Preparation, stability and *in vitro* evaluation of liposomes containing chloroquine

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Desire is the key to motivation, but it is determination and commitment to an unrelenting pursuit of your goal - a commitment to excellence - that will enable you to attain the success you seek.
~Mario Andretti~

Dedicated to my parents

Henri and Erika Nieuwoudt

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LIST OF ABBREVIATIONS

ANOVA – Repeated Measures Analysis of Variance

ARDS – Acute respiratory distress syndrome

CDC – Centres for Disease Control and Prevention

CM – Culture medium

CO_2 – Carbon dioxide

CQ – Chloroquine

DCF – 2', 7' dichlorofluorescein

DCFH – 2', 7'-dichlorodihydrofluorescein

DCFH-DA – 2', 7'-dichlorodihydrofluorescein diacetate

ddH_2O – Double distilled water

DDT – Dichloro-diphenyl-trichloroethane

Eq – Equation

FACS – Fluorescence-activated cell sorter

FCM – Flow cytometry

FDA – Food and Drug Association

Fe^{+3} – Free heme

Fluorescein-DHPE – N-(fluorescein-5-thiocarboyl)-1, 2-diheade-canoyl-sn-glycero-3-phospho-ethanolamine, tri-ethulammonium salt

FSC – Forward scatter

GSH – Glutathione

HEPES – N-(2-hydroxyethyl)piperazine-N'-(-2-ethanesulfonic acid)

HIV – Human immunodeficiency virus

H_2O_2 – Hydrogen peroxide

IM – Intramuscular

iRBC – Infected Red blood cells

IRS – Indoor residual spraying

IV – intravenous

L + CQ – Chloroquine entrapped in liposomes

LP – Lipid peroxidation

LPC – Lysolecithin

MLV – Multilamellar vesicles

MPS – Monoclear phagocytic system

N_2 – Nitrogen gas

NaCl – Sodium chloride

Na_2CO_3 – Sodium bicarbonate

O_2 – Oxygen

PBS – Phosphate buffered saline

PC – Phosphatidylcholine

P. falciparum – Plasmodium falciparum

P value – Probability value

RBC – Red blood cells

RBM – Roll Back Malaria

RES – Reticuloendothelial system

ROS – Reactive oxygen species

RPM – Revolutions per minute

RPMI – Roswell Park Memorial Institute

SEM – Standard error of mean

SSC – Side scatter

SSLs – Sterically stabilized liposomes

UCT – University of Cape Town

ULV – Unilamellar vesicles

UV – Ultra violet

WHO – World Health Organization

WM – Wash medium

LIST OF EQUATIONS

Equation 1:

The equation for the linear standard curve:

y = mx + c

Where y = absorbance of samples; m = slope of the standard curve; x = concentration of samples; c = y intercept of standard curve.

Equation 2:

The equation of the linear standard curve of chloroquine phosphate:

y = 0, 04135x – 0, 03461

Equation 3:

Entrapment efficacy (% EE) was calculated with the following formula:

$$\% \text{ EE} = \frac{\text{Maximum drug concentration – x-value}}{\text{Maximum drug concentration}} \text{ X 100}$$

Equation 4:

The linear standard curve equation for the standardisation of size distribution on a FACSCalibur™:

y = 1.607x + 0.4496 (Slabbert *et al*., 2010)

The y values illustrated the percentage of the particles and the x value the size of the particles (µm).

Equation 5:

Final span values were calculated via the formula:

$$\textbf{Span (µm)} = \frac{\textbf{S95 \% - S5 \%}}{\textbf{S50 \%}}$$

Equation 6:

The percentage parasitemia was calculated with the following formula:

$$\textbf{\% Parasitemia} = \frac{\textbf{Amount of infected red blood cells}}{\textbf{Total amount of red blood cells}} \textbf{ X 100}$$

Equation 7:

The percentage hemolysis was determined with the following equation:

$$\textbf{\% Hemolysis} = \frac{\textbf{Absorbance sample – Absorbance control 0 \%}}{\textbf{Absorbance 100 \% hemolysis}} \textbf{ X 100}$$

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ABSTRACT

Malaria is currently a huge treat worldwide, as far as infections are concerned, and is responsible for thousands of deaths per annum. The dilemma associated with the development of anti-malarial drug resistance over the past few decades should be addressed as a matter of urgency. Novel drug delivery systems should be developed in order to employ new and existing anti-malarial drugs in the treatment and management of malaria. The aim of these delivery systems should include an improvement in the efficacy, specificity, acceptability and therapeutic index of anti-malarial drugs.

Previous studies have suggested that liposomes have the ability to encapsulate, protect and to promote the sustained release of anti-malarial drugs. Two liposome formulations, namely liposomes and chloroquine entrapped in liposomes, were formulated during this thesis and evaluated by conducting a stability study and an *in vitro* study with the main focus on cell viability.

The stability study consisted of a series of stability tests regarding the stability of nine liposome and nine chloroquine entrapped in liposome formulations over a period of twelve weeks. The *in vitro* study included three assays such as a reactive oxygen species assay, a lipid peroxidation assay and a hemolysis assay. The aims of these studies included the manufacturing of liposomes, the incorporation of chloroquine into liposomes, the determination of the stability of the formulations as well as the evaluation of the possible *in vitro* toxicity of liposomes.

Results obtained from these studies revealed that liposomes remained more stable over the stability study period in comparison to chloroquine entrapped in liposomes. The entrapment of chloroquine within liposomes was possible, although the initial entrapment efficiency (%) of 14.55 % was much too low. The production of reactive oxygen species occurred to a small extent in the red blood cells and the infected red blood cells. Equal amounts of reactive oxygen species (%) was observed within both the red blood cells and the infected red blood cells with a maximum value of 23.27 % in the presence of the chloroquine entrapped in liposomes at varying concentrations. Red blood cells experienced the highest degree of lipid peroxidation (%) in the presence of chloroquine, at varying concentrations, entrapped in liposomes. The maximum amount of lipid peroxidation (%) was 79.61 %. No significant degree of hemolysis (%) was observed in the red blood cells neither in the presence of the liposomes nor in the presence of the chloroquine entrapped in liposomes at varying concentrations.

It can be concluded that liposomes are a more stable formulation and have less toxic effects on red blood cells and infected red blood cells in comparison to the chloroquine entrapped in

liposome formulations. Future studies should investigate the possibility of a more stable and less toxic chloroquine entrapped in liposome formulation.

Key words: Malaria, liposomes, chloroquine (CQ), reactive oxygen species (ROS), lipid peroxidation (LP), hemolysis.

UITTREKSEL

Malaria is ‘n groot bedreiging wêreldwyd en is vir duisende sterftes per jaar verantwoordelik. Weerstandbiedendheid teenoor anti-malaria geneesmiddels het gedurende die laaste paar dekades ontwikkel en aandag moet so spoedig as moontlik aan hierdie problem geskenk word. Innoverende geneesmiddelaflewerings-sisteme moet ontwikkel word om reeds bestaande en nuwe anti-malaria middels effektief af te lewer vir die behandeling van malaria. Die hoofklem van hierdie innoverende afleweringsisteme moet val op die verbetering van die effektiwiteit, spesifisiteit en terapeutiese indeks van hierdie middels.

Vorige studies het getoon dat liposome oor die vermoë beskik om anti-malaria middels te enkapsuleer, te beskerm en om verlengde vrystelling van die middels te bevorder. Hierdie studie het gehandel oor twee liposoom preparate. Die een preparaat het uit skoon liposome bestaan terwyl die tweede preparaat bestaan het uit liposome met chlorokien. Hierdie preparate was vervolgens onderwerp aan ‘n stabiliteitsstudie en ‘n *in vitro*, sel-lewensvatbaarheid, studie.

Die stabiliteitsstudie het bestaan uit ‘n reeks stabiliteitstoetse aangaande die stabiliteit van nege monsters van elk van die bogenoemde twee preparate oor ‘n tydperk van twaalf weke. Die *in vitro* studie het bestaan uit die meting van drie aspekte van die selkulture wat aanduidend is van sel-lewensvatbaarheid. Die omvang van die vorming van reaktiewe suurstof spesies, lipied peroksidasie en die gevolglike selhemolise was bepaal. Die hoof oogmerke van hierdie studie het ingesluit die vervaardiging van liposome, chlorokien bevattende liposome, stabiliteitsbepaling van die preparate en ook die bepaling van die potensiële *in vitro* toksisiteit van die preparate.

Die resultate het getoon dat die liposoom preparaat meer stabiel was as die chlorokien bevattende liposoom preparaat. Die chlorokien enkapsuleringseffektiwiteit van die liposome was ook bepaal. Die aanvanklike enkapsuleringseffektiwiteit was 14.55 %. Die produksie van reaktiewe suurstof spesies het in lae vlakke binne die rooibloedselle en die geïnfekteerde rooibloedselle plaasgevind. Gelyke hoeveelhede van die reaktiewe suurstof spesies kon binne beide die rooibloedselle en geïnfekteerde rooibloedselle waargeneem word, met ‘n maksimum waarde van 23.27 % in die teenwoordigheid van chlorokien bevattende liposoom preparate. Die chloroquine bevattende liposome het meer lipied peroksidasie (%) veroorsaak in the teenwoordigheid van rooibloedselle, met ‘n maksimum waarde van 79.61 %. Geen beduidende % hemolise kon by die rooibloedselle in die teenwoordigheid van liposome of in die teenwoordigheid van chlorokien bevattende liposome waargeneem word nie.

Liposome, in vergelyking met chlorokien bevattende liposome, is ‘n stabieler preparaat met minder toksiese effekte op rooibloedselle en geïnfekteerde rooibloedselle. Die moontlikheid van

‘n stabieler en ‘n minder toksiese chlorokien bevattende preparaat moet in toekomstige studies ondersoek word.

Sleutelwoorde: Malaria, liposome, chlorokien (CQ), reaktiewe suurstof spesies (ROS), lipied peroksidasie (LP), hemolise

INTRODUCTION AND AIM OF STUDY

Malaria is a disease caused by a deadly protozoan parasite which is responsible for a high rate of morbidity and mortality among children (RBM, 2009; Snow *et al*., 2004). The World health organization (WHO) has conducted a survey regarding the spraying of indoor insecticides in order to reduce and/or prevent the transmission of malaria (Park, 1936; De Meillon, 1936). Problems have unfortunately been encountered with this approach, which in turn led to a major increase in the amount of reported malaria cases (Mouchet *et al*., 1997).

New malaria prevention strategies have since been implemented but none of them has the ability to optimally manage malaria (Kroeger *et. al*., 1999). *P. falciparum* malaria has also developed drug resistance to most of the anti-malarial drugs, including the first-line drugs such as chloroquine, during the last couple of years (CDC, 2004). *P. falciparum*, however, has a complex life cycle which could provide a suitable target for anti-malarial drug treatment with the aid of novel drug delivery systems.

Liposomes are synthetic, spherical, microcapsule aggregates consisting of lipid bilayers which are separated by aqueous and buffer compartments. They have the ability to entrap both hydrophilic and hydrophobic drugs and to improve the bioavailability, diminish the toxicity, enhance the efficacy and to improve the therapeutic index of the entrapped drugs (Couvreur *et al*., 1991; Redziniak & Perrier, 1996; Sharma & Sharma, 1997; Wang, 2005). The encapsulation method was used, in particular, to incorporate chloroquine within the liposomes. Liposomes were evaluated in terms of their ability to act as an effective drug delivery system for the treatment and management of malaria.

The specific objectives of this study include:

- a literature study on liposomes as a drug delivery system;
- the manufacturing of liposomes;
- the evaluation of liposomes in terms of their morphology and size;
- entrapment of chloroquine within liposomes;
- the evaluation of chloroquine entrapped in liposomes in terms of their morphology, size and entrapment efficiency percentage;
- a stability study on liposomes and chloroquine entrapped in liposomes, and
- the evaluation of the possible *in vitro* toxicity of liposomes by conducting assays regarding reactive oxygen species (ROS), lipid peroxidation (LP) and the hemolysis of red blood cells.

CHAPTER 1

Malaria

1.1) Introduction

The term malaria originated from the Italian phrase 'mal'aria', which means 'bad air'. Charles Louis Alphonse Laveran, which was a French army surgeon during the 19th century, observed parasites in the blood of one of his patients which were suffering from malaria. The fact that mosquitoes transmit malaria was discovered by a British medical officer in Hyderabad India named Dr. Ronald Ross. Additionally the Italian professor Giovanni Battista Grassi confirmed that *Anopheles* mosquitoes were the only transmitters of human malaria (Snow *et al*., 1999; Breman *et al*., 2001).

Malaria is known to be responsible for more that 300 million severe infections annually and claims at least one million lives per annum worldwide (RBM, 2009). During 2008, 243 million malaria-cases were reported and as a result lead to 863 000 casualties. More than 40 % of the world's population, generally those living in the poorest countries, are at risk of contracting malaria (Snow *et al*., 1999; Breman *et al*., 2001; WHO, 2010). The annual cost of anti-malarial drug treatments in Africa is approximately $12 billion (Snow *et al*., 2004).

Malaria is widely distributed throughout the tropic and sub-tropic regions of the world and mainly in Africa and Asia (WHO, 2009). Several areas in the tropic regions are endemic malaria areas. The majority of malaria cases occur in Afghanistan, Brazil, Cambodia, China, India, Indonesia, Sri Lanka, Thailand and Vietnam as illustrated in Figure 1.1. (Snow *et al*., 1999; Breman *et al*., 2001). Ninety percent of the 2414 deaths, which occur daily in Sub-Saharan Africa, are caused by malaria (WHO, 2009). Malaria in the Sub-Saharan region is also the greatest cause of morbidity and mortality among children, which accounts for about 25 % of fatalities in children below the age of five (Snow *et al*., 2004). Every thirty seconds malaria claims the life of a child living in Africa (RBM, 2009). Malaria has the ability to cause severe anemia in pregnant women who live in highly malarious regions (WHO, 2009). This may likely lead to the premature birth of the baby, a low birth weight or even fetal death (Beeson *et al*., 2001; WHO, 2009).

There are no concrete evidence thus far regarding an interaction among malaria and HIV. An increase in clinical malaria attacks along with increased parasitemias within semi-immune HIV-infected Ugandan adults, have however been established. Statistics suggest that non-

immune adults, which are co-infected with HIV and living in South Africa, have a higher risk of contracting severe malaria (French & Gilks, 2000).

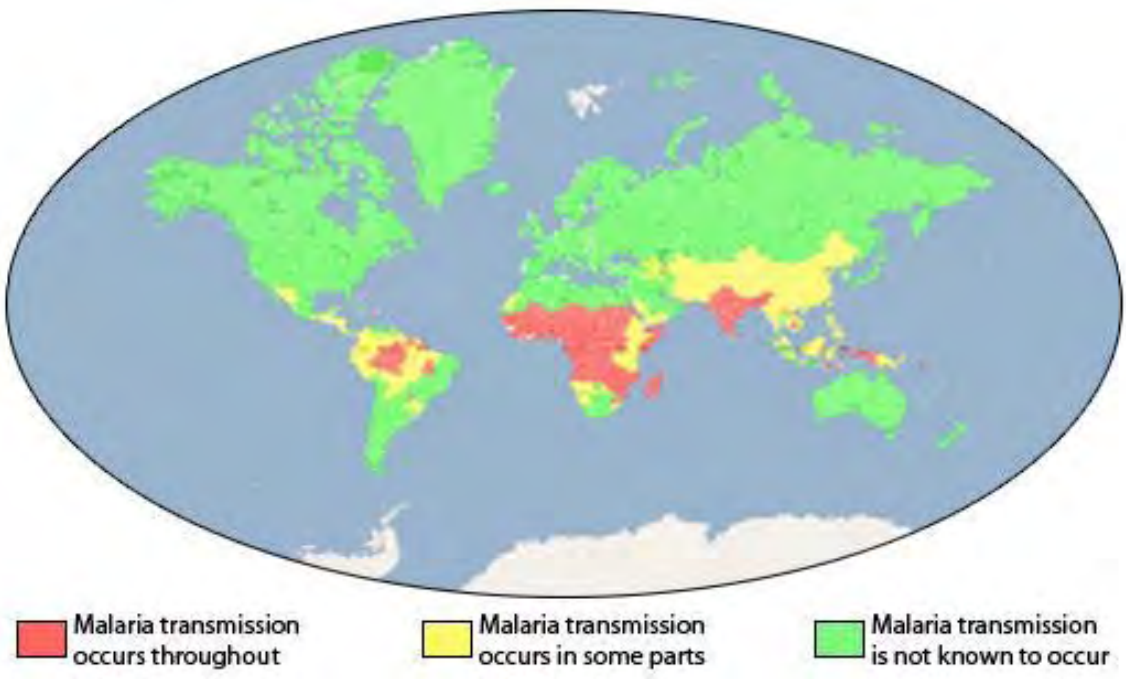


Figure 1.1: The specific malaria transmission areas and endemic countries. The figure was adapted from the Centres for Disease Control and Prevention (CDC) website, www.cdc.gov/malaria/about/distribution.html

1.2) The current malaria problem

The World Health Organization (WHO) conducted a survey in South Africa and India during the 1930's in order to establish whether the spraying of indoor insecticides had the ability to reduce the transmission of malaria (Park, 1936; De Meillon, 1936). During the 1950's and 1960's malaria was suppressed and significantly reduced within Asia, Europe, Latin America, Southern Africa and the Middle East by means of the indoor residual spraying (IRS) of dichloro-diphenyl-trichloroethane (DDT) (Gramiccia & Hampel, 1972; Payne, 1976; Zahar, 1985). Since the employment of IRS the mortality amongst children have been eliminated from 1945 to 1952 in Guyana and have decreased by 50% between 1946 to 1956 in Sri Lanka (Giglioli, 1972; Global Health Council, 2003). The possibility of over 700 million malaria infections has been eliminated during a period of 20 years by means of the malaria suppression campaign (WHO, 2006).

The employment of IRS, in order to control malaria, has been reduced considerably during the 1980's in accordance to a global agreement to rather employ long term control programs. A number of countries have since experienced major relapses in malaria cases. As a result 10

000 people were killed by malaria during 1987-1988 in Madagascar alone (Mouchet *et al*., 1997). The prevention and management of malaria by means of IRS have been attempted by only 12 countries in southern and eastern Africa during the year 2003 (WHO, 2006).

Malaria prevention strategies, which include insecticides, as well as mosquito bed nets which have been treated with insectices, have since been implemented. Several problems have been encountered with these strategies such as the increased development of vector resistance to insecticides as well as a lack in the community's commitment towards the employment of mosquito bed nets. The mosquito bed nets are furthermore unsuccessful in low and unstable malaria transmission regions where mosquito bites occur in the early hours of the mornings and early at night (Kroeger *et al*., 1999).

Significant research, involving the development of an efficient malaria vaccine, has been under way for the past three decades. Research has temporarily been delayed as a result of an inadequate understanding of the mechanisms involved and the degree of protection offered by the immune system. Inadequate finances and the complexity of the malaria parasite causes further delays. The formulation of an effective vaccine is at best years away (Richie & Saul, 2002).

It has been stated that malaria is re-emerging based on the fact that it has resurfaced in areas which have previously been malaria free. More deaths are currently associated with malaria than 40 years ago in spite of the universal economical development (Olliaro *et al*., 1996).

1.3) Etiology

Malaria is a disease caused by a deadly protozoan parasite, which is transmitted by female *Anopheles* mosquitoes infected with the parasite (RBM, 2009). Only 30-50 of the 430 known *Anopheles* species have the ability to transmit malaria. The successful development of the malaria parasite within the mosquito depends on several factors. The most important factors are: the humidity (higher temperatures accelerate parasite growth in the mosquito), the ambient temperature and whether the *Anopheles* mosquito can survive long enough to allow the parasite to complete its life cycle within the mosquito host. In contrast to the human host, the mosquito does not suffer noticeably from the presence of the parasites (CDC, 2004).

The malaria parasite belongs to the genus *Plasmodium*. There are more than 100 known species of the *Plasmodium* parasite. Only four of these species and in particular *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* are known to infect humans (CDC, 2004). The drug reaction, geographical distribution, morphologic appearance, immune response and recrudescence patterns differ from one specie to the next (Snow *et al*.,

2004). *P. falciparum* is the most deadly of the four species and it is responsible for almost all of the malaria cases documented in Sub-Saharan Africa. *P. falciparum* is responsible for approximately 700 000 to 2.7 million fatalities in humans per annum. These fatalities mainly occur within Africa (CDC, 2004).

1.4) Life cycle of malaria parasites

P. falciparum has a complex life cycle, which fortunately provides suitable targets for anti-malarial drug treatment, which will be discussed thoroughly by specifically referring to Figure 1.2.

The life cycle of the malaria parasite starts when the sporozoites enter the bloodstream:

Exo-erythrocyte cycle

1. The infected female *anopheles* mosquito takes a blood meal and injects sporozoites from her salivary glands into the human bloodstream (Yamauchi *et al*., 2007).

2. The sporozoites migrate to the liver in a matter of 30 minutes where they then penetrate the liver cells (hepatocytes). The sporozoites remain within the liver cells for 9-16 days where asexual reproduction occurs (Mota *et al.,* 2001).

   Tens of thousands of merozoites are produced within the liver cells from each sporozoite (Sturm *et al*., 2006).

Erythrocyte cycle

3. The merozoites invade the RBC (red blood cells) by means of complex invasion processes. Asexual division then occurs within the RBC in order to produce thropozoites, which are referred to as the “ring forms” during their early stages (Miller *et al*., 2002).

4. The trophozoites mature inside of the RBC as a result of highly active metabolic processes which entail: host cytoplasm ingestion, glycolysis of large quantities of imported glucose as well as the proteolysis of hemoglobin into essential amino acids. The toxic byproduct of hemoglobin degradation namely heme is not degradable by the malaria parasites. The malaria parasites consequently polymerize the heme to hemozoin (malaria pigment) which are then stored within the malaria parasites’ food vacuoles (Miller *et al*., 2002).

5. Numerous rounds of nuclear division, which does not include cytokinesis, results in the development of schizonts at the end of this trophic stage (Cowman & Crabb, 2002; Tolia *et al*., 2005).

6. Approximately 20 merozoites are enclosed within each mature schizont. These merozoites are released after the lysis of RBC in order to further invade uninfected RBC. The invation consequently leads to flu like symptoms. This recurring intra-erythrocyte cycle persists for about 48 hours in *P. falciparum* infections (Cowman & Crabb, 2002; Tolia *et al*., 2005).

7. A small fraction of the merozoites inside of the RBC eventually differentiate in order to produce micro (male) - and macro (female) gametocytes. These gametocytes have no additional activity in the human host but are vital for the transmission of malaria to new hosts via female *Anopheles* mosquitoes. A few erythrocyte cycles generally occur prior to the production of gametocytes. The erythrocyte cycle and gametocytogenisis take 48 hours and 10-12 days respectively in *P. falciparum* infections (Eksi *et al*., 2006).

Sporogonic cycle

8. A female *Anopheles* mosquito takes a blood meal from an infected individual, which might lead to the consumption and collection of gametocytes in its midgut (Eksi *et al*., 2006).

9. The macrogametocytes form macrogametes in the midgut. Microgametes are furthermore produced when the microgametocytes are exflaggeltated (Eksi *et al*., 2006).

10. A zygote is produced after the fusion and fertilization of these gametes (Eksi *et al.*, 2006).

11. The zygote is subsequently transformed into an ookinete which penetrates the wall of a cell in the midgut of the mosquito and then develops into an oocyst (Eksi *et a*l., 2006). Various sporozoites are produced during sporogony and takes place within the oocyst (Baruch, 1999).

12. When the oocyst ruptures the sporozoites migrate to the salivary glands of the mosquito. Sporozoites are visible within the salivary glands of the mosquito after 10-18 days. The mosquito continues to be infective for 1-2 months afterwards (Baruch, 1999).

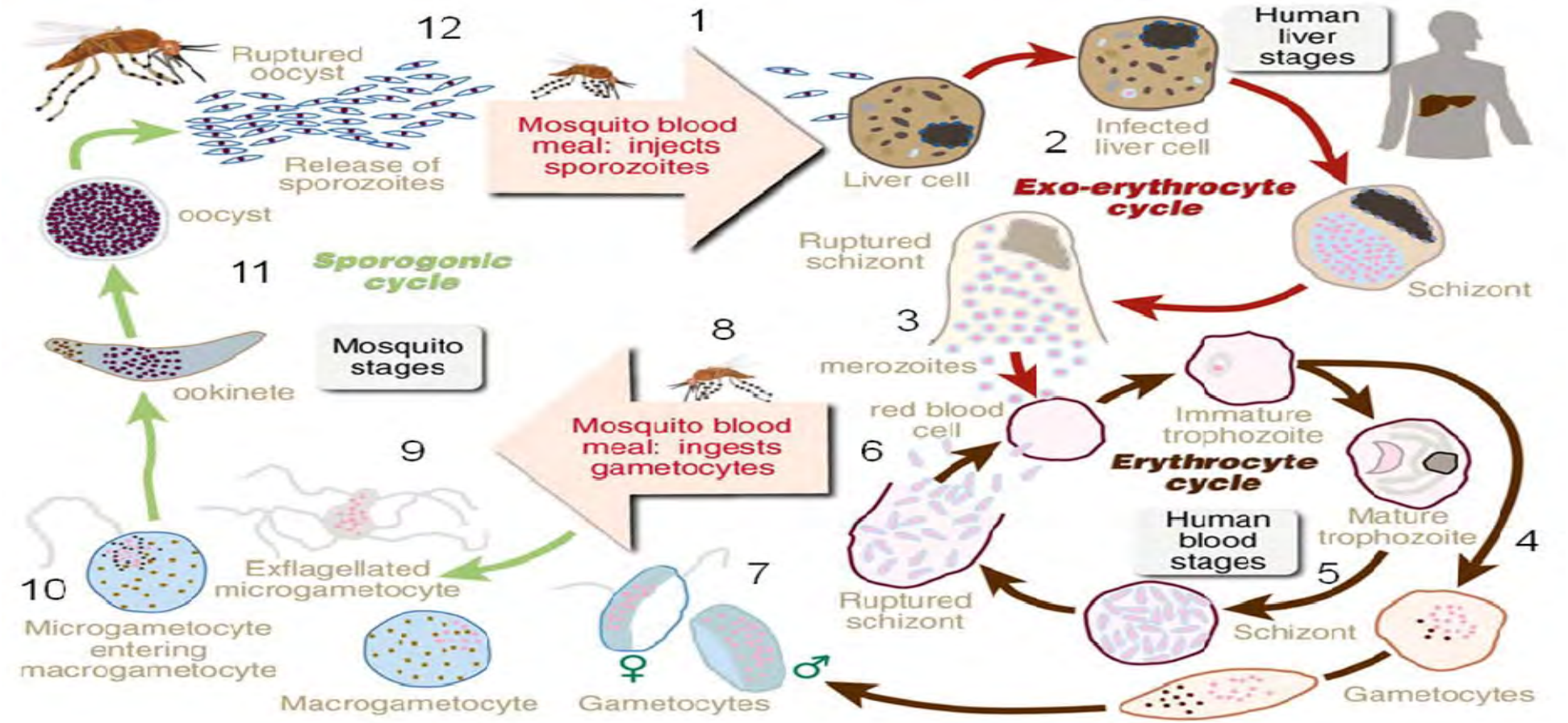


Figure 1.2: The life cycle of the malaria parasite *P. falciparum.* **The figure was adapted from the Proceedings of the National Academy of Sciences of the United States of America (PNAS) web site, www.pnas.org/content/104/29/11865.figures-only**

1.5) Signs and symptoms

P. falciparum has the ability to cause severe cases of malaria, which results in major blood loss (anemia) due to its multiplication in the red blood cells. This parasite is capable of blocking tiny blood vessels in the brain, which can lead to a complication referred to as cerebral malaria. This is a serious condition and can be fatal (CDC, 2004).

The incubation period is the time subsequent to the bite of an infectious *Anopheles* mosquito. The incubation period generally varies between 7 to 30 days, depending on the *Plasmodium* specie. *P. falciparum* typically has an incubation period of 9 to 14 days. The malaria symptoms only emerge once the incubation period has been completed (CDC, 2004; RBM, 2009; Merck Manual, 2009).

Malaria can be categorized as uncomplicated malaria or severe malaria.

1.5.1) Uncomplicated malaria

This type of malaria is seldom observed and may last for 6 to 10 hours. The attack consists of three stages namely:

- a cold stage – the patient has a cold awareness and start to tremble;
- a hot stage – the patient develops a high fever, complains of headaches and starts vomiting. This stage may also induce convulsions in small children;
- a sweating stage – the patient sweats excessively whilst his/her temperature returns to normal and leaves the patient feeling exhausted (CDC, 2004; Miller *et al*., 2002).

The malaria attack may occur every third day by means of “quartan” parasites such as *P. malariae* and every second day by means of “tertial” parasites such as *P. falciparum*, *P. vivax* and *P. ovale* (CDC, 2004).

1.5.2) Severe malaria

Severe malaria is the result of complications which occur during *P. falciparum* infections. A delay in the treatment of uncomplicated malaria, the application of ineffective drug therapy and/or the application of effective drug therapy at incorrect dosages could result in severe malaria (Durrheim *et al*., 2001). The abovementioned complications may include irregularities within the blood of a patient or severe malfunction of the organs or an irregular metabolism. Symptoms associated with severe malaria include:

- cerebral malaria in combination with convulsions, impaired awareness, unusual behavior, loss of consciousness or additional neurological abnormalities;
- severe anemia as a result of hemolysis (red blood cell destruction);
- hemoglobinuria (hemoglobin within the urine) attributable to hemolysis;
- pulomonary edema (fluid buildup within the lungs) or acute respiratory distress syndrome (ARDS);
- irregularities associated with the coagulation of blood together with thrombocytopenia (blood platelet reduction);
- cardiovascular collapse and distress (CDC, 2004; Miller *et al*., 2002).

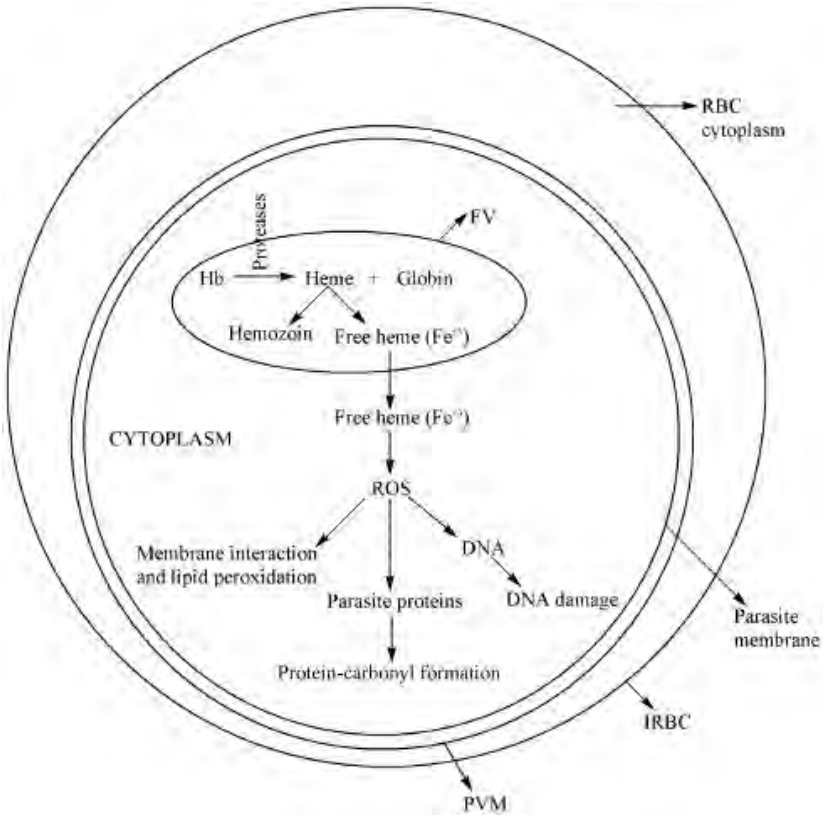
Individuals with no resistance or a low immunity towards malaria are more likely to contract severe malaria. These individuals include people living in low or no malaria transmission regions as well as pregnant women and small children living in high malaria transmission regions (CDC, 2004).

Severe malaria should be treated urgently since it is a medical emergency (CDC, 2004).

1.6) Heme metabolism of *P. falciparum*

Plasmodium falciparum obtains hemoglobin from the erythrocytes through pinocytosis during the intra-erythrocytic stage of its lifecycle. The hemoglobin is subsequently stored within a peripheral tubular structure located inside the *P*. *falciparum* parasite, namely the cytostome. The cytostome consist of various small tubular vesicles which contains hemoglobin. The acidic food vacuole is produced by the merger of all these small tubular vesicles within the cytostome (Yayon *et al*., 1984). Oxidation of hemoglobin to methemoglobin occurs within the acidic pH of the food vacuole (Figure 1.3). The methemoglobin is subsequently hydrolyzed to free heme (Fe^{+3}) and denatured globin by means of aspartic proteases. Cysteine proteases and metallopeptidase, which contains zinc, is responsible for the additional denaturation of globin to small peptides (Eggleson *et al*., 1999). These peptides are then supposedly transported toward the parasitic cytoplasm by means of the peptide transporter which is situated within the parasite's food vacuole membrane (Rubio &Cowman, 1996; Kolakovich *et al*., 1997). The peptides are further hydrolyzed by cytosolic exopeptidase to amino acids. The amino acids are essentially used by the malaria parasite for its protein biosynthetic requirements (Rudzinska *et al*., 1965; Sherman, 1998).

The accumulation of free heme (Fe^{+3}) within the parasitic food vacuole may possibly reach toxic levels of 300-500 mM which might enable the production of reactive oxygen species (ROS) (Wright *et al*., 2001). The toxic heme may additionally have fatal consequences due to the fact that it may induce oxidative stress within the *P*. *falciparum* parasite (Vincent, 1989; Schmitt *et al*., 1993; Kumar, S. & Bandyopadhyay, 2005). Free heme (Fe^{+3}) might have the ability to change the lipid organization and membrane permeability of the parasitic membrane, it may also further induce lipid peroxidation due to its lipophilic molecular character (Ryter & Tyrrell, 2000; Stojiljkovic *et al*., 2001). The oxidation of membrane components, via free heme (Fe^{+3}), in turn encourages the lysis of cells and eventually the death of the parasites (Schmitt *et al*., 1993).



Hb – Hemoglobin

FV – Food vacuole

IRBC – Infected red blood cell

PVM – Parasitophorous vacoular membrane

ROS – Reactive oxygen species

Figure: 1.3 The different mechanisms regarding the free heme (Fe^{+3}) toxicity within malaria parasites. The figure was adapted from Kumar *et al*., 2007.

