

Chapter 4

Experimental methods, results and discussions

This chapter contains the experimental design and methods used during the course of this study, as well as the results and discussions pertaining to those experiments.

4.1. Introduction

Malaria kills more than 1 million people each year with between 350-500 million reported cases each year. The spread of resistance to many current antimalarials have been rampant (WHO, 2009). Drugs that have been the staple of malaria treatment for years have become ineffective; drugs like chloroquine will soon need to be replaced (Wongsrichanalai *et al.*, 2002; Foley & Tilley, 1997). Amodiaquine (sometimes referred to as AQ) is structurally related to chloroquine. Both are 4-aminoquinolines and the antimalarial effects are thought to be the same. Both are effective against blood schizonts. Amodiaquine has been proven effective even in chloroquine resistant malaria (Hawley *et al.*, 1996; Winstanley *et al.*, 1990). Unfortunately amodiaquine has fallen into disuse, due to the toxicity it revealed in a few rare cases (Olliaro & Mussano, 2009). Very few effective new drugs have come onto the world market in the past few years. Slow drug development in this regard has moved the focus to overcoming resistance in other ways. The use of lipid based drug delivery systems has proven to be quite effective in this regard, even being able to reduce toxicity of the drugs. Liposomes are one of these promising drug delivery systems (Drulis-Kawa & Dorotkiewicz-Jach, 2010; Sharma & Sharma, 1997). Liposomes can be produced in a myriad of ways and can be made to entrap both lipophilic and hydrophilic drugs such as amodiaquine (New, 1990). Experiments done in the following chapter include a solubility study of amodiaquine, stability studies on the liposomal drug delivery system, as well as toxicity studies with the drug delivery system.

4.2. Experimental design

This study was divided into two parts; firstly the preparation, characterisation and stability of liposomes, and amodiaquine entrapped liposomes. The steps that were followed are showed in Figure 4.1. The second part of the study was an *in vitro* study which included toxicity studies. The second part of this study is illustrated in Figure 4.2.

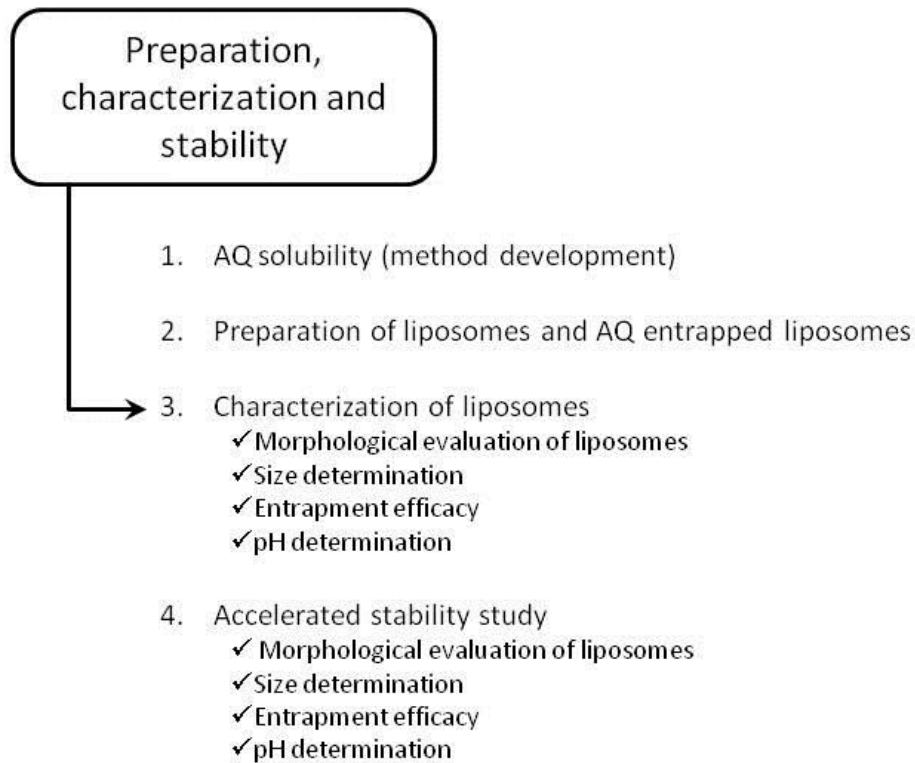


Figure 4.1: Part one in the experimental design. The preparation, characterization and stability as laid out in the steps followed in this study.

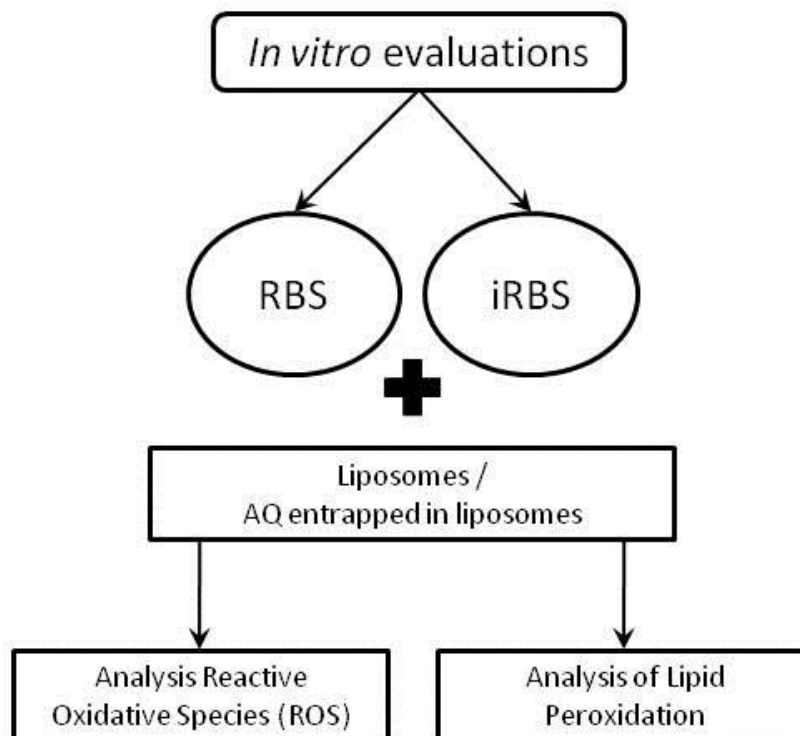


Figure 4.2: Part two in the experimental design. *In vitro* evaluations, as laid out in the steps followed in this study.

4.3. Preparation, characterisation and stability of liposomes containing amodiaquine

As described earlier the formulation and manufacturing of different liposomal formulations will be discussed, as was completed during the course of the research.

4.3.1. Solubility study of amodiaquine (method development)

Even though water is described as the preferred solvent for amodiaquine according to the WHO (Basco, 2007), very little is described about the solubility of amodiaquine, especially when different pH environments are involved. It was important to optimise the method for determining the amount of amodiaquine in solution before being able to prepare liposomes and continuing on to stability studies. It is also of the utmost importance to see if the formulation would mimic physicochemical circumstances for the *in vitro* assays, as to be able to draw accurate conclusions regarding the toxicity of the formulations (New, 1990).

According to the USP, UV-spectroscopy is an easy and reliable technique to determine the concentration of amodiaquine. The maximum peak of absorbance in water is reported to be at 342 nm (United States Pharmacopeia, 2010). The solubility of amodiaquine hydrochloride dissolved in water and different buffers was investigated when using a wide pH range as the drug needs to be in solution in a buffer to be added to the liposomes (New, 1990).

4.3.1.1. Apparatus and materials

The results in this study were attained by using a Shimadzu UV-1800 (Japan) UV-spectrophotometer. Amodiaquine (Hydrochloride) was purchased from Industrial Analytical (South Africa) (Batch number: AMQ0902003). The water used was prepared with a Milipore™ MilliQ® Ultrapure Water Purification System (USA).

4.3.1.2. Method

Calibration curves were constructed by using at least nine different concentrations (concentration range was between 5 mg/100 ml and 0.5 mg/100 ml) of amodiaquine in each different pH environment. The absorbance curve in each pH is illustrated in Figure 4.3. The concentrations used to make up the calibration curves were done in triplicate.

Six amber tubes (20 ml each) were filled with 10 ml buffer with an excess of amodiaquine powder. The tubes were attached to a rotating axis (54 rpm) which was placed in a water bath with a temperature of 37 °C. The vials were left for 24 hours until equilibrium was reached. Thereafter, the solutions were filtered through 0.8/0.2 µm Supor® membranes to remove any undissolved particles. The following dilutions were made after filtrations:

- Dilution of amodiaquine in distilled water: 1 ml to 100 ml then 5 ml to 100 ml.
- Dilution of amodiaquine in pH 1.13: 1 ml to 100 ml then 5 ml to 100 ml.
- Dilution of amodiaquine in pH 4.5: 1 ml to 100 ml then 5 ml to 100 ml.
- Dilution of amodiaquine in pH 6.8: 1 ml to 100 ml then 7 ml to 100 ml.

The dilutions were made in two steps in order to reduce the amount of solvents used. The concentrations of the filtered solutions were determined.

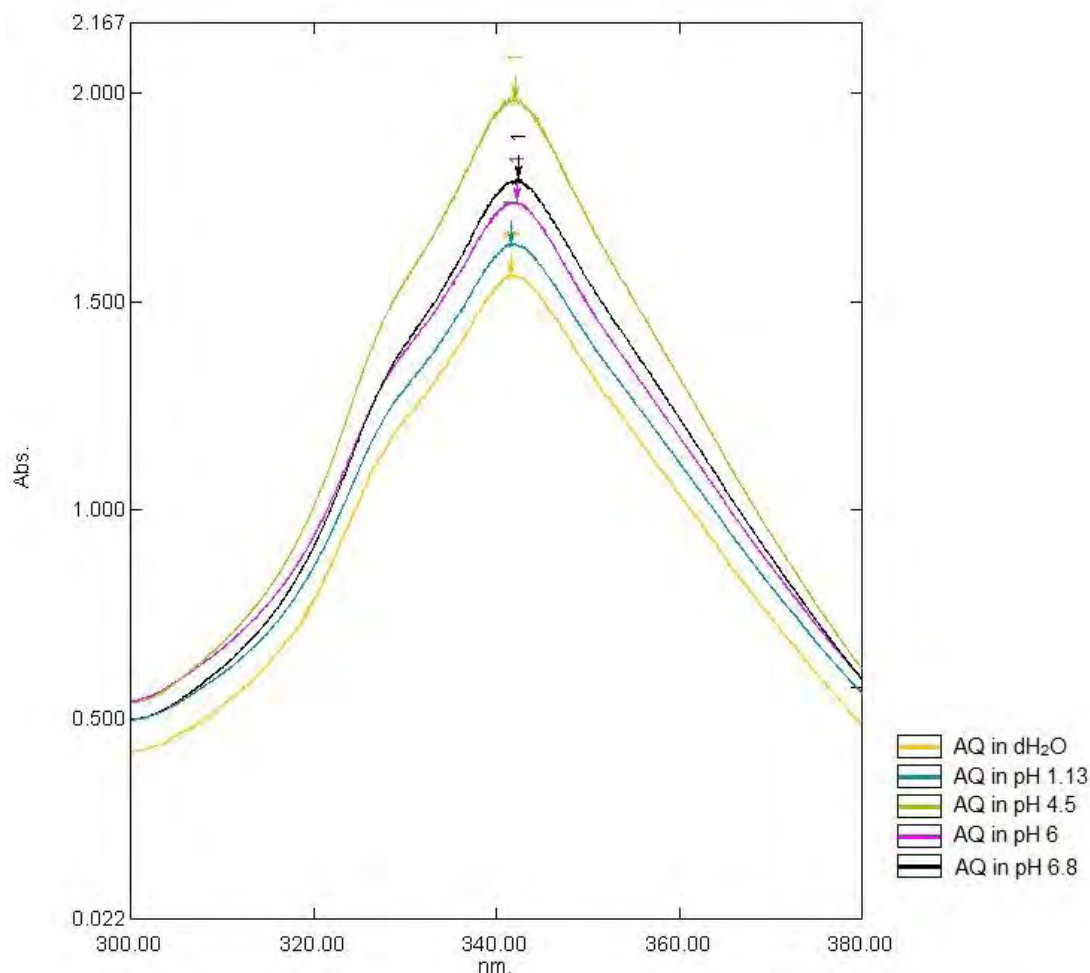


Figure 4.3: An illustration of the absorbance curves created by AQ in different pH values.

4.3.1.3. Results and discussion

Figure 4.4 shows the calibration curves as described in the method section. The specific r^2 values as well as the equation of the curves can be seen in Table 4.1.

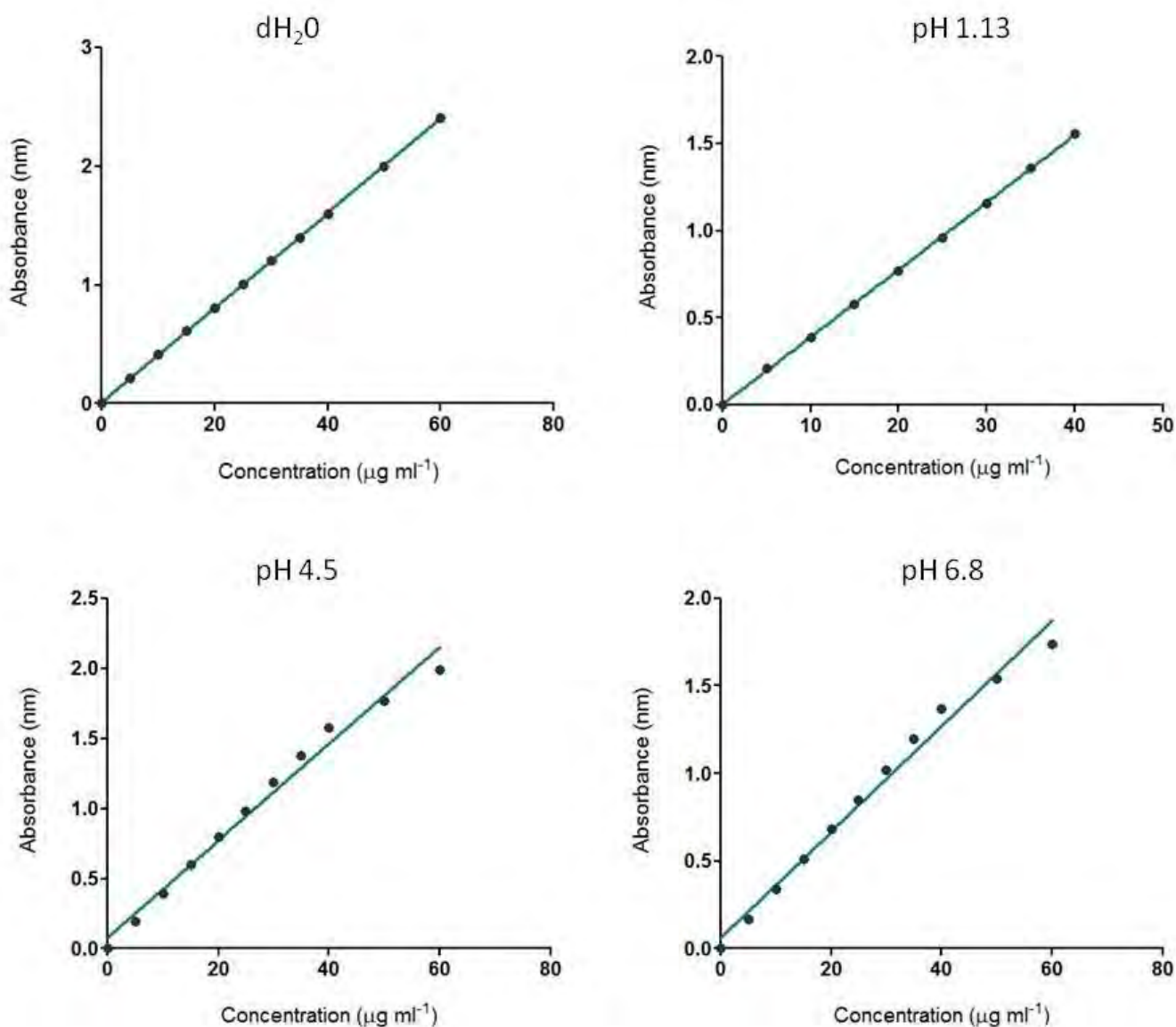


Figure 4.4: The calibration curves of amodiaquine in different pH values

The solubility of amodiaquine in the different pH-ranges varies significantly. Table 4.1 shows the results of the solubility study of amodiaquine. The calibration curves obtained were found to be linear for all the concentrations with correlation coefficients of 0.9999, 0.9997, 0.9843 and 0.9855 for the dH₂O, the 1.13, 4.5 and 6.8 pH-buffers, respectively.

