Liposomes showed lower hemolytic activity (Figure 2B). Concentration dependent hemolysis is observed with no significant difference when compared to initial readings. Daily measurements of Pheroid™ vesicles compared to liposomes differed significantly in hemolytic activity.

Hemolytic activity of MQ entrapped Pheroid™ vesicles and liposomes at an entrapment efficacy of 60% were determined at a drug delivery system concentration of 0.05% (Figure 3). Hemolytic activity of MQ was measured over 144 hours for both the control and in liposomes. The control of MQ showed no hemolysis over 144 hours (Figure 3A). In contrast, Pheroid™ vesicles illustrated a concentration dependent increase in hemolytic activity. Concentration between 2.5 µg/ml and 12.5 µg/ml showed hemolytic activity with significant difference between the initial values and 24 and 48 hours for concentrations above 0.75 µg/ml (p value < 0.005%) (Data not shown). As seen in Figure 3B, the hemolytic activity of liposomes increased with an increase in concentration and over time. P values of less than 0.005% were observed at 10 µg/ml and 12.5 µg/ml from 24 hours to 144 hours compared to the initial readings. Figure 3C illustrates the difference in hemolytic activity of the control, Pheroid™ vesicles and liposomes after 48 hours incubation. Pheroid™ vesicle at the highest concentration MQ after 48 hours incubation, showed hemolysis of 55.42 ± 3.32%, liposomes only 24.08±1.06% with no hemolysis seen at the control. Comparison of the control and liposome formulation at 48 hours gave a p value of 0.1712. An overall increase in hemolytic activity of Pheroid™ is observed compared to liposomes and control with p-values of 0.0073 and 0.0053 respectively.

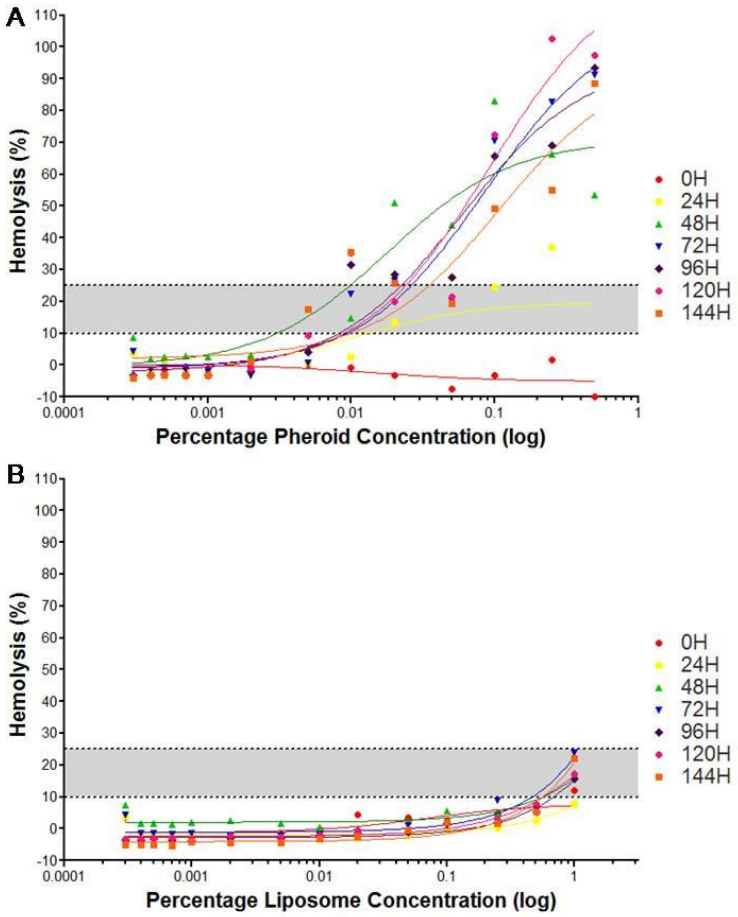

Figure 2 Comparison of log concentration-hemolysis curves by spectrophotometric analysis of Pheroid™ vesicles (A) and liposomes (B) over 7 days. Measurements under 10% are non-hemolytic and above 25% are hemolytic. Each point represents the mean ± SEM of triplicate measurements. Data was transformed and normalized before sigmoidal dose response curve was fitted.