Malaria parasites contain exceptional heme detoxification systems in order to avoid free heme (Fe^{+3}) toxicity (Slater *et al*., 1991). The toxic heme within the parasite's food vacuole is converted to non-toxic hemozoin (malaria pigment) by the so-called hemozoin formation mechanism, which is considered the main heme detoxification system within the malaria parasite (Pandey *et al*., 1995; Sherman, 1998). The hemozoin pigment is compiled by heme units which are linked together via an iron-carboxylate bond. This bond is formed through the

connection between the central ferric iron molecules of one of the heme units and the propionate side chain of another heme (Slater *et al*., 1991; Pagola *et al*., 2000). The hemozoin pigment is released into the human bloodstream subsequent to the rupture of the infected erythrocytes at the end of the erythrocytic cycle of the malaria parasite's lifecycle. The released hemozoin pigment is then deposited in the tissues of the infected human host (Pandey *et al*., 1995; Pandey & Tekwani, 1996; Sullivan *et al*., 1996).

Most of the antimalarial drugs, which specifically target the blood schizont stage of the malaria parasite's lifecycle, are only active against the hemozoin formation mechanism of the malaria parasite (Kishimoto et al., 1968; Peters, 1971). The hemozoin formation process presents the opportunity to design specific inhibitors of target sites within the malaria parasite (Ridley, 2002). A poor understanding of the hemozoin formation mechanism thus far have however hindered the abovementioned attempts regarding the design of specific inhibitors. The catalization process concerning hemozoin formation depends on numerous factors such as:

- the enzyme heme polymerase, which is suggested to be present within the trophozoites of *P. falciparum* malaria. This enzyme is responsible for the conversion of heme to hemozoin within the acidic environment;
- a spontaneous chemical process known as heme polymerization, which requires no parasitic material; and
- the autocatalytic heme conversion process, which have been mediated by preformed hemozoin and the engagement of phospholipids (Slater & Cerami, 1992; Chou & Fitch, 1992; Egan *et al*., 1994; Bendrat *et a*l., 1995; Dorn *et. al*., 1995; Adams *et al*., 1996).

A lot of speculation still exists on the exact mechanism of hemozoin formation. Mediators, which have been associated with hemozoin formation over the last decade, include: histidine-rich proteins, lipids, parasitic lysate and preformed hemozoin templates (Fitch & Chou, 1996; Martiney *et al*., 1996; Sullivan *et al*., 1996).

1.7) Reactive oxygen species and lipid peroxidation

1.7.1) Reactive oxygen species

Reactive oxygen species (ROS) are unstable chemically reactive free radicals which contains unpaired electrons. These unpaired valence shell electrons facilitates the extreme reactivity of ROS by rapidly reacting with other molecules or radicals in order to obtain stability. ROS is continuously produced within cells as the natural by-product of normal oxygen metabolism

(de Zwart *et al*., 1999; Grune *et al*., 2000; Kohen & Nyska, 2002). Environmental factors such as cigarette smoke, herbicides, pollution and radiation may enhance ROS production (Kohen & Nyska, 2002; Koren, 1995). Various pathophysiological conditions which include a deficiency in antioxidant vitamins, exposure to radioactive radiation or ultraviolet (UV) light, hyperoxia, hypoxia, immunological disorders, inflammation and the metabolism of alcohol or drugs causes an increase in ROS production (Chan *et al*., 1999). The occurrence of intracelllur ROS is mainly associated with the auto-oxidation process of oxy-hemoglobin. This process leads to the production of hydrogen peroxide (H_2O_2) by means of dismutation along with the production of superoxide (Misra & Fridovich, 1972). Enzymes which are included within the antioxidant system in order to prevent cellular injury are: catalase, glutathione, peroxidase and superoxide dismutase (Nagababu *et al*., 2003). Reduced glutathione (GSH) is the most important antioxidant responsible for the scavenging of ROS. GSH is the key element within the enzymatic antioxidant system and it also maintains the redox potential inside of the cells (Meister & Anderson, 1983; De Flora *et al*., 2001).

ROS have the ability to oxidize assorted cellular molecules which may lead to DNA damage and the oxidation of polydesaturated fatty acids within lipids (lipid peroxidation). It may also lead to the oxidation of amino acids within proteins, the inactivation of antioxidant enzymes and ultimately apoptosis (cell death) (Hershko *et al*., 1998; Droge, 2002).

1.7.2) Lipid peroxidation

Lipid peroxidation (LP) can be defined as the oxidative degradation of the unsaturated lipids of the cell membranes. It is one of the major mechanisms associated with cellular damage due to free radical reactions caused by ROS as discussed above (Kappus, 1985; Cheeseman, 1993; Rice-Evans, 1994).

1.8) Anti-malarial drugs

Antil-malarial drugs can be divided into numerous classes according to their specific mechanism of action. **Tissue schizonticides** are drugs which eliminate developing or dormant liver stages of the malaria parasite. **Blood schizonticides** are drugs which destroy malaria parasites within the erythrocytes of the host. **Gametocides** are drugs which prevent the transmission of the malaria parasites to the mosquitoes by killing the sexual stages of the malaria parasite (Rosenthal, 2004).

Quinolones and its derivates are essential anti-malarial drugs since they have the ability to inhibit the hemozoin formation process within malaria parasites. The most significant anti-

malarial drugs belonging to this group include: chloroquine, amodiaquine, amopyroquine, tebuquine, mepacrine, pyronaridine, halofantrine, quinine, epiquinine, quinidine and bisquinoline as summarized in Tabel 1.1. (Slater, 1993)

Table 1.1: A comparison between the inhibiting effect of different quinolones on hemozoin formation as well as their efficacy against different strains of *P. falciparum* (Hawley *et al*., 1998; Portela *et al*., 2004)

Anti-malarial drugs	**Aggregation of hematin** IC_{50} **(μM)**	**Anti-malarial action**	
		IC_{50} **(nM)** **Chloroquine sensitive *P. falciparum***	IC_{50} **(nM)** **Chloroquine resistant *P. falciparum***
Chloroquine	24.4	14.0	192.1
Amodiaquine	15.1	7.8	18.5
Amopyroquine	29.5	5.3	11.5
Tebuquine	52.7	9.5	13.1
Pyronaridine	64.4	5.7	9.1
Mepacrine	41.0	12.9	43.3
Mefloquine	46.9	23.4	9.4
Halofantrine	184.5	5.8	2.8
Quinine	64.8	34.2	81.2
Epiquinine	-	3471	1179
Quinidine	24.0	21.5	50.6
Bisquinoline	97±8	123±25	25±3

1.8.1) Chloroquine

Chloroquine (Figure 1.4) has always been the preferred first-line drug in the treatment and management of malaria, especially against *P. falciparum*. This drug has been used since the 1940's due to its affordability and tolerability (Wernsdorfer and Payne, 1991). Its efficacy against *P. falciparum* malaria has however been compromised by drug resistance during the last couple of years. It is none the less still the preferred drug in the treatment of chloroquine sensitive *P. falciparum* malaria (Wongsrichanalai *et al*., 2002; Rosenthal, 2004).

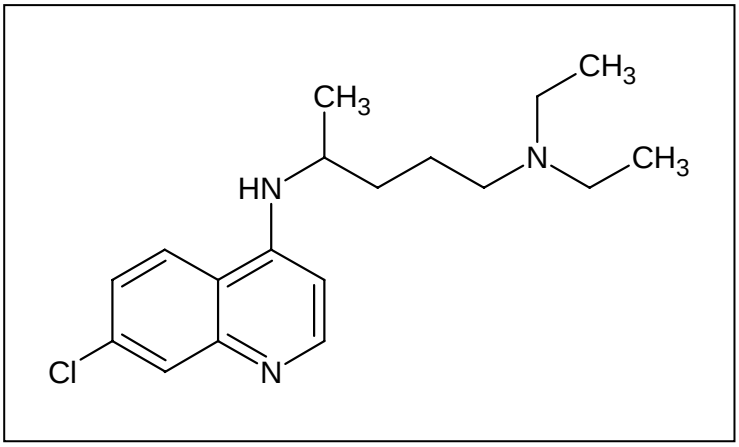


Figure 1.4: Structure of chloroquine, $C_{18}H_{26}ClN_3$ [1, 4-Pentanediamine, N^4 – (7-chloro-4-quinolinyl)-N^1, N^1 -diethyl-7-Chloro-4-[[4-(diethylamino)-1-methylbutyl]amino]quinoline (USP, 2010)].

Chloroquine bears a resemblance to 4-aminoquinoline and is available in the form of a 100 mg/150 mg phosphate salt or sulfate tablet, a syrup which contains 50 mg/5 ml chloroquine phosphate or sulphate as well as intramuscular (IM) and intravenous (IV) injections. Chloroquine is an amphiphilic weak base which is a water soluble drug with a molecular weight of 319.872 g/mol and a pKa value of 10.1. The drug is known to accumulate within the food vacuole of the parasite due to a pH gradient (Yayon *et al*., 1984; Rosenthal, 2004; WHO, 2006; USP, 2010).

1.8.1.1) Pharmacokinetics

The drug is quickly and almost entirely absorbed from the gastrointestinal tract (Rosenthal, 2004). Chloroquine reaches its peak plasma concentration at approximately 30 minutes after the oral administration of a single 10 mg/kg dose and is significantly higher than the therapeutic level required for chloroquine-sensitive *P. falciparum* parasites (RBM, 2010). Within 3 hours

chloroquine achieves its maximum plasma concentration and is quickly distributed throughout the body (White, 1998; Rosenthal, 2004). Chloroquine has a tissue binding tendency. It readily binds to melanin containing tissues of the skin and eyes for example; it also has the tendency to concentrate within erythrocytes. Chloroquine has the benefit of increasing its peak plasma concentration within parasitic erythrocytes (White, 1998; WHO, 1990; RBM, 2010). Chloroquine has a terminal half life that ranges from 30 to 60 days (Rosenthal, 2004).

Only 30 % of chloroquine is slowly and partially metabolized by the liver via the de-ethylation of its side chain in order to form monodesethyl- and disdesethylchloroquine. The de-ethylation of chloroquine is followed by its dealkylation. Desethylchloroquine has similarities to chloroquine with regards to its pharmacokinetic profile and anti-malarial action. The drug elimination process of chloroquine occurs gradually and it exhibits an elimination half-life of approximately 10 days. Chloroquine is primarily excreted within the urine (70 %), with desethylchloroquine accounting for 25 % of the total excreted drug (WHO, 1990; White, 1998; Rosenthal, 2004).

1.8.1.2) Pharmacology

Chloroquine is an extremely effective blood schizonticide and reasonably efficient against the gametocytes of *P. vivax*, *P. Ovale* and *P. malariae*. Chloroquine is ineffective against the gametocytes of *P. falciparum* and inactive against the liver stage parasites within the host (Rosenthal, 2004).

Uncertainty still exists concerning chloroquine's exact mechanism of action. Chloroquine most likely inhibits the polymerization of heme, a parasitic toxin resulting from the degradation of hemoglobin, into hemozonin by accumulating inside *P. falciparum's* food vacuole. Chloroquine accumulation increases the internal pH in the parasitic food vacuole to the alkaline nature of the compound. This results in the obstruction of the conversion process, where toxic heme is converted to hemozoin, by inhibiting the biocrystallization of hemozoin. The parasites are therefore poisoned by the excess heme which reaches toxic levels and in turn lead to parasitic cell lysis and eventually the death of the parasites. Drug resistance however, renders chloroquine ineffective against *P. falciparum* (Rosenthal, 2004; WHO, 2006).

Chloroquine may furthermore have other possible mechanismes of action which include its interference with the biosynthesis of parasitic nucleic acids as well as the formation of a chloroquine-heme or chloroquine-DNA complex (WHO, 2006).

1.8.1.3) Drug treatment regimes

The main objectives regarding anti-malarial drug treatment include:

- the prevention of mortality;
- the prevention of malaria related complications;
- the reduction of malaria transmission by eliminating the parasitemia and;
- to limit the appearance and spread of drug resistance (Department of Health, 2002).

A dosage regime of 25 mg/kg chloroquine over a 3 day time period should be administered to adults and children who require chloroquine treatment. An initial dose of 10 mg/kg should be administered followed 6 to 8 hours later with a 5 mg/kg dose along with a 5 mg/kg dose during each of the following 2 days. An additional treatment regime requires an initial dose (on the first day) of 10 mg/kg followed by a 10 mg/kg dose on the second day and a 5 mg/kg dose on the third day. Table 1.2 provide specific information regarding the daily dosage regime for each specific age group in accordance to their weight (RBM, 2010).

Table 1.2: The recommended dossages for chloroquine treatment (RBM, 2010).

		Amount of tablets					
		100 mg tablets			150 mg tablets		
Weight (kg)	**Age (Years)**	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
5-6	< 4 months	0.5	0.5	0.5	0.5	0.25	0.25
7-10	4-11 months	1	1	0.5	0.5	0.5	0.5
11-14	1-2	1.5	1.5	0.5	1	1	0.5
15-18	3-4	2	2	0.5	1	1	1
19-24	5-7	2.5	2.5	1	1.5	1.5	1
25-35	8-10	3.5	3.5	2	2.5	2.5	1
36-50	11-13	5	5	2.5	3	3	2
50+	14+	6	6	3	4	4	2

The chemoprophylactic treatment of *P. falciparum* malaria by means of chloroquine requires a weekly single dose of 5 mg/kg or 10 mg/kg per week, divided into 6 daily doses (RBM, 2010).

The single prophylactic treatment of chloroquine is recommend in regions which contains chloroquine sensitive parasites; these regions include the Dominican Republic, parts of Ecuador, Haiti and Tajikistan. Chloroquine may be recommended in combination with a daily proguanil dose of 200 mg in regions which contain chloroquine resistant *P. falciparum* parasites. The regions include parts of the Arabian Peninsula, parts of Asia, Namibia, Mauritania and parts of Colombia (RBM, 2010).

It has been established that the daily chloroquine dosage regime of 100 mg in adults have a higher efficacy in comparison to the weekly chloroquine dosage regime. The daily dosage regime isn't as suitable as the weekly dosage regime when dealing with long-term travellers, due to the fact that double dosages are administered with the daily dosage regime which will probably result in adverse effects. It has however not been established whether the proguanil-

chloroquine combination has a difference in efficacy when comparing the daily dosage regime with the weekly dosage regime. A proguanil-chloroquine combination tablet is available in numerous countries. The tablet consists of 100 mg chloroquine and 200 mg proguanil hydrochloride and has the ability to increase compliance in adults. Table 1.3 provide specified information regarding the weekly dosage regime for each specific age group according to their weight (RBM, 2010; Ward, 1999).

Table 1.3: The recommended dosages required for the chemoprophylactic treatment of chloroquine (RBM, 2010).

		Amount of tablets per week	
Weight (kg)	**Age (Years)**	**100 mg tablets**	**150 mg tablets**
5-6	< 4 months	0.25	0.25
7-10	4-11 months	0.5	0.5
11-14	1-2	0.75	0.6
15-18	3-4	1	0.75
19-24	5-7	1.25	1
25-35	8-10	2	1
36-50	11-13	2.5	2
50+	14+	3	2

1.8.1.4) Adverse effects

Severe adverse reactions are very seldomly experienced following the administration of chloroquine according to acceptable dosage regimes. In fact chloroquine is generally well tolerated even with the long-term employment of the drug. Pruritus on the other hand is commonly experienced by dark-skinned people. Calamine lotions occasionally alleviate the unbearable symptoms. Alternative drug therapy should thus be considered in future malaria infections in these patients (Rosenthal, 2004).

Symptoms which are frequently experienced after the administration of chloroquine include abdominal pain, anorexia, blurred vision, gastrointestinal symptoms, headache, malaise, urticaria, nausea and vomiting. These symptoms can be avoided by having a meal prior to the administration of chloroquine. Susceptible individuals may experience sudden acute porphyria and psoriasis attacks. Symptoms which seldomly occur include alopecia, agranulocytosis, bleaching of the hair, exfoliative dermatitis, hypotension, leukopenia and neurological disorders such as neuromyopathy, ototoxicity, polyneuritis, psychosis and convulsions. Permanent visual impairment caused by the accumulation of chloroquine within the retina of the eye, due to the prolonged use of elevated chloroquine dosages, is a rare and distinguished complication (WHO, 1990; Rosenthal, 2004; RBM, 2010). Severe hypotension, cardiac arrest and respiratory arrest can be the result of rapid intravenous infusions or large intramuscular injections of chloroquine hydrochloride (Rosenthal, 2004).

1.8.1.5) Resistance

Anti-malarial drug resistance occurs when a parasitic strain is capable of survival and/or multiplication in spite of the presence of an anti-malarial drug which have been administered within the suggested dosage. Anti-malarial drug resistance may also occur when the anti-malarial drug is administered in a dosage higher than the suggested dose provided that it remains endurable for the host (WHO, 1973).

The development and distribution of drug resistance can be attributed to numerous factors. These factors relate to the interactions that exist between the drug, the human host and the malaria parasite. The rapidity at which drug resistance develops mainly depends on the drug's molecular mechanism. Drug resistance is additionally enhanced, especially within high transmission areas, by drugs with extended terminal elimination half-lives. An increase in drug pressure also contributes to the development of drug resistance. When drugs are administered at sub therapeutic dosages malaria parasites might possibly be exposed to inadequate drug levels which may lead to the development of drug resistant parasites. Two main parasitic factors, which are associated with the development of drug resistance, include the transmission intensity of malaria via the malaria parasites and the specific *Plasmodium* species involved. The specific host immunity level as well as the unnecessary or widespread use of anti-malarial drugs may contribute to the development of drug resistance (Watkins & Mosobo, 1993).

During the last couple of years the malaria parasite started to develop a resistance to most of the drugs used in the anti-malarial treatment regime. *P. falciparum* resistance to chloroquine was first documented during the late 1950's and early 1960's in Southeast Asia, Oceania and South America (CDC, 2004). Drug resistance has also been suspected in Thailand since 1957

and has been established in patients in Colombia and Thailand during the 1960's. During 1978 resistance were documented in East Africa and between 1978 and 1988 in all of the tropical African countries (Trape *et al*., 1998). Since then malaria's resistance to chloroquine has increased and spread rapidly all over the world. According to literature 50% of patients living in Ethiopia and most East African contries experience symptoms and recrudescent parasitemias within 14 days following anti-malarial drug treatment. The *P. falciparum* parasite has furthermore gained resistance to almost all anti-malarial drugs which are currently available (CDC, 2004). The occurrence of drug resistance of various *P. falciparum* strains against many of the single regime anti-malarial drugs can be attributed to ineffective dosage regimes and/or patient non-compliance (Goodman & Gillman, 2001).

P. falciparum's resistance to chloroquine could be explained as a persistent ejection of the drug by the parasite. A suboptimal concentration of the drug inside of the parasite's food vacuole is the result of this efflux system, which occurs 40 to 50 times quicker amongst resistant parasites in comparison to sensitive parasites (Krogstad, 1987; Foley & Tilley, 1997. As a result chloroquine can't hinder the parasite's heme polimerazion (Foley & Tilley, 1997).

1.9) Conclusion

Malaria is a re-emerging deadly disease which is mainly caused by *P. falciparum* and accounts for more than 300 million severe infections annually. Malaria is currently a huge threat worldwide and claims at least one million lives per annum globally. It needs to be diagnosed and managed as soon as possible in order to prevent morbidity and mortality, especially within children and pregnant women.

A clear understanding of the heme toxicity and its detoxification system, by means of the hemozoin formation mechanism within the malaria parasite, will definitely help to design new and effective antimalarial drugs.

The development of malaria vaccines in the near future could effectively resolve the ongoing malaria transmission and infections.

Patient non-compliance as well as the widespread use of anti-malarial drugs may be a contributing factor regarding *P. falciparum's* drug resistance to chloroquine. Chloroquine is a well tolerated and affordable drug which could be used more extensively in combination with other anti-malarial drugs with the aid of novel delivery systems.

CHAPTER 2

Liposomes as colloidal drug delivery system

2.1) Introduction

A lack in economical encouragement hinders new drug discovery and development of new drugs for the treatment and control of parasitic diseases. Less than 1 % of the 1223 novel drug developments between 1975 and 1996 were introduced to the markets for the treatment of tropical infections such as malaria. A disregard towards the discovery of new anti-parasitic drug formulations continued until the year 2000. Only 0.1 % of the global health research community were committed to anti-parasitic disease drug development during this time (Pink *et al*., 2005). The development of novel delivery systems for the delivery of existing anti-parasitic drugs for the treatment of tropical infections poses a real challenge. The aim of novel delivery systems is to improve the efficacy, specificity, acceptability and therapeutic index of existing anti-parasitic drugs (Date *et al*., 2007).

In order to design novel drug delivery systems for the treatment of malaria there are certain aspects that needs to be considered:

- the interactions between the parasite and the host cell,
- the type of biological barriers which needs to be traversed by the drug to enable the targeting of organs, tissue and cells,
- detailed information regarding the type of receptors which are present on parasite infected cells,
- changes that occur within infected cells after parasitic invasion or due to the succession of the disease,
- the absence specific receptors and/or antigens on the surface of the parasite and
- the pathophysiology associated with the infection (Tempone *et al*., 2004).

The main objective when designing a novel drug delivery systems should be to promote the modulation of the pharmacokinetic and physicochemical properties of the anti-parasitic drugs. This will result in improved biospecificity (targetability) as opposed to bioavailability which will

simultaneously lead to the reduction of adverse effects associated with the specific drug (Date *et al*., 2007).

Colloidal drug delivery systems are significant due to their versatility and various benefits. Many scientists have investigated their potential for applications for effective drug delivery (Date *et al*., 2007). During 1861 Graham created the term colloid from the Greek word “Rolla”, which means glue. Colloidal drug delivery systems can be described as systems which consist of a minimum of two components. One of these components, which may be either small particles or large molecules, should be distributed within the other component. A system needs to comply with certain criteria, regarding their size range, in order to be classified as a colloidal drug delivery system: The system should first of all contain an adequate amount of small molecules or particles, which in turn leads to the domination of thermal forces on gravitational forces, which enables the system to remain within a suspension. Secondly the molecules and particles of the system should additionally be adequately large in relation to the dispersed fluid’s molecular dimensions in order to obtain consistent fluidal properties. The size of colloidal drug delivery systems normally ranges between 1 nm and 1 µm. A system may be classified as a colloidal drug delivery system if one of the particles’ dimensions fall within these established size ranges (Burgess, 2006).

2.2) Classification of colloidal drug delivery systems

The physical form and/or functional properties of colloidal drug delivery systems determine their specific classification. Colloidal drug delivery systems may be divided into three groups: **First-generation systems** which include microcapsules and microspheres that have the ability to deliver active substances to their required target. These systems however need to be inserted very close to the required site of action and can therefore not be referred to as carriers. **Second-generation systems** include passive colloidal carriers for example liposomes, nanocapsules and nanospheres as well as active carriers such as temperature-sensitive liposomes and magnetic nanospheres. The active carriers have the ability to release their contents in response to a particular signal. Second-generation systems are typically 1 µm in diameter and either particulates or soluble. These systems have the ability to release active substances to the required target site as well as the ability to carry the active substances to the required target site by means of a general delivery route. **Third-generation systems** include monoclonal antibodies as well as particulate systems of the second-generation (such as liposomes, nanocapsules and nanospheres) which are guided by means of monoclonal antibodies or other ligands. These systems have the ability to recognize the specific target and can be classified as true carriers (Barratt *et al*., 2002).

Table 2.1: Classification and typical applications of colloidal drug delivery systems (Hiemenz, 1986)

Type of delivery system	Mean particle diameter (micrometers)	Classification of delivery system	Typical applications of delivery system
Microspheres, Hydrogels	0.5-20	Alginate, chitosan, gelatin and polymeric hydrogels	Sustained release of active substances
Microparticles	0.2-5	Microspheres, polylactide and polystyrene	Targeted delivery of active substances
Emulsions, Micro-emulsions	0.15-2	Lipid emulsions, oil in water emulsions, water in oil emulsions and micro-emulsions	Targeted and controlled delivery of active substances
Liposomes	30-1000	Bilayer vesicles consisting of phospholipids and polymers	Targeted delivery of active substances
Micelles	3-80	Natural and synthetic surfactant micelles	Targeted delivery of active substances
Nanoparticles	2-100	Inorganic nanoparticles, lipid nanoparticles and polymer nanoparticles	Targeted delivery of active substances as well as navigational appliance *in vivo*
Nanocrystals	2-100	Quantum dots	Imaging agents

Tabel 2.1 provides a summary of the different types of colloidal drug delivery systems which are commonly used. The size of the systems is indicated along with its general classification and typical application. This study focussed on liposomes as a colloidal drug delivery system. The

structural characteristics, manufacturing techniques, mechanism of drug incorporation and different liposome types will be discussed. The application of liposomes as a colloidal drug delivery system for the treatment of malaria will also be investigated.

2.3) Liposomes as a colloidal drug delivery system

Liposomes were first discovered in the early 1960's by A.D. Bangham (Wang, 2005). They were considered to be efficient drug delivery systems due to their high degree of biocompatibility, biodegradability and their ability to carry drugs, which were encapsulated inside of the vesicle, to a targeted cellular site (Redziniak & Perrier, 1996; Wang, 2005).

Biophysicians were the first to make use of these vesicles as biological membrane models. This enabled them to study the physiological properties and in particular the permeability of the vesicles. During 1965 Bangham named these vesicles; he referred to them as liposomes. He revealed that liposomes are closed systems which have the ability to nearly spontaneously develop from natural or synthetic phospholipids. The above mentioned only happens when liposomes are in the presence of an aqueous medium (Redziniak & Perrier, 1996). Liposomes are essential as models for biomembranes, drug formulation and gene delivery. They are also used as vehicles for the delivery of a variety of agents which are used in cancer therapy. Liposomes are particularly efficient in delivering drugs to the phagocytes of the immune system (Wang, 2005).

2.4) Structural characteristics of liposomes

Liposomes are synthetic, spherical, microcapsule aggregates which consist of cholesterol and amphiphilic molecules such as phospholipids (Redziniak & Perrier, 1996; Wang, 2005). Phospholipids belong to the class glycerophospholipids and consist of a polar hydrophilic head (e.g., choline, serine, inositol) and a hydrophobic tail (fatty acids). These phospholipids are the main components of cell membranes. Phospatidylcholine (PC) is the most natural and universally used phosholipid which originates from soyabeans and hen eggs (Patil *et al.*, 2005). Phospholipids function as a selective barrier and holder of the membrane proteins, they are also capable of encapsulating active ingredients in their structure (Redziniak & Perrier, 1996). The dispersion of phospholipids in water causes the hydrophobic tails to aggregate whilst the hydrophilic heads come in contact with the water. The above mentioned causes the creation of a layer that consists of fatty acid tails directed to the inside of the membrane and polar heads directed to the outside. Vesicles are formed when phospholipids swell and hydrate in water. These vesicles are either made of multiple or single concentric bilayers and are either surrounded or divided by single or several aqueous compartments. With consideration to the

size and number of vesicle lamellae, vesicles can be classified as either small or large unilamellar vesicles. Large multilamellar vesicles can be defined as vesicles which consist of various concentric bilayers (Lasic, 1996). Cholesterol is an amphipathic molecule which can be incorporated within phospholipid membranes in ratios of 1:1 and 2:1 of cholesterol to phospatidylcholine. The cholesterol incorportation eradicates normal hydrogen bonding and electrostatic interactions (Patil *et al*., 2005). Liposome vesicles range from 30 µm to 1000 µm in diameters (Sharma & Sharma, 1997).

2.5) Manufacturing of liposomes

Liposomes can be manufactured by various methods, depending on the intended application of the liposomal formulation. All of these methods include the combination of phospholipids with an aqueous phase. Despite all the different methods two methods are mainly employed to manufacture liposomes namely the emulsion method and the hydration method (Chrai *et al*., 2002).

The **emulsion method** includes the dissolution of phospholipids in an organic solvent such as methylene chloride. It is then followed by the controlled addition of phospholipids to a vigorously agitated aqueous medium. The organic solvent is then eradicated under reduced pressure. Extrusion and/or filtration processes determine the size distribution of the resulting liposomes. Liposomal formulations intended for drug delivery include aerosols, dry vesicular powders meant for reconstitution, creams or lotions and semisolid forms such as gels. These formulations also include liposomes which can be administered via the oral, parental and topical routes (Chrai *et al*., 2002).

The **hydration method** comprise of the combination and dissolution of the produced lipids within an organic solvent such as chloroform, methylene chloride, methanol tertiary butanol etc. Evaporation, lyophilization or vacuum drying procedures are subsequently used in order to remove the organic solvent. This is followed by the hydration of the dry cake, film or paste like appearance of the lipid. An ethanol or propylene glycol lipid solution can alternatively be added to the aqueous phase and the solvent then removed by means of dialysis, filtration or chromatography processes. Large multilamellar vesicles are produced before the hydration process. After the hydration process it is possible to reduce the size of the multilamellar vesicles by means of extrusion, homogenization or sonification (Chrai *et al*., 2002).

Unilamellar vesicles (ULV) consist of a single lipid bilayer and an inner aqueous core. ULV can be prepared in various sizes which range from as small as 20 nm to several micrometers in diameter. ULV can be divided into two groups namely small ULV and large ULV. In

comparison to MLV, ULV has a considerably smaller size distribution within a preparation (Margalit & Yerushalmi, 1996).

Multilamellar vesicles (MLV) are the oldest type of liposomes. They consist of concentric shells of lipid bilayers with water between the shells along with an inner aqueous core. These liposomes have the ability to form spontaneously when an appropriate interaction exists between the lipids and water. This is only made possible when the correct use of lipids and technical conditions have been met. MLV typically contain 8-15 concentric shells which range in size from 0.5 to several micrometers in diameter. MLV preparations will therefore typically be fairly heterogeneous in terms of their liposome sizes (Margalit & Yerushalmi, 1996).

Figure 2.1 describes how multilamellar and unilamellar liposome vesicles are produced by means of the hydration method.

1- Addition of water

2- Agitation

3- Extrusion

4- Sonication/Homogenization

5- Small unilamellar vesicles

6- Large unilamellar vesicles

Figure 2.1: An illustration of the manufacturing process of different types of liposome vesicles. This figure was adapted from the Pharmaceutical information for you website, www.pharmainfo.net/reviews/liposome-versatile platform for targeted delivery of drugs

2.6) Mechanism of drug incorporation within liposomes

Drugs are mainly incorporated within liposomes via encapsulation, partitioning and reverse loading. These mechanisms influence the specific performance characteristics of liposomal delivery systems.

ULV and MLV accommodate hydrophilic and hydrophobic drugs in their aqueous and lipid compartments respectively (Margalit & Yerushalmi, 1996). The hydrophobic and hydrophilic layers of liposomes enable the entrapment of hydrophobic molecules within the bilayer and water-soluble materials within the aqueous compartments of the liposomes. The non-polar bilayer enables the entrapment of extremely nonpolar drugs while the encapsulation of polar molecules occurs within the aqueous cavity (Wang, 2005).

2.6.1) Encapsulation

Water soluble drugs are suitable for the encapsulation process which entails the simple hydration of a lipid within an aqueous drug solution. The dissolved drug is passively entrapped within the liposomal interlamellar spaces during the production of the liposomes which in turn leads to a small encapsulated volume. The extent of the drug incorporation within liposomes are determined by the physicochemical properties of the drug, for example its partition coefficient and solubility (Patil *et al*., 2005).

Figure 2.2 illustrates a water soluble drug encapsulated within the aqueous medium of a liposome.

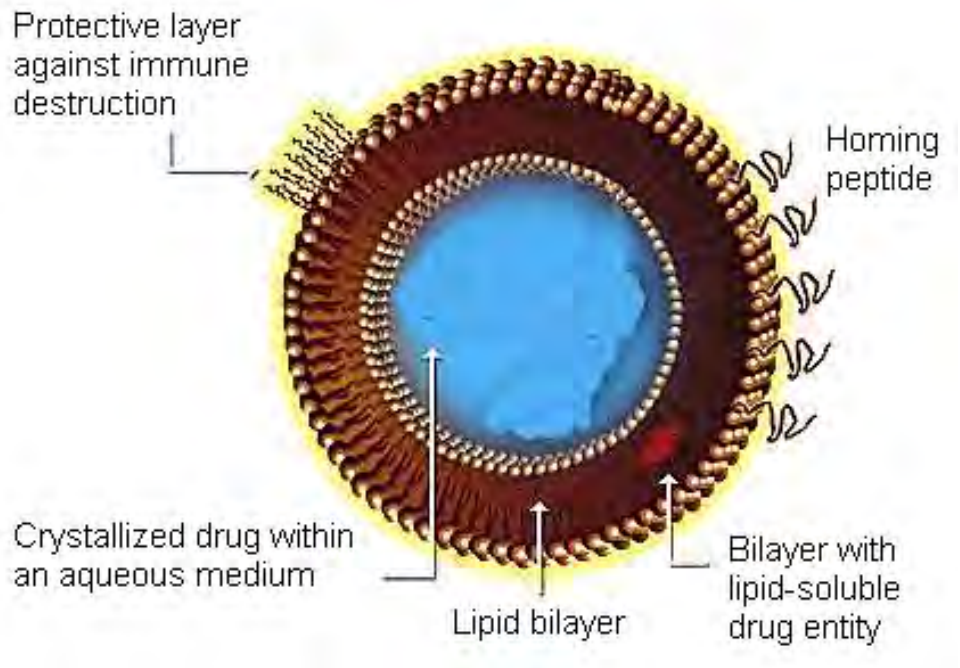


Figure 2.2: An illustration of the appearance of an encapsulated drug within a liposome. The figure was adapted from the Liposomal Encapsulation Technology web site, www.racehorseherbal.com/Infections/LET/let.htm

2.6.2) Partitioning

The partitioning process entails the dissolution of organic soluble drug substances in conjunction with phospholipids in an appropriate organic solvent. This is followed by a drying process and a subsequent addition of the dried solvent to the aqueous phase. Any additional solvent residues are then removed by means of vacuum suction. A soluble drug environment is provided through the acyl chains of the phospholidpids which arer located within the intrabilayer space of the liposome (Chrai *et al*., 2002).

2.6.3) Reverse loading

Drugs which are compatible with both charged and neutral forms may be employed in the reverse loading process, which is based on the environmental pH. The drugs are added to an uncharged aqueous phase in order to permeate through the liposomal bilayers with the aim to finally result within the liposomes. The drug molecules are then charged by adjusting the internal liposomal pH. This leads to drug molecules with impaired lipophilic characteristics. These characteristics unable them to cross the liposomal lipid bilayer and therefore they can only return to the external medium (Gulati *et al*., 1998).

There are various factors which have an effect on the encapsulation efficiency of drugs encapsulated within liposomes. These factors are influenced by the hydrophilic or lipophilic properties of the encapsulated drugs which have a tendency to interact with the membrane bilayer. These liposomal properties include:

- the volume of the aqueous medium;
- the rigidity of the membranes;
- the area of the surface and:
- the techniques used for preparation (Nii *et al.,* 2002).

2.7) Characterization of liposomes

The rigidity of the liposome's bilayer, its charge, morphology (i.e. multilamellar, multivesicular, unilamellar) and size are all factors which influence the *in vivo* and *in vitro* performance of the liposomes. The characterization of liposomes is therefore essential for experimental purposes (Storm & Crommelin, 1998). Liposomes can be characterized in terms of their drug

concentration, cholesterol concentration, osmolarity, pH, phospholipid composition and phospholipid concentration (Barenholz & Crommelin, 1994).