Table 4.1: The results from the solubility study of amodiaquine in a wide pH range.

Solvent	R ² - Calibration curve	Equation for calibration curve	Solubility at 37 °C (mg/ml)
Distilled H ₂ O	0.9999	$y = 0.0298x + 0.0093$	37.1634 ± 1.2395
pH 1.13	0.9998	$y = 0.0387x + 0.0021$	22.4582 ± 0.8983
pH 4.5	0.9843	$y = 0.0395x + 0.0012$	36.3359 ± 0.7904
pH 6.8	0.9855	$y = 0.0345x + 0.007$	15.6052 ± 1.1126

The liposome formulation prepared had to be compatible with cells, but still have as much as possible amodiaquine incorporated into the liposome. Therefore, the pH may be lowered from the standard 7.4, but if the pH is too low, it may damage the erythrocytes that were used for the *in vitro* studies. Erythrocytes are specifically prone to damage because of the large amount of polyunsaturated fatty acids in the cell membrane (Maulik *et al.*, 1998). Therefore, buffer with a pH in the area of 6 was used for the preparation of liposomes to ensure that the amodiaquine concentration (0.5% m/v) could be achieved without the amodiaquine precipitating from the solution.

4.3.2. Manufacturing of liposomes and amodiaquine entrapped liposomes

The manufacturing of liposomes was done according to the film hydration method which gives multilamellar vesicles (MLV's) with a few adaptations (New, 1990). This method is inexpensive and straightforward. It is often used for small scale production of liposomes, as it does not require much expensive equipment.

4.3.2.1. Materials

L-alpha-phosphatidyl choline (batch number: 0001410262) and cholesterol (Batch: 089K89K5312) were acquired from Sigma Aldrich (South Africa). Chloroform was purchased from MERCK (South Africa). A rotary evaporator (Heidolph Laborato 4000, Germany), as well as a rod sonicator (Hilscher UP 100H Ultraschallprozessor) was used in the manufacturing of liposomes.

4.3.2.2. Method

The liposomes were manufactured according to guidelines given by R.R.C. New (New, 1990). A solution of PC and Cholesterol was dissolved in chloroform to create the lipid solution. The amount of PC to cholesterol was 2:3; 1.5% (m/v) PC and 1% (m/v) cholesterol of the final product. The lipid solution was placed into a large round bottomed flask, which was attached to a rotary evaporator. The flask was immersed into a thermostatic water bath set to 30 °C, which is the T_C of this lipid solution. The flask was rotated with the vacuum turned on, to evaporate the solute and to create a thin lipid film on the inside of the flask. The buffer (pH 6), was added to the round bottom flask. A mixed phosphate buffer was prepared for the liposomes; in this case, with a pH of 6 because of reasons stated in Section 4.3.1 (Solubility study). Glass beads (0.5 g) were also added into the round bottom flask to help the agitation of lipid film from the container. The flask was reconnected to the rotary evaporator and rotated at room temperature. This was done until the lipid film was removed from the sides of the flask. The glass beads were removed and the liposomes decanted into another container. To ensure the liposomes were evenly sized, they were sonicated. This involved immersing the liposome container in an ice bath. The mixture was then sonicated for 10 minutes. The liposome mixture was left for 2 hours before it was used; this allowed the liposomes enough time to swell and reach the final size. All experiments used at least two different batches of liposomes; one with a solvent of just the phosphate buffer and the other with 0.5% (m/v) amodiaquine dissolved into the phosphate buffer.

4.3.3. Morphological evaluations of liposomes and amodiaquine entrapped liposomes

The morphology of manufactured liposomes was determined by microscopic methods. The specific method used is referred to as confocal laser scanning microscopy (CLSM). The liposomes were stained with a fluorescent marker.

4.3.3.1. Materials and methods

The structure of the liposomes was determined with a confocal scanning laser (Nikon PCM 2000 equipped with a digital camera DMX 1200, The Netherlands). 100 μ l liposomes were stained with 2 μ l Nile red (1 mg/ml) (Molecular probes Inc., U.S.A.) for 15 minutes in the dark. After that amount of time, 20 μ l of stained liposomes were placed on a microscope slide and covered with a glass coverslip. The slide was viewed under the 60X objective and analysed.

4.3.3.2. Results and discussion

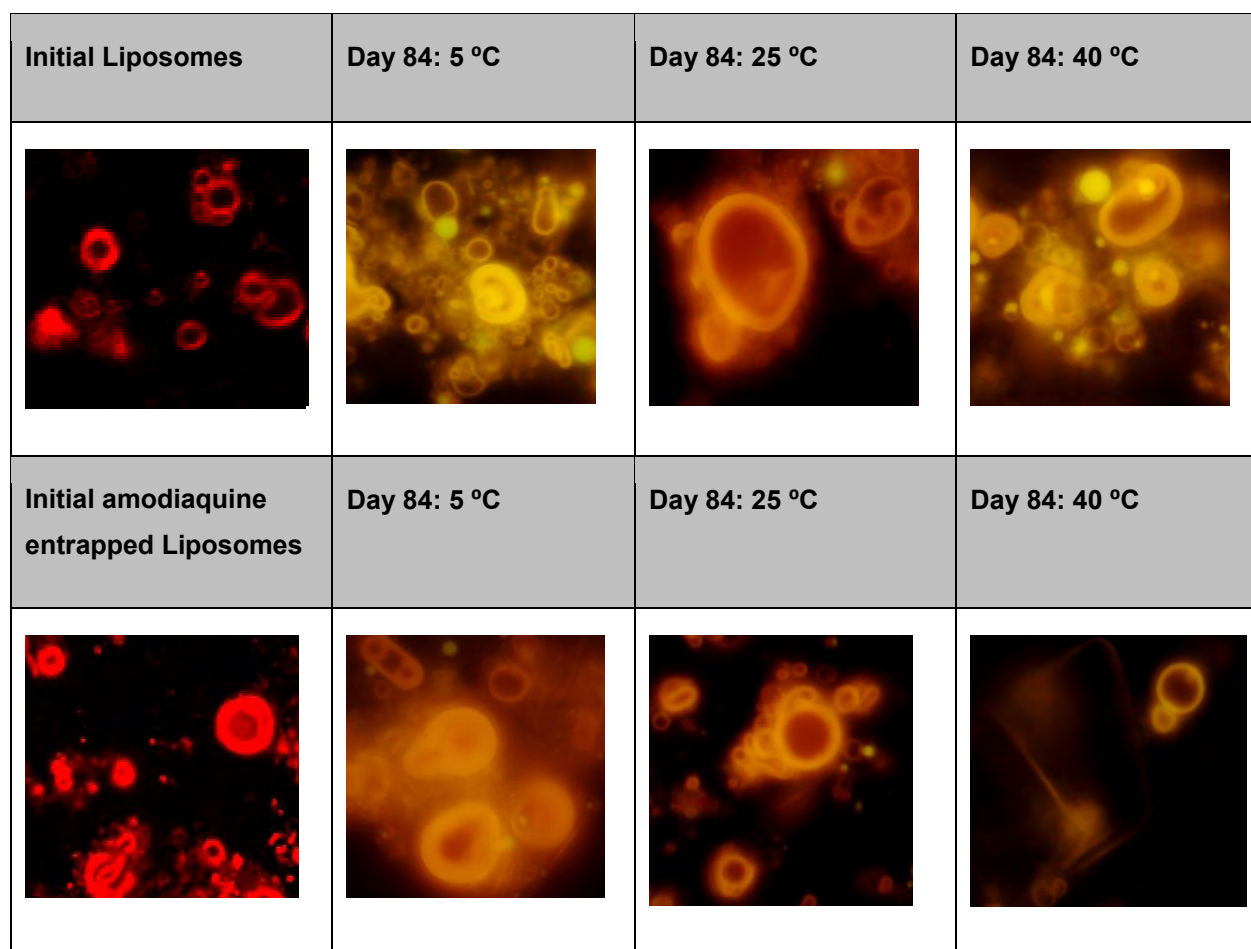


Figure 4.5: Micrograph pictures of liposomes in suspension, as seen under CLSM.

In Figure 4.5, clear red circular structures can be seen. These red structures are the lipid bilayer of the liposome. The inside of the bilayer shows up as black. The black part is the internal aqueous volume of the liposome where the hydrophilic drug is entrapped; in this case it is either amodiaquine in a phosphate buffer or just the phosphate buffer on its own. In the initial stage no difference was seen between amodiaquine entrapped liposomes and regular liposomes. Any of the lipids that did not form liposomes can be seen as tiny coloured spots, with no internal

volume as these just form tiny oil drops. Most of the formulations showed small oil drops after 84 days past during stability testing. At day 84 (40 °C) crystals appeared in the mixture. These crystals may be amodiaquine that precipitated or even crystals formed by the buffer. However, it was more likely to be amodiaquine that precipitated, as the 40 °C liposomes without amodiaquine showed no signs of crystal formation.

4.3.4. Accelerated stability testing

The liposomal formulations were prepared according to the liposome hand shaken method, also known as the film hydration method, as described in section 4.3.2. Two different formulations were prepared; one with amodiaquine, and the other without. The liposomes were decanted into separate amber containers, three containers per formulation, per temperature. The temperatures at which the liposomes were stored; 5 °C, 25 °C (relative humidity of 60%) and 40 °C (relative humidity 75%). The temperatures were controlled by thermostatic ovens (or a refrigerator in the case of the 5 °C). The formulations were tested once a week for the first four weeks, thereafter once every two weeks, until 12 weeks passed. The samples were firstly checked for any visual change, before the size, pH and entrapment efficacy were determined.

4.3.5. Size determination

The size of liposomes can predict certain aspects of the behaviour that the delivery system may present, such as: elimination from the body via RES or the amount of entrapped drug in the aqueous core. Therefore, it is of the utmost importance to do size determinations on liposomes (New, 1990). Flow cytometry has been used with great success in the past to do size determinations accurately (Childers *et al.*, 1989; Vorauer-Uhl *et al.*, 2000). For the purpose of this study, Flow cytometry (FACSCalibur) was used to determine the size of liposomes.

4.3.5.1. Materials

FACSCalibur™ reagents, FACS consumables and a phosphate buffer solution (PBS) were purchased from Scientific Group (South Africa). A Flow Cytometry Size Calibration Kit with Fluorospheres® was acquired from Invitrogen® (The Netherlands).

4.3.5.2. Method

The FACSCalibur™ was calibrated by using polystyrene size calibration beads. After calibration, 30 gates were set up to measure the different sizes of the liposomes according to the amount of FSC that were picked up by the laser. Each gate increased with 0.5 μm in size starting at zero.

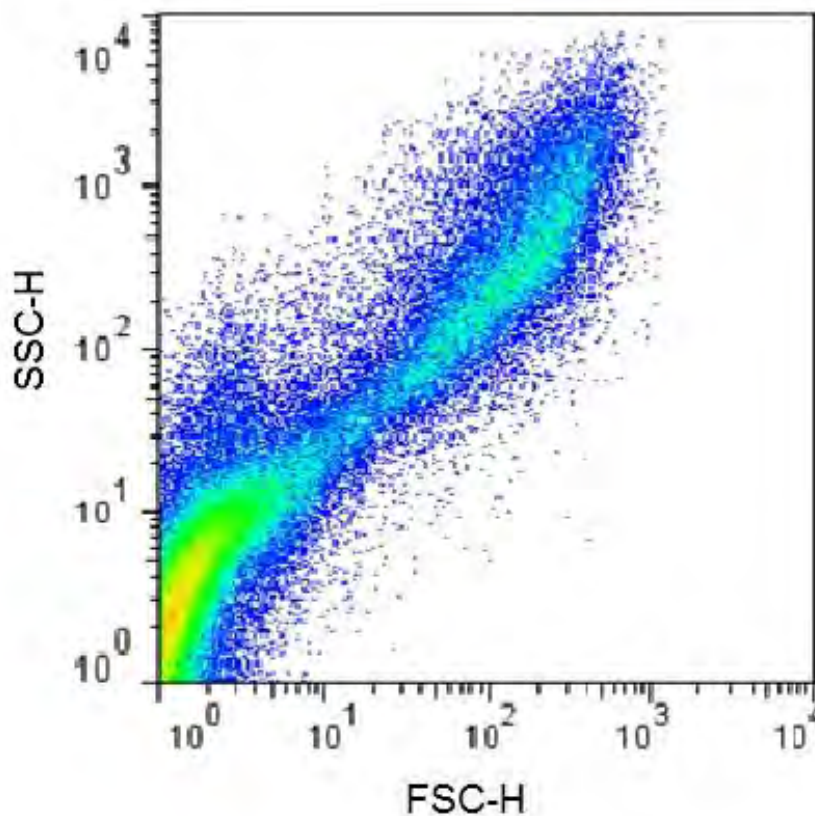


Figure 4.6: A representative sample of the size determination study scatter plot from the FACSCalibur™ before being processed with FlowJo™. The figure portrays both the forward and side scatter as analysed from the size determination sample.

Samples were vortexed for a few seconds to ensure the liposomes were completely diluted. The samples were analysed on the FACS. Forward and side scatter information were plotted on a logarithmic scale. Data of 10 000 cells were collected from each sample. Data were analysed using FlowJo™ (Tree Star Inc. U.S.A.). Front and side scatter of light caused by particles crossing the argon laser, which was set to 488nm, were measured. Data obtained from the FSC and the SSC can be seen in Figure 4.6, which represents a representative sample with liposomes. Figure 4.7 shows a histogram of the same data. In this histogram, the 30 different size gates were drawn (not shown) that illustrate the different particle sizes. Results of this study were expressed in terms of the median particle size ($S_{50\%}$) and the size distribution (Span).

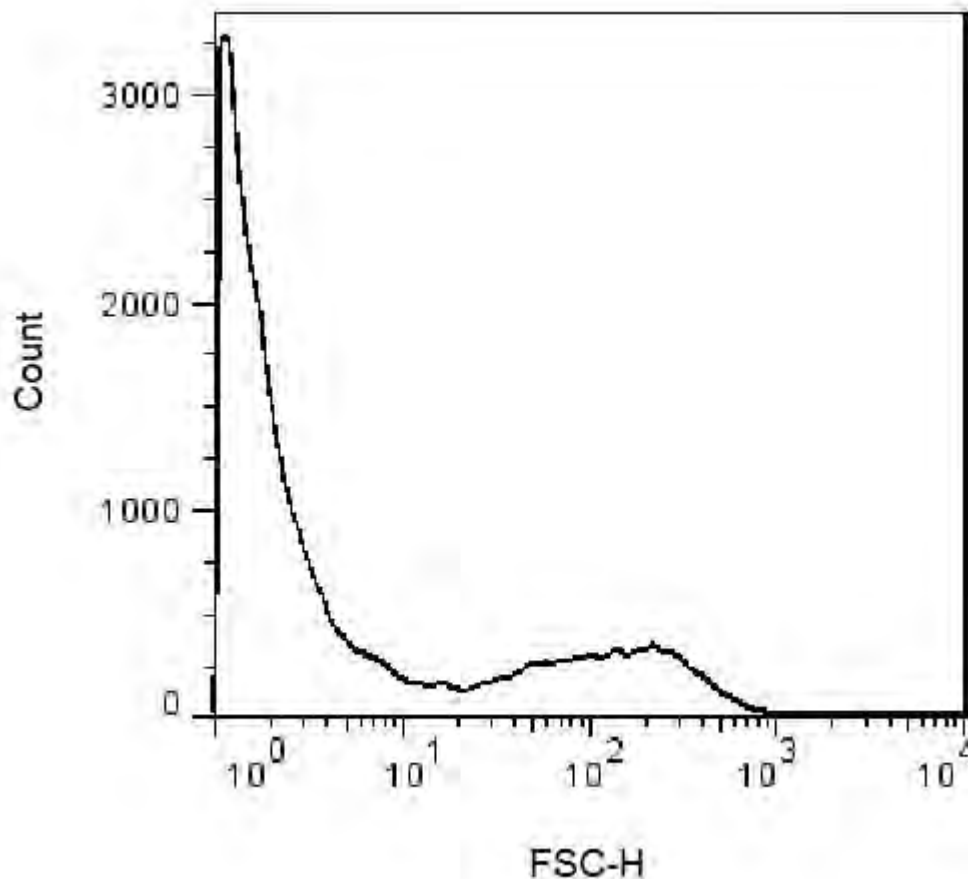


Figure 4.7: The forward scatter plot of a representative liposome size analysis turned into a histogram. The size distribution and span is calculated by adding the different sized gates here (not illustrated as the gates are unclear on such a small scale).