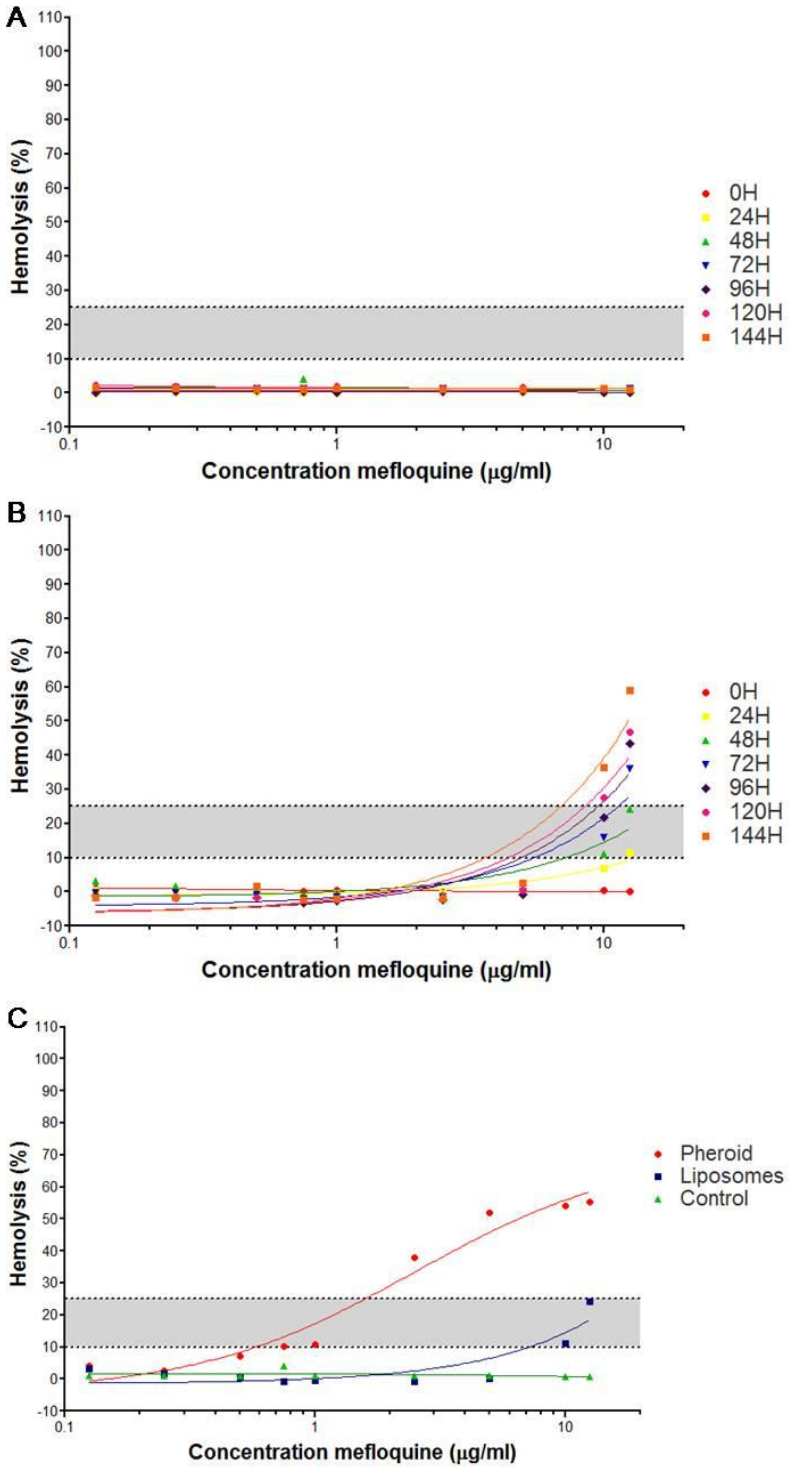

Figure 3 Curves of the log concentration-hemolysis of MQ as control (A) and entrapped in liposomes (B) over 144 hours. Comparison between MQ as control and in combination with a 0.05% Pheroid™ vesicles or liposome concentration after 48 hours incubation (C). Measurements under 10% are non-hemolytic and above 25% are hemolytic. Each point represents the mean ± SEM of triplicate measurements. Data was transformed and normalized before sigmoidal dose response curve was fitted.

3.2.2 ROS analysis

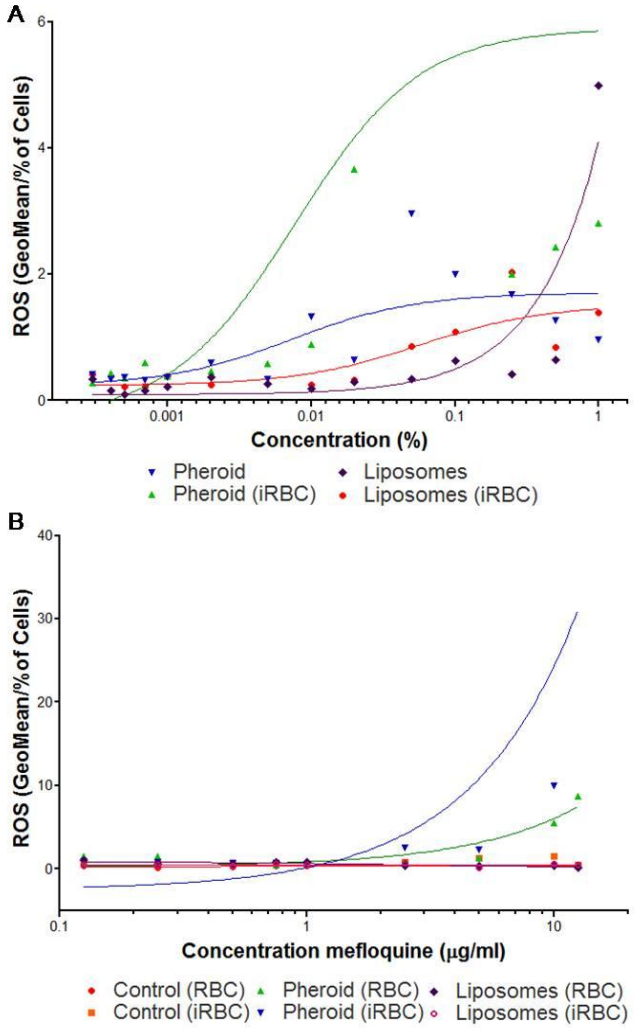

Figure 4 Comparison curves of log concentration plotted against the ratio between the geometric mean of the fluorescent intensity and the amount of cells by flow cytometric analysis of Pheroid™ vesicles and liposomes on infected and uninfected erythrocytes (A). ROS analysis of MQ control and entrapped in liposomes and Pheroid™ vesicles after an 24 hour incubation period with infected and uninfected erythrocytes (B). Each point represents the mean ± SEM of triplicate measurements. Data was transformed and normalized before sigmoidal dose response curve was fitted.

Pheroid™ vesicles and liposomes at different concentrations showed concentration dependent ROS production (Figure 4A). Higher ROS levels were observed in infected erythrocytes but were not statistically different. P-value of 0.0221 indicates significant difference between the Pheroid™ vesicles and liposome formulation as determined on

infected erythrocytes. Determination of ROS levels with the addition of MQ at different concentrations showed dose dependent increase in ROS levels for Pheroid™ vesicles (Figure 4B). Infected erythrocytes showed higher ROS levels than the uninfected samples. Both control samples and liposomes showed no major increase in ROS levels.