Two factors are responsible for the limitation in the shelf-life of liposomes. These factors include physical instability due to drug leakage from the liposomal bilayer as well as the aggregation and fusion of liposomes. Chemical instability due to the oxidation of unsaturated acyl groups or via the hydrolysis of the ester bonds may also lead to a limited shelf life. The selection of low temperatures as well as an environmental pH of 6.5 may possibly reduce hydrolysis. The prevention of oxidation is possible by selecting acyl-chains within the phospholipids, by eliminating oxygen on the inside of the injection vial or by including an anti-oxidant such as vitamin E (Grit *et al*., 1993; Zuidam *et al*., 1996).

2.8) Toxicity of liposomes

Liposomes may be sterilized via gamma irradiation (Woods & Pikaev, 1994; Reid, 1995). This process, however, results in the peroxidation of the unsaturated phospholipids in the liposomes (Zuidam et al., 1995; Stensrud et al., 1996). Peroxidation leads to the production of lysolecithin (LPC) and peroxidised lipids which may enhance the toxic potential of liposomes. These degradation products have the ability to induce hemolysis of red blood cells (Weltzien, 1979; Kobayashi *et al*., 1985). Degradation products such as diverse hydrocarbons, free fatty acids, lysophospholipids and phosphatidic acid has also been identified as a result of the gamma irradiation process (Tinsley & Maerker, 1993; Zuidam *et al*, 1996). Additional toxic effects were experienced with liposomes which consist of negatively charged phospholipids in comparison to liposomes which consist of neutral phospholipids (Juliano *et al*., 1983; Bonté *et al*., 1987; Zbinden *et al*., 1989) The abovementioned is also true for unsaturated phospholipids in comparison to saturated phospholipids (Stensrud *et al*., 1999). The negatively charged liposomes inhibit reversible platelet aggregation which leads to an increased time in whole blood clotting (Juliano *et al*., 1983; Bonté *et al*., 1987; Zbinden *et al*., 1989). The gamma irradiation process has the ability to create sterile, non-toxic, liposomes provided that the liposomal phospholipids are carefully selected (Stensrud *et al*., 1999).

2.9) Types of liposomes

Liposomes can be divided into four different groups in terms of their composition and *in vivo* application namely: conventional liposomes, stablilized liposomes (long-circulating liposomes), immuno-liposomes and cationic liposomes (Allen et. al., 1995; Crommelin et al., 1997; Storm & Crommelin, 1998; Wang, 2005).

Conventional liposomes can be described as lipid vesicles which consist of assorted phospholipids, glycolipids and other lipids with no derivatization in order to enhance the circulation time. Conventional liposomes are primarily metabolized in the liver and spleen by macrophages. They are also slightly metabolized by the reticuloendothelial system (RES) and the monoclear phagocytic system (MPS). The rapid clearance of liposomes had a negative effect on their development as drug delivery systems even though they have been explored to treat diseases that affect the phagocytes of the immune system (Wang, 2005).

Stabilized or long-circulating liposomes were developed to reside in the bloodstream for longer periods and can extravagate to sites in the body where permeability of the vascular wall is more favourable (Allen *et. al*., 1995; Storm & Crommelin, 1998). During the late 1970's attempts were made to modify the biodistribution of conventional liposomes by means of either surface ligands or membrane compositions. The outcome of the attempts revealed the ability to adjust the liposome disposition within the mononuclear phagocytic system which also includes the intra-heptic uptake itself. The best results, however, were achieved by substituting these two lipids with synthetic lipids which contain polymers. When polyethylene glycol was covalently bound to the phospholipid it resulted in the achievement of the longest circulation times. The longest blood circulation time were achieved when the bilayer contained intermediate molecular weights which range from 1500 to 5000 Da (Lasic, 1996). Methods with regards to the stabilization of liposomes are required since conventional liposomes also have stability problems. Sterically stabilized liposomes (SSLs), also known as "stealth liposomes", were a significant development. Synthetic polymers are employed for steric stabilization. Additional approaches include cross-linking membrane components which are covalently bonded, the polymerization of polymerizable lipids and the utilization of uncommonly stable archaebacterial membrane lipids (Wang, 2005). Improved management of colloidal properties are attained by the surface grafting of polymers. Bridging interactions between liposomes can cause the surface absorption of polymers to leak encapsulated molecules or to possibly aggregate liposomes. The enhanced stability is believed to be attributable to the polymer which is attached to the surface which acts as a steric barrier against interacting macromolecules (Lasic, 1996).

Immuno-liposomes have antibodies attached to their surface in order to enhance their binding to target cells (Storm & Crommelin, 1998). Immuno-liposomes are mainly used in tumor targeting in cancer patients (Crommelin *et al*., 1997).

Cationic liposomes are composed of cationic lipids which enable them to interact with negatively-charged DNA (Storm & Crommelin, 1998). Cationic liposomes have also been employed as carriers for chemotherapy drugs since their positive charge enables them to target tumor cells (Wu *et al*., 2007).

2.10) Advantages and disadvantages of liposomes

Liposomes have both advantages and disadvantages associated with their employment. Many of these aspects have been mentioned in the previous sections, but in this section their specific advantages and disadvantages will be discussed in depth.

2.10.1) Advantages

1. Liposomes have numerous properties which are useful for various applications. It is important to consider their structure, chemical composition and colloidal size. The most vital properties of liposomes are their small, uniform and controllable size; their unique membrane and surface characteristics, which include bilayer phase behaviour. The bilayer phase behaviour of liposomes include their permeability and mechanical properties; their charge density; and the presence of either surface-bound or joined polymers, or the connection of exceptional ligands respectively (Redziniak & Perrier, 1996).

2. Liposomes have the ability to improve the bioavailability, diminish the toxicity, enhance the efficacy and to improve the therapeutic index of the entrapped drugs (Wang, 2005).

3. Huge success have been experienced with liposomes during *in vivo* studies due to their lipid composition, the alteration temperature of the melting phase of the phospholipid, the preparation method, their lamellarity, their size and their surface charge (Bakker-Woudenberg, 1995).

4. Liposomes have an adaptability and amendability concerning surface area modification like immuno-liposomes for example (Crommelin *et a*l., 1997).

5. Liposomes have promped research on their application as intracellular targets on bacterial and parasitic infections, due to the fact that they are rapidly removed by the phagocytic cells of the liver and the spleen after intravenous injection and are partially localized in lysosomes (Couvreur *et al*., 1991).

6. Liposomes display unique biological characteristics, when composed of natural lipids, which include biocompatibility and biodegradability along with specific interactions with biological membranes and different cells (Redziniak & Perrier, 1996).

7. Liposomes have the ability to entrap both hydrophilic and hydrophobic drugs due to their amphiphilic character and enormous surface to volume ratios (Couvreur *et al*., 1991).

8. Liposomes can be used for various applications such as solubilizing agents for unsoluble substances, dispersants, sustained-release, the delivery of micro-encapsulated substances, stabilizers, protective agents and micro-reactors (Lasic, 1996).

2.10.2) Disadvantages

1. Depending on the specific composition and/or surface modification liposomes may be manufactured at a high expense (Wang, 2005).

2. The drugs or molecules which are encapsulated within liposomes may start leaking and/or fusing with each other. However, various experiments demonstrated how liposomes were able to rapidly release the encapsulated or membrane-bound molecules when administered intravenously. It has been established that the addition of cholesterol to the liposomal membranes results in a significant reduction in the above mentioned leakage (Redziniak & Perrier, 1996).

3. The phospholipids in liposomes may undergo oxidation and hydrolysis.

4. Liposomes tend to interact poorly with certain drugs (Wang, 2005).

5. Stability problems have been associated with liposomes. It is extremely difficult to maintain stable liposomes throughout circulation whilst trying to selectively enhance the bioavailability of drugs at their specific tissue target area (Lasic, 1996).

6. Liposomes have a short half life.

7. Liposomes have a prolonged onset of action (Wang, 2005).

The disadvantages mentioned pertain to specific types of liposomal formulations. Numerous modifications can be made to the liposomes to limit these disadvantages. The application of liposomes should carefully be considered and as with all formulations, the advantages and disadvantages carefully weighed to achieve an acceptable formulation.

2.11) Applications of liposomes

Liposomes have been succesfully used as carriers for anticancer drugs and antimicrobial agents as well as for the delivery of macromolecules which include DNA and proteins. They have also been used for the delivery of drugs intended for cardiovascular diseases and diabetes (Wang, 2005).

Various liposome formulations have been used in clinical trails of which only a few have been approved by the Food and Drug Association (FDA) as successful liposome-based drugs. The approved formulations include:

- the delivery of doxorubicin (Doxil and Myocet) plus daunorubicin (DaunoXome) as anticancer agents and
- the delivery of amphotericin B (AmBisome, Amphotect, ABELCET) as an antifungal agent (Wang, 2005).

A significant reduction in drug toxicity plus maintained or enhanced efficacy of active compounds has been associated with liposome formulations of these specific drugs (Wang, 2005).

2.12) Liposomes as drug delivery system in the treatment of malaria

The main objectives concerning the encapsulation of anti-malarial drugs in liposomes are:

- to protect the drug from degradation and to promote sustained release and
- to minimize drug associated adverse effects (Date *et al*., 2007).

Various anti-malarial drugs have been encapsulated in liposomes such as ß-artemether, artether and chloroquine. The encapsulation of β-artemether in liposomes has been investigated for the treatment of recrudescent malaria. The report demonstrated the preparation of egg phosphatidylcholine cholesterol liposomes with an entrapment efficiency of 100 %. The report also demonstrated how the recrudescent parasitemia in *Plasmodium Chabaudi*-infected OF1 mice was effectively evaded. The addition of phosphatidylcholine was thought to provoke an immune response against recrudescent malaria (Chimanuka *et al*., 2002).

Liposomes have been employed to safely and effectively deliver primaquine. The accumulation of primaquine, entrapped within negatively charged liposomes, took place in the spleen and the liver. The encapsulated drug's accretion within the spleen and the liver were confirmed to be more significant than its accumulation within the brain, heart, kidneys and lungs. This subsequently led to a reduction in the encapsulated drug's toxicity in comparison to the administration of primaquine on its own. Within 20 minutes subsequent to the perfusion of the encapsulated drug it reached a plateau of 60 % of its initial loading dose (Pirson *et al*., 1980).

The encapsulation of arteether in dipalmitoylphosphatidylcholine, dibehynoylphosphatidylcholine and cholesterol liposomes appeared to remain in the gastrointestinal tract for an extended period of time when administered orally. The drug also demonstrated an elevated bioavailability, an elevated C_{max} and a shorter T_{max} along with an elevated AUC value in comparison to its aqueous suspension. A longer elimination half-life was observed with these liposomes when they were administered intravenously. The liposomes were also found to be within a stable condition with an entrapment efficacy of 100 % during a storage time period of three months (Bayomi *et al*., 1998).

A study investigated the encapsulation of chloroquine in liposomes, with or without surface modification. The efficacy of chloroquine on chloroquine-resistant *Plasmodium berghei* infected mice was determined by tagging antibodies against the infected erythrocytes. The study illustrated how intravenously administered chloroquine completely cured 75 % to 90 % of the *P. berghei* infected animals in 4 to 6 days post infection. The results were achieved by administering chloroquine at a dose of 5 mg/kg of body weight per day. The study concluded that chloroquine resistant malaria may be cured by selectively homing chloroquine to the malaria infected erythrocytes with reduced chloroquine dosages (Owais *et al*., 1995).

The prophylactic and therapeutic efficacy of liposomal chloroquine at a dosage level of 0.8mg/kg chloroquine was increased in contrast to commercial chloroquine formulations. A single intraperitonial injection of a 6 mg/kg dosage of liposomal chloroquine resulted in an efficacy of 100 %. Chloroquine resistant *P*. *berghei* infections could successfully be cured with ongoing stability by the administration of increased chloroquine dosages, per injection, subsequent to liposomal encapsulation. The infections could however not be cured by commercial chloroquine by means of a 7-day drug treatment regimen of 0.8 mg/mouse/day (Peeters *et al*., 1989).

Recent studies illustrated how the incorporation of a *Plasmodium* amino acid sequence, which is capable of recognizing glycosaminoglycans, within blank liposomes has the ability to successfully target the liver of *P. Berghei*-infected mice (Longmuir *et al*., 2006). The intravenous administration of these liposomes resulted in the fast clearance of the peptide-containing liposomes from the circulation. The metabolization of these liposomes occurred almost completely in the liver. According to fluorescence-and electron microscopy non-parenchymal cells and hepatocytes were responsible for the accumulation of the liposomes. Mainly all of the liposomal material was associated with hepatocytes. The liposomal accumulation in the liver was several hundredfold higher in comparison to the heart, lungs and kidneys and 10-fold higher when compared to the spleen. Liposomal binding appeared to be

specific to heparinase sensitive sites during liver slice experiments. The delivery of malaria vaccines has also been attempted with the aid of liposomes (Green *et al*., 1995).

2.13) Conclusion

The liposomal drug delivery system is significant due to its entrapment capabilities, adaptability and reduction in the toxicity potential of encapsulated drugs. The amphiphilic character of liposomes enables them to be exceptional solubilisation systems for numerous compounds. Liposomes may be applied as intracellular targets on bacterial and parasitic infections due to their rapid removal by phagocytic cells within the liver and the spleen. Positive results were obtained by encapsulating the drug chloroquine in liposomes which also illustrate the potential of liposomes as novel drug delivery systems.

Although many advantages are associated with liposomes as novel drug delivery systems several problems still occur with these systems. Their rapid blood clearance, restricted control of encapsulated molecule release and physical as well as chemical instabilities are amongst some of these problems. These systems have obtained limited success in the past due to their short shelf life, stability problems, sustained release, preparation cost and poor interaction with certain drugs. Alteration of the various applicational and design problems usually associated with liposomes may result in an extremely promising novel drug delivery system.

Liposomes have the ability to protect encapsulated anti-malarial drugs from degradation and to promote sustained release. It also has the ability to reduce adverse effects associated with these anti-malarial drugs. Liposomes may therefore have the potential to be recognized as exceptionally effective novel drug delivery systems for the treatment and management of malaria.

CHAPTER 3

Stability of liposomes containing chloroquine:

Methods, Results & Discussion

3.1) Introduction

Stability of a therapeutic system can be defined as the ability of the system to remain stable in terms of drug content, particle size, appearance, form and function for a predetermined time period. The stability of a system is frequently associated with its zeta potential. The zeta potential value of a dispersed system is indicative of the stability of the system in a specific environment. The value of the zeta potential, however, provides inconclusive information regarding the long term stability of a dispersed system. Emulsified systems may undergo several instability processes which influences the quality of the system during the course of time as a result of coalescence, creaming, flocculation, Ostwald ripening, phase separation and rupturing (Welin-berger & Bergenståhl, 2000).

Stability tests need to be performed to determine the stability period (shelf life) and the degradation rate of dispersed systems (Roland *et al*., 2003). This stability study consisted of a series of stability tests regarding the stability of nine liposome and nine chloroquine entrapped in liposome formulations over a period of twelve weeks. Special consideration was given to the pH of the formulation, entrapment efficiency determination with a UV spectrophotometer and the size distribution determination via flow cytometry (FACSCalibur^{TM}).

The main purpose of this study was to manufacture liposomes, to incorporate chloroquine (CQ) into liposomes and to determine the stability of the formulations. In the following sections the preparation and characterization of liposomes and CQ entrapped in liposomes are described. The UV spectrophotometric standardization of CQ phosphate, in order to determine entrapment efficiency, is explained. A detailed experimental design regarding the stabiliy study is given and the methods for determining pH, entrapment efficiency, size distribution with flow cytometry, light and fluorescence microscopy and statistical evaluation are described.

3.2) UV Spectrophotometric standardization of CQ phosphate

A UV spectrophotometer is an apparatus used to examine the interaction between radiation and matter in regards to the wavelengths of photons. It measures visible light as well as the close-to-visible range of infrared and ultraviolet spectrum ranges in particular. The UV spectrophotometer enables the identification of electronic alterations in the various regions of the electromagnetic spectrum (Kajanus *et al*., 1994). The UV spectrophotometric method of the USP was modified and used to determine the UV absorption spectrum of CQ phosphate. The method is based on the dilution of the drug in a suitable solvent. This is followed by the further dilution of the drug with water to obtain different drug concentrations which are then used to standardize the drug (USP, 2010). The absorbance of the CQ phosphate samples were measured at six different UV-wavelengths (340 nm, 341 nm, 342 nm, 343 nm, 344 nm and 345 nm). The different wavelengths were used to establish the wavelength at which maximum absorbance occurred.

3.2.2) Materials

CQ phosphate powder (Annexure D) was purchased from IPCA laboratories limited (India).

3.2.3) Methods

A 100 mg/10 ml CQ phosphate solution was prepared by adding 10 ml double distilled water (ddH_2O) to 100 mg CQ phosphate powder. The solution was then diluted to a 100 ug/ml stock solution by placing 1 ml of the 100 mg/10 ml CQ phosphate solution in a 100 ml volumetric flask and filling it up to volume with ddH_2O. Different CQ phosphate concentrations (55.00 µg/ml, 52.50 µg/ml, 50.00 µg/ml, 47.50 µg/ml, 45.00 µg/ml, 42.50 µg/ml, 40.00 µg/ml, 37.50 µg/ml, 35.00 µg/ml and 32.50 µg/ml) were prepared by further dilution of the stock solution.

Each sample was prepared in triplicate and all the experimental work was performed on a Shimadzu UV-1800 (Japan) UV spectrophotometer. The absorbance of all samples was measured at the six different UV-wavelengths. The experiment was done in triplicate on three different days. Graphs of absorbance versus concentration were constructed for each wavelength, on each of the three experimental days, to produce eighteen linear standard curves. The three graphs for each of the six experimental concentrations were then combined to produce graphs for all six concentrations in the form of six linear standard curves. The mean ± standard error of mean (SEM) was calculated for all of the data obtained from each wavelength. The data were analyzed with linear regression. The best linear standard curve was obtained at a wavelength of 343 nm with a regression value of r = 0.96. The equation of

the standard curve (Figure 3.1) was used to calculate the concentration (x) of the CQ entrapped in liposomes in samples, which will be discussed in section 3.5, in order to establish the entrapment efficiency of each sample.

The equation for the standard curve is: **y = mx + c** *Eq 3.1*

Where y = absorbance of samples; m = slope of the standard curve; x = concentration of samples; c = y intercept of standard curve.

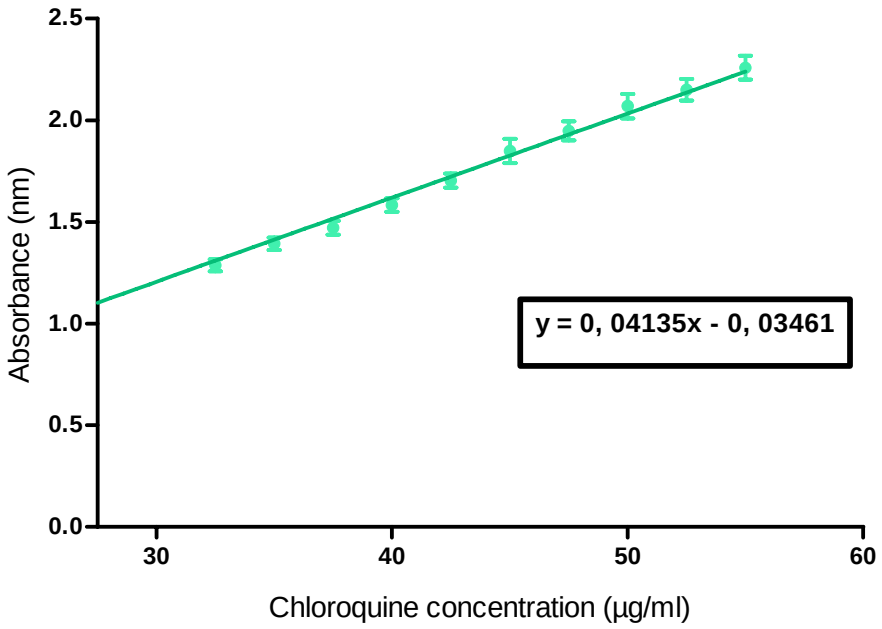


Figure 3.1: Linear standard curve of CQ phosphate at a wavelength of 343 nm. Results are expressed as mean ± SEM (r = 0.96)

3.3) Manufacturing of liposomes and CQ entrapped in liposomes

Liposomes (as discussed in chapter 2) are synthetic, spherical, microcapsule aggregates which consist of cholesterol and amphiphilic molecules such as phospholipids (Redziniak & Perrier, 1996; Wang, 2005). They have the ability to entrap both hydrophilic and hydrophobic drugs as a result of their amphiphilic character and enormous surface to volume ratios (Couvreur *et al*., 1991). In this study liposomes, consisting mainly of phosphatidyl choline and cholesterol, were

manufactured. CQ phosphate was incorporated into the liposomes (reffered to as CQ entrapped in liposomes) during manufacturing.

3.3.1) Materials

Phosphatidyl choline and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA). CQ phosphate was obtained from IPCA laboratories limited (India). Chloroform and phosphate buffered saline (PBS), with a concentration of 10 mM, were obtained from Scientific Group (South Africa) and Invitrogen (United States of America) respectively. The PBS was diluted with ddH_2O to a concentration of 1 mM.

3.3.2) Methods

Liposomes and CQ entrapped in liposomes were manufactured in accordance to a modified version of the film hydration method as described by New, 1990. This method makes use of the gentle, manual, agitation of lipids from the sides of a glass container by suspending the lipids in an aqueous medium (New, 1990).

Table 3.1 & 3.2 illustrates the quantities of the compounds that were used to produce a 200 ml liposome formulation and a 200 ml CQ entrapped in liposome formulation.

Table 3.1: The quantities of the compounds used in the formulation of liposomes.

Compound	**Quantity of compound**
Phosphatidyl choline	**[1.5 %]** = 3 g in 200 ml
Cholesterol	**[1.0 %]** = 2 g in 200 ml
PBS	200 ml
Chloroform	± 100 ml

Table 3.2: The quantities of the compounds used in the formulation of CQ entrapped in liposomes.

Compound	Quantity of compound
Phosphatidyl choline	**[1.5 %]** = 3 g in 200 ml
Cholesterol	**[1.0 %]** = 2 g in 200 ml
CQ phosphate	**[0.5 %]** = 1 g in 200 ml
PBS	200 ml
Chloroform	± 100 ml

The phosphatidyl choline and the cholesterol were individually weighed for each formulation. Two individual flasks were filled with phospatidyl choline and cholesterol for each formulation. CQ was only weighed for the CQ entrapped in liposome formulation. Chloroform was added to the flasks of both formulations in order to dissolve the contents of each flask. The contents of each flask was then equally divided and distributed to two different flasks for each formulation. The chloroform was then extracted from each of the four flasks under vacuum for 15-20 minutes. The lipids could be observed as thin film layers on the inside of each flask after the evaporation process. The lipids were then hydrated by adding 100 ml PBS and a few beads to the flasks. CQ phosphate, which was used for the manufacturing of CQ entrapped in liposomes, was dissolved in 200 ml PBS. The lipids of the two CQ entrapped in liposome flasks were then hydrated by the addition of 100 ml PBS and a few beads to both of the flasks. All four flasks were then individually placed on a Heidolph VV60 Electronic rotation device and rotated at maximum speed of 240 revolutions per minute (rpm) for ± 35 minutes or until the film layers were completely dissolved in the PBS. This resulted in the production of liposomes and CQ entrapped in liposomes with multilamellar vesicles. The beads were then removed from all of the flasks and the contents of the flasks, which contained liposomes and CQ entrapped in liposomes, were transferred to 250 ml Schott bottles. Each of the two liposome formulations in the bottles were placed in a beaker with ice water and then individually sonicated with a UP 100 H Ultraschallprozessor Dr. Hielscher GmbH sonicater, at an amplitude of 50 % and at a cycle setting of 1, for a duration of 20 minutes to reduce the size of the multilamellar vesicles and to produce small unilamellar vesicles (Chrai *et al*., 2002).

3.4) Experimental design

The measuring of pH values and size distribution, which will be discussed in sections 3.5 & 3.7, were conducted and documented for both the initial liposome and chloroquine entrapped in liposome formulations. The entrapment efficiency, which will be discussed in section 3.6, was also determined and documented for the CQ entrapped in liposomes. Light Microscope photos, which will be discussed in section 3.8, were also taken of both the initial liposome and CQ entrapped in liposome formulations. The liposome and CQ entrapped in liposome formulations were then each transferred, in volumes of 20 ml, to nine plastic containers. The nine containers of each formulation, eighteen in total, represented triplicate samples of each formulation for a modified version of Roland *et al's* stability testing at three different temperatures (5°C, 25°C & 40°C) for a time period of twelve weeks. Six of the samples, three liposomes and three CQ entrapped in liposomes, were placed in a refrigerator at a temperature of 5°C. The remaining twelve samples, six liposomes and six CQ entrapped in liposomes, were divided and placed in incubation ovens at temperatures of 25°C and 40°C respectively (Roland *et al*., 2003). Stability tests (i.e. measuring of pH value, entrapment efficiency of the CQ entrapped in liposomes and size determination) were performed on each of the eighteen samples during weeks 1, 2, 4, 6, 8, 10 and 12 of the stability testing.

3.5) Measuring of pH

3.5.1) Materials

Buffer solutions with pH values of 4, 7 and 10 were purchased from Merck Chemicals (South Africa).

3.5.2) Methods

The pH of the samples was measured with a Mettler Toledo MP220 pH meter. The pH meter was calibrated at a pH of 4, 7 and 10 by submerging its probe in the buffer solutions. The pH of all eighteen liposome and CQ entrapped in liposome samples (six at 5°C, six at 25°C and six at 40°C) were measured by placing the probe in each of the samples. The measuring of pH was one of the stability tests which were performed on all eighteen, nine liposomes and nine CQ entrapped in liposomes, samples at three different temperatures on week 1, 2, 4, 6, 8, 10 and 12 of stability as discussed in section 3.4. The preliminary data was processed with Microsoft Excel™ 2010 and Graphpad Prism™ version 5 (GraphPad Software Inc., Dan Diego, CA, USA). The data is presented as mean ± standard error of mean (SEM).

3.6) Entrapment efficiency determination with a UV spectrophotometer

The entrapment efficiency was determined, for the nine CQ entrapped in liposome samples, after the pH of each of the nine liposome and nine CQ entrapped in liposome samples were measured. The nine liposome samples (which served as the control group) were also prepared for analysis with an UV spectrophotometer.

3.6.1) Methods

Liposome and CQ entrapped in liposome samples (1 ml of each of the eighteen), as explained in section 3.4, were transferred to eighteen Eppendorf® tubes. The Eppendorf® tubes were placed in a K Centrifuge, PLC Series, Gemmy Industrial Corp., and centrifuged at a speed of 2000 revolutions per minute (rpm) for 10 minutes. After the centrifugation process was complete a 100 µl of the supernatant was removed from each of the eighteen samples and placed in eighteen small glass beakers and made up to a volume of 10 ml with ddH_20.

The absorbency of the nine liposome samples (three at 5°C, three at 25°C and three at 40°C) were determined via the UV Spectrophotometer on week 1, 2, 4, 6, 8, 10 and 12 of the stability evaluation. The absorbencies of the nine CQ entrapped in liposome samples (three at 5°C, three at 25°C and three at 40°C) were determined, after that of the liposome samples, via the UV Spectrophotometer on week 1, 2, 4, 6, 8, 10 and 12 of the stability testing as discussed in section 3.4.

The equation of the linear standard curve of chloroquine phosphate (Figure 3.1) was used to determine the drug concentrations (x) of the glass beakers which contained the CQ entrapped in liposome samples:

y = 0, 04135x – 0, 03461 *Eq. 3.2*

The y-value represented the average absorbency of the triplicate values at each of the three different temperatures. The calculated x-values were then used to determine the entrapment efficiency of the nine samples on week 1, 2, 4, 6, 8, 10 and 12 of the stability testing.

The maximum possible drug concentration was set at 50 µg/ml which would mean that no drug entrapment had occurred if the free drug concentration was also determined to be 50 µg/ml. The percentage entrapment efficiency (% EE) of each of the nine CQ entrapped in liposome samples were determined with the following formula:

$$\% \text{ EE} = \frac{\text{Maximum drug concentration} - \text{x-value}}{\text{Maximum drug concentration}} \text{ X 100} \qquad \textit{Eq. 3.3}$$

The preliminary data was processed with Microsoft Excel™ 2010 and Graphpad Prism™ version 5 (GraphPad Software Inc., Dan Diego, CA, USA). The data is presented as mean ± standard error of mean (SEM).

3.7) Determination of size distribution via flow cytometry

Flow cytometry (FCM) is a technique which employs the principle of light scattering. FCM is extremely useful in analyzing cells by using a fluorescent marker suspended in a fluid stream (Salzman & Wilder, 1979; Childers & Michalek, 1989; Shapiro, 1995). The technique entails the measurement of individual particles in a continues flow system. The individual particles can be analyzed at a rate of 40 000 events per second. FCM can be considered as a fluorescence microscope that has the ability to detect samples at a signal of forward-scatter (FSC) and side-scatter (SSC). It also has the ability to detect various fluorescent colours (Vorauer-Uhl *et al*., 2000; Hai *et al*., 2004). FCM is an ideal method of analysis due to its accurate and reliable calculation of cell populations and specific cell selections (Shapiro, 1995; Ibrahim & van der Engh, 2003).

The FCM technique can also be employed for the characterization of liposomes in terms of analyzing their size distribution and fusion detection (Childers *et al*., 1989; Anzar *et al*., 2002).

3.7.1) Materials

FACSCalibur™ reagents and consumables were purchased from Scientific group (South Africa). PBS, with a concentration of 10 mM, was obtained from Scientific Group (South Africa) and diluted with ddH_2O to a concentration of 1 mM.

3.7.2) Methods

The FCM analysis was performed on a FACSCalibur™ (BD Biosciences). Each of the samples was analyzed at a flow rate of 1000 events/second.

Nine liposome and nine CQ entrapped in liposome samples (10 µl), as explained in section 3.4, were transferred to eighteen FACS tubes. PBS (500 µl) was added to the content of each of the eighteen FACS tubes. The samples were then vortexed and analyzed with the FACSCalibur™ to determine their size distributions. The size distribution of the nine liposome samples (three at

5°C, three at 25°C and three at 40°C) and the nine CQ entrapped in liposome samples (three at 5°C, three at 25°C and three at 40°C) were determined on week 1, 2, 4, 6, 8, 10 and 12 of the stability testing as discussed in section 3.4. FSC and SSC were collected on a log scale. For each sample 100 000 events were collected. The preliminary data of all twelve weeks were acquired on a MAC-OC computer using CELLQUEST PRO (BD Biosciences) and analyzed with FlowJo version 7.6.1 (Tree star). Figure 3.2 illustrates the forward-scatter and side-scatter distribution of particles which were observed via FlowJo.

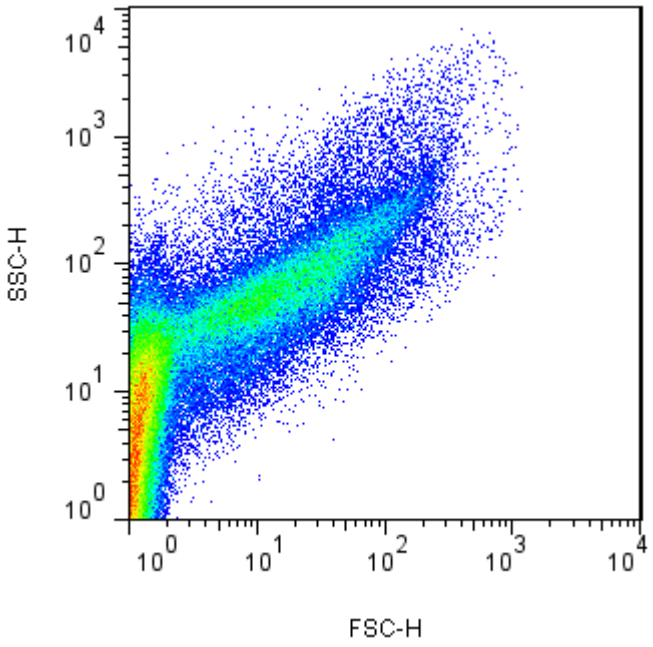


Figure 3.2: Illustration of the forward-scatter and side-scatter distribution of particles.

The preliminary data, which were obtained in FlowJo, were converted to span values. Preliminary span values were calculated by determining the size (µm) of each of the samples at S5 % (smaller than 5 % of the total size value), S50 % (mean) and S95 % (larger than 95 % of the total size value) (Annexure A). The values of each of the percentages were then converted to log y-values and afterwards to log x-values via the linear standard curve equation for the standardisation of size distribution on a FACSCalibur™: **y = 1.607x + 0.4496** *(Eq. 3.4)* (Slabbert *et al*., 2010). The y-values were represented by the percentage of the particles and the x-values the size of the particles (µm). Final span values were calculated with the aid of equation 3.5.

$$\textbf{Span (µm)} = \frac{\textbf{S95 \% - S5 \%}}{\textbf{S50 \%}} \qquad \textit{Eq. 3.5}$$

The final span values, which were obtained for the determination of the size distibution of liposomes and CQ entrapped in liposomes for the duration of the twelve week stability evaluation (as discussed in section 3.5), were then processed with Microsoft Excel™ 2010 and Graphpad Prism™ version 5 (GraphPad Software Inc., Dan Diego, CA, USA). The data is presented as the mean ± standard error of mean (SEM).

3.8) Formulation of CQ entrapped in liposomes at various concentrations

Five different CQ entrapped in liposome formulations were prepared to establish if there were any correlation between size distribution and entrapment efficiency when dealing with a variety of CQ concentrations.

The five CQ entrapped in liposome formulations were also manufactured in accordance to a modified version of the film hydration method as described by New, 1990. Table 3.3 contains the quantities of the compounds (materials were obtained as mentioned in section 3.3.1) that were used to produce the four different CQ entrapped in liposome formulations.

Table 3.3: The quantities of the compounds used in the formulation of various CQ entrapped in liposome preparations.

Compound	Quantity of compound
Phosphatidyl choline	**[1.5 %]** = 0.075 g in 5 ml
Cholesterol	**[1.0 %]** = 0.050 g in 5 ml
CQ phosphate	**[0.2 %]** = 0.010 g **[0.3 %]** = 0.015 g **[0.4 %]** = 0.020 g **[0.5 %]** = 0.025 g
PBS	5 ml
Chloroform	± 2 ml

3.8.1) Methods

The only difference in the manufacturing process when comparing the 200 ml liposome and the 200 ml CQ entrapped in liposome formulations (section 3.3) were the quantities of raw material that were used and the manufacturing time. These formulations were not divided prior to the high vacuum evaporation process of chloroform and stability tests were only conducted once for each sample. Each of the four 5 ml preparations was manufactured in 200 ml flasks, distributed to 10 ml tubes that were placed in beakers with ice water, and individually sonicated for 5 minutes. An extra [0.5 %] CQ entrapped in liposome preparation was manufactured in exactly the same way as the other formulations with the only difference being that it was not sonicated.