Span of each formulation was determined by the following formula:

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

Where $S_{50\%}$ is the median of the particle size. $S_{95\%}$ is equal to the size of the particles larger than 95% of the sample and $S_{5\%}$ is the size of particles smaller than 5% of the entire sample. Therefore, to differentiate, the $S_{50\%}$ or mean of the mixture is the mean size of the entire sample, whereas the size distribution (Span) indicates how wide the particles are separated, or in other words, how widely spread the population sizes differ from each other.

Statistical difference between the initial and the values determined in the accelerated stability studies were determined by one-way ANOVA using the Statistica 9 program (Statsoft, California, U.S.A.). Significant differences in data were determined by Bonferroni post hoc test. A p-value of less than 0.05 was deemed significant, whereas a p-value of less than 0.005 was termed highly significant.

4.3.5.4. Results and discussion

The median size and the span of liposomes manufactured with phosphate buffer (pH 6) are illustrated in Figure 4.8.

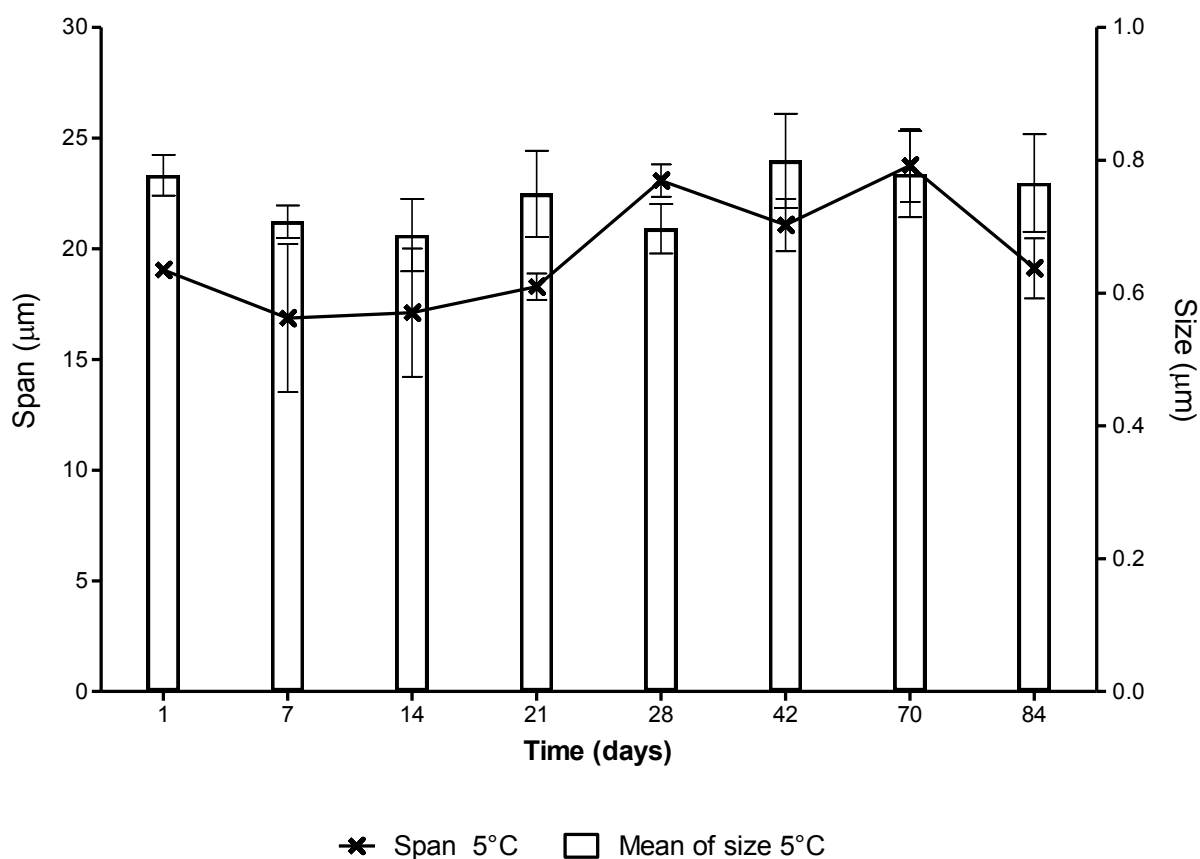


Figure 4.8: Illustrates the median size (in µm) and the size distribution (span in µm) of liposomes manufactured with a buffer of pH 6 at 5 °C over a period of 84 days. Results are shown as mean ± SEM (n=3).

The initial median size of the liposomes at 5 °C was $0.777 \pm 0.031 \mu\text{m}$ with a size distribution (span) of $19.055 \pm 0.305 \mu\text{m}$. The median size of the particles decreased slightly on day 7, 14, 21 and 28. The decrease in size were as follows: $0.707 \pm 0.025 \mu\text{m}$, $0.687 \pm 0.054 \mu\text{m}$, $0.749 \pm 0.065 \mu\text{m}$ and $0.697 \pm 0.037 \mu\text{m}$, respectively. The span of the particles on these days were $16.871 \pm 3.350 \mu\text{m}$, $17.118 \pm 2.893 \mu\text{m}$, $18.294 \pm 0.590 \mu\text{m}$ and $23.085 \pm 0.732 \mu\text{m}$. From day

42 a larger mean particle size was observed when compared to day 1, with $0.799 \pm 0.071 \mu\text{m}$, $0.779 \pm 0.065 \mu\text{m}$ and $0.766 \pm 0.074 \mu\text{m}$ on days 42, 70 and 84. The span varied slightly from day 28, with span readings for day 42, 70 and 84 as follow; $21.074 \pm 1.177 \mu\text{m}$, $23.763 \pm 1.644 \mu\text{m}$ and $19.119 \pm 1.354 \mu\text{m}$, respectively. No significant differences were noticed in the median size of the liposomes during the study in 5°C . There was a significant change in the span values at day 28 ($p = 0.021872$) and a highly significant difference in the span at day 70 ($p = 0.003839$) (see Annexure C).

The results of the liposome formulation with buffer, size test at 25°C over 84 days can be seen in Figure 4.9.

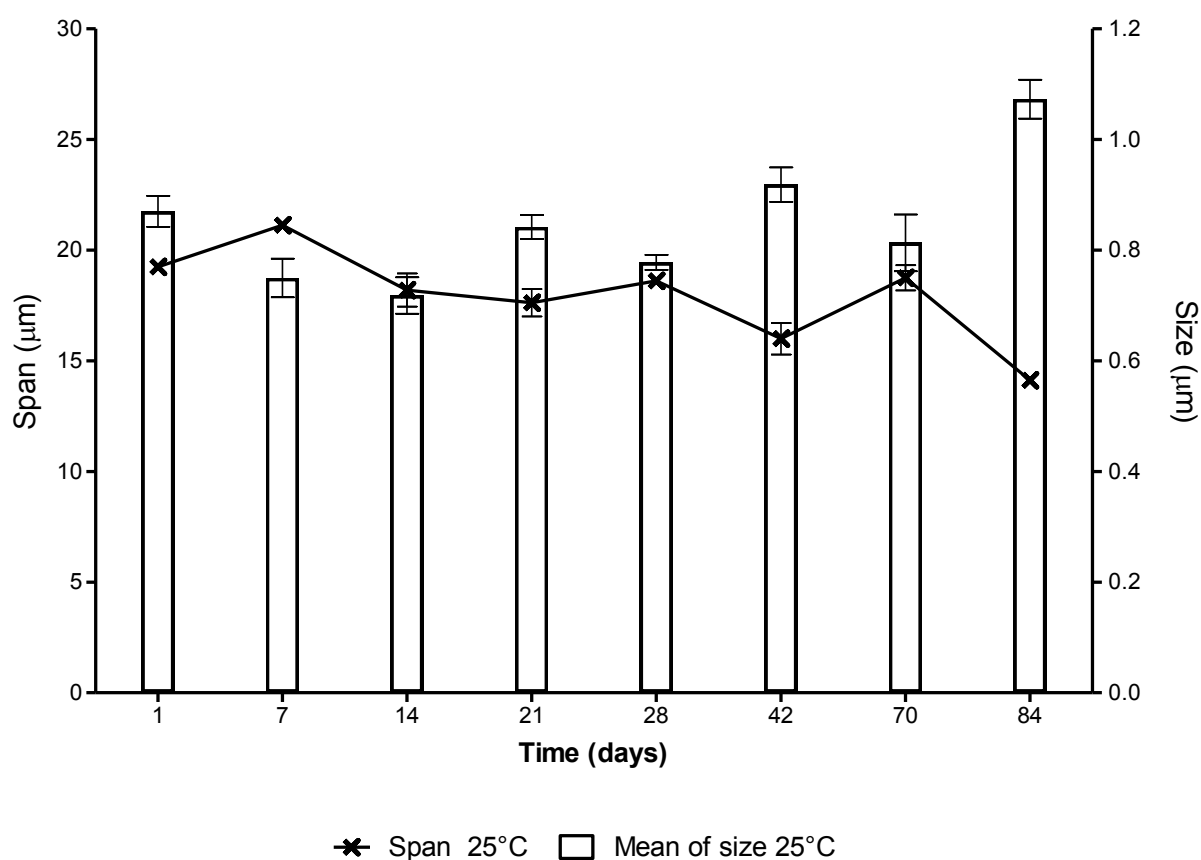


Figure 4.9: Illustrates the median size (in μm) and the size distribution (span in μm) of liposomes manufactured with a buffer of pH 6 at 25°C over a period of 84 days. Results are shown as mean \pm SEM (n=3).

The initial median size of the liposomal formulation at 25°C was $0.870 \pm 0.028 \mu\text{m}$ with a span of $19.247 \pm 0.328 \mu\text{m}$. This was slightly higher than the median size observed at 5°C , but a similar span was observed. There were no significant differences in the median size or span between formulations stored at 5°C and 25°C formulations. The median size values on day 7 - 28 were lower than the initial value (See Annexure C). Day 42 and 84 had the largest median size reading of $0.918 \pm 0.031 \mu\text{m}$ and $1.073 \pm 0.035 \mu\text{m}$ concurrently. These days exhibited the

lowest span values of $16.002 \pm 0.713 \mu\text{m}$. and $14.126 \pm 0.430 \mu\text{m}$. It is unclear why this inverse relationship was observed on these days. There were no significant differences between the initial median size and span at 25°C . The amount of fluctuation in the median size and span seemed to differ as the median size at 25°C was increased, and the median size at 5°C remained constant.

The 40°C liposome stability test according to median size and span is illustrated in Figure 4.10.

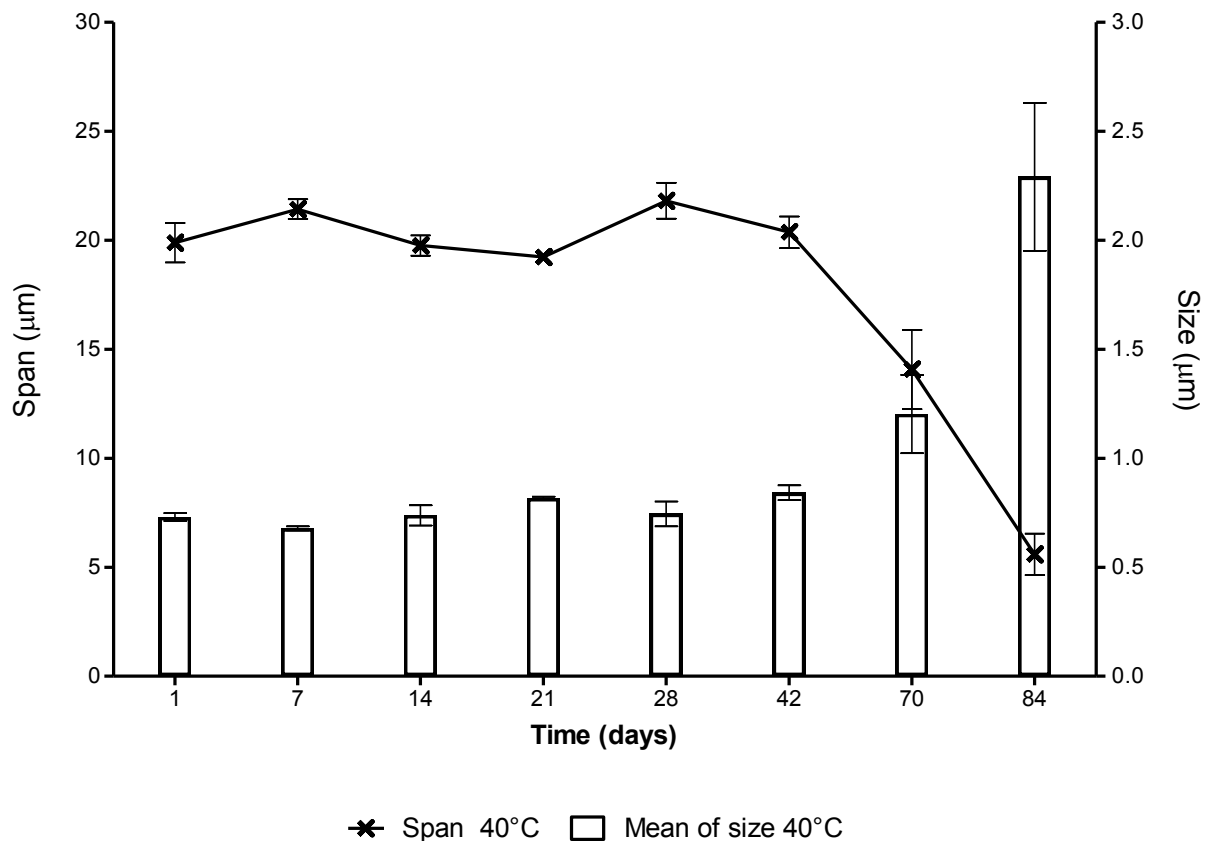


Figure 4.10: Illustrates the median size (in μm) and the size distribution (span in μm) of liposomes manufactured with a buffer of pH 6 at 40°C over a period of 84 days. Results are shown as mean \pm SEM (n=3).

The initial size of the particles started at $0.730 \pm 0.018 \mu\text{m}$ with a span of $19.893 \pm 0.902 \mu\text{m}$. The median size of day 1 at 40°C was smaller than that of day 1 at both 5°C and 25°C , though no significant difference was noted. There was no mentionable difference in the initial span between the three temperatures. The size of the particles at 40°C remained relatively constant until day 42 (See annexure C). A large spike in mean size appeared on day 70 and day 84 ($1.203 \pm 0.180 \mu\text{m}$ and $2.291 \pm 0.339 \mu\text{m}$). The span on day 70 and 84 was $14.068 \pm 1.822 \mu\text{m}$ and $5.594 \pm 0.953 \mu\text{m}$, respectively. The change in median size was significant for day 70 ($p = 0.006998$) and day 84 ($p = 0.00000$) when compared to the initial value. Initially, there was little difference between the formulation in the three different temperatures, but day

70 and 84 showed a huge increase in median size and a decrease in the span at 40 °C, which was not the case at the other temperatures.