3.2.3 Efficacy

The antimalarial activity of MQ did not change with an increase in concentration with a parasitemia of 2.950 ± 0.363% at 0 μM and 3.060 ± 0.313% at a concentration of 12.5 μM. The results of the efficacy study illustrated highly significant lower values of percentage parasitemia of Pheroid™ vesicles and liposomes compared to the control ($p<0.0001$). Pheroid™ vesicles had a parasitemia of 1.047 ± 0.252% and 1.000 ± 0.167% for 0 μM and 12.5 μM MQ concentrations respectively. Liposomes showed a slight decrease in parasitemia from 1.870 ± 0.076% at 0 μM to 1.067 ± 0.117% at 12.5 μM. Pheroid™ vesicles showed a 186% decrease in parasitemia levels and liposomes a 207% compared to the control.

3.2.4 Neurotoxicity

Table 1 Percentage cell death as determined by flow cytometry after a 48 hour incubation period with MQ. A 0.05% Pheroid™ vesicle and liposome concentration was used. Data is shown as mean ± SEM of duplicate readings.

μM	Control	Pheroid™ vesicles	Liposomes
0	14.370 ± 0.770	1.680 ± 1.650	20.590 ± 2.530
0.125	17.550 ± 2.220	4.690 ± 0.066	20.060 ± 2.350
0.25	2.505 ± 2.475	1.595 ± 0.205	14.760 ± 3.580
0.5	7.925 ± 7.905	1.530 ± 0.090	11.270 ± 6.660
0.75	14.640 ± 0.250	2.480 ± 0.090	8.585 ± 5.145
1	16.275 ± 2.565	5.105 ± 0.295	16.100 ± 7.620
2.5	14.660 ± 0.680	0.955 ± 0.955	21.545 ± 0.535
5	16.460 ± 0.370	2.850 ± 2.070	18.335 ± 2.865
10	13.285 ± 0.225	10.705 ± 4.345	11.120 ± 4.900
12.5	13.365 ± 1.055	7.080 ± 1.820	11.530 ± 0.050

Experiment on the neurotoxicity of MQ showed that free MQ and liposome entrapped MQ was much more toxic to the neuroblastoma cells than Pheroid™ vesicles loaded MQ (Table 1). Pheroid™ vesicles increased the viability of neuroblastoma cells after a 24 hour incubation period with MQ. A significant lower amount of dead cells was observed compared to the control ($p<0.0001$) and liposomes ($p=0.0014$).

4. Discussion

The purpose of this study was to evaluate the MQ loaded drug delivery system toxicity on various cells and assays. This included the characterization of the formulation after which the hemolytic activity, ROS analysis, efficacy and neurotoxicity were determined. The results obtained showed that MQ can be entrapped in both Pheroid™ vesicles and liposomes at an efficacy of 63.03% and 62.96% respectively. Because no major difference between the 0.4% and 0.5% Pheroid™ vesicle MQ formulations were seen, 0.5% formulations was used for all experiments. Size determination showed no difference between the Pheroid™ vesicle formulations. In contrast, liposomes showed a variety of sizes with 5.2 μm for the 0.5% MQ formulation.

ROS levels increased with an increase in concentration of both Pheroid™ vesicles and liposomes with higher ROS production with infected erythrocytes. Pheroid™ vesicles illustrated ROS levels significantly higher in comparison with ROS levels generated by liposomes with iRBC. MQ showed no increase in ROS levels *in vitro* but in combination with Pheroid™ showed a dose dependent increase in ROS levels. Lower MQ concentrations, however, showed no increase in ROS levels. The exact mechanism of increased ROS levels in unknown.