The measuring of pH values, size distribution determinations and entrapment efficiency determinations were conducted for all five of the CQ entrapped in liposome formulations (as discussed in sections 3.5, 3.6 and 3.7).

3.9) Light and fluorescence microscopy

3.9.1) Materials

Nile Red dye was purchased from Sigma-Aldrich (St. Louis, MO, USA). First grade frosted 1.2 mm microscope slides and cover slips were obtained from Lasec (South Africa).

3.9.2) Methods

Liposome and CQ entrapped in liposome samples (50 µl), at the three different temperatures after the twelve week stability evaluation, were placed in Eppendorf® tubes. Nile Red dye (1 µl) was added to each of the tubes. The tubes were then incubated for 15 minutes while protected from light. Each of the samples in the tubes was vortexted after a 15 minute incubation period and 20 µl of each sample was then placed on a microscope slide. A cover slip was placed over each microscope slide to evenly distribute the sample across the surface of the microscope slide. Each microscope slide was then inspected under a Nikon Eclipse TE-300 inverted microscope equipped with a DXM 1200 digital camera (Japan).

3.10) Statistical evaluation

The data were non-parametrically analyzed with STATISTICA through Repeated Measures Analysis of Variance (ANOVA) tests to establish if there were any statistically significant differences between the pH of liposomes and CQ entrapped in liposomes, as well as between the size distribution values of liposomes and CQ in entrapped liposomes at temperatures of 5°C, 25°C and 40°C during a time period of twelve weeks. T-tests for Dependant Samples were also conducted with STATISTICA at each of the three temperatures (5°C, 25°C and 40°C) where the initial values and the values obtained at week 12 (for pH as well as for span) were compared to establish if there were any statistically significant differences. Differences were considered to be statistically significant when the probability (p) value was $p < 0.05$.

Spearman Rank Order Correlation tests were non-parametrically conducted with STATISTICA to establish if there were any statistically significant differences between the pH and entrapment efficiency of CQ entrapped in liposomes at temperatures of 5°C, 25°C and 40°C during a time period of twelve weeks. Spearman Rank Order Correlation tests were also non-parametrically conducted with STATISTICA to establish if there were any statistically significant differences between the entrapment efficiency and span of CQ entrapped in liposomes at temperatures of 5°C, 25°C and 40°C during a time period of twelve weeks. Spearman Rank Order Correlation tests were lastly conducted non-parametrically with STATISTICA to establish if there were any statistically significant differences between the size distribution and entrapment efficiency of the five different CQ entrapped in liposome formulations. Differences were considered to be statistically significant when the probability (p) value was $p < 0.05$.

3.11) Results and discussion

3.11.1) Evaluation of pH

The aim of this part of the stability study was to determine the pH of the liposomes and the CQ entrapped in liposome formulations at temperatures of 5°C, 25°C and 40°C over a time period of twelve weeks. Measuring the pH of the formulations provide important information regarding the stability of the formulations.

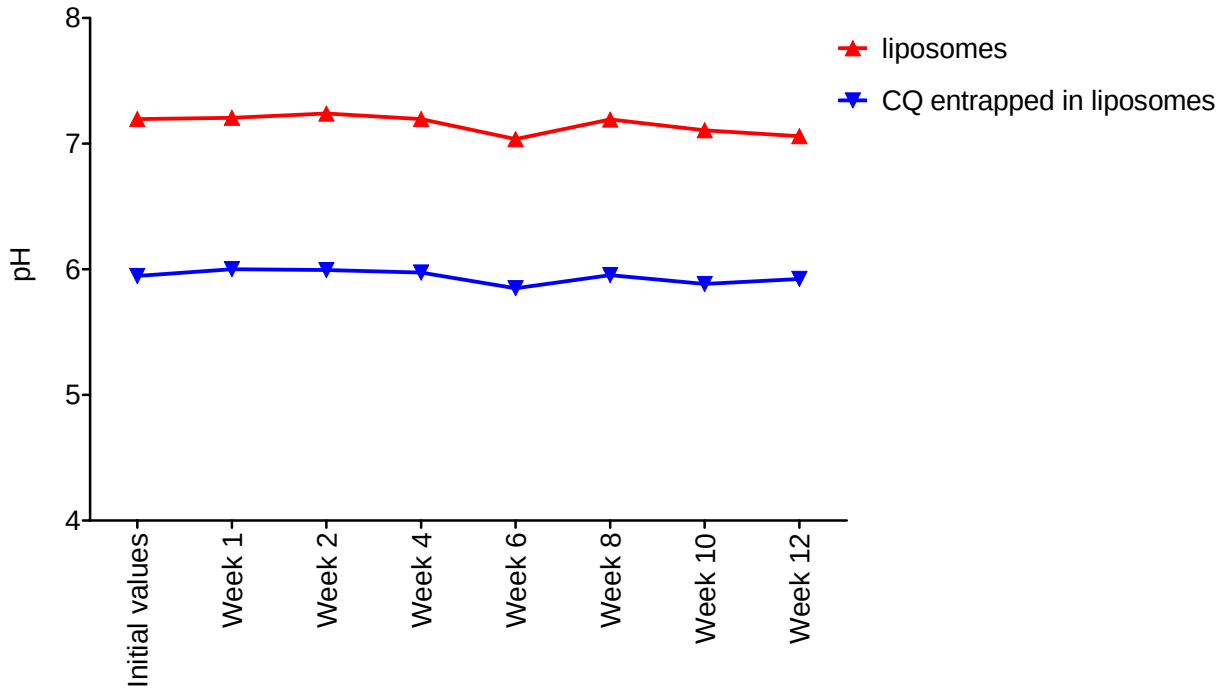


Figure 3.3: pH of liposomes and CQ entrapped in liposomes (mean ± SEM) at 5°C during a time period of twelve weeks (n = 3). The SEM values are too small to be visible on the graph.

Figure 3.3 illustrates the pH of liposomes and CQ entrapped in liposomes during a stability study at 5°C over a time period of twelve weeks. The y-axis represents the pH and the x-axis the twelve week time period. The pH of the liposomes ranged from 7.04 to 7.24 (Annexure B, Table B1) from the initial value to the value obtained at week 12. There was no statistically significant difference between the initial mean (7.20 ± 0.01) and the mean obtained at week 12 (7.06 ± 0.04) with p = 0.11 (Annexure C). The pH of the CQ entrapped in liposomes ranged from 5.85 to 6.00 (Annexure B, Table B1) from the initial value to the value obtained at week 12. There was no statistically significant difference between the initial mean (5.95 ± 0.01) and the mean obtained at week 12 (5.92 ± 0.04) with p = 0.59 (Annexure C). The pH of liposomes in comparison to the pH of CQ entrapped in liposomes was significantly higher over the entire 12 week period with p = 0.000003 (Annexure C). The pH of liposomes as well as the pH of CQ entrapped in liposomes remained more or less constant from their initial values to their values obtained at week 12 which may indicate that both formulations remained in a stable condition at 5°C over the twelve week time period.

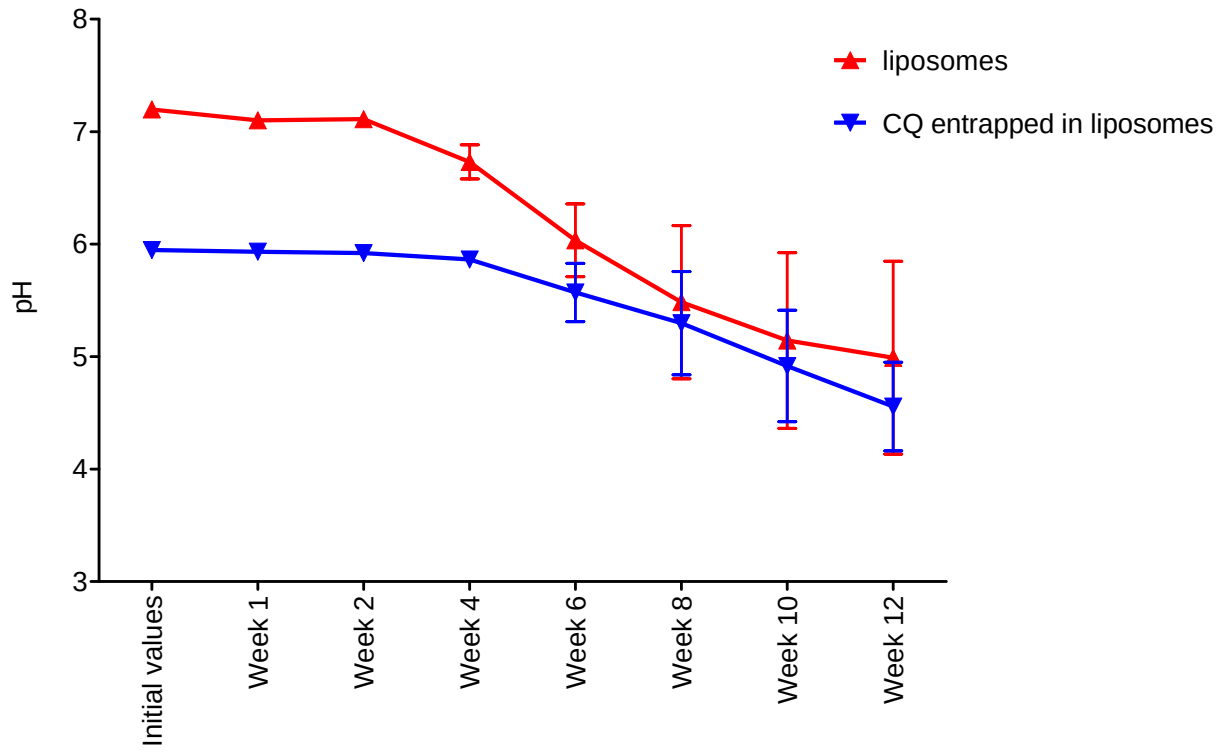


Figure 3.4: pH of liposomes and CQ entrapped in liposomes (mean ± SEM) at 25°C during a time period of twelve weeks (n = 3).

Figure 3.4 illustrates the pH of liposomes and CQ entrapped in liposomes during a stability study at 25°C over a time period of twelve weeks. The y-axis represents the pH and the x-axis the twelve week time period. The pH of the liposomes had decreased from 7.20 to 4.99 (Annexure B, Table B2) from the initial value to the value obtained at week 12. The pH remained constant from the initial value to week 4 and then started to decrease from week 6 to week 12. There was no statistically significant difference between the initial mean (7.20 ± 0.01) and the mean obtained at week 12 (4.99 ± 0.86) with p = 0.12 (Annexure C). The pH of the CQ entrapped in liposomes ranged from 5.95 to 4.56 (Annexure B, Table B2) from the initial value to the value obtained on week 12. The pH remained constant from the initial value to week 4 and started to decrease from week 6 to week 12. There was no statistically significant difference between the initial mean (5.95 ± 0.01) and the mean obtained at week 12 (4.56 ± 0.39) with p = 0.07 (Annexure C). There were no statistically significant difference between liposomes and CQ entrapped in liposomes over the 12 week time period with p = 0.15 (Annexure C). The decrease in pH of both liposomes and CQ entrapped in liposomes, from week 6 to week 12, may possibly be attributed to bacterial contamination which occurred in the formulations. No statistically significant difference could, however, be established between the

intial values and the values obtained on week 12 for both liposomes and CQ entrapped in liposomes.

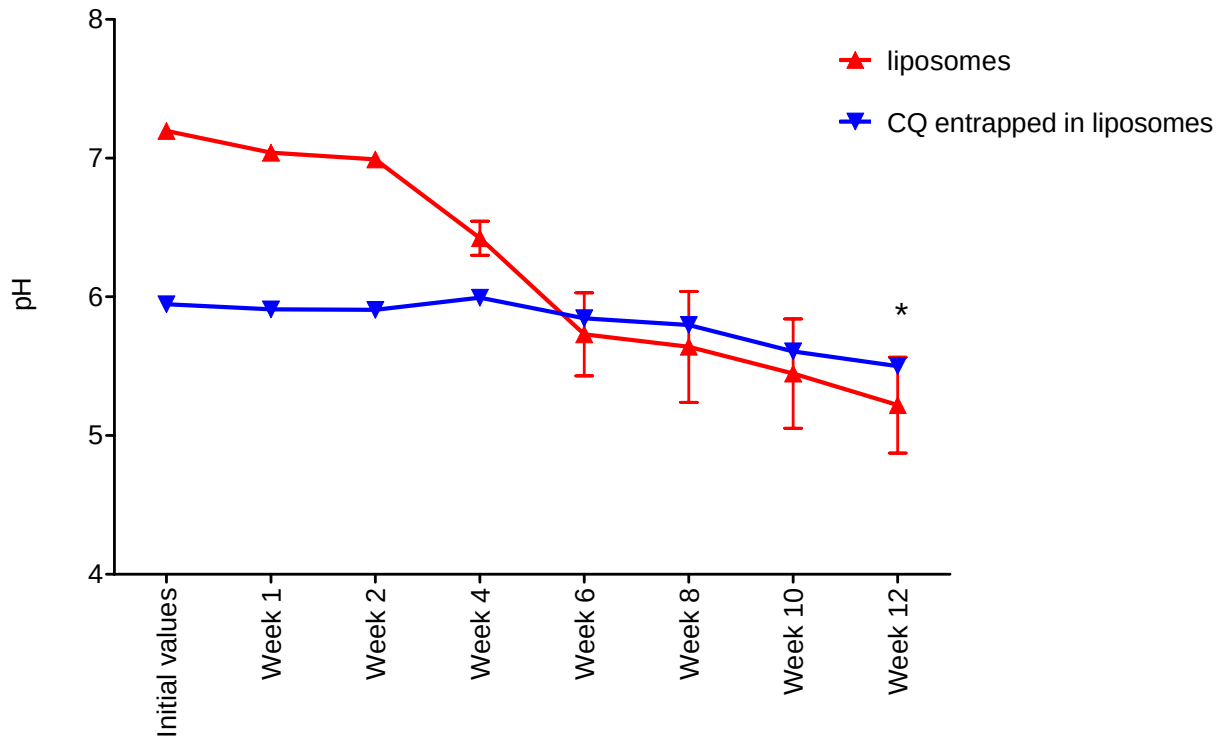


Figure 3.5: pH values of liposomes and CQ entrapped in liposomes (mean ± SEM) at 40°C during a time period of twelve weeks (n = 3). * Indicates a statistically significant difference from the initial values for both formulations (p < 0.05).

Figure 3.5 illustrates the pH of liposomes and CQ entrapped in liposomes during a stability study at 40 °C over a time period of twelve weeks. The y-axis represents the pH and the x-axis the twelve week time period. The pH of the liposomes had decreased from 7.20 to 5.22 (Annexure B, Table B3) from the initial value to the value obtained at week 12. The pH remained constant from the initial value to week 3 and started to decrease from week 4 to week 12. There was a statistically significant difference between the initial mean (7.20 ± 0.01) and the mean obtained on week 12 (5.22 ± 0.35) with a p = 0.03 (Annexure C). The pH of the CQ entrapped in liposomes ranged from 5.99 to 5.50 (Annexure B, Table B3) from the initial value to the value obtained at week 12. The pH remained constant to the initial value until week 4 and started to decrease from week 6 to week 12. There was a statistically significant difference between the initial mean (5.95 ± 0.01) and the mean obtained at week 12 (5.50 ± 0.02) with p = 0.000223 (Annexure C). The pH of liposomes in comparison to the pH of CQ entrapped in

liposomes remained more or less the same from the initial value to week 3. The pH of the liposomes, however, started to rapidly decrease from week 4 to week 12, whilst the pH of CQ entrapped in liposomes only started to decrease from week 6 to week 12. There was no statistically significant difference between liposomes and CQ entrapped in liposomes with $p = 0.11$ (Annexure C). The decrease in pH of both the liposome and the CQ entrapped in liposome formulations at 40°C, from week 4 to week 12 and from week 6 to week 12 respectively, may possibly be attributed to bacterial contamination which occurred in the formulations. None of the above formulations remained stable for the duration of the test period.

The pH of both liposomes and CQ entrapped in liposomes at 5°C remained relatively constant, whereas at 25°C and 40°C decreases were seen at week 4 and week 6 respectively. This could possibly be attributed to bacterial contamination at 25°C and 40°C. Bacterial contamination was not evident at 5°C.

3.11.2) The influence of pH on the entrapment efficiency of CQ entrapped in liposomes

Chemical instability of a formulation, reflected by changes in pH, may lead to changes in the entrapment efficiency of liposomes. In the following section the influence of pH on the entrapment efficiency of CQ entrapped liposomes was determined at temperatures of 5°C, 25°C and 40°C over a time period of twelve weeks.

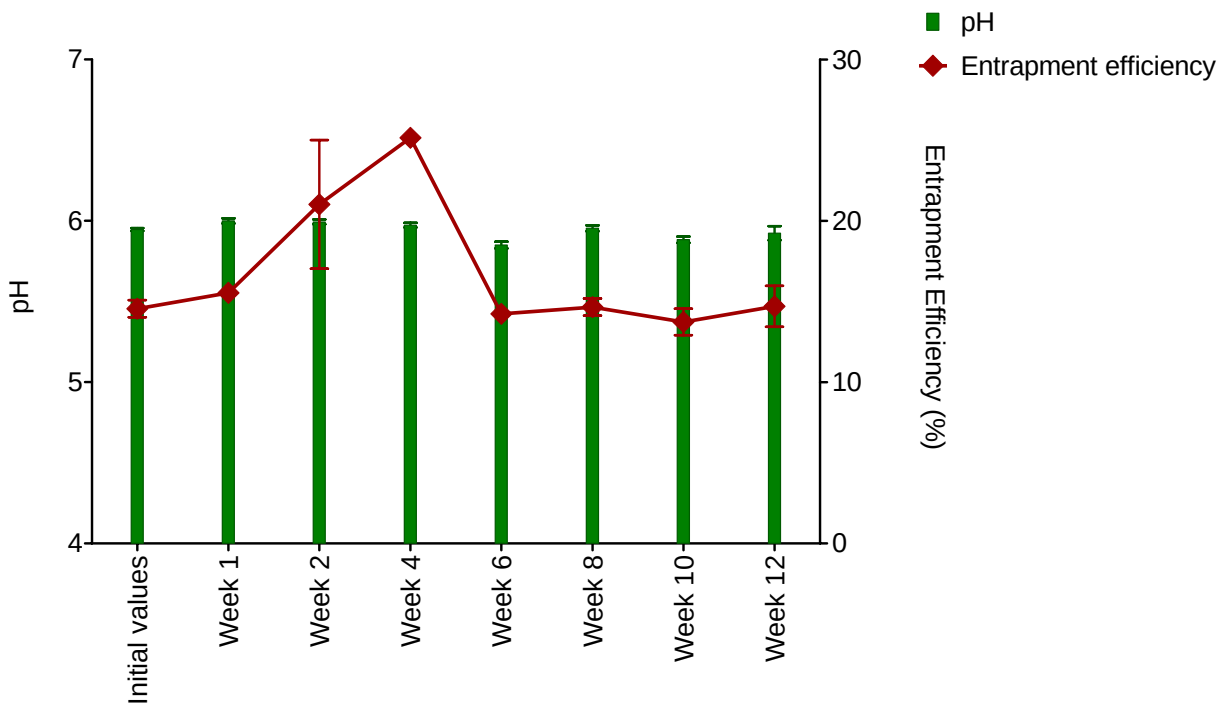


Figure 3.6: Comparison between pH and entrapment efficiency of CQ entrapped in liposomes (mean ± SEM) at 5°C during a time period of twelve weeks (n = 3).

Figure 3.6 illustrates the comparison between pH and entrapment efficiency of CQ entrapped in liposomes during a stability study at 5°C over a time period of twelve weeks. The left y-axis represents the pH and the right y-axis the entrapment efficacy (%) of CQ entrapped in liposomes. The x-axis represents the twelve week time period. As previously discussed, the pH remained more or less constant from the initial value to the value obtained at week 12. The entrapment efficiency ranged from 13.73 % to 25.15 % (Annexure B, Table B4) from the initial value (14.55 ± 0.53 %) to the value obtained on week 12 (14.70 ± 1.27 %). The entrapment efficiency started to increase from the initial value to week 4 (25.15 ± 0.05 %) and then decreased from week 4 to week 6. The entrapment efficiency then remained more or less constant from week 6 to week 12. A statistically significant difference could be observed between the pH and entrapment efficiency of CQ entrapped in liposomes during the twelve week stability study at 5°C. The Spearman Rank Order Correlation (R_s) of 0.43 indicated that $p < 0.05$ (Annexure C). The statistically significant difference suggests a direct correlation between pH and entrapment efficiency during the twelve week time period at 5°C. This correlation could be attributed to the decrease in both pH and entrapment efficiency from week 4 to week 6. It should be noted that although a significant correlation is observed between pH and entrapment efficiency, the overall trend of the data suggests that pH did not have an influence on entrapment efficiency. The low R_s value of 0.43 confirms this finding since it is

generally accepted that a correlation value close to or equal to 1 is considered to be indicative of a positive correlation.

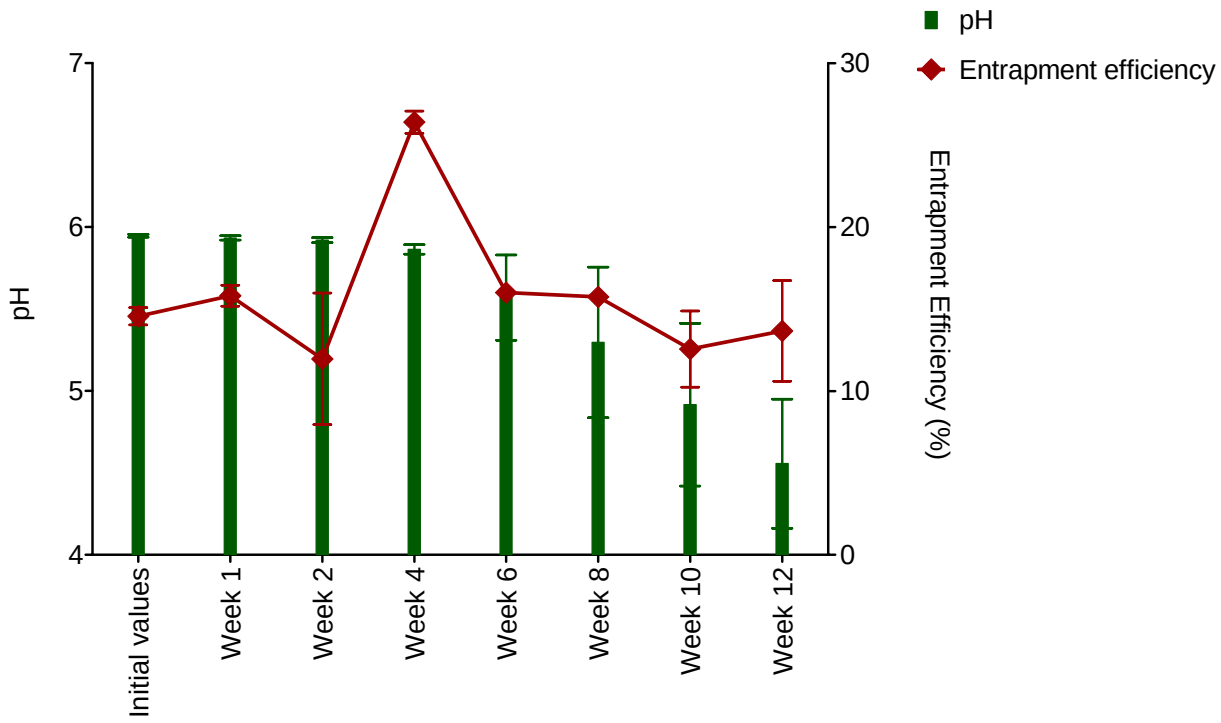


Figure 3.7: Comparison between pH and entrapment efficiency of CQ entrapped in liposomes (mean ± SEM) at 25°C during a time period of twelve weeks (n = 3).

Figure 3.7 illustrates the comparison between pH and entrapment efficiency of CQ entrapped in liposomes during a stability study at 25°C over a time period of twelve weeks. The left y-axis represents the pH and the right y-axis the entrapment efficiency (%) of CQ entrapped in liposomes. The x-axis represents the twelve week time period. The pH remained constant, as previously discussed, from the initial value to week 4 and started to decrease from week 6 to week 12. The entrapment efficiency ranged from 11.96 % to 26.40 % (Annexure B, Table B5) from the initial value (14.55 ± 0.53 %) to the value obtained at week 12 (13.67 ± 3.07 %). Entrapment efficiency decreased from week 1 to week 2 and then increased from week 2 to week 4 (26.40 ± 0.68 %). A decrease in the entrapment efficiency was observed between week 4 and week 10 and thereafter a slight increase from week 10 to week 12. No statistically significant difference could be observed between the pH and entrapment efficiency of CQ entrapped in liposomes during the twelve week stability study at 25°C with a R_s value of 0.05 which indicates that $p > 0.05$ (Annexure C). It can therefore be concluded that pH did not have a significant influence on entrapment efficiency.

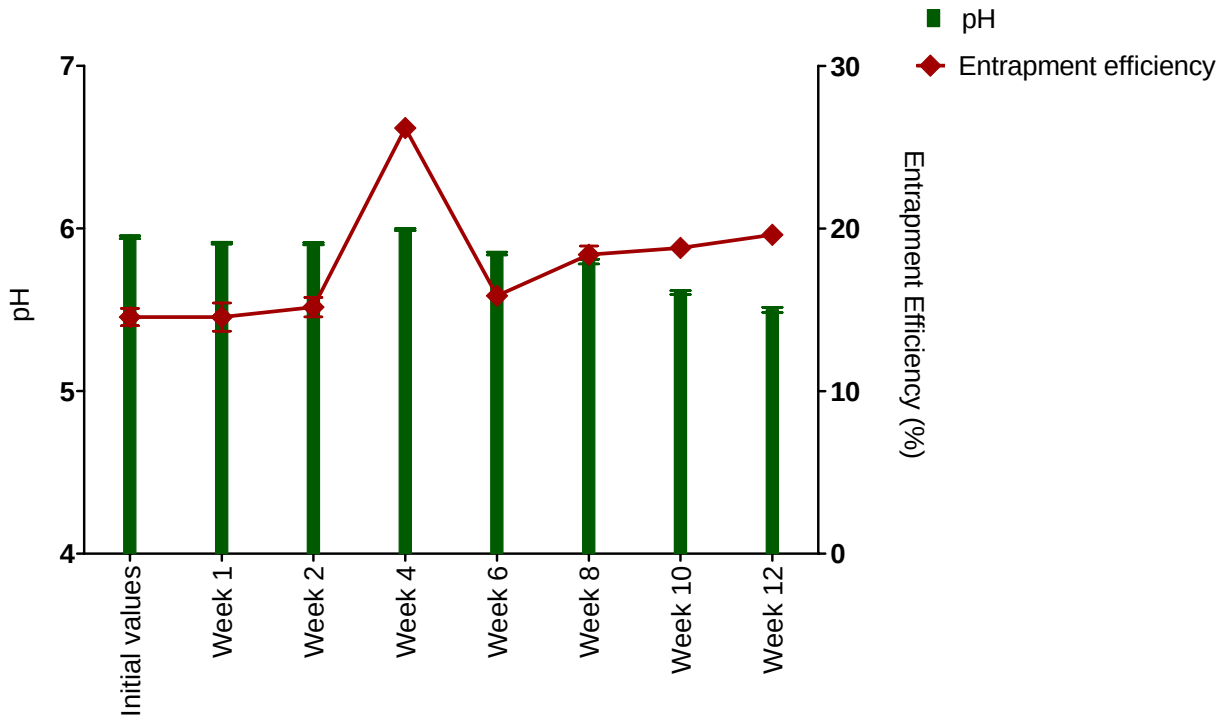


Figure 3.8: Comparison between pH and entrapment efficiency of CQ entrapped in liposomes (mean ± SEM) at 40°C during a time period of twelve weeks (n = 3).

Figure 3.8 illustrates the comparison between pH and entrapment efficiency of CQ entrapped in liposomes during a stability study at 40°C over a time period of twelve weeks. The left y-axis represents the pH and the right y-axis the entrapment efficiency (%) of CQ entrapped in liposomes. The x-axis represents the twelve week time period. The pH remained constant from the initial value to week 4 and started to decrease from week 6 to week 12, as discussed earlier. The entrapment efficiency ranged from 14.55 % to 26.18 % (Annexure B, Table B6) from the initial value (14.55 ± 0.53 %) to the value obtained at week 12 (19.62 ± 0.14 %). Entrapment efficiency remained more or less constant from the initial value until week 2, with an increase in entrapment efficiency between week 2 and week 4 (26.18 ± 0.32 %). A decrease was observed between week 4 and week 6 with a slight increase in entrapment efficiency between week 6 and week 8. The entrapment efficiency remained more or less constant from week 10 to week 12. No statistically significant difference was observed between the pH and entrapment efficiency of CQ entrapped in liposomes during the twelve week stability study at 40°C with a R_s value of -0.27 which indicates that $p > 0.05$ (Annexure C). This indicates that at 40°C, pH did not a have a marked effect on entrapment efficiency.

The amount of the initially entrapped CQ had a low percentage of 14.55 %. The entrapment efficiency at all of the temperatures showed a steady increase from the initial values to peak

levels at week 4. At week 6 the entrapment efficiency appeared to be similar to values determined at week 1 to week 3. The sharp increase in entrapment efficiency seen at week 4 could possibly be attributed to experimental error with the UV spectrophotometric method. There were no marked correlation between the pH and the entrapment efficiency at all of the temperatures which indicates that the pH did not influence the amount of CQ entrapped inside of the liposomes.

3.11.3) Size determination

The stability of a formulation is also determined by its size distribution. This section investigates the span values of the liposome and the CQ entrapped in liposome formulations at temperatures of 5°C, 25°C and 40°C over a time period of twelve weeks.

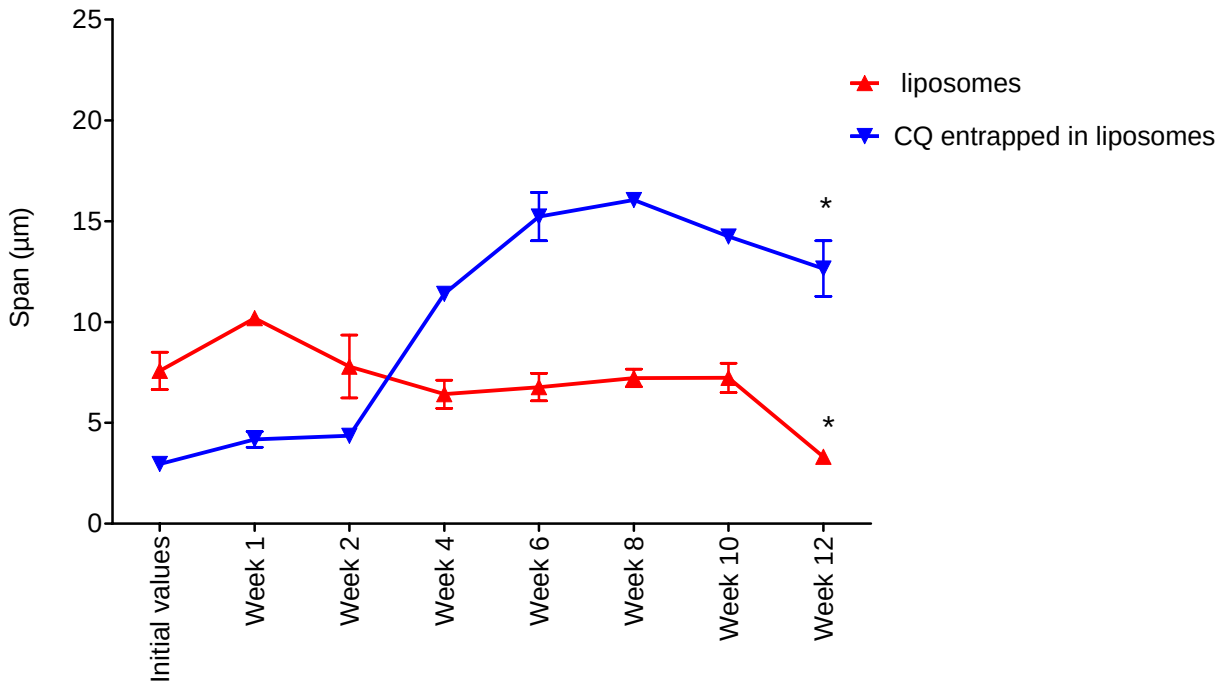


Figure 3.9: Span values of liposomes and CQ entrapped in liposomes (mean ± SEM) at 5°C during a time period of twelve weeks (n = 3). * Indicates a statistically significant difference from the initial values for both formulations ($p < 0.05$).

Figure 3.9 illustrates the span values of liposomes and CQ entrapped in liposomes during a stability study at 5°C over a time period of twelve weeks. The y-axis represents the span (µm) and the x-axis the twelve week time period. The span values of the liposomes ranged from

3.32 µm to 10.19 µm (Annexure B, Table B7) from the initial values to the values obtained at week 12. The span values of liposomes remained more or less constant from the initial value up until week 10 and then started to decrease between week 10 and week 12. There was a statistically significant difference between the initial value (7.58 ± 0.92 µm) and the value obtained at week 12 (3.32 ± 0.09 µm) with p = 0.04 (Annexure C). The span values of the CQ entrapped in liposomes ranged from 2.96 µm to 16.05 µm (Annexure B, table B7) from the initial value to the value obtained at week 12. The span values of the CQ entrapped in liposomes remained more or less constant between week 1 to week 2 and then started to increase between week 2 and week 8 with another slight decrease between week 8 and week 10. There was a statistically significant difference between the initial value (2.96 ± 0.07 µm) and the value obtained at week 12 (12.65 ± 1.38 µm) with p = 0.02 (Annexure C). The span values of both the liposomes and the CQ in liposomes differed from the initial values to the values obtained at week 12. There was a statistically significant difference between liposomes and CQ entrapped in liposomes with p = 0.000809 (Annexure C). A lot of fluctuations in CQ entrapped in liposomes span values were observed during the twelve week time period at 5°C which may indicate that liposomes are a more stable formulation in terms of their span values.