Liposomes with entrapped buffer remained relatively stable over the 12 week course. The liposomes stored at the highest temperature (40 °C) underwent a drastic change on day 70 which indicated a decrease in the stability in terms of the size of the particles. This is one of the disadvantages of liposomes.

Results amodiaquine entrapped in liposome stored at 5 °C over 84 days is shown in Figure 4.11.

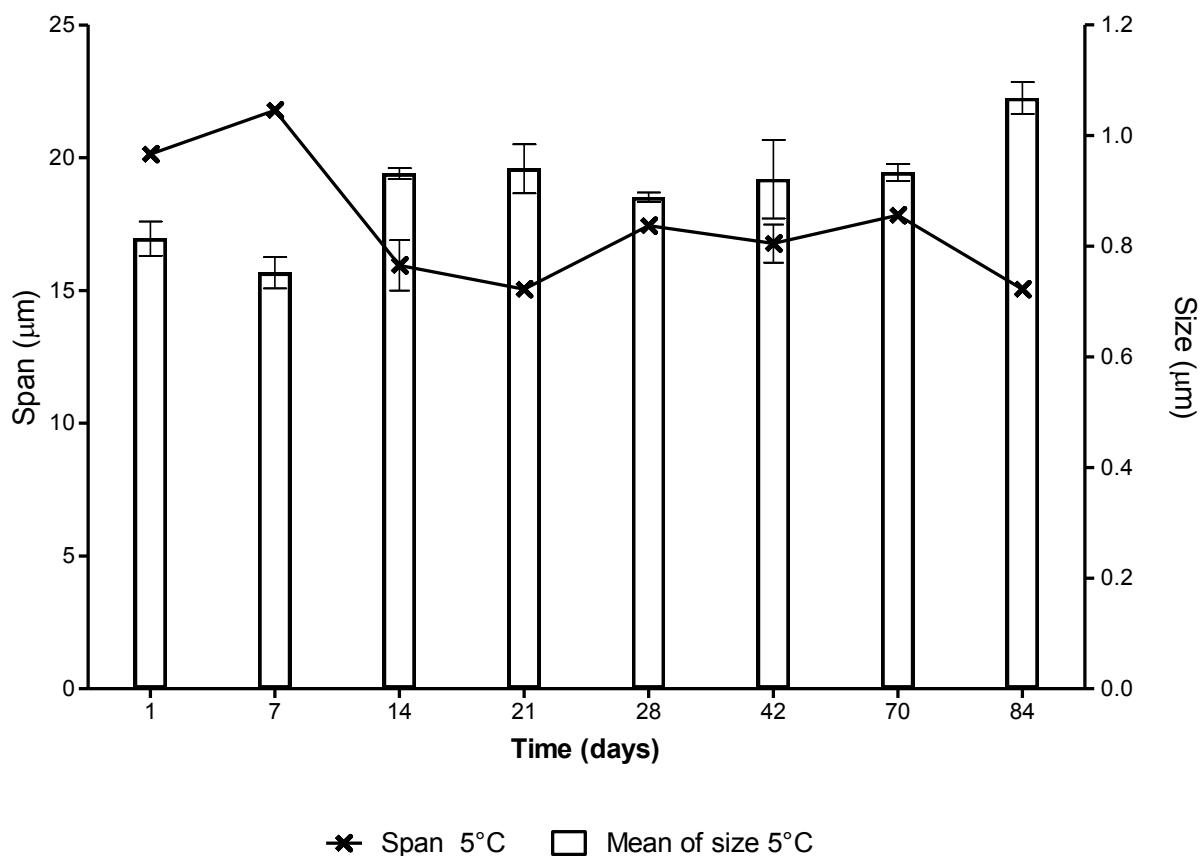


Figure 4.11: Illustrates the median size (in μm) and the size distribution (span in μm) of liposomes manufactured with entrapped amodiaquine with buffer of pH 6 at 5 °C over a period of 84 days. Results are shown as mean ± SEM (n=3).

The initial median size of the formulation was $0.814 \pm 0.031 \mu\text{m}$ with a span of $20.136 \pm 0.304 \mu\text{m}$. From day 14 an increase in median size and a decrease the span was observed when compared to the initial value (See Annexure C). A steady increase in the size of the amodiaquine entrapped liposomes could be seen, with the median size on day 84 being the highest ($1.068 \pm 0.029 \mu\text{m}$ with a span of $15.049 \pm 0.267 \mu\text{m}$). The median size fluctuated

between days 14 and 70, with the span responding conversely. The size of the particles did not vary significantly between day 14 and 70. The size varies by no more than 0.07 μm . There were also no significant differences in the span.

The result of the stability test at 25 °C when the size of liposomes with entrapped amodiaquine is shown in Figure 4.12.

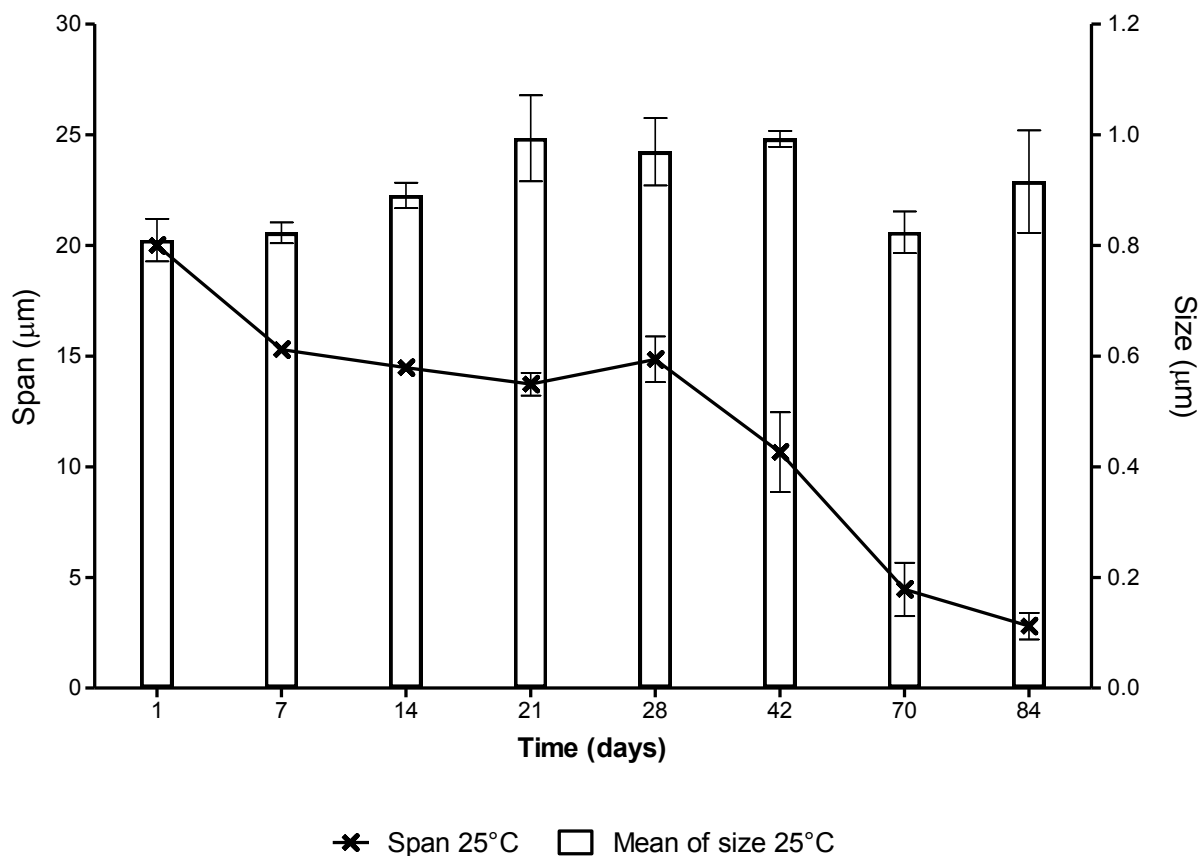


Figure 4.12: Illustrates the median size (in μm) and the size distribution (span in μm) of liposomes manufactured with entrapped amodiaquine with buffer of pH 6 at 25 °C over a period of 84 days. Results are shown as mean \pm SEM (n=3).

The initial size of the particles was $0.810 \pm 0.038 \mu\text{m}$ with a span of $19.999 \pm 0.283 \mu\text{m}$. From day 7 to day 84 the span decreased, with a slight increase in the size of the particles (see Annexure C). The largest median particle size was on day 21 and 42 with sizes of $0.994 \pm 0.078 \mu\text{m}$ and $0.993 \pm 0.014 \mu\text{m}$, respectively. A span of $13.731 \pm 0.515 \mu\text{m}$ on day 21 decreased to $10.658 \pm 1.800 \mu\text{m}$. The size and span on the final day of testing were $0.915 \pm 0.099 \mu\text{m}$ and $2.799 \pm 0.596 \mu\text{m}$, respectively. There was no significant change in particle size when compared to the initial size, but the span on day 42, 70 and 84 deferred significantly with values of 0.000036, 0.00000, and 0.00000, respectively.

The median size between the 5 °C and 25 °C storage conditions did not differ significantly, with the initial and end readings almost the same, but the span at 5 °C decreased and the decrease seen in the 25 °C, span was drastic, almost reaching a tenth of the span initially attained.

In Figure 4.13 the result of the stability test on the size of amodiaquine entrapped liposomes at 40 °C can be seen. The initial size of particles was $0.849 \pm 0.057 \mu\text{m}$ with a span of $17.120 \pm 0.749 \mu\text{m}$.

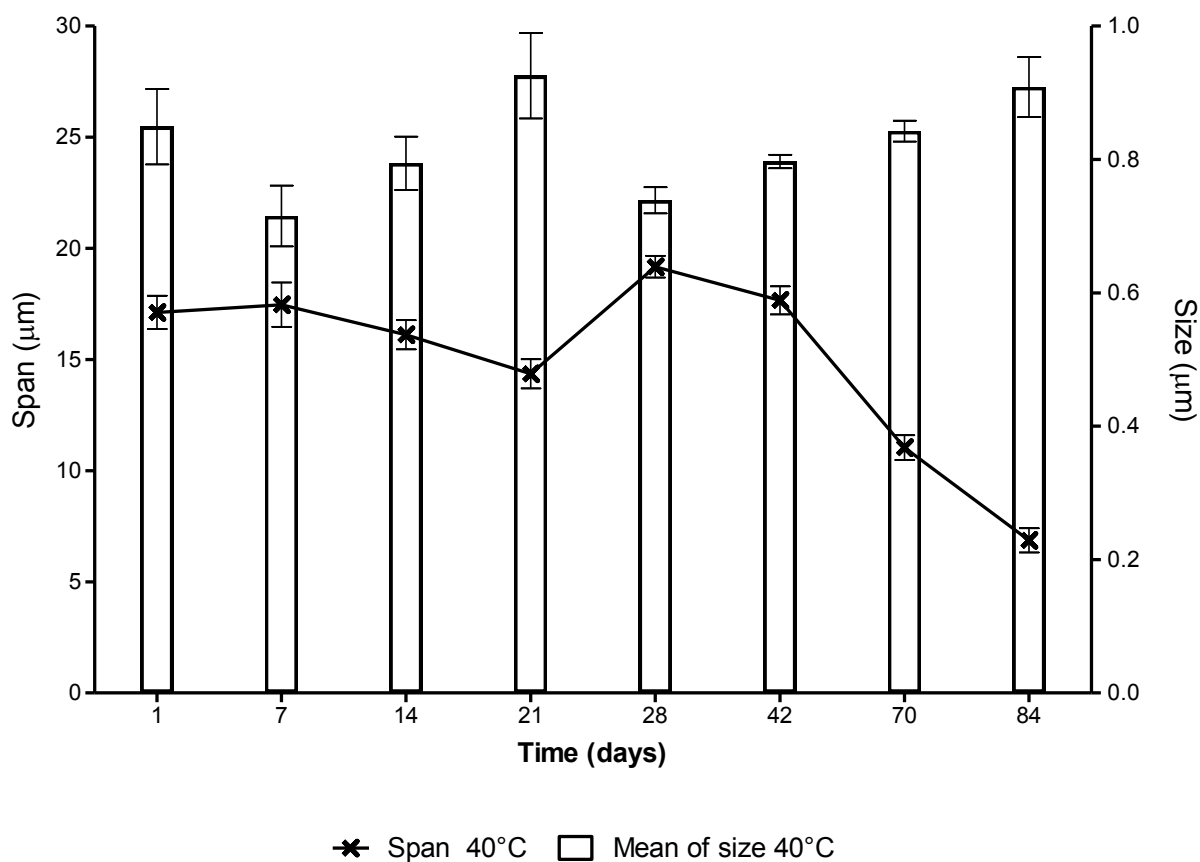


Figure 4.13: Illustrates the median size (in μm) and the size distribution (span in μm) of liposomes manufactured with entrapped amodiaquine with buffer of pH 6 at 40 °C over a period of 84 days. Results are shown as mean \pm SEM (n=3).

The initial median size at 40 °C was $0.850 \pm 0.057 \mu\text{m}$ with a span of $17.120 \pm 0.749 \mu\text{m}$. An increase in size from day 7 to 21 was visible with the values increasing from $0.715 \pm 0.046 \mu\text{m}$ to $0.926 \pm 0.064 \mu\text{m}$. The span decreases over this time from $17.468 \pm 0.997 \mu\text{m}$ to $14.353 \pm 0.654 \mu\text{m}$. A slight decrease in occurred on day 28 ($0.739 \pm 0.020 \mu\text{m}$ with a span of $19.178 \pm 0.494 \mu\text{m}$), but the increase in size could be observed from day 28 until day 84 with the span decreasing. The final median size of the liposomes was $0.909 \pm 0.045 \mu\text{m}$ with a span of 6.866

$\pm 0.539 \mu\text{m}$ (See Annexure C). There was a highly significant change in the span at day 84 ($p = 0.000002$).

Amodiaquine entrapped liposomes seemed less stable at 25 °C and 40 °C from day 42. At 5 °C the median size and span was relatively constant during the last two weeks. The change in size and span was very low when compared to that at the higher temperatures. The incorporation of amodiaquine did not have a significant influence on the size or the span of the liposomes, as both the liposomes and the amodiaquine entrapped liposomes depicted similar initial values.

The increase of particle size and decrease of span that occurred simultaneously could be a result of agglomeration between different liposomes or even a fusion of the liposomal membranes. That unfortunately points to instability of the formulations at higher temperatures. When the liposomes with amodiaquine and without amodiaquine were compared, the reaction to the highest temperature seemed to be the same, but the liposomes without amodiaquine appeared to be more stable at the 25 °C bracket. Both formulations were stable for 84 days at 5 °C.

4.3.6. Determination of pH

The pH of a solution is measured on a numerical scale on which 0 to 7 is considered acidic and between 7 and 14 is considered alkaline. A reading of 7 is considered to be neutral, as it is the point where hydroxyl and hydrogen ion concentrations are about the same (Martin, 1993).

4.3.6.1. Apparatus and method

A WTW, Level 1 inoLab[®] pH-meter was used for pH determinations (Merck (Pty.) Ltd, South Africa). The pH-meter was calibrated using buffer solutions obtained from Merck Chemicals (Pty) Ltd. (Johannesburg), using the manufacturer's auto calibration method. The probe was thoroughly cleaned with distilled water after each different container was tested. The liposomes were tested as soon as being removed from the different climates. Each climate contained three amber bottles per formulation. After being cleaned and no longer used, the probe was stored in a potassium chloride solution (Merck Chemicals (Pty) Ltd., Johannesburg).