Hemolysis, the release of hemoglobin because of an alteration or destruction of the erythrocyte membranes, is used to access the safety of pharmaceutical formulations (57,58). Hemolytic activity of a compound is directly proportional to the amount of hemoglobin released (51). Strong hemolytic activity is illustrated with Pheroid™ vesicles at high concentrations. Values under 10% are deemed to be non-hemolytic and above 25% are hemolytic (58). A Pheroid™ vesicle concentration of 0.05% is non-hemolytic over a 7 day period. MQ illustrated no hemolysis but when entrapped in liposomes, dose dependent hemolytic activity was observed. MQ entrapped in Pheroid™ vesicles was a

more potent hemolytic formulation. The mechanism is not clearly understood, but increased levels of ROS causes modification in cell structures leading to cell damage that may play a role in hemolytic activity and parasite death (59,60). The hemolytic activity of Pheroid™ vesicles may be related to the increased ROS levels observed. ROS levels at high liposome concentrations were slightly elevated with increased hemolytic activity at the same concentrations. After 24 hours incubation with MQ loaded liposomes, no ROS production or hemolysis were observed.

Even though MQ did not show a dose dependent decrease in parasitemia, MQ entrapped in Pheroid™ vesicles and liposomes decreased parasitemia levels. No documented mechanism of action of MQ includes the increasing of ROS production to decrease the parasitemia levels. Liposomes only showed an increase in ROS levels and the lowest parasitemia levels at the highest MQ concentration. However, an overall increase of MQ entrapped in liposomes was seen. The increase in solubility of MQ in the lipid bilayer can also increase the efficacy of MQ. The efficacy of a drug is dependent on the solubility of the compound in the pharmaceutical compound or bodily fluids (61). The higher solubility of MQ in Pheroid™ vesicles could also have contributed to the increase in efficacy. The increase in ROS and hemolysis can contribute to the decrease in parasitemia levels, but the exact mode of action is unknown and should be investigated.

MQ has been proven to be neurotoxic *in vitro* and *in vivo* (12,27). Increase in ROS levels was illustrated *in vitro* with rat cortical neurons when treated with MQ (28). Oxidative stress leads to apoptosis and cell death and can thus be responsible for neurotoxicity (28,30). MQ showed neurotoxicity through an increase in neuroblastoma cell death. Liposomes illustrated no increase in neurotoxicity when compared to the control. The neurotoxicity of MQ was decreased when entrapped in Pheroid™ vesicles with a cell viability of above 92%. Nitrous oxide at sub anesthetic concentrations may have potentially neuroprotective properties (62) and an increase in ROS levels may be hindered by the anti-oxidant effect of α-tocopherol (63,64) present in Pheroid™ vesicles. Further studies should be conducted to evaluate the possible neuroprotective effect of Pheroid™ vesicles.

5. Conclusion

Pheroid™ vesicles was stable and uniform in size with a relatively high entrapment efficacy. Pheroid™ vesicles increased the ROS levels alone and in combination with MQ and may be responsible for the hemolytic activity and efficacy observed. The anti-oxidant, α-tocopherol is known to provide protection against ROS (65), however, in the presence of iron may act as a pro-oxidant, that induces ROS. Increasing ROS levels found in RBC and iRBC can thus partly be attributed to the pro-oxidant effect of α-tocopherol. A neuroprotective effect was seen with Pheroid™ vesicles possibly due to the anti-oxidant effect of α-tocopherol as well as the neuroprotection of nitrous oxide. Liposomes showed less hemolysis and ROS production but no difference in neurotoxicity. Pheroid™ vesicles were more stable and reproducible lipid drug carriers with potential of possible neuroprotection compared to liposomes. It is, therefore, necessary for further *in vitro* and *in vivo* investigations into the possible increase in efficacy and neuroprotective effect of MQ entrapped in Pheroid™ vesicles.

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