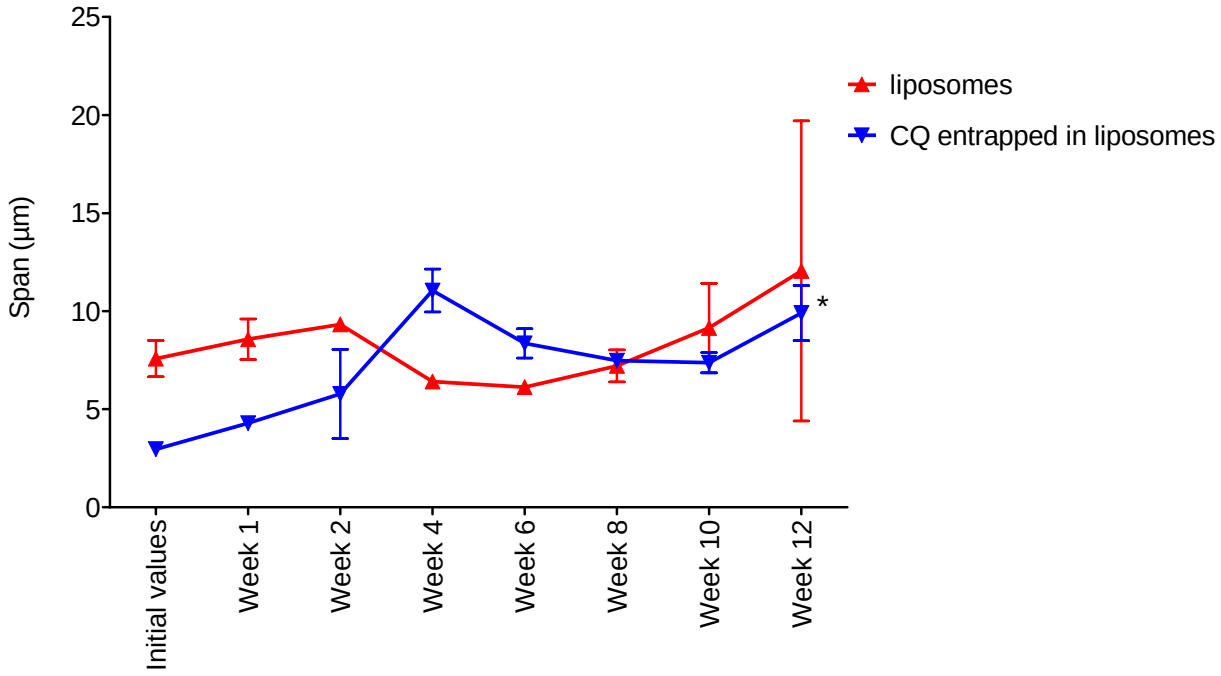


Figure 3.10: Span values of liposomes and CQ entrapped in liposomes (mean ± SEM) at 25°C during a time period of twelve weeks (n = 3). * Indicates a statistically significant difference between the initial and final value of the CQ entrapped in liposome formulation (p < 0.05).

Figure 3.10 illustrates the span values of liposomes and CQ entrapped in liposomes during a stability study at 25°C over a time period of twelve weeks. The y-axis represents the span (µm) and the x-axis the twelve week time period. The span values of the liposomes ranged from 6.13 µm to 12.05 µm (Annexure B, Table B8) from the initial value to the value obtained at week 12. The span values remained more or less constant from the initial value up until week 2 which was followed by a decrease between week 2 and week 4 and an increase between week 8 to week 12. There was no statistically significant difference between the initial value (7.58 ± 0.92 µm) and the value obtained at week 12 (12.05 ± 7.65 µm) with p = 0.61 (Annexure C). The span values of the CQ entrapped in liposomes ranged from 2.96 µm to 11.05 µm (Annexure B, Table B8) from the initial value to the value obtained at week 12. The span values started to increase from the initial value up until week 4 with a decrease between week 4 and week 10 which was followed by another slight increase between week 10 and week 12. There was a statistically significant difference between the initial value (2.96 ± 0.07 µm) and the value obtained at week 12 (9.90 ± 1.40 µm) with a p = 0.04 (Annexure C). The span values of the liposomes, in comparison to the span values of CQ entrapped in liposomes, remained more or less the same from the initial values up until week 2. The only difference was that the CQ entrapped in liposomes showed an increase in the span value during week 4 of the stability

test whilst a decrease was observed with the liposomes. There was no statistically significant difference between liposomes and CQ entrapped in liposomes with p = 0.45 (Annexure C). The span values of the liposomes remained more or less constant during the twelve week time period at 25°C. The liposome formulation therefore remained in a stable condition.

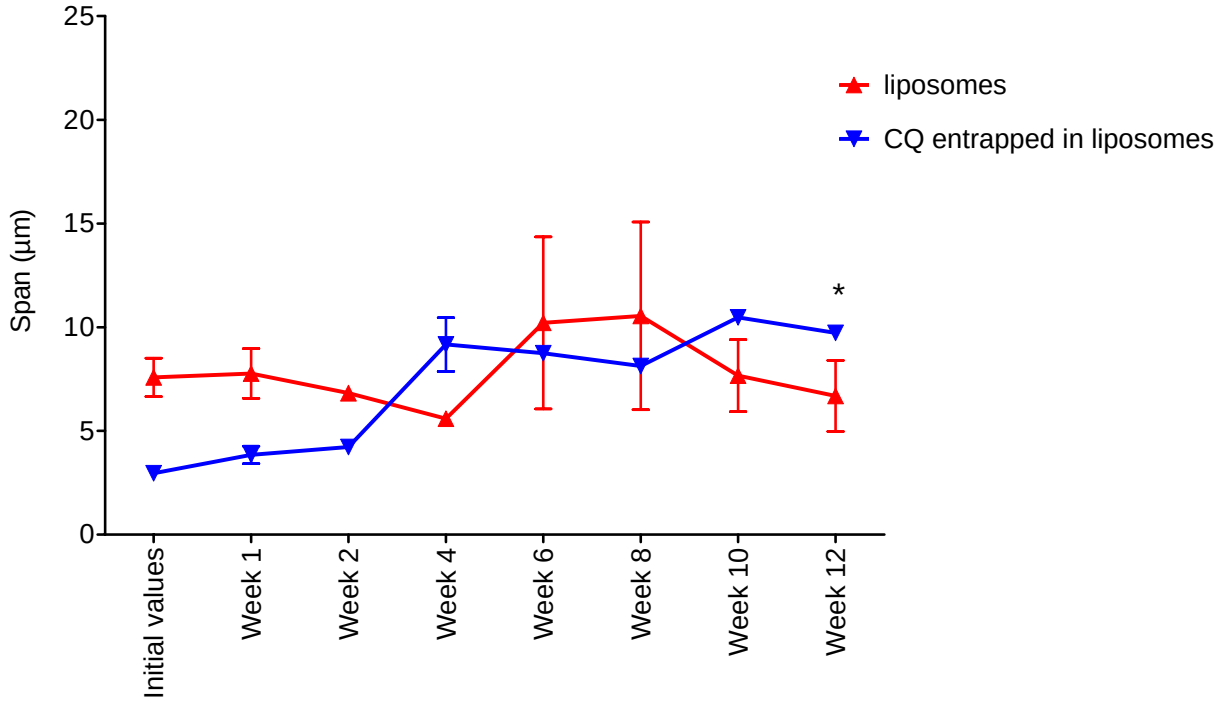


Figure 3.11: Span values of liposomes and CQ entrapped in liposomes (mean ± SEM) at 40°C during a time period of twelve weeks (n = 3). * Indicates a statistically significant difference between the initial and final value of the CQ entrapped in liposome formulation (p < 0.05).

Figure 3.11 illustrates the span values of liposomes and CQ entrapped in liposomes during a stability study at 40°C over a time period of twelve weeks. The y-axis represents the span (µm) and the x-axis the twelve week time period. The span values of the liposomes ranged from 5.59 µm to 10.55 µm (Annexure B, Table B9) from the initial value to the value obtained at week 12. The span values remained more or less constant from the initial value up until week 4 and then started to increase between week 4 and week 8 which was then followed by a decrease in the span value between week 8 and week 12. There was no statistically significant difference between the initial value (7.58 ± 0.92 µm) and the value obtained at week 12 (6.69 ± 1.71 µm) with p = 0.41 (Annexure C). The span values of the CQ entrapped in liposomes ranged from

2.96 µm to 10.47 µm (Annexure B, Table B9) from the initial value to the value obtained at week 12. The span values remained more or less constant from the initial value up until week 2 and then started to increase between week 2 and week 4. The span values remained more or less constant between week 4 and week 8 which was followed by an increase between week 8 and week 10 and remained more or less stable between week 10 and week 12. There was a statistically significant difference between the initial value (2.96 ± 0.07 µm) and the value obtained at week 12 (9.72 ± 0.31 µm) with $p = 0.001450$ (Annexure C). There was no statistically significant difference between liposomes and CQ entrapped in liposomes with $p = 0.69$ (Annexure C). The span values of only the liposomes remained more or less constant during the twelve week time period at 40°C. The liposome formulation therefore remained in a stable condition.

3.11.4) Influence of span on entrapment efficiency of CQ entrapped in liposomes

This part of the stability study's main goal was to evaluate the relationship between the span value variances and entrapment efficiency of CQ entrapped in liposomes at temperatures of 5°C, 25°C and 40°C over a time period of twelve weeks. The size of the span values of the CQ entrapped in liposomes may have an effect on the entrapment efficiency of CQ within the liposomes.

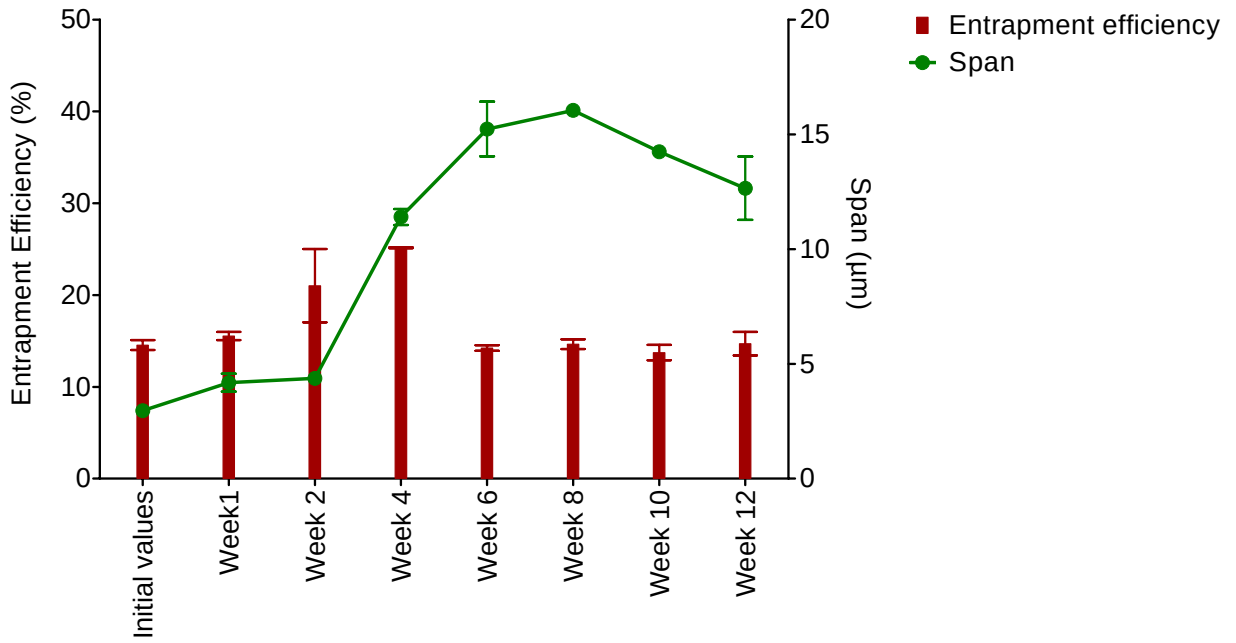


Figure 3.12: Comparison between entrapment efficiency and span of CQ entrapped in liposomes (mean ± SEM) at 5°C during a time period of twelve weeks (n = 3).

Figure 3.12 illustrates the comparison between entrapment efficiency and span of CQ entrapped in liposomes during a stability study at 5°C over a time period of twelve weeks. The left y-axis represents the entrapment efficiency (%) and the right y-axis the span (µm) of CQ entrapped in liposomes. The x-axis represents the twelve week time period. The entrapment efficiency, as discussed earlier, started to increase from the initial value up until week 4 and started to decrease between week 4 and week 6 which then remained more or less constant between week 6 and week 12. The span values, as discussed earlier, remained more or less constant from the initial value up until week 2 and started to increase between week 2 and week 8 it then decreased between week 8 and week 10. No statistically significant difference could be observed between the entrapment efficiency and span of CQ entrapped in liposomes during the twelve week stability study at 5°C with a R_s value of -0.14 which indicates that $p > 0.05$ (Annexure C).

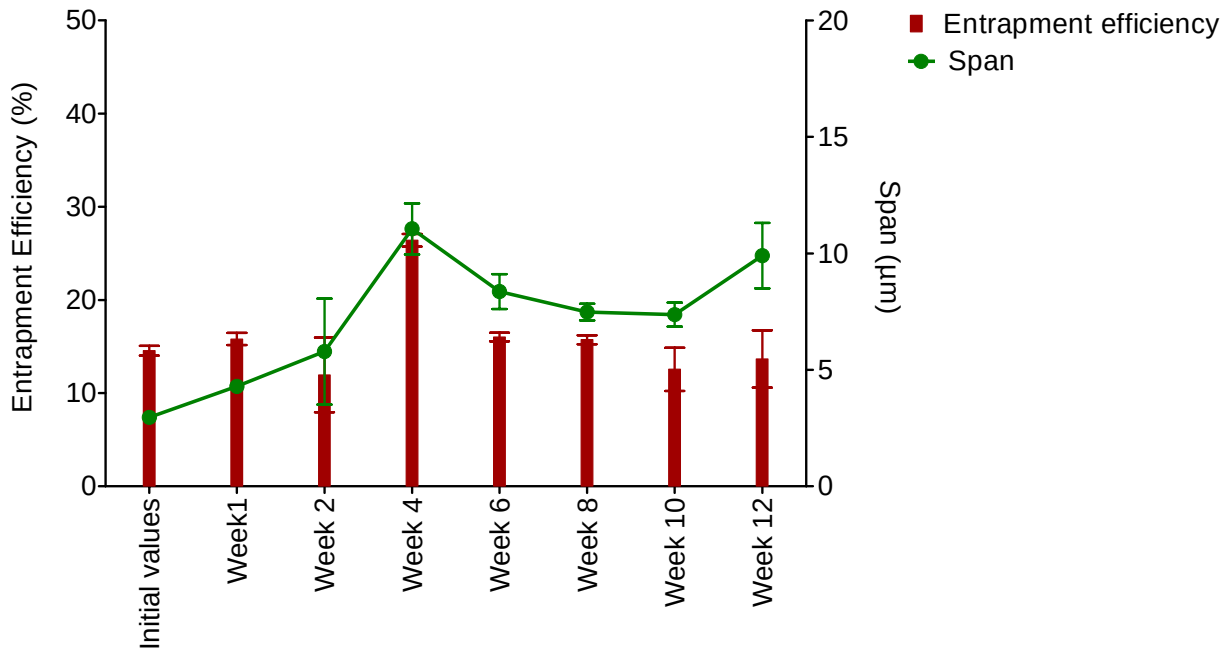


Figure 3.13: Comparison between entrapment efficiency and span of CQ entrapped in liposomes (mean ± SEM) at 25°C during a time period of twelve weeks (n = 3).

Figure 3.13 illustrates the comparison between entrapment efficiency and span of CQ entrapped in liposomes during a stability study at 25°C over a time period of twelve weeks. The left y-axis represents the entrapment efficiency (%) and the right y-axis the span (µm) of CQ entrapped in liposomes. The x-axis represents the twelve week time period. Entrapment efficiency, as previously discussed, decreased between week 1 and week 2 and then started to increase between week 2 and week 4. Entrapment efficiency then decreased between week 4 and week 10 and then increased slightly between week 10 and week 12. The span values, as previously discussed, started to increase from the initial value to week 4, decreased from week 4 to week 10 and increased slightly from week 10 to week 12. A statistically significant difference could be observed between the entrapment efficiency and span of chloroquine entrapped in liposomes during the twelve week stability study at 25°C with a R_s value of 0.59 which indicates that $p < 0.05$ (Annexure C).

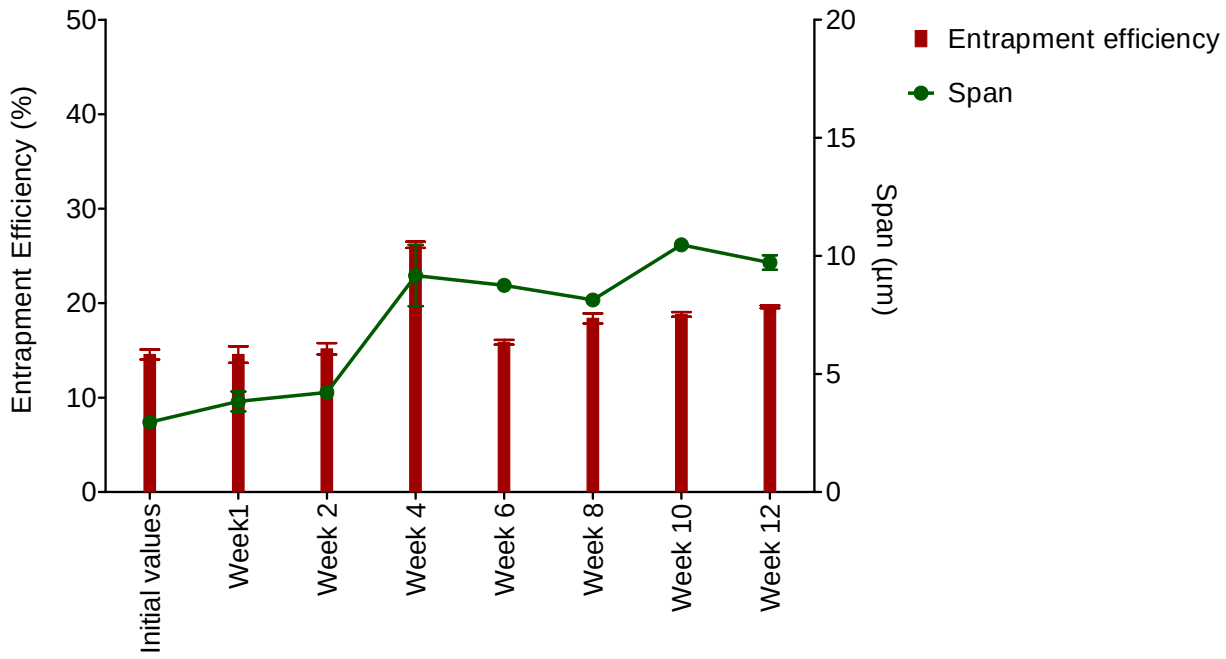


Figure 3.14: Comparison between entrapment efficiency and span of CQ entrapped in liposomes (mean ± SEM) at 40°C during a time period of twelve weeks (n = 3).

Figure 3.14 illustrates the comparison between entrapment efficiency and span of CQ entrapped in liposomes during a stability study at 40°C over a time period of twelve weeks. The left y-axis represents the entrapment efficiency (%) and the right y-axis the span (µm) of CQ entrapped in liposomes. The x-axis represents the twelve week time period. As earlier discussed, the entrapment efficiency remained more or less constant between the initial value and week 2 and then started to increase between week 2 and week 4. Entrapment efficiency then decreased between week 4 and week 6 then slightly increased between week 6 and week 8 and remained more or less constant between week 10 and week 12. As earlier discussed, the span values remained more or less constant between week 1 and week 2, started to increase between week 2 and week 4 and remained more or less constant between week 4 to week 8. Span then increase between week 8 and week 10 and remained more or less stable between week 10 and week 12. A statistically significant difference could be observed between the entrapment efficacy and span of CQ entrapped in liposomes during the twelve week stability study at 40°C with a R_s value of 0.74 which indicates that $p < 0.05$ (Annexure C).

There appeared to be no direct significant relationship between the size of liposomes and the entrapment efficiency of liposomes at a storage temperature of 5°C. A direct correlation was however observed at both 25°C and 40°C. Which therefore means that as the size of the

liposomes increased the entrapment efficiency of the liposomes increased as well. The amount of CQ entrapped within the liposomes, however, remained very low.

In an effort to determine whether the amount of CQ entrapped inside of the liposomes could be increased, different CQ entrapped in liposome formulations were manufactured. The relationship between the span value variances and the entrapment efficiency of the formulation was then evaluated.

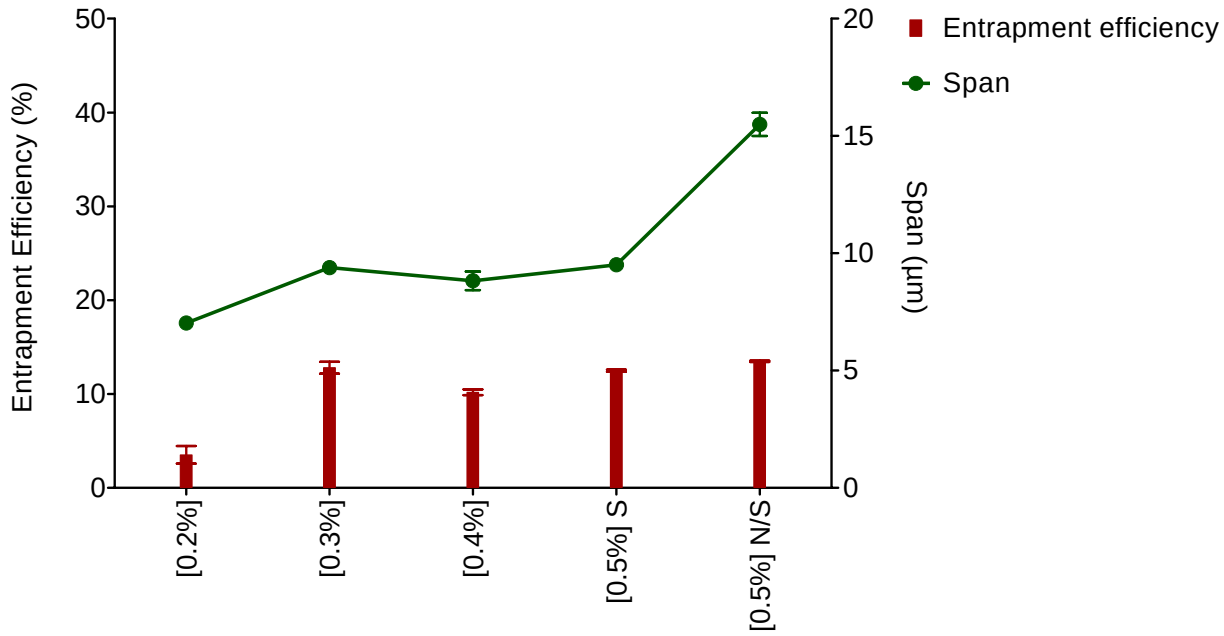


Figure 3.15: Comparison between entrapment efficiency and span (mean ± SEM) of various CQ entrapped in liposome formulations (n = 3).

Figure 3.15 illustrates the comparison between the entrapment efficiency and span of different CQ entrapped in liposome formulations. The left y-axis represents the entrapment efficiency (%) and the right y-axis the span (µm) of the various CQ entrapped in liposome formulations. The x-axis represents the five different CQ entrapped in liposome formulations. The value of the [0.2 %] formulation was 3.51 % in terms of entrapment efficiency and 7.02 µm in terms of span (Annexure B, Table B10). The value of the [0.3 %] formulation was 12.81 % in terms of entrapment efficiency and 9.39 µm in terms of span (Annexure B, Table B10). The value of the [0.4 %] formulation was 10.18 % in terms of entrapment efficiency and 8.82 µm in terms of span (Annexure B, Table B10). The value of the [0.5 %] formulation, which was sonicated (S), was 12.47 % in terms of entrapment efficiency and 9.51 µm in terms of span (Annexure B, Table B10). The value of the [0.5 %] formulation, which was not sonicated (N/S), was 13.47 %

in terms of entrapment efficiency and 15.49 µm in terms of span (Annexure B, Table B10). There were no statistically significant differences between any of the formulations in terms of their entrapment efficiency and span with R_s values of -1.00, 0.50, -1.00, -0.50 and 0.50 respectively which indicates that $p > 0.05$ (Annexure C).

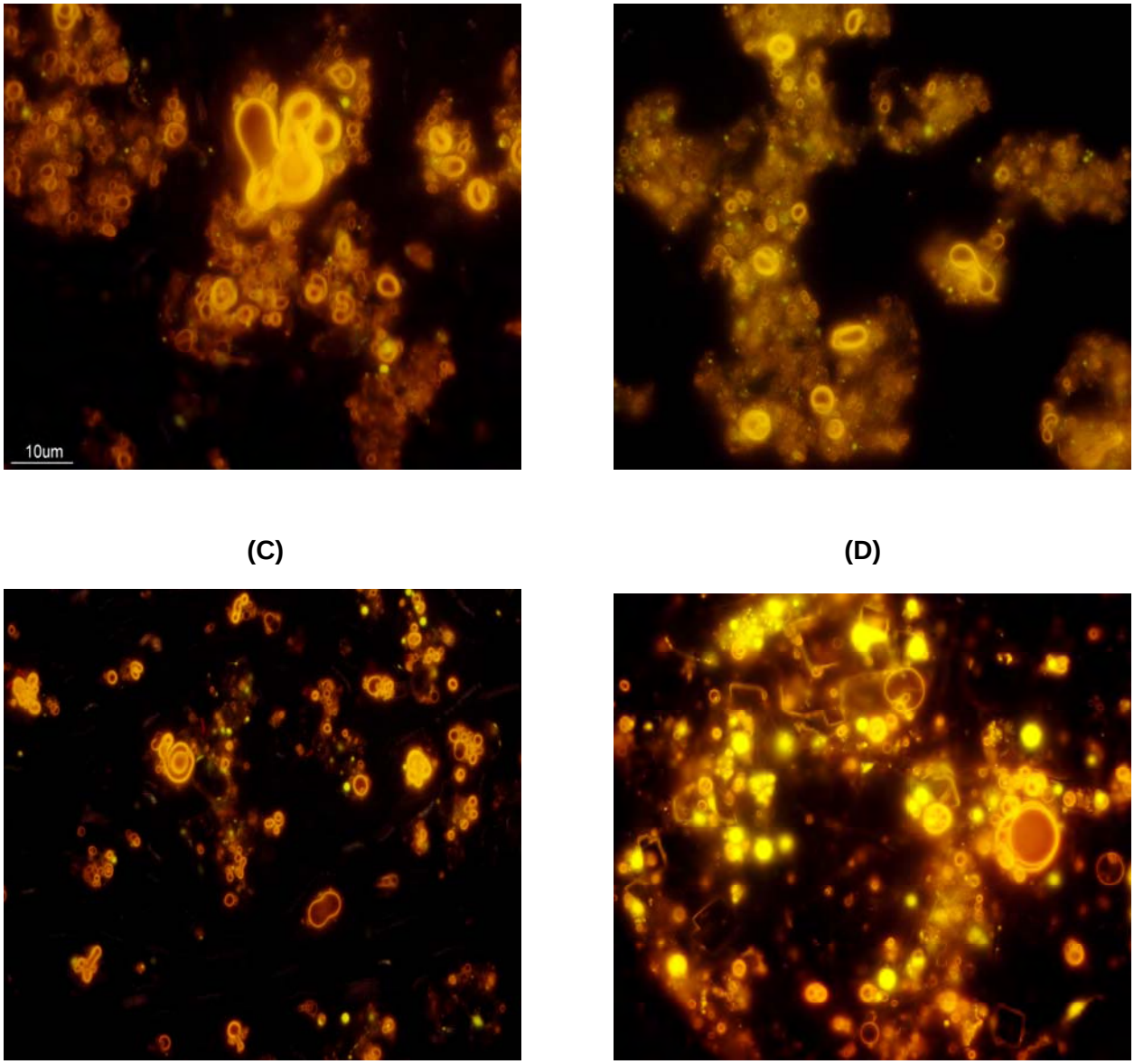
Incorporation of [0.2 %] CQ resulted in a low entrapment efficiency (3.51 ± 0.93 %), whereas, [0.3 %] (12.81 ± 0.63 %), [0.4 %] (10.18 ± 0.31 %) and [0.5 %] (12.47 ± 0.11 %) resulted in slightly higher entrapment efficiencies. The size of the formulations increased slightly with increased CQ concentrations [0.2 %] (7.02 ± 0.14 µm), [0.3 %] (9.39 ± 0.21 µm), [0.4 %] (8.82 ± 0.40 µm) and [0.5 %] (9.51 ± 0.11 µm). All the formulations were sonicated to reduce the size of the vesicles. In order to determine whether this had an influence on the entrapment efficiency another [0.5 %] CQ entrapped in liposome formulation was prepared and not sonicated. This resulted in liposomes with a large span (15.49 ± 0.49 µm), but with no increase in entrapment efficiency (13.47 ± 0.06 %). There were no statistically significant differences which indicate that the size of the vesicles did not have an influence on their entrapment efficiency.

3.11.5) Morphological evaluation

Light microscope photos were taken of the initial liposome and initial CQ entrapped in liposome formulation. Light microscope photos were also taken of the liposome and CQ entrapped in liposome formulations after being subjected to twelve weeks of stability testing at temperatures of 5°C, 25°C and 40°C. The morphological evaluation of liposomes and CQ entrapped in liposomes are essential in determining whether the formulations remained in a stable condition over a time period of twelve weeks.

The appearance of the initial liposome formulation, as described in section 3.3.2, and liposome samples which were exposed to temperatures of 5°C, 25°C and 40°C, as mentioned in section 3.4, are shown in image 3.1, photos A to D, a particle size scale bar of 10 µm is included for comparative purposes.

(A) **(B)**



(C) **(D)**

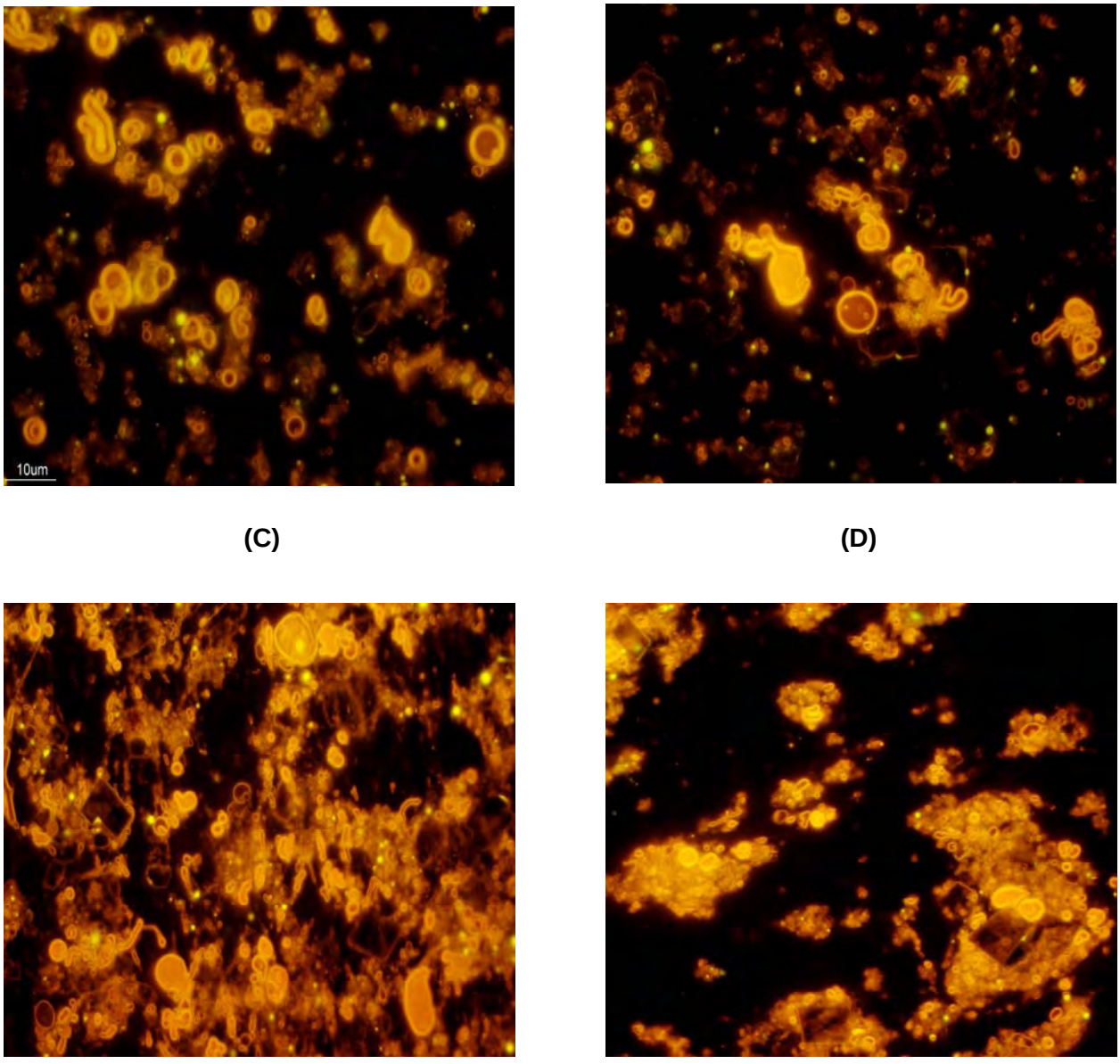
Image 3.1: Light microscope photos of the initial liposomes (A), liposomes at 5°C (B), liposomes at 25°C (C) and liposomes at 40°C (D) after being subjected to twelve weeks of stability testing.

Spherical liposome vesicles can be observed in image A of the initial liposome sample, in image B at 5°C after being subjected to twelve weeks of stability testing and in image C at 25°C after

being subjected to twelve weeks of stability testing. Crystal formation can clearly be observed in Image D at 40°C after being subjected to twelve weeks of stability testing. Oil droplets are visible as green dots.

The appearance of the initial CQ entrapped in liposome formulation, as described in section 3.3.2, and CQ entrapped in liposome samples which were exposed to temperatures of 5°C, 25°C and 40°C, as mentioned in section 3.4, are shown in image 3.2, photos A to D, a particle size scale bar of 10 µm is included for comparative purposes.

(A) **(B)**



(C) **(D)**

Image 3.2: Light microscope photos of the initial CQ entrapped in liposomes (A), CQ entrapped in liposomes at 5°C (B), CQ entrapped in liposomes at 25°C (C) and CQ

entrapped in liposomes at 40°C (D) after being subjected to twelve weeks of stability testing.

Spherical liposome vesicles can be observed in image A of the initial CQ entrapped in liposome sample and in image B at 5°C after being subjected to twelve weeks of stability testing. Crystal formation can clearly be observed in image C at 25°C after being subjected to twelve weeks of stability testing and in image D at 40°C after being subjected to twelve weeks of stability testing. Oil droplets are visible as green dots.