4.3.6.2. Statistical analysis

Statistical differences between the initial values and the values determined in the accelerated stability studies were determined by one-way ANOVA using the Statistica 9 program (Statsoft, California, U.S.A.). Significant differences in data were determined by Bonferroni post hoc test. A p-value of less than 0.05 was deemed significant, whereas a p-value of less than 0.005 was termed highly significant.

4.3.6.3. Results and discussion

The results of the pH-data gathered over the period of 84 days can be seen in Table 4.2 and Table 4.3. Table 4.2 contains the data of the liposomes prepared with just the pH 6 buffers. Table 4.3 contains the data of the amodiaquine entrapped liposomes.

The starting pH for the liposomes at 5 °C, 25 °C and 40 °C were 6.01 ± 0.015 , 5.85 ± 0.009 and 5.88 ± 0.007 , respectively. The final values: 5.89 ± 0.024 , 5.85 ± 0.015 and 5.91 ± 0.006 in each different temperature, as well as Figure 4.14 illustrate that there was little or no difference in starting pH values (see Annexure D for full data). This indicated that the liposomes remained stable for 84 days. None of the values were statistically significant.

Table 4.2: The pH of the liposomes with buffer (pH 6). Results represented as mean \pm SEM (n=3).

Time (Days)	Liposomes		
	5 °C	25 °C	40 °C
1	6.01 \pm 0.015	5.85 \pm 0.009	5.88 \pm 0.007
7	5.76 \pm 0.320	5.96 \pm 0.020	6.00 \pm 0.035
14	6.03 \pm 0.025	5.86 \pm 0.023	5.89 \pm 0.032
21	5.96 \pm 0.019	5.98 \pm 0.009	5.97 \pm 0.021
28	5.99 \pm 0.015	5.89 \pm 0.021	5.88 \pm 0.015
42	6.05 \pm 0.010	5.91 \pm 0.012	5.96 \pm 0.020
56	5.98 \pm 0.012	5.85 \pm 0.012	5.93 \pm 0.007
70	6.02 \pm 0.001	5.87 \pm 0.006	5.84 \pm 0.015
84	5.89 \pm 0.024	5.85 \pm 0.015	5.91 \pm 0.006

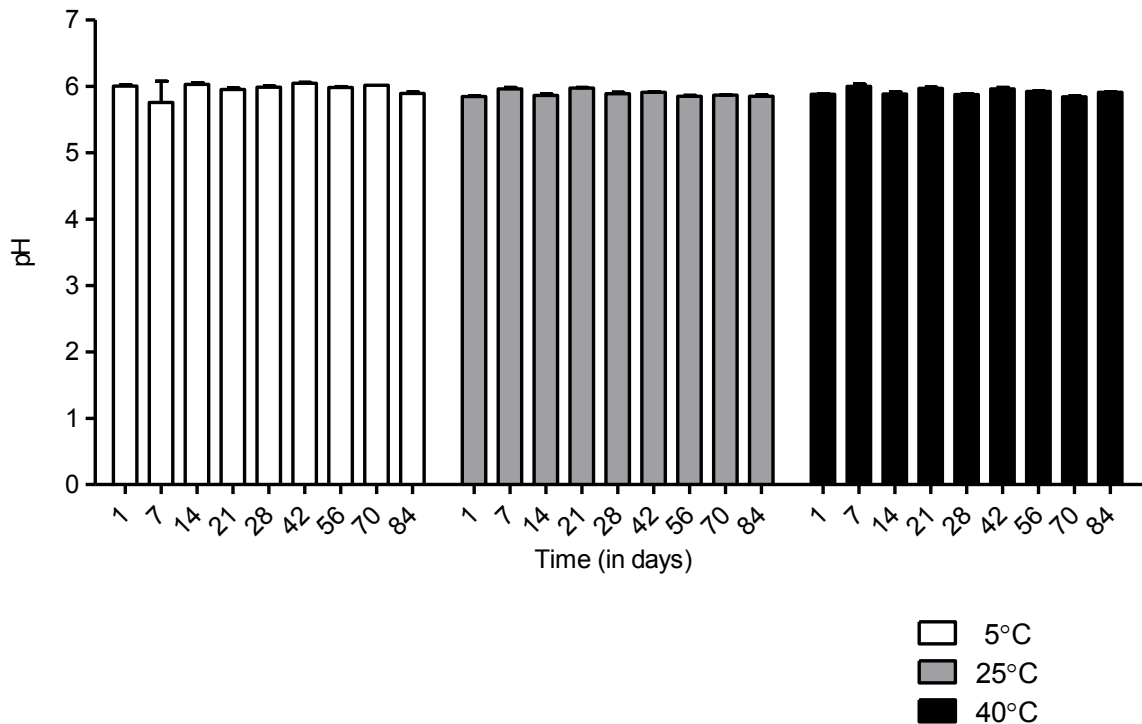


Figure 4.14: The pH for liposomes manufactured with just a pH 6 buffer at three different temperatures over a period of 84 days. Results are shown as mean \pm SEM (n=3).

The starting pH-values in the different temperatures for amodiaquine entrapped liposomes were 5.94 ± 0.013 (in 5 °C), 5.86 ± 0.023 (in 25 °C) and 5.91 ± 0.007 (in 40 °C) as can be seen in Table 4.3. Figure 4.15 show that even though there was a small fluctuation between pH-values during individual weeks, the pH never changed by more than 0.2. This constant pH is considered to be an indication of stability. The final pH-values on day 84 were 5.90 ± 0.010 for the 5 °C, 5.85 ± 0.030 for the 25 °C and 5.96 ± 0.018 in 40 °C. None of the values were statistically significant

Table 4.3: The pH in the liposomes with AQ entrapped. Results represented as mean \pm SEM (n=3).

Time (Days)	Liposomes with amodiaquine entrapped		
	5 °C	25 °C	40 °C
1	5.94 \pm 0.013	5.86 \pm 0.023	5.91 \pm 0.007
7	6.13 \pm 0.006	6.08 \pm 0.013	6.01 \pm 0.003
14	6.00 \pm 0.010	5.91 \pm 0.018	5.99 \pm 0.007
21	6.02 \pm 0.010	5.99 \pm 0.015	5.93 \pm 0.022
28	5.96 \pm 0.006	5.98 \pm 0.018	5.90 \pm 0.006
42	6.01 \pm 0.003	5.87 \pm 0.013	5.89 \pm 0.007
56	5.99 \pm 0.015	5.82 \pm 0.003	5.88 \pm 0.020
70	6.07 \pm 0.020	5.83 \pm 0.010	5.91 \pm 0.013
84	5.90 \pm 0.010	5.85 \pm 0.030	5.96 \pm 0.018

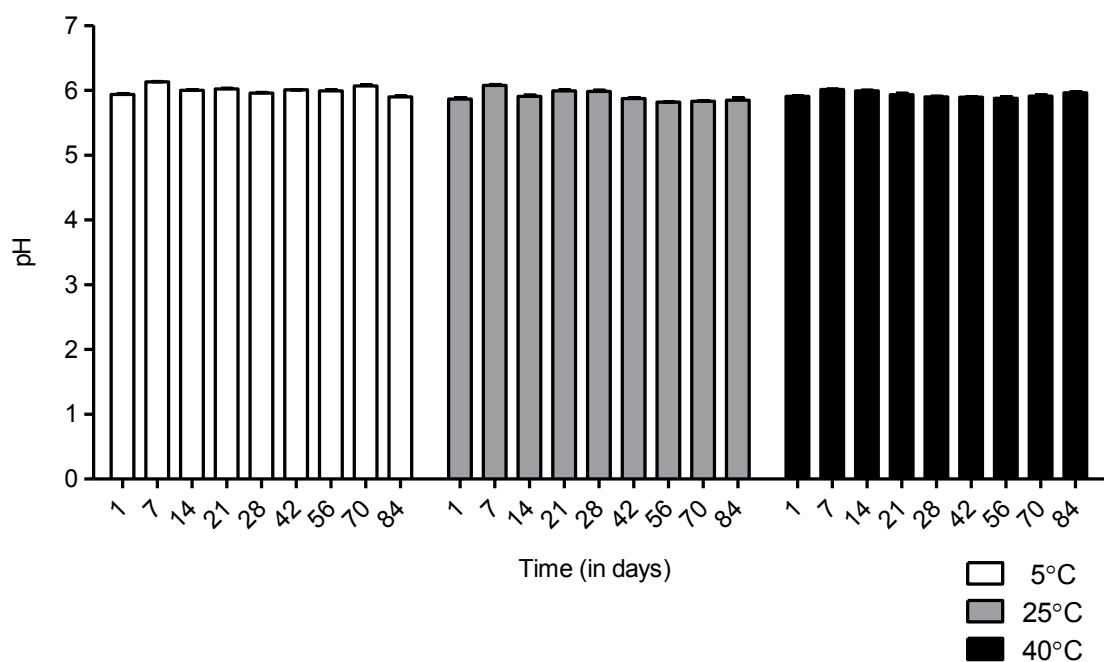


Figure 4.15: The pH for AQ entrapped liposomes in three different temperatures over a period of 84 days. Results are shown as mean \pm SEM (n=3).

The amodiaquine entrapped liposomes depicted a lower starting pH than the liposomes made with just the buffer, but that can be accounted for by the amodiaquine dissolved in the buffer. Dissolved amodiaquine lowers the pH of the solution (United States Pharmacopeia, 2010). However, both formulations' pH-values remained constant relative to their own starting pH values. This indicated stability to a certain degree, in terms of pH.

4.3.7. Entrapment efficacy and leakage

The entrapment of water soluble drugs in MLV's are reported to be in the area of 30% when the hand shaken method is used. If the entrapment efficacy decreases, that points to leakage of the drug from the liposome (New, 1990).

4.3.7.1. Apparatus and method

The entrapment efficacy (EE%) of the liposomes was determined by UV-spectroscopy using the Shimadzu UV-1800 (Japan) UV-spectrophotometer. A calibration curve was created for a pH 6 in which the liposome formulation was made. The absorbance maximum for amodiaquine is at 342nm (United States Pharmacopeia, 2010).

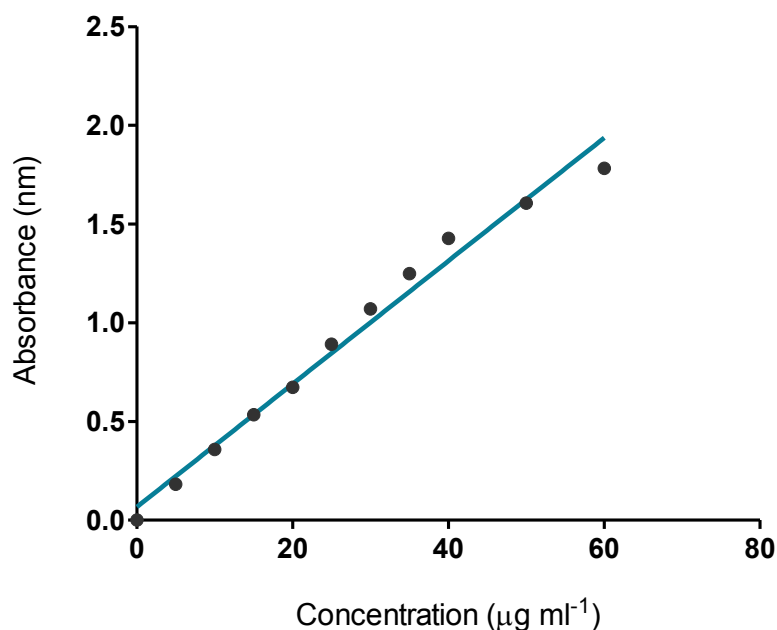


Figure 4.16: The calibration curve used to determine the concentration of AQ in a pH of 6. ($n=3$) $r^2 = 0.9832$. $y = 0.0357x - 0.005$.

On the day of the test 1 ml of each of the different vials was decanted into smaller Eppendorf 1.5 ml tubes, which were then centrifuged for 10 minutes at 2000 rpm in a benchtop centrifuge plc-05 series (Interbiolab Inc., U.S.A). An amount of the clear supernatant was diluted with the original production buffer to be able to obtain an accurate reading on the UV-spectrophotometer. The amount of supernatant used, as well as the amount of buffer used for the dilution needed to be changed during the course of the study as the amount of detectable amodiaquine diminished.

The formula for entrapment efficacy (Maestrelli *et al.*, 2005) is as follows:

$$EE\% = \left(\frac{\text{Initial drug load} - \text{Unentrapped drug}}{\text{Initial drug load}} \right) \times 100$$

4.3.7.2. Statistical analysis

Statistical differences between the initial and the values determined in the accelerated stability studies were determined by one-way ANOVA using the Statistica 9 program (Statsoft, California, U.S.A.). Significant differences in data were determined by Bonferroni post hoc test.

A p-value of less than 0.05 was deemed significant, whereas a p-value of less than 0.005 was termed highly significant.

4.3.7.3. Results and discussion

The %EE of the liposomes with amodiaquine at 5 °C can be seen in Figure 4.17.

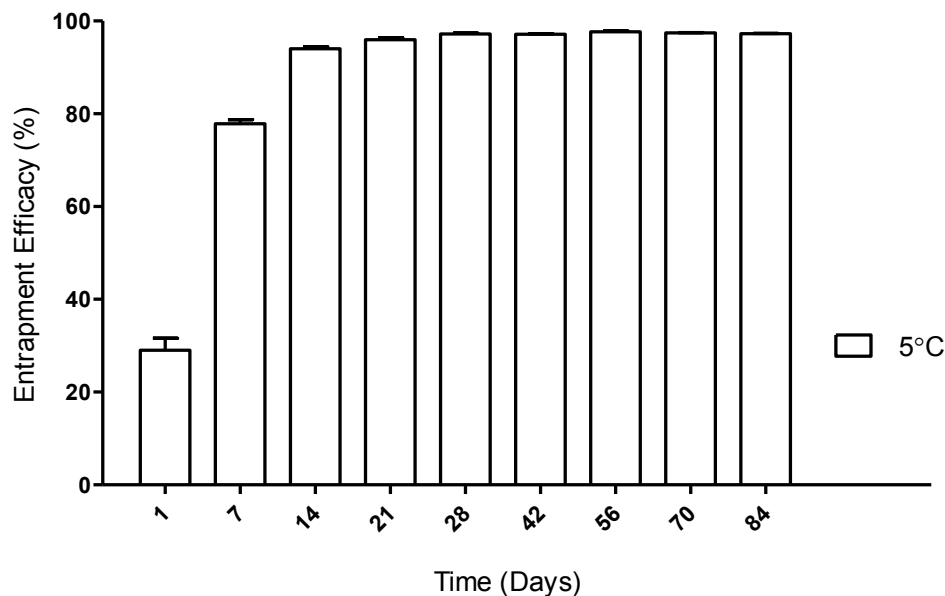


Figure 4.17: The entrapment efficacy of amodiaquine in the liposome formulation at 5 °C. Results are shown as mean ± SEM (n=3).

The initial %EE was $29.038 \pm 2.599\%$, thereafter it increased every week from day one. On day 7 the %EE increased to $77.824 \pm 0.934\%$ and on to $94.024 \pm 0.407\%$ on day 14. By day 28 the %EE reached $97.208 \pm 0.160\%$. The %EE did not drop below 97%, with the final reading on day 84 of $97.283 \pm 0.035\%$ (see Annexure E for raw data).

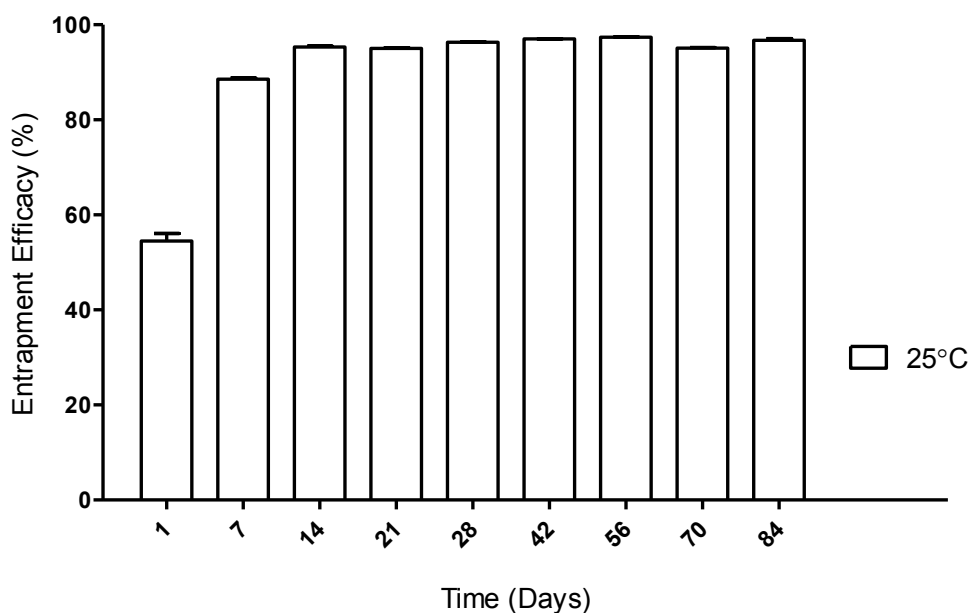


Figure 4.18: The entrapment efficacy of amodiaquine in the liposome formulation at 25 °C. Results are shown as mean ± SEM (n=3).

The %EE at 25 °C started at $54.482 \pm 1.617\%$ on day 1, which increased to $88.562 \pm 0.233\%$ on day 7, $95.023 \pm 0.187\%$ on day 21 and on to $96.331 \pm 0.065\%$ on day 28. The %EE never dropped below 95% thereafter, with a final reading of $96.723 \pm 0.366\%$ on day 84. These results were illustrated in Figure 4.18.

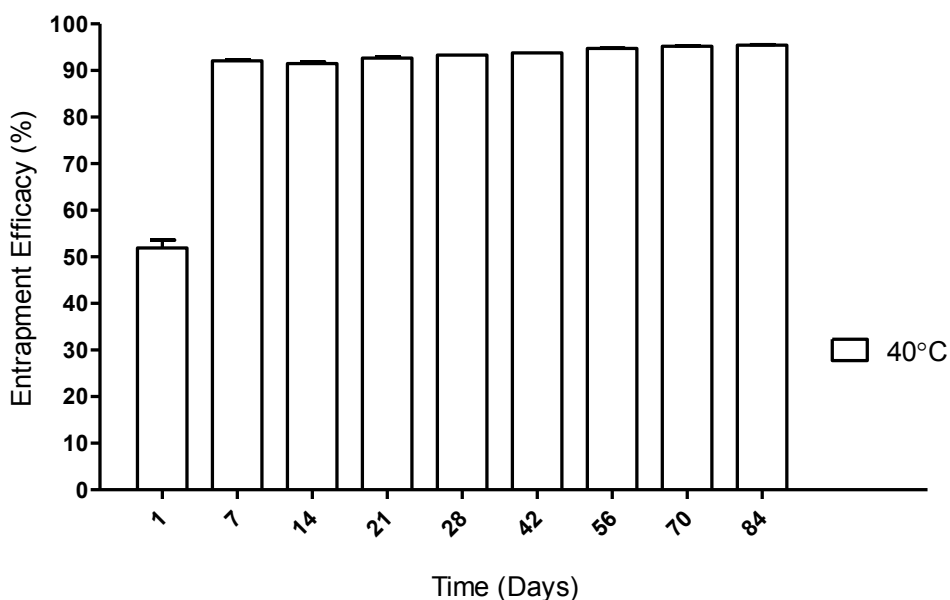


Figure 4.19: The entrapment efficacy of amodiaquine in the liposome formulation at 40°C. Results are shown as mean ± SEM (n=3).

The %EE at 40 °C was $51.914 \pm 1.683\%$ on day 1, which increased to $92.063 \pm 0.233\%$ by day 7. This increase continued until day 84 at $95.425 \pm 0.108\%$. These results are illustrated in Figure 4.19.

Initial EE% for the different temperatures was in the area of 30% as was reported for normal MLV's described by New (1990). All of the samples depicted a steady increase in EE% since day one. None of the samples varied significantly from one another ($P = 0.4038$). Normally liposomes EE% do not increase significantly after production, except if specific changes were made in pre-formulation, for example creating a different pH inside liposomes for active loading of drug. As this was not the case with these liposomes, other reasons were sought. There does not seem to be any decrease of the EE%. That points to the fact that no leakage of the drug took place.

During the course of the study the amodiaquine tended to precipitate slowly from solution. This effect has not been clearly documented. After four weeks (after day 28), small grains were noticed in the amodiaquine entrapped liposome suspension after being shaken. At first the liposomes with entrapped drug were a bright yellow colour. The colour slowly started fading to a dull yellow as time went by. This could possibly account for the high EE% readings after a few weeks, as the UV-spectrophotometer can only detect a drug as long as it is dissolved in the medium. That would mean the increase in entrapment efficacy measured did not reflect an actual increase. It was also important to remember that the precipitated amodiaquine would be completely entrapped, as the amodiaquine cannot cross the liposomal membrane if it is not in solution.

4.4. *In vitro* cultivation of *P. falciparum*

Trager and Jensen (1976) were the first to describe the continuous cultivation of *P. falciparum* *in vitro* in 1970. The basics of the methods they described are still used today. This method imitates the conditions necessary for the parasite in the vertebrate host. This is also the phase that is associated with the pathology of the parasite, as well as the target for many therapies. Thus, the erythrocytic phase of the parasite is recreated for study (Schuster, 2002). Some of the first techniques required the use of human serum and erythrocytes suspended in a Roswell Park Memorial Institute (RPMI) 1640 buffer. These techniques became impractical and different types of serum and serum replacements were tested, for example Albumax I until Albumax II combined with hypoxanthine gave the required parasitemia levels (Schuster, 2002; Ringwald *et al.*, 1999).

4.4.1. Materials

RPMI 1640 buffer, gentamicin solution, HEPES, hypoxanthine, sodium chloride, sodium bicarbonate and D-(+)-glucose powder were purchased from Sigma Aldrich® (South Africa). Ethylenediaminetetraacetic acid (EDTA) vacutainers which were used for the collection of blood were obtained from BD Biosciences® (South Africa). Albumax® II, which was used for serum replacement, was imported from Gibco (New Zealand). A “special gas” mixture in the ratio of 5% carbon dioxide (CO₂), 5% oxygen (O₂) and balanced with nitrogen (N₂) was purchased from Afrox (Potchefstroom, South Africa). The containers and culture flasks used were purchased from Scientific group (South Africa).

4.4.2. Cultivation

The cultivation method was adapted from the continuous culture method described by Trager and Jensen (1976). A culture medium is firstly made by mixing the reagents in the amounts as illustrated in Table 4.4, up to 100 ml with water for injection.

Table 4.4: Amounts of the reagents used to make the cultivation medium.

Reagents	Amount
D-(+)-glucose powder	0.4g
RPMI 1640 buffer	1.04g
HEPES	0.6g
Hypoxanthine	0.0044g
Gentamicin solution (40 mg/ ml)	0.12 ml
Sodium bicarbonate (5% solution)	4.2 ml
Albumax® II	0.5g

The reagents were dissolved in the sterile water under aseptic conditions inside a 4ft, class II laminar flow hood. The mixture was filtered through a 0.22 µm filter with the help of a vacuum pump. The culture medium can be made into a wash medium, which is used for the preparation of erythrocytes. The only difference between cultivation medium and wash medium is that wash medium does not contain Albumax® II.

A⁺ whole blood was collected in EDTA vacutainers to stop blood coagulation. The whole blood was washed by adding wash medium, and centrifuging the mixture for 8 minutes at 2000 rpm. The supernatant was removed by use of a vacuum pump. This was repeated three times to remove all the leucocytes. The last time the erythrocytes were suspended in the wash mixture and preserved at 4 °C. Ethics approval was obtained from the Ethics Panel of the North-West University (NWU-0008-08-S5) (Can be viewed in Annexure A).

The parasites cultures were kept in airtight culture flasks under the special gas mixture. Parasites were continually cultivated by replacing the culture medium three times a week and adding erythrocytes once a week to maintain the parasitemia and hematocrit around 5% consistently. The cultures were kept at 37 °C.

4.5. Microscope evaluation and determination of parasitemia

Microscopic determination of the amount of malaria parasites present in a population of erythrocytes is dependent on the principle that mature erythrocytes do not contain any DNA material, whereas malaria parasites do (Cohen, 1982). Therefore, a thin or thick blood smear on a microscope slide can be stained with a DNA specific stain, such as Giemsa, to reveal malaria parasites inside the erythrocytes. The amount of parasites on the slide can be counted and the parasitemia for the culture determined (Basco, 2007). This process was completed to ensure the culture remained at the correct parasitemia for experimental work, which required specific levels to be accurate, as well as ensuring the successful cultivations of the parasites *in vitro*.

4.5.1. Materials

Sodium phosphate, potassium phosphate and Giemsa solution were acquired from Sigma Aldrich® (South Africa). Methanol was purchased from MERCK (South Africa). 1.2 mm frosted, microscope slides were purchased from Lasec (South Africa).

4.5.2. Methods

A thin blood smear was made on the microscope slide by placing a drop of erythrocytes from the culture on the edge of the slide. Another slide was used to spread the erythrocytes evenly across the surface of the slide. The slide was left to dry completely. The erythrocytes were fixed on the microscope slide with methanol. Sodium phosphate and potassium phosphate were

made into a buffer at a pH of 7.2. The Giemsa stain was prepared according to instruction. One part Giemsa stain was added to four parts of the phosphate buffer and put onto the blood smear. After 7 minutes the stain was removed under running water. The slides were left to air dry and analysed under a light microscope, with an enlargement of at least 60x. The parasitemia was determined by counting the total amount of erythrocytes as well as the amount of erythrocytes that were infected by *P. falciparum*. The formula for determining parasitemia is as follows:

$$\% \text{ Parasitemia} = \left(\frac{\text{Infected erythrocytes}}{\text{Total amount of erythrocytes}} \right) \times 100$$

The parasitemia was used to ensure that the parasitemia of the cultures were within acceptable parameters.

4.6. *In vitro* studies (Flow cytometric determination of reactive oxygen species and lipid peroxidation)

Erythrocytes are often used in *in vitro* studies of new drug delivery systems, as erythrocytes are one of the most abundant components in blood. Erythrocytes are specifically susceptible to oxidative damage since there are many sources of oxidants that the erythrocytes are exposed to as a carrier of oxygen, as well as erythrocytes cell membranes having a high concentration of polyunsaturated fatty acids (Maulik *et al.*, 1998).

4.6.1. Analysis of reactive oxygen species (ROS)

A few methods have been used to determine the amount of ROS in biological systems. The basis for all of these tests are that the ROS will interact with other molecules that have a high affinity to it, thus, other compounds that will easily undergo oxidation. DCFH-DA is a fluorescein derivative that is non-fluorescent, but after being oxidised by peroxide, the product of this interaction dichlorofluorescein (DCFH) is highly fluorescent. This fluorescence can be measured and quantified by flow cytometry. The higher the concentration peroxide, the more fluorescence will be measured (Halliwell & Whiteman, 2004; Sarkar *et al.*, 2005; Wang & Joseph, 1999) .

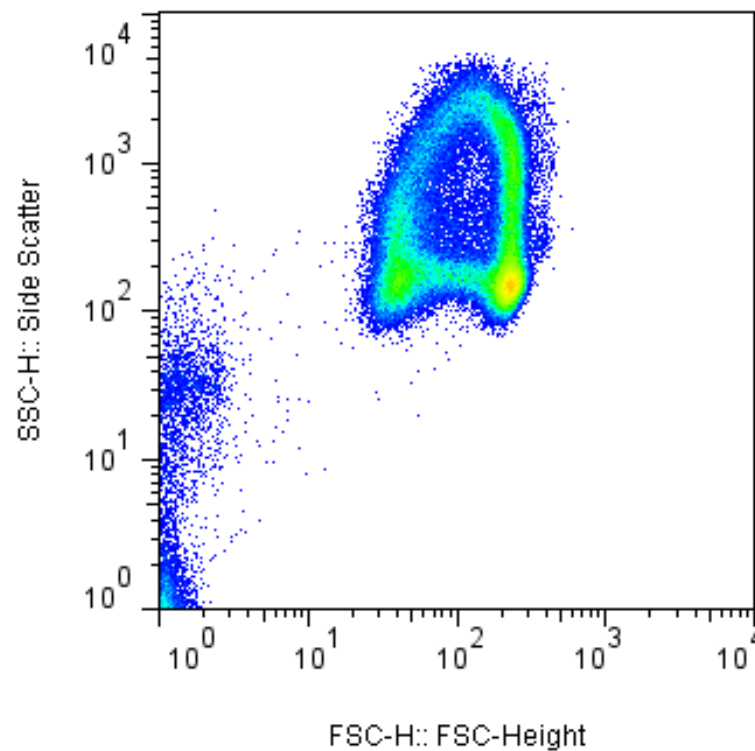


Figure 4.20: A representative sample of a scatter plot as obtained from the FACSCalibur™ before being processed with FlowJo™. The figure portrays both the forward and side scatter when a ROS analysis was done on erythrocytes.

4.6.1.1. Materials

DCFH-DA was purchased from Sigma Aldrich® (South Africa). FACSCalibur™ reagents, FACS consumables and a phosphate buffer solution (PBS) were procured from Scientific Group (South Africa).

4.6.1.2. Method

This method was done according to methods described by Amer *et al.* (2004) and Du Plessis *et al.* (2010). Erythrocytes were prepared the same as for erythrocytes for cultivation, with the exception that the final hematocrit was reduced to be between 1% and 1.5% in final samples. Erythrocytes infected with *P. falciparum* were also tested, with a parasitemia of between 1% and 1.5% and the same hematocrit as the uninfected cells. Samples were composed of 100 μ l erythrocytes or infected erythrocytes and 100 μ l of solute. The solute mixture differed in concentration of liposomes or liposomes with entrapped amodiaquine. Figure 4.20 illustrates data as compiled by FACS analysis and Figure 4.21 portrays the same data after being processed by FlowJo™ software.

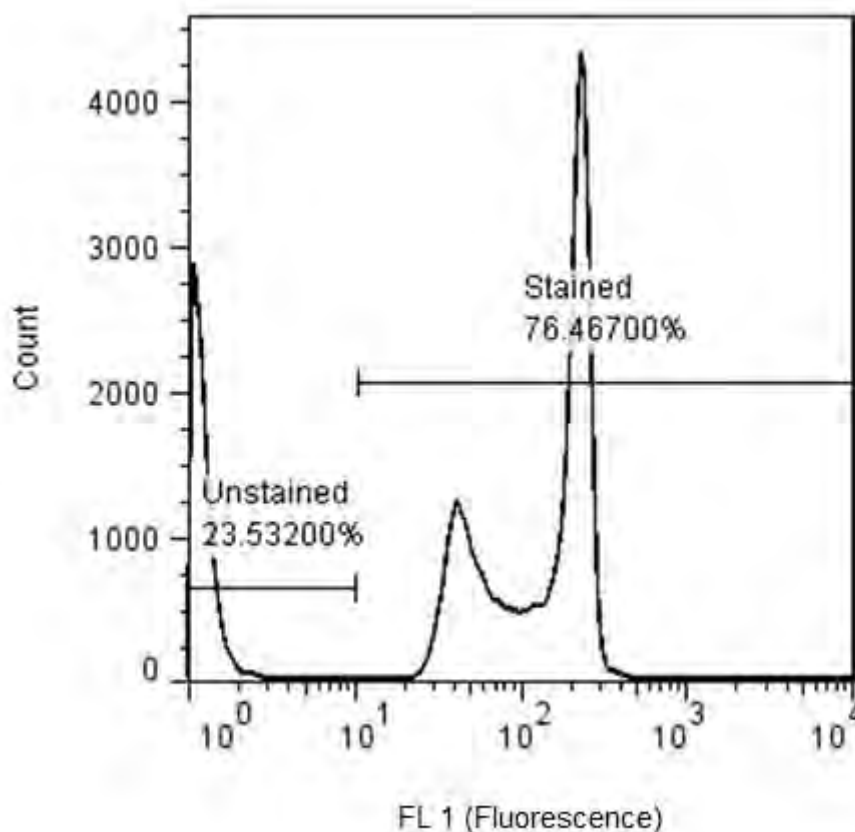


Figure 4.21: The fluorescence histogram of a representative erythrocyte sample, illustrating fluorescent species (Stained) and non-fluorescent species (Unstained) after processing with FlowJo™.