3.12) Conclusion

The pH of both the liposome and CQ entrapped in liposome formulations remained stable for a period of twelve weeks at a temperature of 5°C, but not at temperatures of 25°C and 40°C. Liposome formulations also remained stable for a period of twelve weeks at temperatures of 25°C and 40°C in terms of their span values. CQ entrapped in liposomes did not remain stable over the twelve week period in terms of their span values. A directly proportional relationship was observed between the entrapment efficiency and the pH of CQ entrapped in liposomes over the twelve week period at a temperature of 5°C. Statistically significant differences were also observed between the entrapment efficiency and span of CQ entrapped in liposomes during the twelve week period at temperatures of 25°C and 40°C. No statistically significant differences were observed, with varying CQ concentrations, between any of the different CQ entrapped in liposome formulations in terms of their entrapment efficiencies and span. Based on the evidence it may be reasonable to conclude that the CQ entrapped in liposome formulation remained stable over the twelve week period at a temperature of 5°C whilst the liposome formulation remained stable over the twelve week period at temperatures of 5°C and 25°C in terms of their morphology.

CHAPTER 4

In vitro evaluation of liposome toxicity:

Methods, Results & Discussion

4.1) Introduction

All the *in vitro* assays currently in use are based on Trager & Jensen's (1976) *in vitro* culture method. The *in vitro* sensitivity of *P*. *falciparum* parasites to existing drugs and new drug candidates can be established by this culture method. Human malaria parasites are directly exposed to drugs in culture plates in all of these *in vitro* methods. Numerous *in vivo* and *in vitro* bioassays are available which are based on continuous culture of reference strains of *P. falciparum (*Basco, 2007).

Three assays were conducted during this *in vitro* study. Two of the assays included ROS and LP which were analyzed via flow cytometry with a FACSCalibur^{TM}. An assay on hemolysis were also conducted which were analyzed via spectrophotometry with a Biotek EL X 800 plate reader.

The main purpose of this study was to evaluate the possible *in vitro* toxicity of liposomes. In the following sections the cultivation of *P. falciparum* and the maintenance of the cell cultures are described. A detailed experimental design regarding the three *in vitro* toxicity assays is given along with the methods used to conduct the ROS assay, LP assay and hemolysis assay respectively. The statistical evaluation as well as an in depth discussion of each assay is also described.

4.2) Manufacturing of liposomes and CQ entrapped in liposomes

A 10 ml liposome and a 10 ml CQ entrapped in liposome formulation were prepared for this *in vitro* study. In this study liposomes, consisting mainly of phosphatidyl choline and cholesterol, were manufactured (as discussed in section 3.4 of chapter 3). CQ phosphate was incorporated into the liposomes (reffered to as CQ entrapped in liposomes) during manufacturing.

4.3) Cultivation of *Plasmodium falciparum*

The continuous cultivation of *P. falciparum* was originally described by Trager and Jensen as the candle jar method (Trager and Jensen 1976; Basco, 2007). *In vitro* growth of *Plasmodium* requires mimicking the conditions during the parasite's erythrocytic cycle (Schuster, 2002).

4.3.1) Materials

The CQ-resistant strain (RSA 11) were supplied by The University of Cape Town (UCT) (South Africa). D-(+)-glucose powder, gentamycin solution, HEPES [(N-(2-hydroxyethyl)piperazine-N'-(-2-ethanesulfonic acid)], hypoxanthine, Roswell Park Memorial Institute (RPMI) – 1640 powder, sodium bicarbonate (Na_2CO_3), sodium chloride (NaCl) and sorbitol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Albumax II® serum was purchased from Gibco (New Zealand). Sterile water for injection was purchased from Medirex Pharmacy (Potchefstroom, South Africa). A three component gas mix, 5 % oxygen (O_2), 5 % carbon dioxide (CO_2) and 90 % nitrogen gas (N_2) were obtained from Afrox (Germiston, South Africa). Liquid nitrogen was purchased from Afrox (Potchefstroom, South Africa). Culture flasks and other consumables were purchased from Scientific Group (South Africa). Giemsa® stain, sodium phosphate and potassium phosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Merck Chemicals (South Africa) and first grade frosted 1.2 mm microscope slides were purchased from Lasec (South Africa).

4.3.2) Methods

P. falciparum was cultivated according to a modified version of the cultivation method described by Trager and Jensen (1976). All of the procedures discussed below were performed under sterile conditions by employing a 4ft, class II, laminar flow hood (Laboratory and Air Purification Systems, Midrand, South Africa).

A CQ-resistant *P. falciparum* strain (RSA 11) was used in this study. The strain was stored in liquid nitrogen for long-term storage purposes. The parasite samples were thawed in a Sonorex Digital 10P Bandelin (Germany) water bath at 37°C. The contents were then transferred, under sterile conditions, to 15 ml centrifuge tubes. A 12 % and 1.6 % NaCl solution were respectively added slowly and thoroughly mixed with the contents in the centrifuge tubes for ten to twenty seconds. The contents were then transferred to 75 cm^3 culture flasks. Fresh red blood cells (RBC) (± 0.5 ml) and preheated culture medium (10 ml) were added to the contents in the culture flasks and gassed for approximately 30 seconds with a gas mixture which comprised of

5 % O_2, 5 % CO_2 and 90 % N_2. The culture flasks were subsequently sealed airtight and incubated at 37°C.

A 100 ml *in vitro* culture medium of *P. falciparum* (Table 4.1) was prepared for cultivation purposes.

Table 4.1: The quantities of the compounds used to produce the culture medium.

Compound	Quantity of compound
RPMI 1640 medium	1.04 g/100 ml
D-(+)-glucose	0.4 g/100 ml
HEPES	0.6 g/100 ml
Hypoxantine	0.0044 g/100 ml
Gentamycin (40mg/ml)	0.12 ml/100 ml
Sodium bicarbonate	4.2 ml/100 ml of a 5 % solution
Albumax II®	0.5 g/100 ml

All of the reagents were dissolved in sterile water for injection and then filtered under vacuum through a 0.22 µm filter. A wash medium was also prepared and was used to wash freshly obtained human RBC for the removal of its leukocytes. The wash medium consisted of the same ingredients as the culture medium with the exception of Albumax II®.

4.4) Blood collection and preparation

The cultivation procedures were performed biweekly which entailed that the culture medium in the culture flasks was replaced with fresh (pre-heated) culture medium. A few drops of blood were also added to the culture flasks once a week during one of the cultivation procedures. The culture flasks were gassed after each cultivation procedure (as discussed before) and maintained at 37°C.

The parasitemia of each of the culture flasks were determined by preparing thin blood smears of the parasite cultures. The thin blood smears were prepared by placing a drop of the parasite culture from each culture flask on a microscope slide. The samples were then smeared across

the length of the slides by placing a second slide on the drop of parasite culture from each of the initial slides. The second slide was moved backwards to spread the red blood cells across the width of the slide and then moved forward to smear a thin film of red blood cells on each of the initial slides. The slides were then left to air dry. A Giemsa® solution which consisted of 100 µl Giemsa® stain and 400 µl of a phosphate buffer (0.65 g sodium phosphate and 0.41 g potassium phosphate to one liter of water) were prepared for each microscope slide. The air dried blood smears were then fixed with 96 % methanol and stained with the Giemsa® solution for 10 to 15 minutes. Each of the thin blood smear slides was rinsed, after staining, under running water to remove all of the unbound stain. The microscope slides were then left to air dry and afterwards viewed under a Nikon Eclipse TE-300 inverted light microscope equipped with a DXM 1200 digital camera (Japan). Each microscope slide was divided into ten fields. Infected versus uninfected erythrocytes were then counted in the ten fields of each microscope slide. The percentage parasitemia of each slide was calculated with the following formula:

$$\textbf{\% Parasitemia} = \frac{\textbf{Amount of infected red blood cells}}{\textbf{Total amount of red blood cells}} \textbf{ X 100} \qquad \textit{Eq. 4.1}$$

A parasitemia of 1.0 % - 1.5 % needed to be maintained in the culture flasks for experimental purposes.

Fresh RBC were obtained from human whole blood (group A^+) and collected in 10 ml EDTA-vaccutainers which contained an anticoagulant. The blood washing procedure consisted of repeated steps of blood and wash medium mixing, centrifugation and aspiration. A PLC Series Gemmy Industrial Corporation centrifuge was used for this procedure. The removal of leucocytes is essential since their presence is detrimental to the growth of parasites. The whole blood was washed with the wash medium and then centrifuged at a speed of 2000 rpm for eight minutes and the buffy coat/supernatant was removed by means of aspiration. This procedure was repeated thrice in an attempt to remove all the leukocytes. After the washing procedure the red blood cells were resuspended in wash medium and stored at 4°C in a refrigerator. Ethics approval was obtained from the Ethics Committee of the North-West University, approval number of NWU – 0008 – 08 – S5 (Annexure D).

4.5) Experimental design

FCM (as discussed in chapter 3, section 3.7) is a technique based on the principle of light scattering. FCM is extremely useful in analyzing viable, single cells by using a fluorescent marker suspended in a fluid stream (Salzman & Wilder, 1979; Childers & Michalek, 1989; Shapiro, 1995). The nature and the degree of cellular damage during *in vitro* studies can also be identified via FCM (Eisenbrand *et al*., 2002). The FCM analysis for both the ROS assay and the LP assay were performed on a FACSCalibur™ (BD Biosciences). Each of the samples was analyzed at a flow rate of 1000 events/second.

Hemolysis (destruction of red blood cells) provides a common method for the assessment of cytotoxicity (Hadbagy *et al*., 2003). A spectrophotometric analysis of the hemolysis assay was performed with a Biotek EL X 800 plate reader.

Red blood cell (RBC) samples (14) and infected red blood cell (iRBC) samples (14) were prepared in duplicate, each in their own 96-well plate. Each of the total of 28 samples per well plate consisted of 6 control samples (consisting of cells with either wash medium or culture medium) and 22 experimental samples (consisting of cells with different liposome concentrations, different CQ entrapped in liposome concentrations and a [0.5 %] CQ concentration. The samples were prepared and afterwards incubated at 37°C for 24 hours.

4.5.1) Materials

CQ phosphate was purchased from IPCA laboratories limited (India). PBS, with a concentration of 10 mM, was obtained from Scientific Group (South Africa) and diluted with ddH_2O to a concentration of 1 mM. The CQ-resistant strain (RSA 11) were supplied by The University of Cape Town (UCT) (South Africa). Fresh RBC were obtained from human whole blood (group A^+).

4.5.2) Methods

The initial parasitemia of the CQ-resistant strain of *P. falciparum* (RSA 11) was determined for each culture flask by preparing thin blood smears. The smears were then viewed under a light microscope. The parasitemia of each culture flask was subsequently calculated (as discussed in section 4.2.2). The contents of the culture flask, which contained the correct parasitemia for the experiment, were placed in a 10 ml falcon tube. The percentage parasitemia and hematocrit of the contents in the falcon tube were adjusted, under sterile conditions, to

approximately 1.5 % and 3.0 % respectively. The hematocrit of the washed, fresh RBC (as discussed in section 4.2.2) were adjusted to 3.0 %.

All of the procedures discussed below were performed under sterile conditions by employing a 4ft, class II, laminar flow hood (Laboratory and Air Purification Systems, Midrand, South Africa). Fourteen different samples (100 µl) were prepared in duplicate, for the RBC and for the iRBC, for incubation in 96-well plates. The six control samples of RBC and iRBC were made up to 200 µl with wash medium (WM) and culture medium (CM) respectively. The difference between the control samples will be discussed in the respective results sections. The experimental samples, which were also made up to 200 µl, consisted of five different liposome samples (with concentrations of 0, 25, 50, 75 and 100 %) in duplicate, five different CQ entrapped in liposome samples (with concentrations of 0, 25, 50, 75 and 100 %) in duplicate and one [0.5 %] CQ sample in duplicate. A [0.5 %] CQ phosphate solution was prepared by dissolving 0.05 g CQ phosphate powder in 10 ml PBS. The liposome samples were prepared by adding the manufactured liposome formulation to the cells and by diluting the samples to their specific concentrations with PBS. The CQ entrapped in liposome samples were prepared by adding the manufactured CQ entrapped in liposome formulation to the cells and then diluting the samples to their specific concentrations with some of the manufactured liposome formulation. The well plates were gassed (as discussed in section 4.3.2) with the three component gas mixture and placed in an incubation oven at 37°C for 24 hours. The samples of the hemolysis assay (which were only prepared for RBC) were also prepared in duplicate in a 96-well plate, with control and experimental samples, and incubated for 24 hours.

4.6) ROS assay

ROS (as discussed in chapter 1 section 1.7) are unstable, chemically reactive, free radicals which contains unpaired electrons and facilitates the extreme reactivity of ROS, by rapidly reacting with other molecules or radicals in order to obtain stability (de Zwart *et al*., 1999; Grune *et al*., 2000; Kohen & Nyska, 2002). ROS have the ability to oxidize assorted cellular molecules which may lead to DNA damage and the oxidation of polyunsaturated fatty acids within lipids (LP) (Hershko *et al*., 1998; Droge, 2002). ROS production was detected during this *in vitro* study by using the dye 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is a stable, cell-permeable, non-fluorescent compound which is converted to 2', 7'- dichlorodi-hydrofluorescein (DCFH) by intracellular esterases as soon as cell penetration occurs. This leads to the entrapment of the dye within the cell. The dye is then converted to the highly fluorescent 2', 7' dichlorofluorescein (DCF) due to cellular oxidation as a result of ROS. DCF produces green fluorescence, in reaction to excitation at 488 nm, proportional to the intracellular level of ROS. The intracellular accumulation of ROS, as well as the overall oxidative stress in

cells, is therefore influenced by changes in the fluorescence of DCF (Crow, 1997; Wang & Joseph, 1999; Gomes *et al*., 2005).

The main goal was to investigate the effect of CQ [0.5 %], plus different liposomes and CQ entrapped in liposomes concentrations, on the production of ROS which occurs within RBC and iRBC. The applicability of FCM in the determination of the production of ROS within RBC and iRBC were also investigated

4.6.1) Materials

PBS, with a concentration of10 mM, was obtained from Scientific Group (South Africa) and diluted with ddH_2O to a concentration of 1 mM. DCFH-DA dye was purchased from Invitrogen (United Stated of America). Hydrogen peroxide (H_2O_2) (30 %) solution was obtained from Sigma-Aldrich (St. Louis, MO, USA) and diluted to a 50 mM stock solution.

4.6.2) Methods

The two well plates containing 28 samples each (discussed in section 4.5) were removed from the incubation oven after 24 hours. Each of the samples (50 µl) was transferred to FACS tubes. A stock solution of the dye in methanol (10 mM) was prepared. The dye was used at a final concentration of 10 µm. H_2O_2 (80 µl) was added to all of the positive controls (to initiate ROS production) and incubated in the dark at room temperature for 60 minutes. After the 60 minutes of incubation DCFH-DA dye was added to the positive controls and all of the other samples except for the cell controls. All of the samples were then incubated in the dark at room temperature for 30 minutes. After the 30 minutes of incubation each sample was vortexed and analyzed by flow cytometry via a FACSCalibur^{TM}. A 488-nm argon laser was used for excitation. Forward scatter (FSC) and side scatter (SSC) were collected on a log scale. A 100 000 events were recorded for each sample. The preliminary data of all of the samples were acquired on a MAC-OC computer using CELLQUEST PRO (BD Biosciences) and analyzed with FlowJo version 7.6.1 (Tree star). Results were plotted on a histogram which illustrated the amount of DCFH fluorescence (FL1) and the amount of cells counted (count). Gates (named M1 and M2) was set on the histogram (Figure 4.1). The first gate (M1) was set according to the cell control (cells without any dye added) and presented as a baseline (background) fluorescence value within the first decade (10^1). The second gate (M2) was also set on the cell control and represented the fluorescence of the dye. The geometric means of fluorescence for DCFH were calculated from the respective histograms for both M1 and M2.

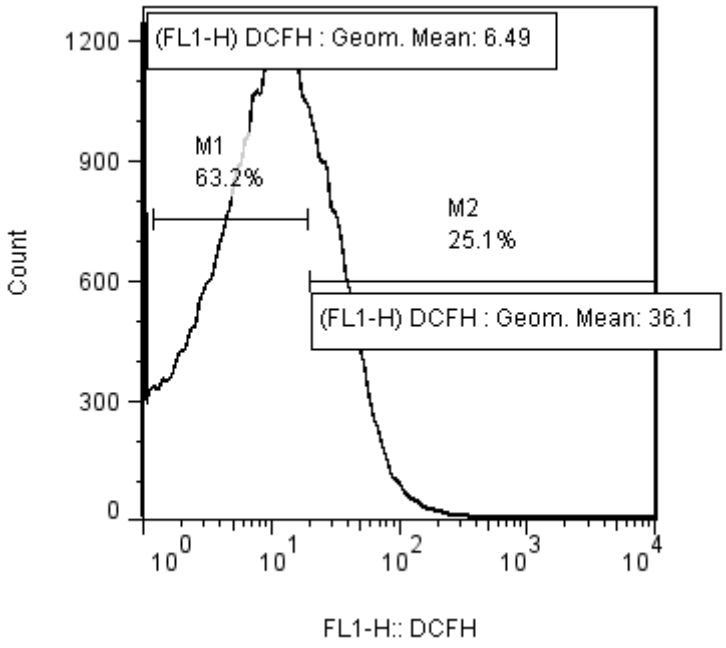


Figure 4.1: Histogram indicating the amount of fluorescence (FL1-H: DCFH) versus the amount of cells (count) of a representative sample. The gates (M1 and M2) are indicated on the plot.

The geometric means were processed with Microsoft Excel™ 2010 and Graphpad Prism™ version 5 (GraphPad Software Inc., Dan Diego, CA, USA). The data is presented as the mean ± standard error of mean (SEM).

4.7) LP assay

LP (as discussed in chapter 1 section 1.7) is the oxidative degradation of the unsaturated lipids of the cell membranes and is one of the major mechanisms associated with cellular damage due to free radical reactions caused by ROS (Kappus, 1985; Cheeseman, 1993; Rice-Evans, 1994). Extreme sensitivity to oxidation is associated with the polyunsaturated fatty acid residues of phosholipids. Individual cell membranes can be labeled with lipophilic fluorescein molecules in order to measure lipid peroxidation within different cell membranes. The fluorescence of the lipophilic fluorescein molecules can then be measured with flow cytometry. The fluorescence of fluorescein is diminished when a reaction occurs between fluorescein and peroxyl radicals (Makrigiorgos *et al*, 1997; Marnett, 2000). LP was detected during this *in vitro* study by using the dye N-(fluorescein-5-thiocarboyl)-1,2-diheade-canoyl-sn-glycero-3-phosphoethanolamine, tri-ethulammonium salt (fluorescein-DHPE).

The main goal was to investigate the effect of CQ [0.5 %], plus different liposomes and CQ entrapped in liposomes concentrations, on LP which occurs RBC and iRBC. The applicability of

FCM in the rapid and sensitive detection of lipid peroxidation within RBC and iRBC, exposed to oxidative stress, were also investigated

4.7.1) Materials

PBS, with a concentration of 10 mM, was obtained from Scientific Group (South Africa) and diluted with ddH_2O to a concentration of 1 mM. Fluorescein-DHPE dye was purchased from Invitrogen (United Stated of America). Hydrogen peroxide (H_2O_2) (30 %) solution was obtained from Sigma-Aldrich (St. Louis, MO, USA) and diluted to a 50 mM stock solution.

4.7.2) Methods

The two well plates containing 28 samples each (discussed in section 4.5.2) were removed from the incubation oven after 24 hours. Each of the samples (50 µl) was transferred to FACS tubes. A stock solution of the dye (10 mM) in ethanol was prepared. The dye was used at a final concentration of 5µm. Fluorescein-DHPE dye was added to the negative controls (which demonstrated normal lipid peroxidation within cells) and all of the samples, except for the cell controls. The samples were then incubated in the dark at room temperature for 60 minutes. After the 60 minutes of incubation H_2O_2 (10 mM) were added to all of the positive controls (to initiate LP production) and incubated in the dark at room temperature for 30 minutes. After the 30 minutes of incubation each sample was vortexed and analyzed by flow cytometry via a FACSCalibur™. A 488-nm argon laser was used for excitation. Forward scatter (FSC) and side scatter (SSC) were collected on a log scale. A 100 000 events were recorded for each sample. The preliminary data of all of the samples were acquired on a MAC-OC computer using CELLQUEST PRO (BD Biosciences) and analyzed with FlowJo version 7.6.1 (Tree star). Results were plotted on a histogram with Fluorescein-DHPE (FL1) on the x-axis and the amount of cells counted (count) on the y-axis. Gates (named M1 and M2) were set on the histogram (Figure 4.2). The geometric means of fluorescence for Fluorescein-DHPE were calculated from the respective histograms for both M1 (which indicates the normal fluorescence percentage) and M2 (which indicates the fluorescence percentage of the sample). In this assay a decrease in Fluorescein-DHPE is indicative of an increase in LP. The gates (M1 and M2) were set on the cell control (cells without any dye added).

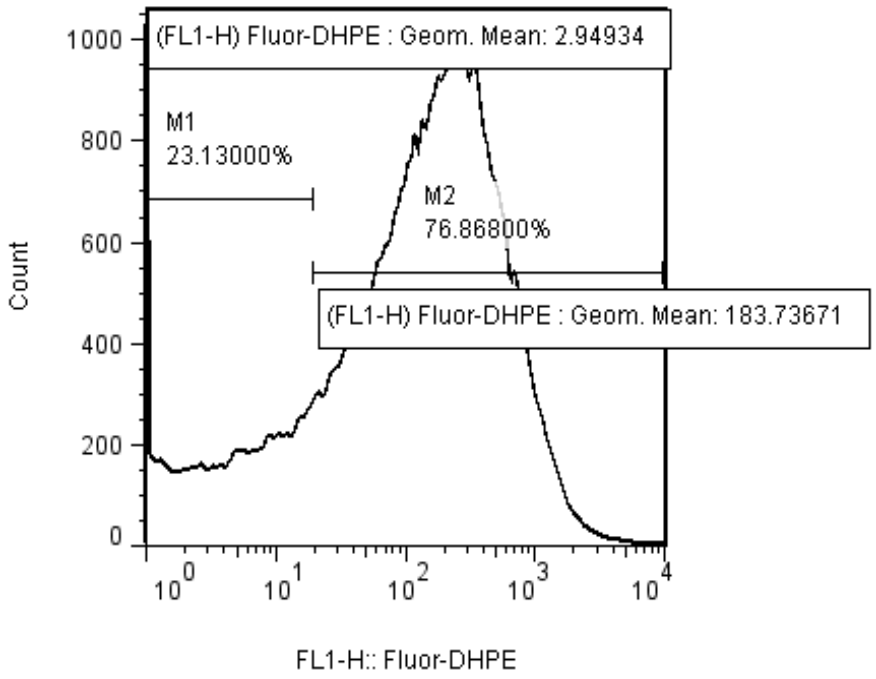


Figure 4.2: Histogram indicating the amount of fluorescence (FL1-H: Fluor-DHPE) versus the amount of cells counted (count) in a representative sample. **The gates (M1 and M2) are indicated on the plot.**

The geometric means were processed with Microsoft Excel™ 2010 and Graphpad Prism™ version 5 (GraphPad Software Inc., Dan Diego, CA, USA). The data was inverted and is presented as the mean ± standard error of mean (SEM).

4.8) Hemolysis assay

Hemolysis is defined as the destruction of RBC due to oxidative stress, caused by oxygen species, or due to membrane damage (when the antioxidant enzymes, which are the free radical scavengers, are depleted and can therefore not protect the RBC from oxidative damage) (Trotta *et al*., 1981; CDC, 2004; Tabatabaie & Floyd, 1994; Miller *et al.*, 2002). The oxygen species, specifically O_2^-, reacts with methemoglobin and oxyhemoglobin within the RBC, which then leads to the precipitation of hemoglobin. The precipitation of hemoglobin therefore promotes hemolysis (Lynch *et al*., 1976; Sutton *et al*., 1976).

This assay consisted of a mixture of test solutions with RBC. The hemolysis of the test solutions were then compared to the hemolysis obtained from a negative control with normal saline (Reed & Yalkowsky, 1985). The main goal was to investigate the effect of different liposomes and CQ entrapped in liposomes concentrations on the incidence of RBC hemolysis.

4.8.1) Methods

The well plate, containing the hemolysis samples as discussed in section 4.5.2, was removed from the incubation oven. The well plate was then placed in a Sigma Laborzentrifugen, 3K 15 Series (Gemmy Industrial Corp.), at a speed of 2000 rpm and a temperature of 25°C for 15 minutes. After the centrifugation process was complete, 100 µl of the supernatant was removed from each of the twenty eight samples and placed in another 96-well plate. The supernatant of all the samples were read by a Biotek EL X800 plate reader at 540 nm and used to evaluate the relative hemolysis. Controls included RBC + ddH_20 (positive controls representing 100 % RBC hemolysis) and a blank consisting of culture medium. The blank was subtracted from all measurements:

The percentage hemolysis for each sample was then determined with the following equation:

$$\textbf{\% Hemolysis} = \frac{\textbf{Absorbance sample – Absorbance control 0 \%}}{\textbf{Absorbance 100 \% hemolysis}} \textbf{ X 100} \qquad \textit{Eq. 4.2}$$

The data was processed with Microsoft Excel™ 2010 and Graphpad Prism™ version 5 (GraphPad Software Inc., Dan Diego, CA, USA). Graphpad was used to perform log transformations and normalizations of the data. The data is presented as the mean ± standard error of mean (SEM).

4.9) Statistical evaluation

The data were non-parametrically analyzed with STATISTICA by means of Univariate Tests of Significance (2-way ANOVA tests) to establish if there were any statistically significant differences between the RBC and the iRBC of both the ROS assay and the LP assay. Tukey HSD tests were also performed, with STATISTICA, between the different liposomes and different CQ entrapped in liposomes concentrations for the RBC and the iRBC. These tests were conducted for both the ROS assay and the LP assay to establish if there were any statistically significant differences between the different liposomes and different CQ entrapped in liposomes concentrations. Differences were considered to be statistically significant when the probability (p) value was $p < 0.05$.

Univariate Tests of Significance (2-way ANOVA tests) were also performed with STATISTICA for the hemolysis assay to establish if there were any statistically significant differences between the liposomes and CQ entrapped in liposomes. A Kruskal-Wallis test was also performed with STATISTICA to establish if there were any statistically significant differences

between the different liposome and different chloroquine entrapped in liposome concentrations. Differences were considered to be statistically significant when the probability (p) value was $p < 0.05$.

4.10) Results and discussion

4.10.1) Evaluation of ROS assay

The aim of this assay was to investigate the effect of CQ [0.5 %], plus different liposome and CQ entrapped in liposome concentrations, have on the production of ROS. The percentage of ROS production within RBC and iRBC was determined and compared to one another. The ROS assay is necessary to establish whether the liposome concentrations and the CQ entrapped in liposome concentrations have toxic effects on RBC and iRBC.

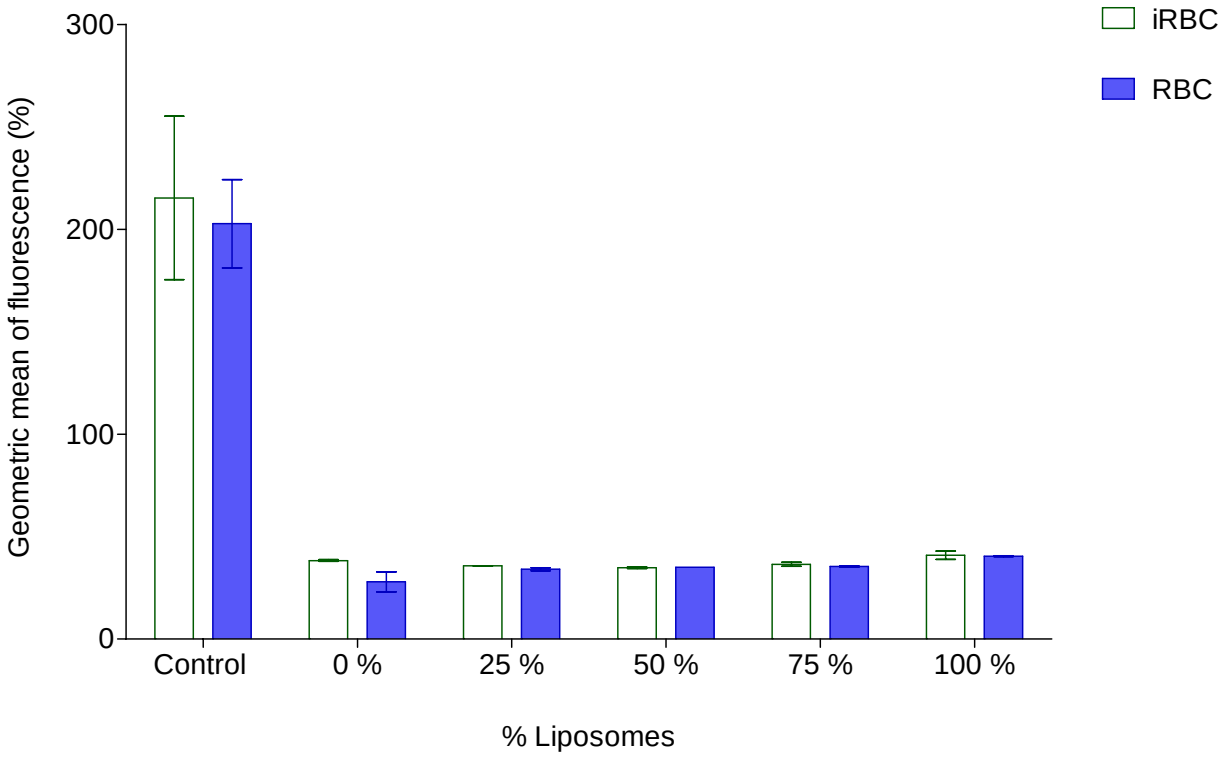


Figure 4.3: Geometric means of fluorescence (%) within RBC and the iRBC in the presence of the control group and five liposome concentrations obtained from the ROS assay.

Figure 4.3 illustrates the geometric means of fluorescence within RBC and the iRBC in the presence of the control group and five liposome concentrations obtained from the ROS assay. The y-axis represents the geometric mean of fluorescence (%) and the x-axis the control group and the five liposome concentrations. The control (10mM H_2O_2) induced high levels of intracellular ROS in both the RBC (202.66 ± 21.59 %) and the iRBC (215.23 ± 39.86 %) groups. Significantly lower intracellular ROS levels were observed in the RBC and the iRBC, in the presence of the different liposome concentrations in comparison to their intracellular ROS levels in the presence of the control. There was a slight increase in the fluorescence of the RBC in the presence of the 100 % concentration (40.43 ± 0.27 %) in comparison to its fluorescence in the presence of the 0 % concentration (27.93 ± 4.88 %), but this was not significant with $p > 0.05$ (Annexure C). The iRBC, in conjunction with the different liposome concentrations, resulted in slightly higher levels of intracellular ROS in comparison to RBC but this was also not significant with $p > 0.05$ (Annexure C). The intracellular levels of ROS in the RBC and the iRBC (Annexure B, Table B11) therefore remained more or less the same in the presence of the five different liposome concentrations.

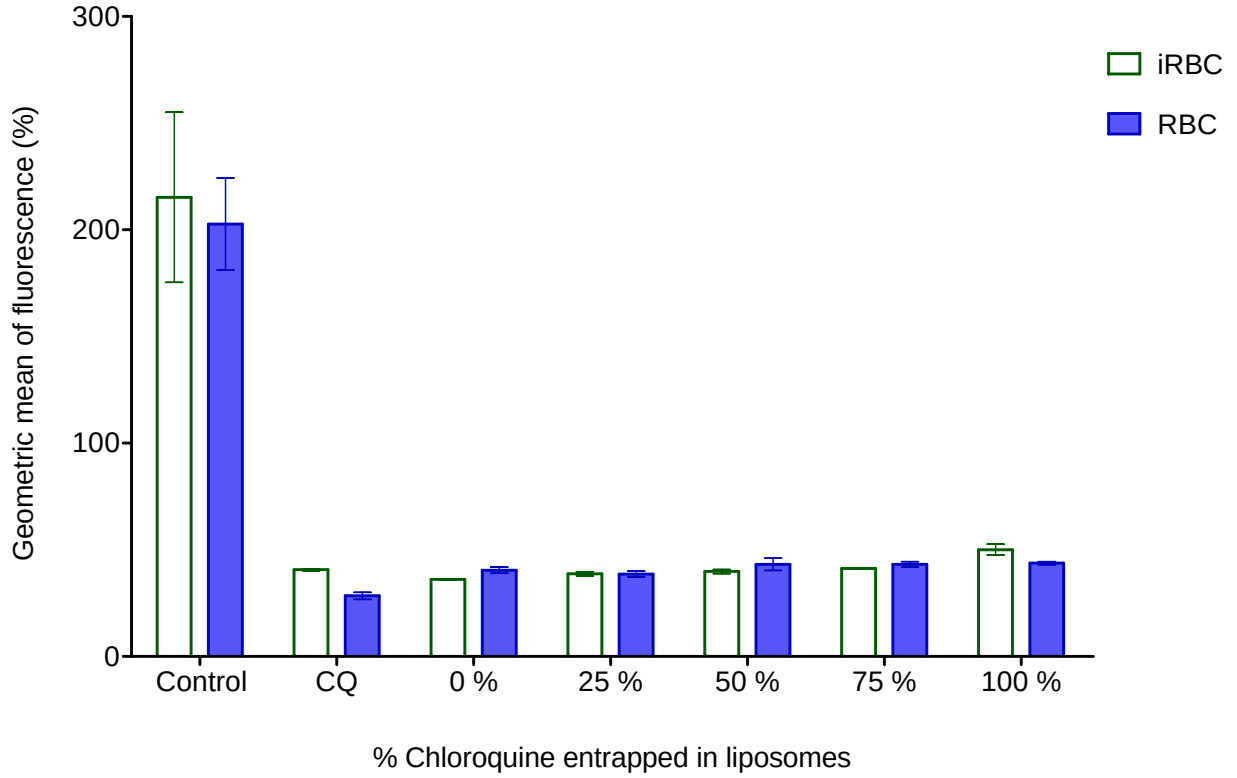


Figure 4.4: Geometric means of fluorescence (%) within RBC and iRBC in the presence of the control group, CQ [0.5 %] and five CQ entrapped in liposome concentrations obtained from the ROS assay.

Figure 4.4 illustrates the geometric means of fluorescence within RBC and iRBC in the presence of the control group, CQ [0.5 %] and CQ entrapped in liposomes obtained from the ROS assay. The y-axis represents the geometric means of fluorescence and the x-axis the control groups, CQ [0.5 %] and five CQ entrapped in liposome concentrations. The control (10mM H_2O_2) induced high levels of intracellular ROS in both the RBC (202.66 ± 21.59 %) and the iRBC (215.23 ± 39.86 %) groups. Significantly lower intracellular ROS levels were observed in the RBC and the iRBC, in the presence of CQ and the different CQ entrapped in liposome concentrations, in comparison to the control. There was a slight increase in the fluorescence of the iRBC in the presence of the 100 % concentration (50.08 ± 2.57 %) when compared to its fluorescence in the presence of the 0 % concentration (36.09 ± 4.88 %), but this was not significant with $p > 0.05$ (Annexure C). A slightly higher fluorescence was observed in the iRBC in the presence of CQ (40.66 ± 0.39 %) when compared to the fluorescence observed within the RBC in the presence of CQ (28.45 ± 1.65 %). The iRBC in conjunction with the CQ and the different CQ entrapped in liposome concentrations resulted in slightly higher levels of intracellular ROS in comparison to RBC, but this was also not significant with $p > 0.05$

(Annexure C). The intracellular levels of ROS in the RBC and the iRBC (Annexure B, Table B12) remained more or less the same in the presence of CQ and the five different CQ entrapped in liposome concentrations.