The liposomes were manufactured using the lipid film hydration method as described in section 4.3.2. The mixtures were placed in 96 well plates and incubated under the special gas mixture (5% CO₂, 5% O₂ and 90% N₂) for 24 hours at 37 °C. All samples were done in duplicate. FACS samples were prepared by extracting 100 µl of a sample and adding 500 µl PBS in a falcon tube. DCFH-DA dye was added to each sample to a final concentration of 10 µl per 500 µl sample. The samples were incubated in the dark for 30 minutes before analysing with the FACSCalibur at 488 nm. Control samples were also prepared, consisting of a cell control without any DCFH-DA dye namely (1), a negative control which had no liposomes or drug added, just erythrocytes with the DCFH-DA dye and (2); a positive control with erythrocytes treated with 4mM H₂O₂ for an hour before adding the DCFH-DA dye.

Forward and side scatter information were plotted on a logarithmic scale. The data of 10 000 cells were collected from each sample. An example of this type of graph can be seen in Figure 4.20. The data were analysed using FlowJo™ software which was acquired from Tree Star Inc. (U.S.A.). Gates were set up to differentiate between marked (fluorescent) and unmarked cells.

Figure 4.21 is an example of gates that were set up for this experiment. The geometric mean of each gate was determined with the FlowJo™ software. As described in the literature, the amount of fluorescence caused after the non fluorescent DCFH-DA is converted to fluorescent DCFH, is measured and can be quantified to find the amount of ROS active in the solution.

4.6.1.3. Statistical analysis

Statistical differences between the initial and the values determined in the accelerated stability studies were determined by one-way ANOVA using the Statistica 9 program (Statsoft, California, U.S.A.). Significant differences in data were determined by Bonferroni post hoc test. A p-value of less than 0.05 was deemed significant, whereas a p-value of less than 0.005 was termed highly significant.

4.6.1.4. Results and discussion

In the first experiment the amount of liposomes was diluted with a mixed phosphate buffer (the same as was used to create the liposomes) and added to erythrocytes and infected erythrocytes. The results of the ROS analysis can be seen in Figure 4.22. The numbers on the graph denote the percentage solution of liposomes which were added to the erythrocytes before incubation.

The data used were all expressed in terms of the non-stained cells, thus giving the non-stained cells a value of 1. The non-stained cells served as an isotype control (background fluorescence) to accurately express changes in DCFH fluorescence. The negative control of erythrocytes gave a value of 0.663 ± 0.019 and the negative control of the infected erythrocytes gave a reading of 0.970 ± 0.200 . This indicated low levels of intracellular ROS, as expected. The 0% liposomes mixture responded by showing high levels of intracellular ROS in erythrocytes, with a value of 1.655 ± 0.025 . This does not seem to be the case in infected erythrocytes as the value was just slightly elevated above the negative control (1.018 ± 0.008). The high levels of ROS observed for the erythrocytes were probably due to experimental error. Liposomes between 20% and 60% dilution seemed to have little or no effect on intracellular ROS in both erythrocytes and infected erythrocytes. In the erythrocyte population the amount of ROS was higher at the 80% and 100% dilution, with values of 1.111 ± 0.024 and 1.212 ± 0.087 compared to the negative control. In contrast to this, the values of ROS in the infected erythrocyte populations were constantly lower than the negative control (see Annexure F for full data).

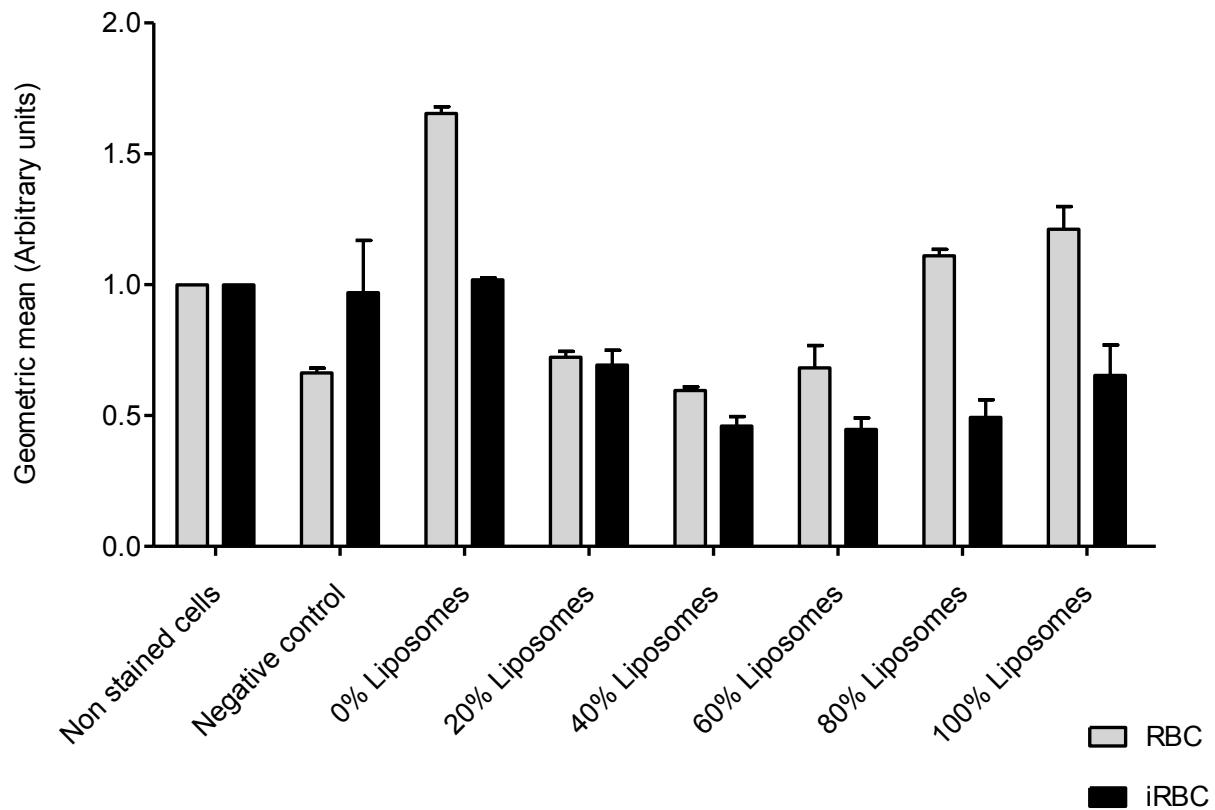


Figure 4.22: The amount of intracellular ROS detected in erythrocytes (RBC) and *P. falciparum* infected erythrocytes (iRBC). The numbers on the x-axis denote the concentration of liposomes added to the RBS solution before incubation. Results are shown as mean \pm SEM (n=2) and a factor of the unstained cells.

It would seem that higher concentrations of liposomes caused an increase in the amount of ROS in the uninfected erythrocytes. The amount of ROS in the infected erythrocytes seemed to remain relatively constant regardless to the amount of liposomes added to the erythrocytes. This may be accounted for by the fact that the *Plasmodium* parasites that infect the erythrocytes have their own systems to protect themselves and the host cell against ROS (Becker *et al.*, 2004).

Except for the 0% liposomes, the ROS caused by the other samples were below the amount of ROS in the negative sample, meaning that the amount of ROS detected was no more than would normally be detected, as cells are normally slightly being oxidised even if the system is in balance (Kohen & Nyska, 2002). Only two of the concentrations did not show any significant differences when compared to the negative control in the erythrocytes. These two formulations were the 0% and the 20%. In the infected erythrocyte population, the only formulation not to differ significantly to the negative control was the 0% concentration formulation.

In the next experiment the amount of liposomes was kept constant. The variable was the amount of amodiaquine entrapped liposomes (AQLiposomes) which were added. The 100% refers to 100% amodiaquine entrapped liposomes whereas the 0% is just liposomes with no entrapped drug. This was done to determine if the addition of amodiaquine to liposomes would increase the toxicity of the amodiaquine to the erythrocytes. The results of this ROS analysis can be seen in Figure 4.23.

Once again the data were expressed in terms of the non-stained cells, thus giving the non-stained cells a value of 1. The negative control of RBS gave a value of 2.659 ± 0.113 and the negative control of the infected erythrocytes showed a reading of 2.039 ± 0.056

The 0% AQLiposome formulation resulted in higher levels of intracellular ROS when compared to the negative control (2.180 ± 0.130). Low levels of intracellular ROS were observed for all the other amodiaquine dilutions in both erythrocytes and infected erythrocytes (See Annexure F). The lowest readings in each population were 40% AQLiposomes in the erythrocyte population (1.521 ± 0.020) and the 60% AQLiposomes in the infected erythrocyte population (1.120 ± 0.336) with the exception of amodiaquine (AQ) (just amodiaquine without any liposomes) which showed a reading of 1.530 ± 0.034 and 0.774 ± 0.027 in the erythrocyte and infected erythrocyte populations respectively. In this experiment all the values from both erythrocyte populations varied significantly from the values of the negative control, except for the 0% concentration in the infected erythrocyte population.

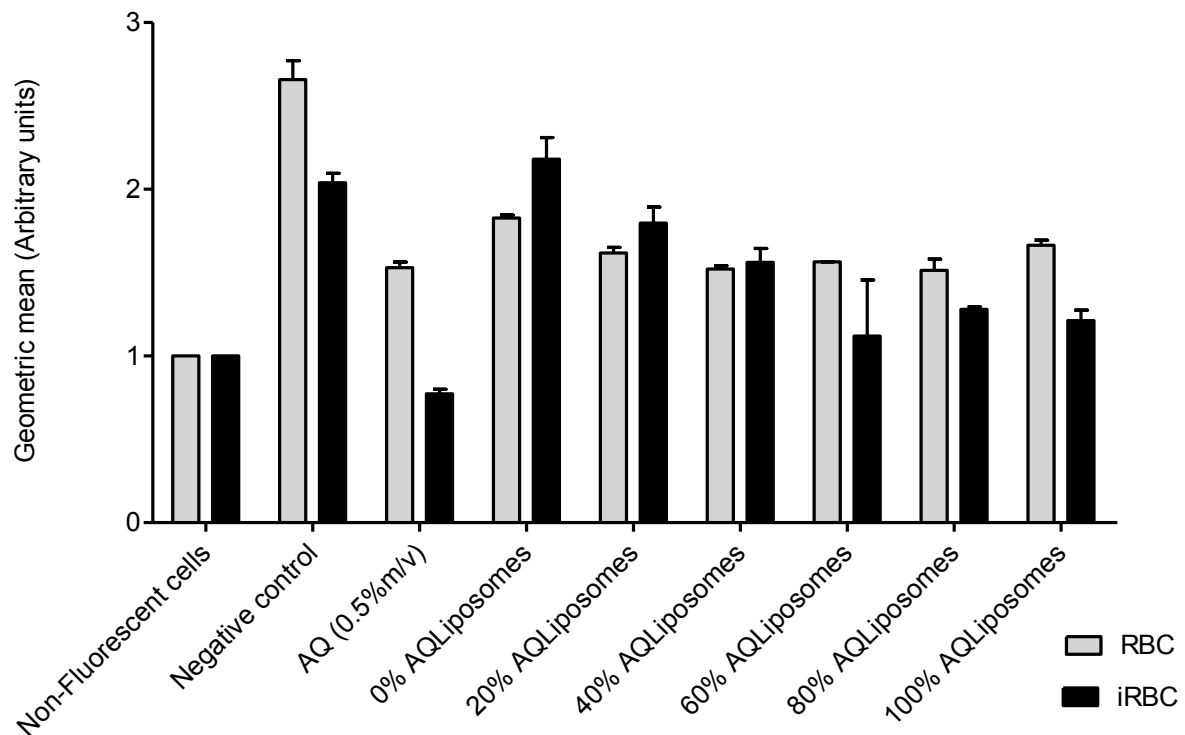


Figure 4.23: The amount of intracellular ROS detected in erythrocytes (RBC) and *P. falciparum* infected erythrocytes (iRBC). The numbers on the x-axis denote the concentration of liposomes with entrapped amodiaquine then diluted with liposomes with no entrapped drug, which are added to the RBS solution before incubation. Results are shown as mean \pm SEM (n=2) and a factor of the unstained cells.

These results show that an increase in the amount of amodiaquine does not in fact cause an increase in the amount of ROS. In the erythrocyte population, the amount of intracellular ROS decreased as the concentration of AQLiposomes increased. The amount of ROS in the infected erythrocyte population showed almost no variation. This could be due to the defence mechanisms that *P. falciparum* has against ROS (Becker *et al.*, 2004).

In both experiments the amount of ROS detected was close to the negative control and the unstained cell controls, with the exception of the negative control and the amodiaquine solution, in the case of erythrocyte population. This indicated that neither liposomes or amodiaquine entrapped liposomes had any toxic effects on erythrocytes or infected erythrocytes in terms of the formation of ROS.

4.6.2. Analysis of Lipid peroxidation

Methods to determine the amount of lipid peroxidation on a cell by cell basis have been a challenging prospect until the combination of flow cytometry and the lipophilic probe known as N-(fluorescein-5-thiocarbonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine or in short Fluorescein-DHPE. The flow cytometer can analyse on a cell by cell basis. The probe makes it possible to differentiate between cells that are undergoing lipid peroxidation and cells that are not (Maulik *et al.*, 1998). This method was specifically designed for erythrocytes, as erythrocytes are prone to lipid peroxidation because of the unsaturated fatty acids in the cell membrane.

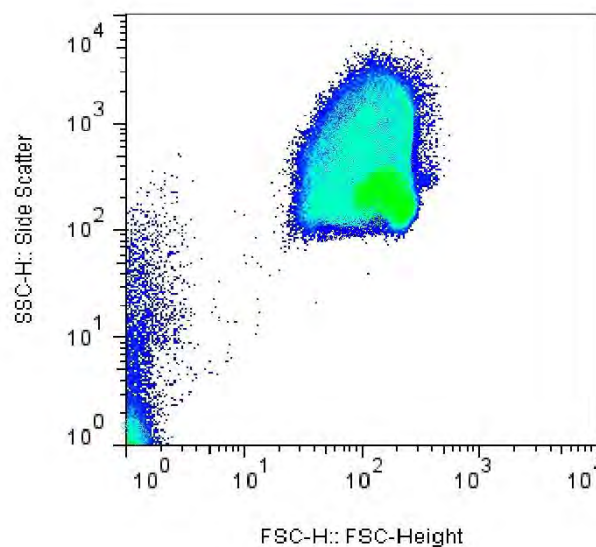


Figure 4.24: A representative sample of a scatter from the FACSCalibur™ before being processed with FlowJo™. The figure portrays both the forward and side scatter when a lipid peroxidation analysis was done.

4.6.2.1. Materials

Fluorescein-DHPE was purchased from Sigma Aldrich® (South Africa). FACSCalibur™ reagents, FACS consumables and a phosphate buffer solution (PBS) were procured from Scientific Group (South Africa).

4.6.2.2. Method

This method was done according to methods described by Amer *et al.* (2004) and Du Plessis *et al.* (2010). Erythrocytes were prepared the same as erythrocytes for cultivation, with the

exception that the final hematocrit was reduced to be between 1% and 1.5% in final samples. Erythrocytes infected with *P. falciparum* were also tested, with the parasitemia of between 1% and 1.5%, and the same hematocrit as the uninfected cells. Samples were of 100 μ l erythrocytes or infected erythrocytes and 100 μ l of solute to be tested, which were different concentrations of liposomes as well as liposomes with entrapped amodiaquine. The liposomes were manufactured using the lipid film method as described in section 4.3.2. The mixtures were placed in 96 well plates and incubated under the special gas mixture (5% CO₂, 5% O₂ and 90% N₂) for 24 hours at 37 °C. Figure 4.24 illustrates data as compiled by FACS analysis and Figure 4.25 portrays the same data after being processed by FlowJo™ software.

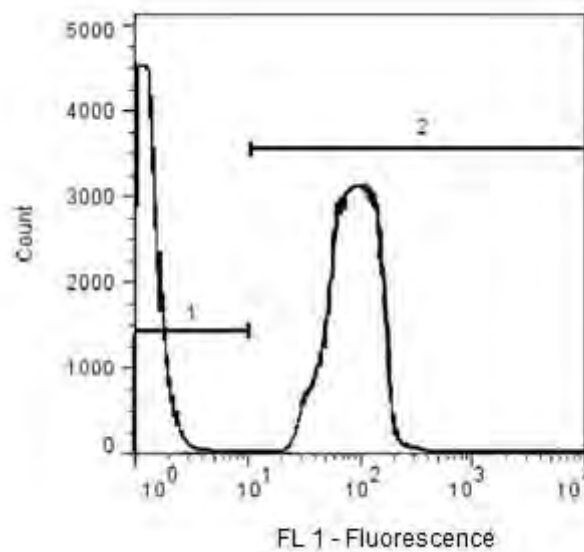


Figure 4.25: The fluorescence (FL 1) histogram of a representative erythrocyte sample, fluorescent species (2) and non-fluorescent species (1) after processing with FlowJo™.

All samples were done in duplicate. FACS samples were prepared by extracting 100 μ l of sample and adding 500 μ l PBS in a falcon tube. Fluorescein-DHPE dye was added to each sample to a final concentration of 50 μ l in 500 μ l sample. The samples were incubated in the dark for 60 minutes, before analysing with the FACSCalibur. Control samples were also prepared, consisting of a cell control without any Fluorescein-DHPE dye, a negative control which had no liposomes or drug added, another sample with just erythrocytes and Fluorescein-DHPE dye and a positive control which consisted of erythrocytes treated with 4mM H₂O₂ for an hour before adding the Fluorescein-DHPE dye.

Forward and side scatter information was plotted on a logarithmic scale. Data of 10 000 cells were collected from each sample. An example of this graph can be seen in Figure 4.24. The data were analysed using FlowJo™ software which was acquired from Tree Star Inc. (U.S.A.).

Gates were set up to differentiate between stained (fluorescent) and unstained cells. An example of a graph can be seen in Figure 4.25. The geometric mean of each gate was determined with the FlowJo™ software. The Fluorescein-DHPE binds to cells and makes it possible to differentiate between cells that undergo lipid peroxidation and those that do not. Cells that do not undergo lipid peroxidation are highly fluorescent and the cells that are affected by lipid peroxidation are not stained.

4.6.2.3. Statistical analysis

Statistical differences between the initial and the values determined in the accelerated stability studies were determined by one-way ANOVA using the Statistica 9 program (Statsoft, California, U.S.A.). Significant differences in data were determined by Bonferroni post hoc test. A p-value of less than 0.05 was deemed significant, whereas a p-value of less than 0.005 was termed highly significant.

4.6.2.4. Results and discussion

In the first experiment to determine lipid peroxidation, the amount of liposomes added to the mixture of erythrocytes, varied as a percentage of the added sample.

It is important to remember that lipid peroxidation measures the amount of damage done to the lipid bilayer of the cells being tested. The data used were all expressed in terms of the non-stained cells. Lipid peroxidation is reflected by cells that do not show fluorescence, thus the graph data were also inverted (this was done by dividing one with the readings from experiments and multiplying by 100). This gave the non-stained cells a value of 100%. The negative control of erythrocytes gave a value of $37.683 \pm 1.601\%$ and the negative control of the infected erythrocytes produced a reading of $49.078 \pm 1.343\%$ (see Annexure G).

The increasing concentration of liposomes in the erythrocyte population did not cause a significant lipid peroxidation, when compared to the negative control (see Annexure G). The 0% liposome formulation did not cause lipid peroxidation, in either populations of erythrocytes or infected erythrocytes with values of $33.765 \pm 0.072\%$ and $44.704 \pm 4.323\%$, respectively. In the erythrocyte population the increase in liposomes produced a slight increase in the amount of lipid peroxidation detected, with the 100% liposome formulation giving a reading of $60.072 \pm 0.191\%$.

Lipid peroxidation in the infected erythrocyte population seemed to be constant (with the exception of the 0% liposome formulation) with the highest reading at 100% liposome formulation (74.539 ± 9.535) and the lowest at the 60% liposome formulation (68.922 ± 3.197). Only two of the concentrations did not show any significant differences when compared to the negative control in the erythrocytes, these two formulations were the 0% and the 20%. In the infected erythrocyte population, the only formulation not to differ significantly to the negative control, was the 0% concentration formulation.

The amount of lipid peroxidation appears to be higher in the infected erythrocytes when compared to the erythrocyte populations. This damage can be attributed to the *Plasmodium* parasites in the erythrocytes.

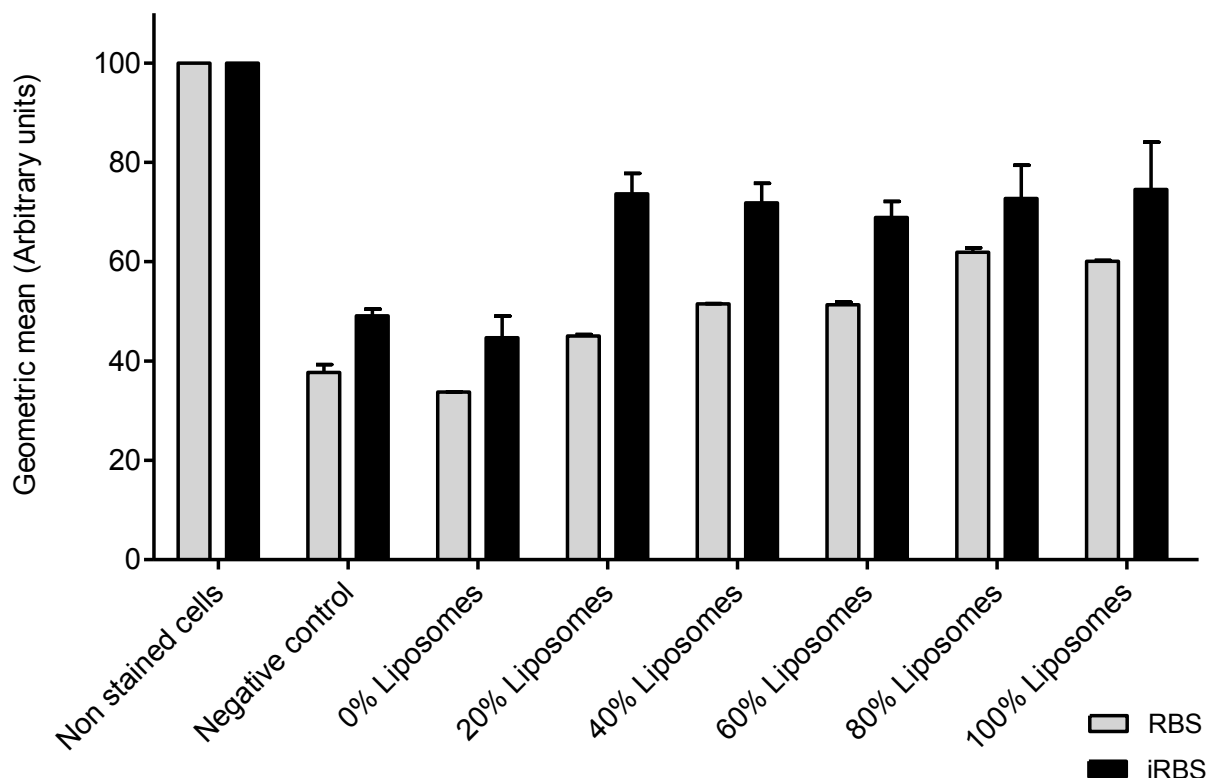


Figure 4.26: The amount of lipid peroxidation detected in erythrocytes (RBC) and *P. falciparum* infected erythrocytes (iRBC). The numbers on the x-axis denote the concentration of liposomes added to the RBS solution before incubation. Results are shown as mean \pm SEM ($n=2$), a factor of the unstained cells and are also inverted ($1/x$).

In the second lipid peroxidation experiment, the amount of liposomes were kept constant with the amount of amodiaquine entrapped liposomes added being the variable. The results of that experiment can be seen in Figure 4.27.

Once again it is important to remember that lipid peroxidation measures the amount of damage done to the lipid bilayer of the cells being tested. The data used were all expressed in terms of

the non-stained cells. Lipid peroxidation is reflected by the cells that do not show fluorescence, thus the graph data were also inverted (this was done by dividing one with the readings from experiments and multiplying by 100). This gave the non-stained cells a value of 100%. The negative control of erythrocytes gave a value of $37.683 \pm 1.601\%$ and the negative control of the infected erythrocytes gave a reading of $49.078 \pm 1.343\%$.

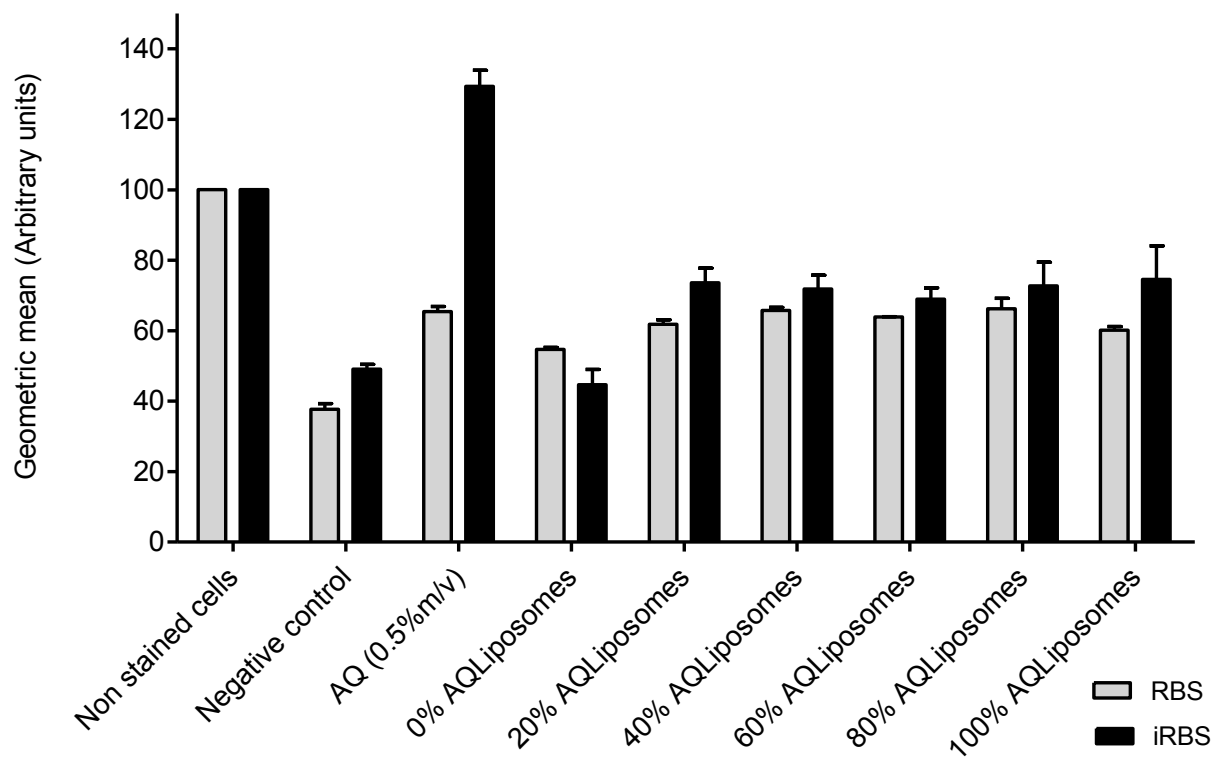


Figure 4.27: The amount of lipid peroxidation detected in different solutions of erythrocytes (RBC) and *Plasmodium falciparum* infected erythrocytes (iRBC). The numbers on the x-axis denote the concentration of liposomes with entrapped amodiaquine then diluted with liposomes with no entrapped drug, which were added to the RBS solution before incubation. Results are shown as mean \pm SEM (n=2), a factor of the unstained cells and are also inverted ($1/x$).

In the both the RBS and iRBS populations, the amodiaquine (0.5% m/v) caused lipid peroxidation, but the amount caused in the iRBS was exceptionally high, to the extent that the amount of lipid peroxidation caused in this group was more than twice as much as the negative control with a value of $129.403 \pm 4.534\%$. This may be due to the mechanism of action that amodiaquine exhibits towards the *Plasmodium* parasites, in the process damaging erythrocytes that contain the parasites. The amount of lipid peroxidation detected was higher than the negative control throughout the entire experiment, remaining relatively constant as the AQLiposome crossed the 20% mark through to 100% AQLiposomes. The highest value in the erythrocytes was 80% AQLiposomes with a reading of 66.213 ± 2.941 and in the infected erythrocytes the highest reading was $74.539 \pm 9.535\%$, given by the 100% AQLiposome formulation. The 0% AQLiposome formulation seemed to have caused the least amount of lipid

peroxidation, but still caused more than the negative control with values of $54.723 \pm 0.553\%$ in the erythrocyte population and $44.704 \pm 4.323\%$ in the infected erythrocyte population. In this experiment all the values from both erythrocyte populations varied significantly from the values of the negative control, except for the 0% concentration in the infected erythrocyte population.

4.7. Conclusion

Amodiaquine is a relatively insoluble drug, especially if and when the pH of the solution reaches neutral. The solubility of amodiaquine increases as the pH becomes more acidic, but this creates a problem for the formulation of liposomes, as liposomes are normally made with neutral buffers. The pH of the buffer had to be lowered to accommodate the solubility of amodiaquine. A pH of 6 was chosen as this pH was still viable for use with cell cultures (cell cultures prefer a pH in the area of 7.4) as well as being able to dissolve amodiaquine to effective levels. An amodiaquine concentration of 0.5% m/v could then easily be attained.

A couple of formulations could be made to test the stability of the liposomes with entrapped amodiaquine because a stability test with amodiaquine entrapped liposomes has not yet been done. The different factors that were tested included: morphology, pH, entrapment efficacy, and size, as drastic changes in any of these factors may cause the drug delivery system to fail. The stability tests were run under accelerated stability study methods. The studies were done over 12 weeks at 5 °C, 25 °C (relative humidity of 60%) and 40 °C (relative humidity 75%). The results of these tests showed that the pH of the different solutions stayed relatively constant. The size of the liposomes increased over time, with the span decreasing. This could mean that the liposomes agglomerated into larger groups or the liposomes swelled. However, this did not indicate stability at higher temperatures. The liposomes at the lowest temperature (5 °C) remained constant for both formulations, indicating stability. The entrapment efficacy study revealed that most of the liposomes increased in size, the entrapment efficacy increased, but this could also be explained by the fact that amodiaquine tends to precipitate from solutions as time goes by. The reason for this is unknown.

Toxicity studies on erythrocytes followed the stability testing. Two groups of erythrocytes were tested, erythrocytes infected with *P. falciparum* and erythrocytes with no infection. Different concentrations of liposomes and liposomes with entrapped amodiaquine were tested to see if the levels of ROS increased or signs of lipid peroxidation occurred (which is signs of damage in the cell membranes). None of the formulations caused any significant increases in either ROS or lipid peroxidation. It can, therefore, be concluded that liposomes containing amodiaquine do not have any toxic effects on erythrocytes or infected erythrocytes, in terms of ROS or lipid peroxidation.