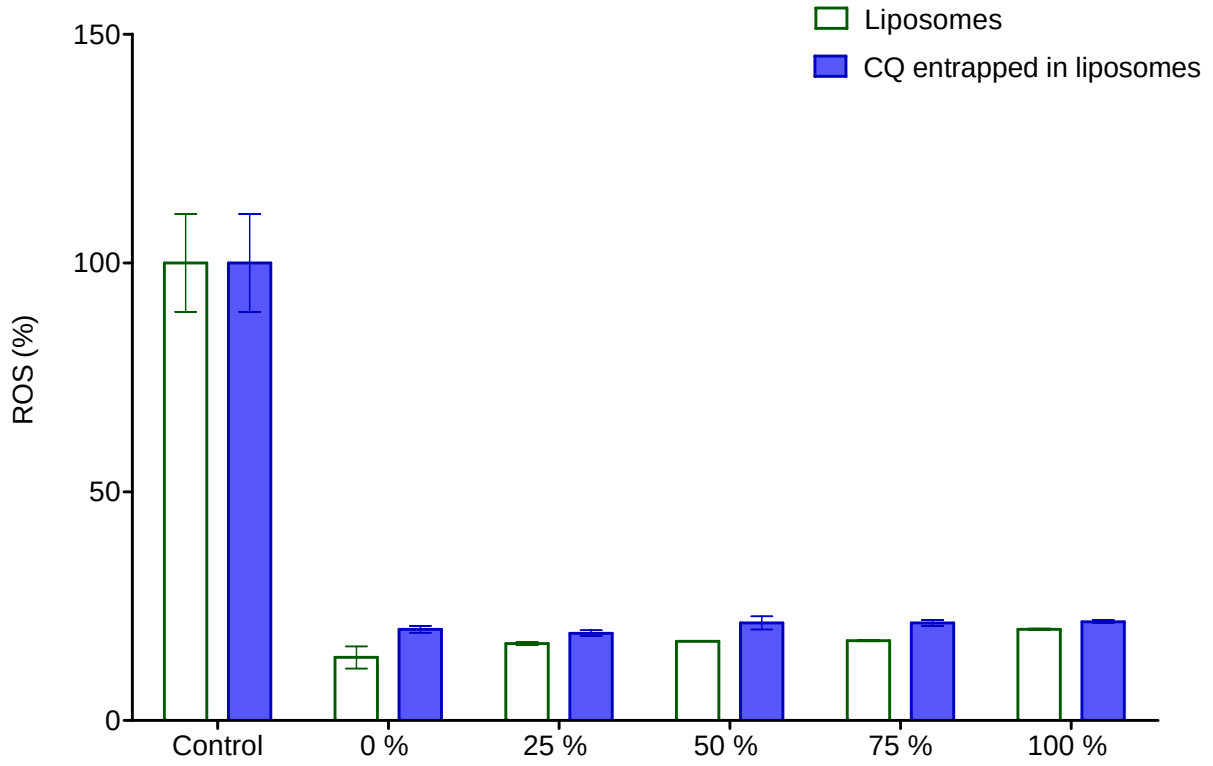


Figure 4.5: Comparison between the ROS (%) within RBC, in terms of its ROS (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes at five different concentrations.

Figure 4.5 illustrates the comparison between the ROS (%) within RBC, calculated in terms of its ROS (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes. The y-axis represents ROS (%) and the x-axis the liposomes and the CQ entrapped in liposome concentrations. The control group represented 100 % ROS in the RBC which was significantly higher than the ROS (%) in the RBC in the presence of the different liposome and chloroquine entrapped in liposome concentrations which ranged from 13.78 % in the presence of the 0 % liposome concentration to 21.31 % in the presence of the 50 % CQ entrapped in liposome concentration (Annexure B, Table B13). P = 0.000217 (Annexure C) was obtained from the comparison between the amount of ROS (%) which was observed in the RBC in the presence of all the different liposome and different CQ entrapped in

liposome concentrations to the amount of ROS (%) observed in the presence of the control. This indicated a statistically significant difference. A slightly higher degree of ROS (%) was observed in the RBC in the presence of CQ entrapped in liposomes in comparison to the ROS (%) observed in the RBC in the presence of only liposomes with $p > 0.05$ (Annexure C), which indicated no statistically significant difference. The production of ROS in the RBC therefore remained more or less the same in the presence of the different liposome and five different CQ entrapped in liposome concentrations.

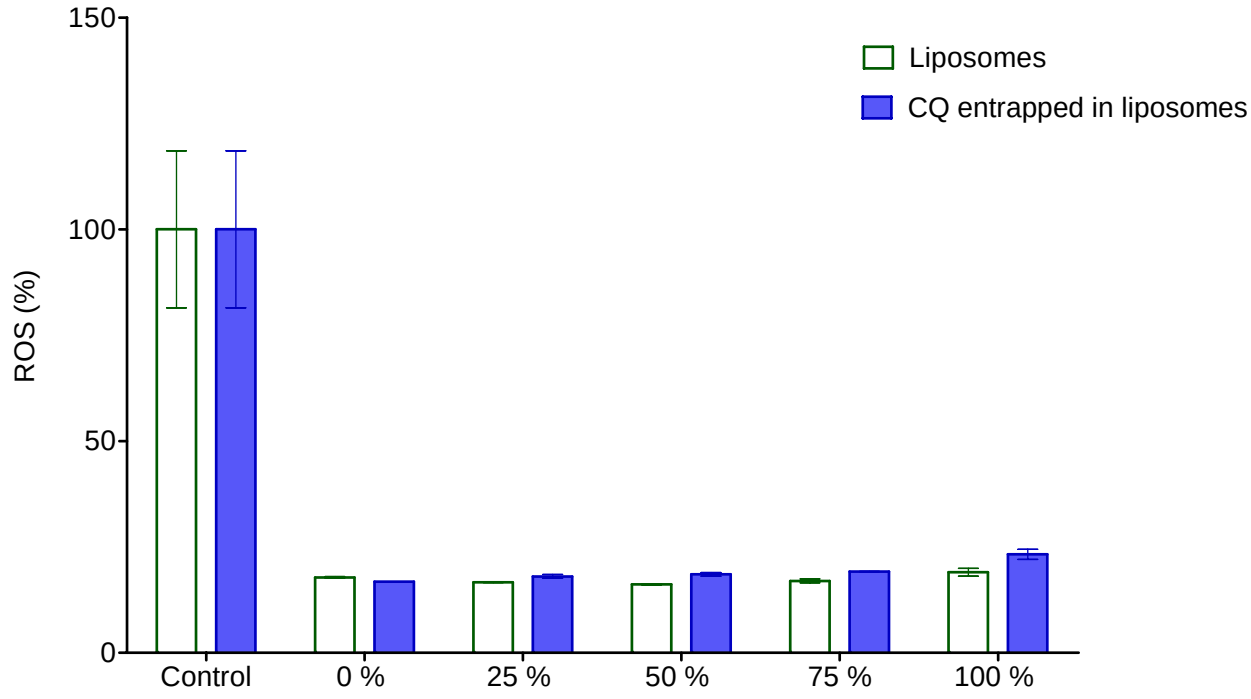


Figure 4.6: Comparison between the ROS (%) within iRBC, in terms of its ROS (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes at five different concentrations.

Figure 4.6 illustrates the comparison between the ROS (%) within iRBC, calculated in terms of its ROS (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes. The y-axis represents ROS (%) and the x-axis the liposomes and CQ entrapped in liposome concentrations. The control group represented 100 % ROS in the RBC which was significantly higher than the ROS (%) observed in the RBC in the presence of the different liposome and CQ entrapped in liposome concentrations which ranged from 16.16 % in the presence of the 50 % liposome concentration to 23.27 % in the presence of the 100 % CQ

entrapped in liposome concentration (Annexure B, Table B14). P = 0.000217 (Annexure C) was obtained from the comparison between the amount of ROS (%) observed in the iRBC in the presence of all the different liposome and different CQ entrapped in liposome concentrations to the amount of ROS (%) observed in the presence of the control. This indicated a statistically significant difference. A slightly higher degree of ROS production occurred in the iRBC in the presence of CQ entrapped in liposomes when comparing it to the ROS production obtained in the presence of only the liposomes with $p > 0.05$ (Annexure C), which indicated no statistically significant difference. The production of ROS in the iRBC therefore remained more or less the same in the presence of the five different liposome and five different CQ entrapped in liposome concentrations.

The lowest amount of ROS (%) were observed in the RBC in the presence of the 0 % liposome concentration (13.78 ± 2.41 %) and the highest ROS (%) in the presence of the 50 % CQ entrapped in liposome concentration (21.31 ± 1.43 %). The iRBC experienced the lowest ROS (%) in the presence of the 50 % liposome concentrations (16.16 ± 0.15 %) and the highest ROS (%) in the presence of the 100 % CQ entrapped in liposome concentration (23.27 ± 1.19 %). No statistically significant differences were obtained between the ROS (%) within the RBC and the iRBC in the presence of the different liposome and different CQ entrapped in liposome concentrations with $p > 0.05$. The ROS (%) obtained within the iRBC were slightly higher in comparison to those of the RBC. A general $p = 0.43$ (Annexure C) was, however, obtained from the comparison between the RBC and the iRBC. There were therefore no statistically significant differences between the RBC's and the iRBC's ROS (%). The production of ROS thus remained more or less the same within the RBC and the iRBC. Evidence suggested that the production of ROS only slightly occurred within the RBC and the iRBC, which could mean that there was a problem with the ROS (%) analysis. This will be discussed in more detail in section 4.10.2 of the LP assay

4.10.2) Evaluation of LP assay

The LP assay provides important information regarding the toxicity potential of liposomes and CQ entrapped in liposomes in RBC and iRBC. The main goal was to investigate the effect of CQ [0.5 %], plus different liposome and CQ entrapped in liposome concentrations, on LP (due to oxidative stress) in RBC and iRBC. The % LP within RBC and iRBC was determined and compared to one another.

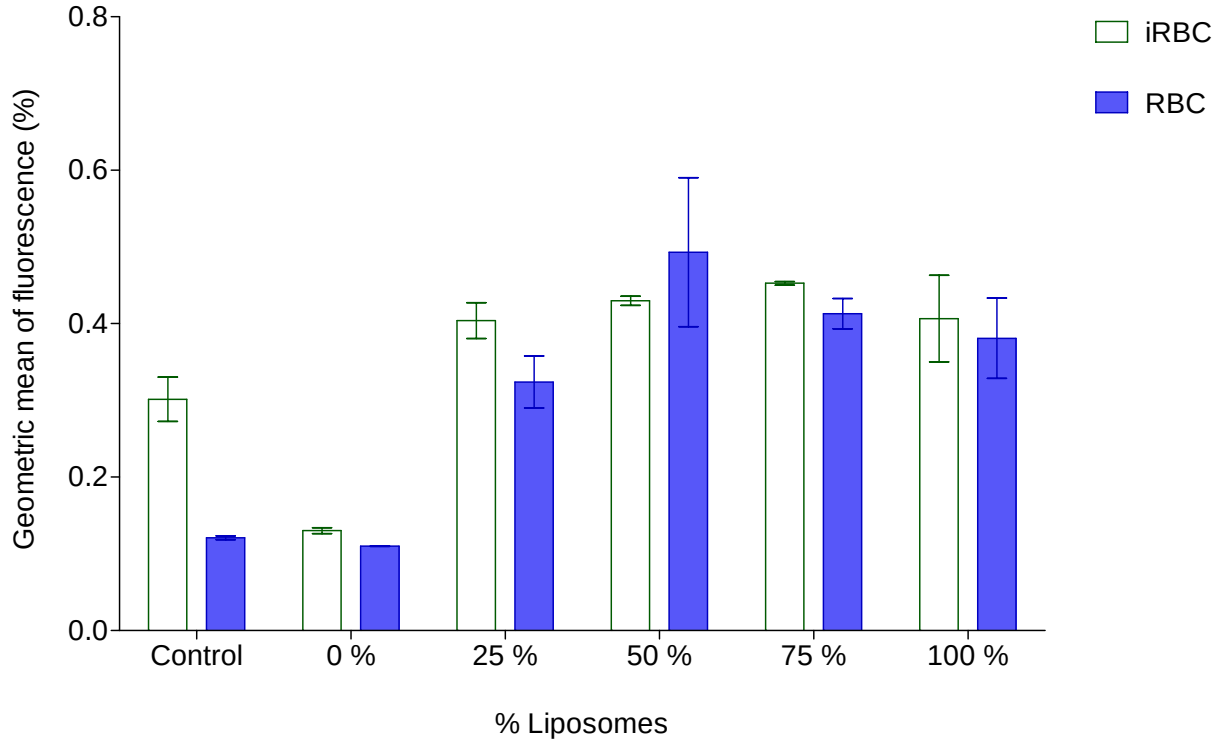


Figure 4.7: Geometric means of fluorescence (%) within RBC and iRBC in the presence of the control group and five liposome concentrations obtained from the LP assay.

Figure 4.7 illustrates the geometric means of fluorescence within RBC and iRBC in the presence of the control group and liposome concentrations obtained from the LP assay. The y-axis represents the geometric mean of fluorescence and the x-axis the control group and the liposome concentrations. The control (which consisted of either culture medium or wash medium) demonstrated low levels of LP (with the exception of the elevated value of the iRBC's in the presence of the control which could possibly be attributed to an experimental error) in both the RBC (0.12 ± 0.003 %) and the iRBC (0.30 ± 0.03 %) groups. Significantly higher levels of LP (Annexure B, Table B15) were observed in the RBC and the iRBC in the presence of the liposomes when compared to their LP in the presence of the control, with the exception of the 0 % concentration. There were increases in the fluorescence of the iRBC in the presence of the 25 % (0.40 ± 0.020 %), 50 % (0.43 ± 0.01 %), 75 % (0.45 ± 0.002 %) and 100 % (0.41 ± 0.06 %) liposome concentration in comparison to its fluorescence in the presence of the 0 % (0.13 ± 0.004) liposomes concentrations. There were also increases in the fluorescence of the RBC in the presence of the 25 % (0.32 ± 0.03 %), 50 % (0.49 ± 0.10 %), 75 % (0.41 ± 0.02 %) and 100 % (0.38 ± 0.05 %) liposome concentrations in comparison to its fluorescence obtained in the presence of the 0 % (0.11 ± 0.0001 %) liposome concentration. Statistically significant differences with p = 0.000185 (Annexure C) were obtained when comparing LP within both the

RBC and the iRBC in the presence of the 25 % liposome concentration, 50 % liposome concentration, 75 % liposome concentration and 100 % liposome concentration to their LP obtained in the presence of the 0 % liposome concentration. No statistically significant differences were observed between the LP in the RBC and the iRBC in the presence of the abovementioned four liposome concentrations. A statistically significant difference was, however, observed between the LP (%) in the RBC and the iRBC in the presence of the 0 % liposome concentration with $p = 0.03$ (Annexure C). The LP was lower in both the RBC and the iRBC in the presence of the 0 % liposome concentration than it was in the presence of the other four concentrations. Statistically significant differences were observed between the comparison of RBC and iRBC with $p < 0.05$ (Annexure C). LP therefore remained more or less the same in the RBC and the iRBC in the presence of the four highest liposome concentrations.

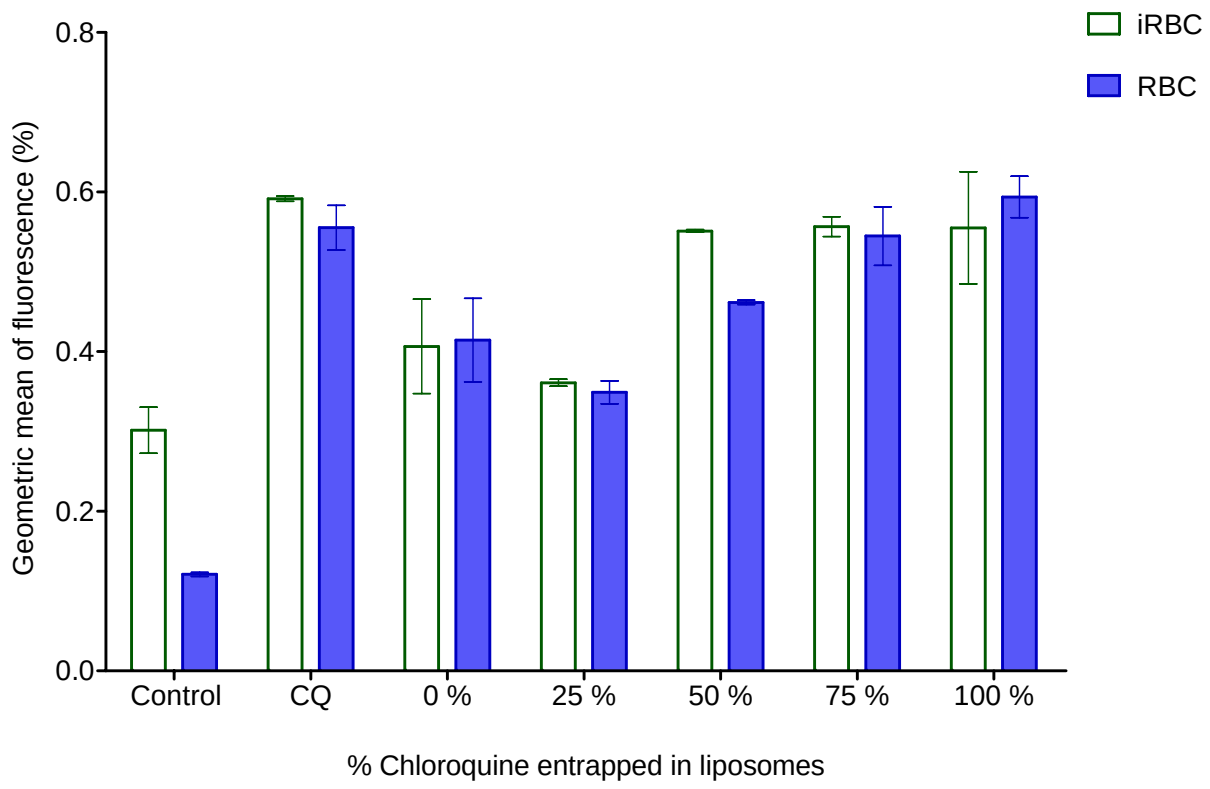


Figure 4.8: Geometric means of fluorescence within RBC and iRBC in the presence of the control group, CQ [0.5 %] and five CQ entrapped in liposome concentrations obtained from the LP assay.

Figure 4.8 illustrates the geometric means of fluorescence within RBC and iRBC in the presence of the control group, CQ [0.5 %] and CQ entrapped in liposome concentrations

obtained from the LP assay. The y-axis represents the geometric means of fluorescence and the x-axis the control group, CQ [0.5 %] and CQ entrapped in liposome concentrations. The control (which consisted of either culture medium or wash medium) demonstrated low levels of LP (with the exception of the elevated value of the iRBC's in the presence of the control which could possibly be attributed to an experimental error) in both the RBC (0.12 ± 0.003 %) and the iRBC (0.30 ± 0.03 %) groups. Significantly higher LP values (Annexure B, Table B16) were obtained in both the RBC and the iRBC in the presence of liposomes when compared to their LP obtained in the presence of the control. Increases in fluorescence within iRBC were observed in the presence of the CQ [0.5 %] (0.59 ± 0.003 %), 50 % (0.55 ± 0.002 %), 75 % (0.56 ± 0.01 %) and 100 % (0.56 ± 0.07 %) CQ entrapped in liposome concentrations, when compared to its fluorescence obtained in the presence of the 0 % (0.41 ± 0.06 %) and 25 % (0.36 ± 0.004 %) CQ entrapped in liposome concentrations. Increases in fluorescence were also observed within the RBC in the presence of the CQ [0.5 %] (0.56 ± 0.03 %), 50 % (0.46 ± 0.003 %), 75 % (0.55 ± 0.04 %) and 100 % (0.59 ± 0.03 %) CQ entrapped in liposome concentrations, when compared to its fluorescence in the presence of the 0 % (0.41 ± 0.05 %) and 25 % (0.35 ± 0.01 %) CQ entrapped in liposome concentrations. Statistically significant differences were established within both the RBC and the iRBC in terms of their fluorescence in the presence of the 25 % CQ entrapped in liposome concentration in comparison to their fluorescence obtained in the presence of the 0.5 % CQ concentration with $p = 0.02$ (Annexure C). A statistically significant difference was also observed within the RBC when comparing the LP in the presence of 25 % CQ entrapped in liposomes to the LP obtained in the presence of the 100 % CQ entrapped in liposomes with $p = 0.01$ (Annexure C). The comparison between the LP obtained within the RBC in the presence of the 25 % CQ entrapped in liposomes and its LP obtained in the presence of the 75 % CQ entrapped in liposomes with $p = 0.03$ (Annexure C) also indicated a statistically significant difference. Statistically significant differences were observed when comparing the RBC to the iRBC with $p < 0.05$ (Annexure C). Only one statistically significant difference was observed when comparing the RBC with the iRBC. The LP therefore remained more or less the same in the iRBC in the presence of CQ and the five different CQ entrapped in liposome concentrations.

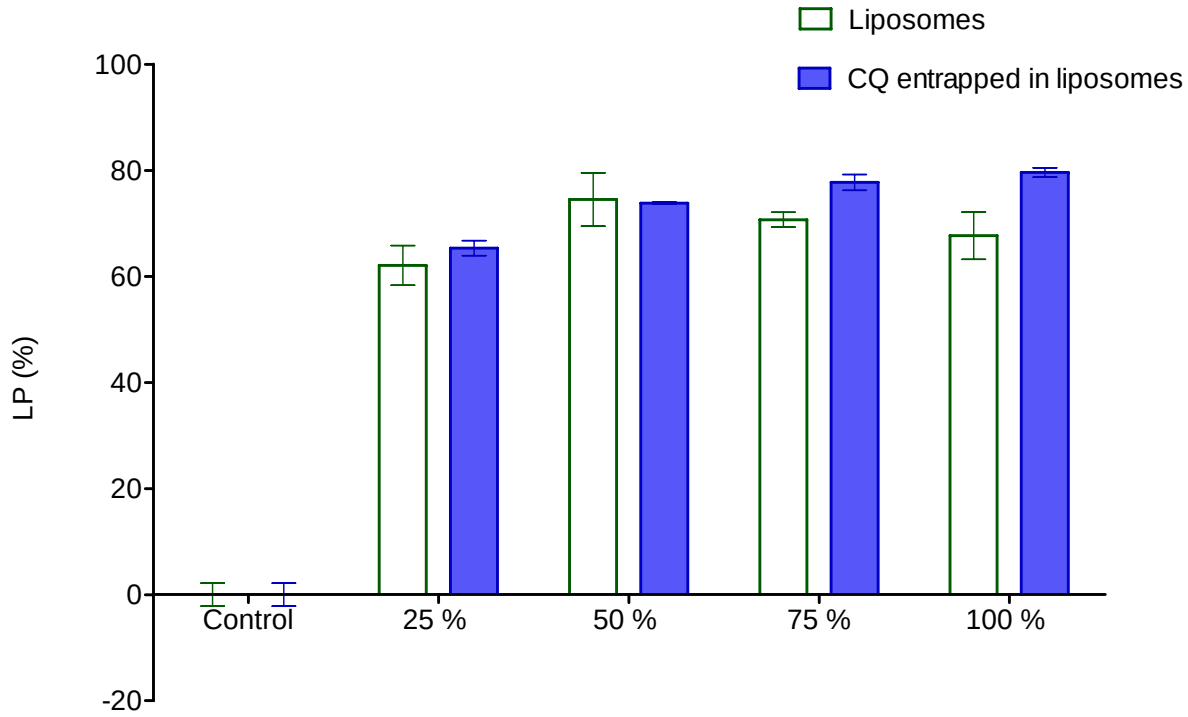


Figure 4.9: Comparison between the LP (%) within RBC, in terms of its LP (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes at five different concentrations.

Figure 4.9 illustrates the comparison between the LP (%) within RBC, in terms of its LP (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes at different concentrations. The y-axis represents the LP (%) and the x-axis the liposomes and CQ entrapped in liposome concentrations. The % LP within the RBC in the presence of the control group represents 0 % LP which is lower than the LP (%) obtained within the RBC in the presence of the different liposome and CQ entrapped in liposome concentrations, which ranged from 62.10 % in the presence of the 25 % liposome concentration to 79.61 % in the presence of the 100 % CQ entrapped in liposome concentration (Annexure B, Table B17). The LP within the RBC in the presence of the 0 % concentration for both the liposomes and the CQ entrapped in liposomes is not presented on the graph, since no LP (%) was observed at this concentration. The comparison between the LP obtained in the RBC in the presence of the different liposome and different CQ entrapped in liposome concentrations resulted in $p > 0.05$ (Annexure C) which indicated no statistically significant differences. The LP obtained in the RBC in the presence of CQ entrapped in liposomes seemed to have a slightly higher LP (%) than the LP obtained in the presence of the liposomes with $p > 0.05$ (Annexure C), which indicated no statistically significant difference. The LP remained more or less the same in the RBC in the presence of the different liposome and five different CQ entrapped in liposome concentrations.

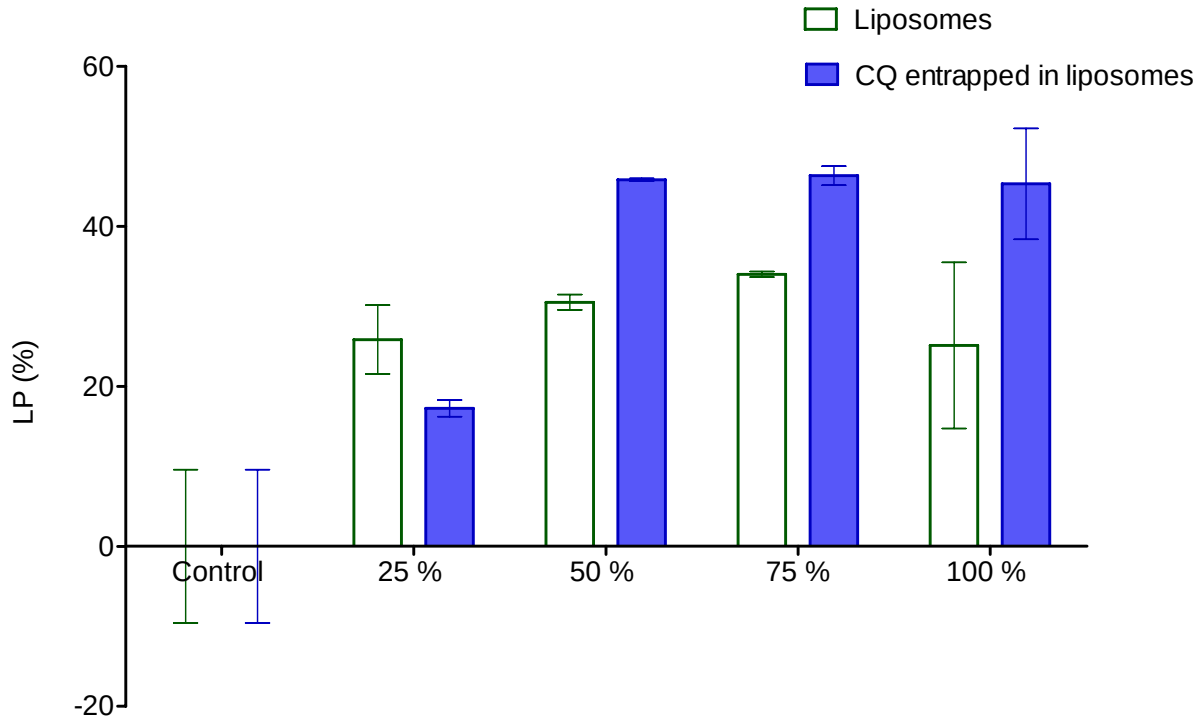


Figure 4.10: Comparison between the LP (%) within iRBC, in terms of its LP (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes at five different concentrations.

Figure 4.10 illustrates the comparison between the LP (%) within iRBC, in terms of its LP (%) in the presence of the control group, in the presence of liposomes and CQ entrapped in liposomes at different concentrations. The y-axis represents the LP (%) and the x-axis the liposomes and CQ entrapped in liposome concentrations. The % LP within the iRBC in the presence of the control group represents 0 % which is lower than the LP (%) obtained in the presence of the different liposome and CQ entrapped in liposome concentrations, which ranged from 17.24 % in the presence of the 25 % CQ entrapped in liposomes concentration to 46.32 % in the presence of the 75 % CQ entrapped in liposomes concentration (Annexure B, Table B18). The LP (%) within the iRBC in the presence of the 0 % concentration of both the liposomes and the CQ entrapped in liposomes is not presented on the graph, since no LP (%) was observed at this concentration. The comparison between the LP (%) obtained within the iRBC in the presence of the different liposomes and different CQ entrapped in liposomes resulted in $p < 0.05$ (Annexure C) which indicated statistically significant differences. The LP (%) within the iRBC in the presence of CQ entrapped in liposomes seemed to have a slightly higher LP (%) than the LP (%) in the presence of the liposomes with $p < 0.05$ (Annexure C), which indicated statistically significant differences. The LP (%) therefore varied in the iRBC in the presence of the different liposome and five different CQ entrapped in liposome concentrations.

The lowest LP (%) within the RBC was obtained in the presence of the 25 % CQ entrapped in liposomes concentration (62.10 ± 3.74 %) and the highest LP (%)in the presence of the 100 % CQ entrapped in liposome concentration (79.61 ± 0.89 %). The lowest LP (%) within the iRBC was obtained in the presence of the 25 % CQ entrapped in liposome concentration (17.24 ± 1.02 %) and the highest LP (%) in the presence of the 75 % CQ entrapped in liposome concentration (46.32 ± 1.20 %). No statistically significant differences were obtained within the RBC in the presence of the different liposomes and different CQ entrapped in liposome concentrations with $p > 0.05$ (Annexure C). Statistically significant differences were, however, obtained within the iRBC when comparing its LP (%) in the presence of the different liposomes to its LP (%) in the presence of different CQ entrapped in liposome concentrations with $p < 0.05$ (Annexure C). The LP (%) obtained in the RBC were higher in comparison to those of the iRBC. A general p-value of 0.000001 was obtained when comparing the RBC and iRBC. There were therefore a few statistically significant differences between the RBC's and the iRBC's LP (%). The LP (%) thus varied between the RBC and the iRBC. Based on the evidence it can be stated that the LP (%) occurred more in the RBC in the presence of the CQ entrapped in liposome concentrations. This could indicate that chloroquine was responsible for the high concentrations of LP in the RBC. LP, however, is one of the results of ROS formation. The absence of ROS formation could indicate that the dye did not stain the cells effectively. The ROS assay method should therefore be re-evaluated in future studies.

4.10.3) Evaluation of Hemolysis assay

The aim of this assay was to investigate the effect of different liposomes and different CQ entrapped in liposome concentrations on the incidence of RBC hemolysis. The % hemolysis of the different liposome concentrations were compared to that of the different CQ entrapped in liposome concentrations. The cytotoxicity potential of liposomes and CQ entrapped in liposomes on RBC can be determined via the hemolysis assay.

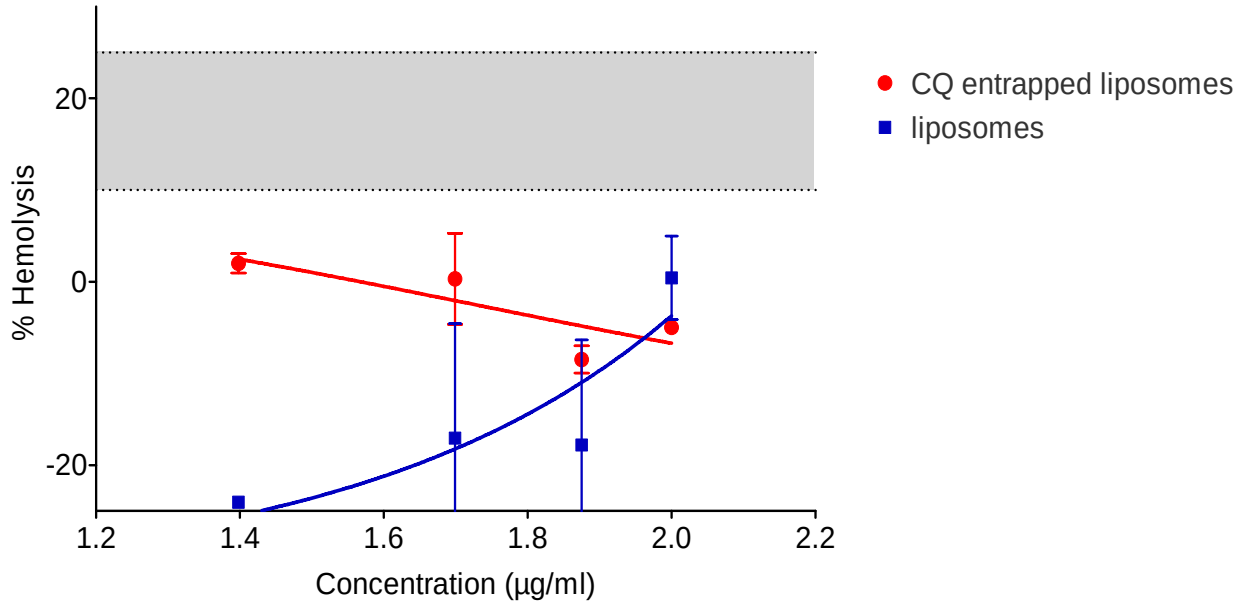


Figure 4.11: Hemolysis of RBC containing different concentrations of liposomes and CQ entrapped in liposomes. Hemolysis is rated as significant from 10 % upwards as indicated by the grey area.

Figure 4.11 illustrates the hamolysis of RBC containing different concentrations of liposomes and CQ entrapped in liposomes. The y-axis represents the % hemolysis and the x-axis the different concentrations (µg/ml). The initial data was normalized and transformed with graphpad (Annexure B, Table B19). The % hemolysis observed in the RBC in the presence of the different liposome concentrations were individually compared with each other. The % hemolysis observed in the RBC in the presence of CQ entrapped in liposomes at varying concentrations was compared in a similar way. No statistically significant differences were observed between the % hemolysis observed in the RBC in the presence of any of the different liposome concentrations and the different CQ entrapped in liposome concentrations with $p > 0.05$ (Annexure C). The % hemolysis observed in the RBC in the presence of the different liposome concentrations were compared to the % hemolysis observed in the RBC in the presence of the different CQ entrapped in liposome concentrations. $P = 0.03$ (Annexure C) was obtained which indicated a statistically significant difference between the % hemolysis observed in the RBC in the presence of liposomes and the % hemolysis observed in the RBC in the presence of CQ entrapped in liposomes. The % hemolysis observed in the RBC in the presence of CQ entrapped in liposomes caused a higher % hemolysis in comparison to the % hemolysis observed in the RBC in the presence of liposomes. The % hemolysis, however, is

only significant from 10 % upwards as indicated with the grey area on the graph. Therefore it can be concluded that the % hemolysis observed in the RBC in the presence of the liposomes and in the presence of CQ entrapped in liposomes did not have an effect on the % hemolysis of the RBC.

4.11) Conclusion

The production of ROS remained more or less the same in the RBC and the iRBC. It can be stated that the production of ROS occurred in both the RBC and the iRBC but only to a small extent. This suggests that no significant ROS production occured in either the RBC or the iRBC. LP occurred in both the RBC and the iRBC in the presence of CQ [0.5], liposomes and CQ entrapped in liposomes at varying concentrations. The amount of LP (%), however, differed from the RBC when compared tothat in the iRBC. RBC experienced the highest degree of LP (%) in the presence of CQ entrapped in liposomes at varying concentrations. No significant % hemolysis of RBC was observed in the presence of neither the liposomes nor in the presence of CQ entrapped in liposomes at varying concentrations.

SUMMARY AND FUTURE PROSPECTS

The aim of this thesis included the evaluation of CQ entrapped in liposomes in terms of their size, morphology and entrapment efficiency by being subjected to twelve weeks of stability testing. The *in vitro* toxicity of liposomes was also investigated by conducting assays with regards to ROS, LP and the hemolysis of RBC.

The pH of liposomes and CQ entrapped in liposomes remained more or less constant during a twelve week time period at 5°C which may indicate that both formulations remained in a stable condition. The pH of liposomes and CQ entrapped in liposomes did not remain constant during a twelve week time period at 25°C which may be attributed to bacterial contamination. No statistically significant difference could be established between the intial values and the values obtained at week 12 for neither the liposomes nor for the CQ entrapped in liposomes at 25°C. The pH of liposomes and CQ entrapped in liposomes did not remain constant during a twelve week time period at 40°C which may also be attributed to bacterial contamination. A statistically significant difference was observed between liposomes and CQ entrapped in liposomes at that temperature. None of the above formulations remained stable for the duration of the stability testing. Contamination of formulations due to bacteria can adversely affect the properties of the formulations leading to gas production, colour and odour changes, hydrolysis of fats and oils and pH changes in the formulation. Knowledge on the pH of formulations is essential for the entrapment of CQ. CQ has a pH of 7.4, indicating that it is more or less a neutral drug which could be entrapped within the liposomes due to its low permability of the liposomal bilayers. The interior pH of the liposomes need to be buffered in order to obtain a high drug concentration within the liposomes (Lee *et al*., 1998; Fahr *et al*., 2005; Fritze *et al*., 2006). Qiu *et al*., have established that more than 90 % of CQ could be released from liposomes when the internal pH of the liposomes was buffered at a pH between 5.5 and 6.5. Bacterial growth assays should be performed in the future in order to limit bacterial contamination which affects the pH of the formulation and in turn leads to a lower drug concentration within the liposomes.

Statistically significant differences were observed between both liposomes and CQ entrapped in liposome's span values during a twelve week time period at 5°C. A lot of fluctuations in CQ entrapped in liposome's span values were observed during a twelve week time period at 5°C in comparison to that of the liposomes which could indicate that liposomes are a more stable formulation at 5°C in terms of their span values. A statistically significant difference was observed with CQ entrapped in liposomes at 25°C over a twelve week time period whilst the liposome formulation remained in a stable condition. A statistically significant difference was also observed with the span values of the CQ entrapped in liposomes over a twelve week time period at 40°C. The span values of only the liposome formulation remained more or less

constant during a twelve week time period. Flow cytometry was used to determine the span values of both liposome and CQ entrapped in liposome formulations. Qiu *et al*., have established that high levels of drug accumulation are possible when the mass ratio of CQ and that of phosphatidylcholine, soybean phosphatidylcholine (SPC) in particular, is too high. High drug accumulation levels could explain why the size distribution of the CQ entrapped in liposomes increased over a twelve week time period, whilst the size distribution of the liposomes remained more or less the same. Experiments should therefore be conducted with different CQ and phosphatidyl choline mass ratios in order to establish which ratio presents with the ideal size distribution.

A statistically significant difference was observed between pH and entrapment efficiency during a twelve week time period at 5°C which could indicate a directly proportional relationship between the two variables. No statistically significant difference was observed between the pH and entrapment efficiency of CQ entrapped in liposomes during the twelve week stability study at 25°C and 40°C. There was also no statistically significant difference observed between the entrapment efficiency and span of CQ entrapped in liposomes during the twelve week stability study at 5°C. Statistically significant differences were, however, observed between the entrapment efficiency and span of CQ entrapped in liposomes during the twelve week stability study at 25°C and 40°C. There were no statistically significant differences between any of the different CQ entrapped in liposome formulations, with different CQ concentrations, in terms of their entrapment efficiency or span. The manufacturing of liposomes, with different CQ concentrations did not improve the entrapment efficiency. It was also apparent that sonication did not have an effect on the entrapment efficiency either; there was no statistically significant difference between the sonicated and non-sonicated formulations. The internal pH of the liposomes should be buffered, as discussed earlier, in order to obtain the maximum drug amount within the liposomes. The higher the amount of the drug encapsulated within the liposomes the higher the entrapment efficiency. The entrapment efficiency of CQ entrapped in liposomes can also be increased by the use of a negatively charge phosphate group, ie. Dipalmitoylphosphatidylglycerol (DPPG) during the manufacturing process of CQ entrapped in liposomes. The positively charged CQ easily interacts with the negatively charged phosphate group which increases the membrane-water partition coefficient of CQ (Ceh & Lasic, 1997).

Spherical liposome vesicles were observed in both the liposome and CQ entrapped in liposome formulations. Crystal formation occurred at 40°C in the liposome formulations and at 25°C and 40°C in the CQ entrapped in liposome formulation. Liposomes are therefore more stable than CQ entrapped in liposomes at 25°C in terms of their morphology.

Liposomes remained more stable over the twelve week stability study in comparison to CQ entrapped in liposomes. The stability of CQ entrapped in liposomes can, however, be improved by manufacturing an optimal CQ entrapped in liposomes formulation. This may be achieved by correcting the above mentioned shortcomings which were encountered with the CQ entrapped in liposomes formulation.

No statistically significant differences were observed in the amount of intracellular ROS (%) which occured in either the RBC or the iRBC in the presence of CQ and the five different CQ entrapped in liposome concentrations. There were no statistically significant differences noticed in the amount of intracellular ROS (%) observed in either the RBC or the IRBC in the presence of the different liposome concentrations. There were also no statistically significant differences observed between the RBC's and the iRBC's ROS (%). The production of ROS therefore remained more or less the same within the RBC and the iRBC. Very small amounts of ROS were produced in the RBC and the iRBC, however, it did occur in the presence of all of the liposome and CQ entrapped in liposome concentrations. This could mean that the liposomes protected the cells from possible ROS production caused by the addition of CQ. It could also mean that the dye did not stain the cells effectively which led to inaccurate results.

Statistically significant differences which presented with similar p-values, in the LP assay, were observed between the RBC and the iRBC in the presence of the 0.5 % CQ concentration and in the presence of the 25 % CQ entrapped in liposome concentration. Statistically significant differences were observed in the LP (%) in RBC in the presence of 25 % CQ entrapped in liposomes when compared to its LP (%) in the presence of the 75 % and 100 % CQ entrapped in liposome concentrations. Only one statistically significant difference was observed with the iRBC in comparison to the three observed with the RBC. Statistically significant differences were observed in LP (%) of both the RBC and the iRBC in the presence of the 25 %, 50 %, 75 % and the 100 % liposome concentrations in comparison to both the RBC and the iRBC LP (%) in the presence of the 0 % liposome concentration. The LP (%) within the RBC and the iRBC in the presence of the 0 % liposome concentrations was a lot lower than the LP (%) within the RBC and the iRBC in the presence of the other four liposome concentrations (which indicated no statistically significant differences). A statistically significant difference was observed within the RBC and the iRBC LP (%) when comparing their LP (%) in the presence of the 0 % liposome concentration. Statistically significant differences were obtained when comparing the LP (%) in the RBC and the iRBC in the presence of the control group to their LP (%) in the presence of most of the liposome and CQ entrapped in liposome concentrations. This implies that LP (%) occurred mostly in the RBC and the iRBC in the presence of most of the liposome and CQ entrapped in liposome concentrations. A few statistically significant differences were noticed between the LP (%) obtained within the RBC's and the iRBC's. The

LP (%) remained more or less the same in the iRBC in the presence of CQ and the five different CQ entrapped in liposome concentrations. The LP (%) varied between the RBC and the iRBC. The LP (%) observed in the RBC and the iRBC varied from one another in the presence of the control. The LP (%) obtained within the RBC were higher in comparison to those of the iRBC. The most LP (%) therefore occurred within RBC in the presence of CQ entrapped in liposomes at varying concentrations. This could mean that CQ had an influence on the LP (%) obtained in the RBC and the iRBC, seeing that the LP (%) within the RBC and the iRBC are lower in the presence of the liposome concentrations. Very high levels of LP were obtained within the cells (iRBC & RBC) in comparison to the low levels of ROS (%).

No statistically significant differences were observed in the % hemolysis of the RBC in the presence of any of the different liposome concentrations or any of the different CQ entrapped in liposome concentrations. A statistically significant difference was noticed between the % hemolysis of the RBC in the presence of the liposomes and CQ entrapped in liposomes (which caused a higher percentage of hemolysis). None of these percentages of hemolysis were however significant. This indicates that neither the liposomes nor the CQ entrapped in liposomes had a negative effect on cell viability.

Liposomes could successfully be manufactured and characterized. The entrapment of CQ within liposomes was possible, although the EE % was much too low for effective dosaging purposes. Stability problems were encountered with liposomes and CQ entrapped in liposomes over the twelve week stability testing period. Future studies should include the investigation of possible methods to improve the encountered stability problems. It would also be of great value to conduct drug type/liposome compatibility studies with the main focus on drug characteristics such as lipophilicity/hydrophilicity, pK_a, molecular size etc. The ROS assay method should be re-evaluated in future studies. The *in vivo* toxicity of liposomes and CQ entrapped in liposomes should also be investigated in future studies with specific reference to the cell viability of RBC and iRBC.

ANNEXURE A

Calculation of span values at S5 %, S50 % and S95 %

The following table presents the S5 %, S50 % and S95 % and span (µm) values of the initial formulations of liposomes and chloroquine entrapped in liposomes (L + CQ).

Depth	Name	Statistic	#Cells	log y	log x	new x	Span
Liposomes	initial samples		100000				
>	5	1.08687		0.036178	-0.25726	0.553015	5.777835
>	50	2.10308		0.322856	-0.07887	0.833931	
>	95	41.96056		1.622841	0.730082	5.371328	
Liposomes	initial samples		100000				
>	5	1.14978		0.060615	-0.24206	0.572721	8.816706
>	50	3.67011		0.564679	0.071611	1.179264	
>	95	132.1931		2.121209	1.040205	10.96995	
Liposomes	initial samples		100000				
>	5	1.10877		0.044841	-0.25187	0.559922	8.149219
>	50	2.56448		0.408999	-0.02526	0.943485	
>	95	83.60307		1.922222	0.91638	8.24859	
L + CQ	initial sample		100000				
>	5	1.14591		0.059151	-0.24297	0.571521	2.821196
>	50	56.02291		1.748366	0.808193	6.429729	
>	95	311.7896		2.493862	1.272098	18.71105	
L + CQ	initial samples		100000				
>	5	1.16623		0.066784	-0.23822	0.577806	2.998892
>	50	48.64835		1.687068	0.770049	5.889096	
>	95	299.2349		2.476012	1.260991	18.23857	
L + CQ	initial samples		100000				
>	5	1.14941		0.060475	-0.24214	0.572606	3.06566
>	50	44.0882		1.644322	0.743449	5.539224	
>	95	281.392		2.449312	1.244376	17.55398	

The following tables present the S5 %, S50 % and S95 % and span (µm) values of liposomes and chloroquine entrapped in liposomes (L + CQ) during a twelve week time period at 5°C.

Depth	Name	Statistic	#Cells	log y	log x	regte x	Span
Liposomes 5°C	Week 1		100000				
>	5	1.06532		0.02748	-0.26268	0.546165	10.5591
>	50	1.77674		0.249624	-0.12444	0.750861	
>	95	87.31444		1.941086	0.928118	8.474582	
Liposomes 5°C	Week 1		100000				
>	5	1.06548		0.027545	-0.26264	0.546217	9.993932
>	50	1.8903		0.276531	-0.1077	0.780374	
>	95	85.18258		1.930351	0.921438	8.345223	
Liposomes 5°C	Week 1		100000				
>	5	1.06232		0.026255	-0.26344	0.545208	10.02505
>	50	1.61819		0.20903	-0.1497	0.708432	
>	95	74.02789		1.869395	0.883507	7.647276	

L + CQ 5°C	Week 1		100000				
>	5	1.13957		0.056741	-0.24447	0.569551	4.890745
>	50	17.33753		1.238987	0.491218	3.098974	
>	95	235.808		2.372559	1.196614	15.72584	
L + CQ 5°C	Week 1		100000				
>	5	1.19415		0.077059	-0.23182	0.586376	3.554696
>	50	30.38874		1.482713	0.642883	4.39423	
>	95	247.4981		2.393572	1.20969	16.20653	
L + CQ 5°C	Week 1		100000				
>	5	1.17581		0.070337	-0.23601	0.580755	4.093119
>	50	23.43181		1.369806	0.572623	3.737863	
>	95	239.5404		2.379379	1.200858	15.88027	

Liposomes 5°C	Week 2		100000				
>	5	1.28403		0.108575	-0.21221	0.613462	4.687641
>	50	9.3068		0.9688	0.323087	2.104199	
>	95	122.7817		2.089134	1.020245	10.47719	
Liposomes 5°C	Week 2		100000				
>	5	1.06626		0.027863	-0.26244	0.546465	9.06339
>	50	1.84316		0.265563	-0.11452	0.768206	
>	95	71.88897		1.856662	0.875583	7.50902	
Liposomes 5°C	Week 2		100000				
>	5	1.06156		0.025945	-0.26363	0.544965	9.629034
>	50	1.61198		0.20736	-0.15074	0.706739	
>	95	69.46101		1.841741	0.866298	7.350183	

L + CQ 5°C	Week 2		100000				
>	5	1.13925		0.056619	-0.24454	0.569452	4.72496
>	50	18.78932		1.273911	0.51295	3.257994	
	95	241.5572		2.38302	1.203124	15.96334	
L + CQ 5°C	Week 2		100000				
>	5	1.13276		0.054138	-0.24609	0.567431	4.45416
>	50	21.45395		1.331507	0.548791	3.538271	
>	95	250.4725		2.39876	1.212919	16.32746	
L + CQ 5°C	Week 2		100000				
>	5	1.16965		0.068056	-0.23743	0.57886	3.915796
>	50	27.06006		1.432329	0.61153	4.08818	
>	95	256.9101		2.409781	1.219777	16.58734	

Liposomes 5°C	Week 4		100000				
>	5	1.11578		0.047579	-0.25017	0.562123	5.021017
>	50	7.60669		0.881196	0.268572	1.855976	
>	95	111.7495		2.048246	0.994801	9.881009	
Liposomes 5°C	Week 4		100000				
>	5	1.07229		0.030312	-0.26091	0.548386	7.144677
>	50	3.80572		0.580437	0.081417	1.206193	
>	95	99.0471		1.995842	0.962192	9.166246	
Liposomes 5°C	Week 4		100000				
>	5	1.07634		0.031949	-0.25989	0.549674	7.095772
>	50	3.74491		0.573441	0.077064	1.194163	
>	95	96.57468		1.984863	0.95536	9.023185	

L + CQ 5°C	Week 4		100000				
>	5	1.06834		0.028709	-0.26191	0.547128	12.00542
>	50	4.11452		0.614319	0.102501	1.266196	
>	95	236.3504		2.373556	1.197235	15.74834	
L + CQ 5°C	Week 4		100000				
>	5	1.07178		0.030106	-0.26104	0.548224	11.4039
>	50	3.85494		0.586018	0.08489	1.215877	
>	95	205.0051		2.311765	1.158783	14.41396	
L + CQ 5°C	Week 4		100000				
>	5	1.07864		0.032877	-0.25932	0.550405	10.79564
>	50	4.47231		0.650532	0.125035	1.33363	
>	95	217.343		2.337146	1.174577	14.9478	

Liposomes 5°C	Week 6		100000				
>	5	1.09722		0.040294	-0.2547	0.556286	5.509031
>	50	5.85611		0.767609	0.19789	1.577212	
>	95	100.4216		2.001827	0.965916	9.245193	
Liposomes 5°C	Week 6		100000				
>	5	1.07114		0.029846	-0.2612	0.54802	7.811691
>	50	3.00627		0.478028	0.01769	1.041574	
>	95	90.81567		1.958161	0.938743	8.684473	
Liposomes 5°C	Week 6		100000				
>	5	1.07682		0.032143	-0.25977	0.549827	7.004709
>	50	3.59212		0.555351	0.065806	1.163607	
>	95	91.08608		1.959452	0.939547	8.700556	

L + CQ 5°C	Week 6		100000				
>	5	1.05168		0.021884	-0.26616	0.541803	17.49573
>	50	1.36229		0.13427	-0.19622	0.636469	
>	95	146.1566		2.164819	1.067342	11.67729	
L + CQ 5°C	Week 6		100000				
>	5	1.05174		0.021908	-0.26614	0.541823	14.76481
>	50	1.47587		0.169048	-0.17458	0.668989	
>	95	121.6936		2.085268	1.017839	10.41932	
L + CQ 5°C	Week 6		100000				
>	5	1.05322		0.022519	-0.26576	0.542297	13.43379
>	50	1.59991		0.204096	-0.15277	0.703442	
>	95	113.777		2.056055	0.999661	9.992188	

Liposomes 5°C	Week 8		100000				
>	5	1.06077		0.025621	-0.26383	0.544713	7.976492
>	50	3.04596		0.483724	0.021235	1.05011	
>	95	94.82157		1.976907	0.950409	8.920905	
Liposomes 5°C	Week 8		100000				
>	5	1.07901		0.033025	-0.25922	0.550522	6.472506
>	50	3.99542		0.601562	0.094563	1.243262	
>	95	89.35931		1.95114	0.934374	8.597546	
Liposomes 5°C	Week 8		100000				
>	5	1.07791		0.032583	-0.2595	0.550173	7.236117
>	50	3.37262		0.527967	0.048766	1.118836	
>	95	90.17333		1.955078	0.936825	8.646199	

L + CQ 5°C	Week 8		100000				
>	5	1.05261		0.022267	-0.26592	0.542101	16.33862
>	50	1.39016		0.143065	-0.19075	0.64454	
>	95	134.1944		2.127734	1.044265	11.073	
L + CQ 5°C	Week 8		100000				
>	5	1.05314		0.022486	-0.26578	0.542271	15.71391
>	50	1.45043		0.161497	-0.17928	0.661789	
>	95	131.644		2.119401	1.03908	10.94157	
L + CQ 5°C	Week 8		100000				
>	5	1.05468		0.023121	-0.26539	0.542765	16.09204
>	50	1.47175		0.167834	-0.17534	0.667826	
>	95	138.4348		2.141245	1.052673	11.28945	

Liposomes 5°C	Week 10		100000				
>	5	1.05422		0.022931	-0.26551	0.542617	8.644492
>	50	2.55989		0.408221	-0.02575	0.942434	
>	95	90.89983		1.958563	0.938994	8.689481	
Liposomes 5°C	Week 10		100000				
>	5	1.0698		0.029303	-0.26154	0.547594	6.251157
>	50	4.35897		0.639384	0.118098	1.312497	
>	95	91.95677		1.963584	0.942118	8.752216	
Liposomes 5°C	Week 10		100000				
>	5	1.06534		0.027488	-0.26267	0.546172	6.799745
>	50	3.89521		0.590531	0.087698	1.223765	
>	95	93.9104		1.972714	0.947799	8.867464	

L + CQ 5°C	Week 10		100000				
>	5	1.04806		0.020386	-0.26709	0.540642	14.08614
>	50	1.41328		0.150228	-0.18629	0.65119	
>	95	108.7189		2.036305	0.987371	9.713394	
L + CQ 5°C	Week 10		100000				
>	5	1.05218		0.02209	-0.26603	0.541964	14.61017
>	50	1.56911		0.195653	-0.15803	0.694984	
>	95	126.9246		2.103546	1.029213	10.6958	
L + CQ 5°C	Week 10		100000				
>	5	1.04997		0.021177	-0.2666	0.541255	14.01853
>	50	1.48628		0.172101	-0.17268	0.671921	
>	95	113.1996		2.053845	0.998286	9.960602	

Liposomes 5°C	Week 12		100000				
>	5	1.43243		0.156073	-0.18266	0.656667	3.1626
>	50	40.09242		1.603062	0.717774	5.22124	
>	95	271.55		2.43385	1.234754	17.16936	
Liposomes 5°C	Week 12		100000				
>	5	1.29318		0.111659	-0.21029	0.616179	3.468079
>	50	34.33471		1.535733	0.675876	4.74107	
>	95	268.7401		2.429332	1.231943	17.05859	
Liposomes 5°C	Week 12		100000				
>	5	1.34154		0.127604	-0.20037	0.630418	3.331708
>	50	36.91037		1.567148	0.695425	4.959356	
>	95	271.1481		2.433207	1.234354	17.15354	

L + CQ 5°C	Week 12		100000				
>	5	1.05114		0.021661	-0.2663	0.54163	9.953386
>	50	1.43642		0.157281	-0.1819	0.657804	
>	95	65.53769		1.816491	0.850586	7.089011	
L + CQ 5°C	Week 12		100000				
>	5	1.0518		0.021933	-0.26613	0.541842	13.52852
>	50	1.37303		0.13768	-0.1941	0.639586	
>	95	99.53812		1.997989	0.963528	9.194497	
L + CQ 5°C	Week 12		100000				
>	5	1.05302		0.022437	-0.26581	0.542233	14.47429
>	50	1.44326		0.159345	-0.18062	0.659752	
>	95	115.6029		2.062969	1.003963	10.09167	

The following tables present the S5 %, S50 % and S95 % and span (µm) values of liposomes and chloroquine entrapped in liposomes (L + CQ) during a twelve week time period at 25°C.

Depth	Name	Statistic	#Cells	log y	log x	regte x	span
Liposomes 25°C	Week 1		100000				
>	5	1.07643		0.031986	-0.25987	0.549703	9.821814
>	50	1.71015		0.233034	-0.13476	0.733223	
>	95	75.65248		1.878823	0.889373	7.751281	
Liposomes 25°C	Week 1		100000				
>	5	1.07761		0.032462	-0.25958	0.550078	9.374307
>	50	1.7383		0.240125	-0.13035	0.74071	
>	95	71.65375		1.855239	0.874698	7.493721	
Liposomes 25°C	Week 1		100000				
>	5	1.08985		0.037367	-0.25652	0.553958	6.511107
>	50	4.80408		0.68161	0.144375	1.394359	
>	95	107.2726		2.030489	0.983752	9.632781	

L + CQ 25°C	Week 1		100000				
>	5	1.14815		0.059999	-0.24244	0.572216	4.607371
>	50	19.62513		1.292813	0.524712	3.347436	
>	95	242.3298		2.384407	1.203987	15.99509	
L + CQ 25°C	Week 1		100000				
>	5	1.13081		0.05339	-0.24655	0.566823	4.344923
>	50	22.06262		1.343657	0.556352	3.600407	
>	95	247.5911		2.393735	1.209792	16.21032	
L + CQ 25°C	Week 1		100000				
>	5	1.16856		0.067651	-0.23768	0.578524	3.919789
>	50	26.71329		1.426727	0.608044	4.0555	
>	95	254.1254		2.405048	1.216831	16.47523	

Liposomes 25°C	Week 2		100000				
>	5	1.06534		0.027488	-0.26267	0.546172	9.416641
>	50	2.01043		0.303289	-0.09105	0.810875	
	95	82.51936		1.916556	0.912854	8.181891	
Liposomes 25°C	Week 2		100000				
>	5	1.06606		0.027782	-0.26249	0.546402	9.380329
>	50	2.17012		0.336484	-0.07039	0.850375	
>	95	88.12074		1.945078	0.930602	8.523195	
Liposomes 25°C	Week 2		100000				
>	5	1.0667		0.028042	-0.26233	0.546606	9.172966
>	50	1.9162		0.282441	-0.10402	0.787011	
>	95	75.88077		1.880132	0.890188	7.765828	

L + CQ 25°C	Week 2		100000				
>	5	1.10252		0.042386	-0.2534	0.557956	10.31564
>	50	5.19805		0.71584	0.165675	1.464453	
>	95	234.337		2.369841	1.194923	15.66472	
L + CQ 25°C	Week 2		100000				
>	5	1.13889		0.056482	-0.24463	0.56934	3.842626
>	50	28.62345		1.456722	0.626709	4.233596	
>	95	263.1641		2.420227	1.226277	16.83746	
L + CQ 25°C	Week 2		100000				
>	5	1.16908		0.067844	-0.23756	0.578685	3.193735
>	50	39.31463		1.594554	0.712479	5.157975	
>	95	268.5707		2.429059	1.231773	17.05189	

Liposomes 25°C	Week 4		100000				
>	5	1.07977		0.033331	-0.25903	0.550764	7.006319
>	50	3.82637		0.582787	0.082879	1.210262	
>	95	96.69611		1.985409	0.955699	9.030243	
Liposomes 25°C	Week 4		100000				
>	5	1.1168		0.047975	-0.24992	0.562442	6.007559
>	50	4.67153		0.669459	0.136813	1.370293	
>	95	92.67271		1.966952	0.944214	8.794557	
Liposomes 25°C	Week 4		100000				
>	5	1.09803		0.040614	-0.2545	0.556541	6.230396
>	50	4.28957		0.632414	0.113761	1.299454	
>	95	90.28154		1.955599	0.937149	8.652654	

L + CQ 25°C	Week 4		100000				
>	5	1.06729		0.028282	-0.26218	0.546794	12.17272
>	50	3.09094		0.490091	0.025196	1.059733	
>	95	183.3502		2.263281	1.128613	13.44662	
L + CQ 25°C	Week 4		100000				
>	5	1.06553		0.027566	-0.26262	0.546232	12.12536
>	50	3.19209		0.504075	0.033899	1.081182	
>	95	187.9587		2.274062	1.135322	13.65595	
L + CQ 25°C	Week 4		100000				
>	5	1.07083		0.029721	-0.26128	0.547922	8.860975
>	50	2.17442		0.337343	-0.06985	0.851423	
>	95	81.07307		1.908877	0.908075	8.092357	

Liposomes 25°C	Week 6		100000				
>	5	1.0902		0.037506	-0.25644	0.554068	6.293763
>	50	4.14655		0.617687	0.104597	1.272321	
>	95	88.76228		1.948228	0.932563	8.561755	
Liposomes 25°C	Week 6		100000				
>	5	1.08861		0.036872	-0.25683	0.553565	6.324007
>	50	4.22188		0.625506	0.109462	1.286655	
>	95	90.91501		1.958636	0.939039	8.690384	
Liposomes 25°C	Week 6		100000				
>	5	1.11511		0.047318	-0.25033	0.561913	5.760502
>	50	4.58323		0.661172	0.131656	1.354117	
>	95	85.46301		1.931778	0.922326	8.362309	

L + CQ 25°C	Week 6		100000				
>	5	1.06592		0.027725	-0.26252	0.546357	8.75817
>	50	2.39854		0.379947	-0.04334	0.905016	
>	95	87.28237		1.940927	0.928019	8.472645	
L + CQ 25°C	Week 6		100000				
>	5	1.06314		0.02659	-0.26323	0.54547	9.413869
>	50	2.15402		0.33325	-0.0724	0.846443	
>	95	87.96429		1.944306	0.930122	8.513776	
L + CQ 25°C	Week 6		100000				
>	5	1.07681		0.032139	-0.25978	0.549824	6.904671
>	50	3.04323		0.483335	0.020992	1.049524	
>	95	76.36205		1.882878	0.891896	7.796442	

Liposomes 25°C	Week 8		100000				
>	5	1.08561		0.035674	-0.25758	0.552615	6.406993
>	50	3.84367		0.584746	0.084098	1.213664	
>	95	84.90926		1.928955	0.920569	8.32855	
Liposomes 25°C	Week 8		100000				
>	5	1.08732		0.036357	-0.25715	0.553157	6.375215
>	50	4.08493		0.611185	0.10055	1.260522	
>	95	89.22091		1.950467	0.933956	8.589257	
Liposomes 25°C	Week 8		100000				
>	5	1.06374		0.026835	-0.26308	0.545661	8.842837
>	50	2.88965		0.460845	0.006998	1.016243	
>	95	105.4772		2.023159	0.97919	9.532134	

L + CQ 25°C	Week 8		100000				
>	5	1.06406		0.026966	-0.263	0.545763	7.99563
>	50	2.51058		0.399774	-0.03101	0.931096	
>	95	79.43886		1.900033	0.902572	7.990462	
L + CQ 25°C	Week 8		100000				
>	5	1.06146		0.025904	-0.26366	0.544933	7.655775
>	50	2.79711		0.44671	-0.0018	0.995867	
>	95	82.31161		1.915461	0.912172	8.169066	
L + CQ 25°C	Week 8		100000				
>	5	1.08268		0.0345	-0.25831	0.551687	6.789931
>	50	4.733		0.675137	0.140346	1.381485	
>	95	112.6755		2.051829	0.997031	9.931877	

Liposomes 25°C	Week 10		100000				
>	5	1.07878		0.032933	-0.25928	0.550449	6.074246
>	50	4.43178		0.646578	0.122575	1.326096	
>	95	89.49196		1.951784	0.934775	8.605485	
Liposomes 25°C	Week 10		100000				
>	5	1.06741		0.028331	-0.26215	0.546832	7.770127
>	50	3.12157		0.494373	0.027861	1.066255	
>	95	93.30372		1.969899	0.946048	8.831773	
Liposomes 25°C	Week 10		100000				
>	5	1.07205		0.030215	-0.26097	0.54831	13.59345
>	50	4.23208		0.626554	0.110114	1.288589	
>	95	294.6635		2.469326	1.25683	18.06468	

L + CQ 25°C	Week 10		100000				
>	5	1.07313		0.030652	-0.2607	0.548654	7.197297
>	50	3.35629		0.525859	0.047455	1.115461	
>	95	89.01575		1.949467	0.933333	8.576961	
L + CQ 25°C	Week 10		100000				
>	5	1.07865		0.032881	-0.25932	0.550408	6.570048
>	50	3.884		0.589279	0.086919	1.221572	
>	95	89.00301		1.949405	0.933295	8.576197	
L + CQ 25°C	Week 10		100000				
>	5	1.0803		0.033544	-0.2589	0.550932	8.336899
>	50	4.2648		0.629899	0.112196	1.294779	
>	95	139.5385		2.144694	1.054819	11.34538	

Liposomes 25°C	Week 12		100000				
>	5	1.26622		0.102509	-0.21599	0.608153	3.481986
>	50	32.65721		1.513979	0.662339	4.595568	
>	95	257.4708		2.410728	1.220366	16.60986	
Liposomes 25°C	Week 12		100000				
>	5	1.17478		0.069957	-0.23624	0.580439	5.362027
>	50	28.5503		1.455611	0.626018	4.22686	
>	95	441.8663		2.645291	1.366329	23.24498	
Liposomes 25°C	Week 12		100000				
>	5	1.0667		0.028042	-0.26233	0.546606	27.30857
>	50	1.55437		0.191554	-0.16058	0.690914	
>	95	330.84		2.519618	1.288126	19.41448	

L + CQ 25°C	Week 12		100000				
>	5	1.04484		0.01905	-0.26792	0.539608	12.24961
>	50	1.68188		0.225795	-0.13927	0.725657	
>	95	103.6426		2.015538	0.974448	9.428621	
L + CQ 25°C	Week 12		100000				
>	5	1.03879		0.016528	-0.26949	0.537661	10.05945
>	50	1.72816		0.237584	-0.13193	0.738018	
>	95	78.98023		1.897518	0.901007	7.961723	
L + CQ 25°C	Week 12		100000				
>	5	1.07671		0.032099	-0.2598	0.549792	7.395948
>	50	2.72327		0.435091	-0.00903	0.979425	
>	95	76.31683		1.88262	0.891736	7.793568	

The following tables present the S5 %, S50 % and S95 % and span (µm) values of liposomes and chloroquine entrapped in liposomes (L + CQ) during a twelve week time period at 40°C.

Depth	Name	Statistic	#Cells	log y	log x	new x	Span
Liposomes 40°C	Week 1		100000				
>	5	1.09924		0.041093	-0.25421	0.556923	6.150559
>	50	5.39932		0.732339	0.175942	1.499485	
>	95	109.9121		2.041046	0.990321	9.779595	
Liposomes 40°C	Week 1		100000				
>	5	1.08391		0.034993	-0.258	0.552077	7.048568
>	50	4.18938		0.62215	0.107374	1.280483	
>	95	106.2877		2.026483	0.981259	9.577649	
Liposomes 40°C	Week 1		100000				
>	5	1.07855		0.03284	-0.25934	0.550376	10.11489
>	50	1.85287		0.267845	-0.1131	0.770722	
>	95	85.1978		1.930428	0.921486	8.346151	

L + CQ 40°C	Week 1		100000				
>	5	1.16133		0.064956	-0.23936	0.576294	3.176889
>	50	45.44672		1.657503	0.751651	5.644827	
>	95	306.4044		2.486295	1.26739	18.50928	
L + CQ 40°C	Week 1		100000				
>	5	1.10839		0.044693	-0.25196	0.559803	4.620883
>	50	23.11049		1.363809	0.568892	3.705884	
>	95	284.7555		2.454472	1.247587	17.68426	
L + CQ 40°C	Week 1		100000				
>	5	1.1332		0.054307	-0.24598	0.567568	3.738626
>	50	32.61222		1.51338	0.661967	4.591627	
>	95	286.0423		2.45643	1.248805	17.73395	

Liposomes 40°C	Week 2		100000				
>	5	1.07244		0.030373	-0.26088	0.548434	7.09733
>	50	3.64655		0.561882	0.069871	1.174548	
>	95	94.20199		1.97406	0.948637	8.884587	
Liposomes 40°C	Week 2		100000				
>	5	1.07966		0.033287	-0.25906	0.550729	6.613044
>	50	4.26033		0.629443	0.111912	1.293935	
>	95	98.0303		1.99136	0.959403	9.107576	
Liposomes 40°C	Week 2		100000				
>	5	1.07589		0.031768	-0.26001	0.549531	6.752656
>	50	3.97267		0.599082	0.09302	1.238852	
>	95	94.72202		1.976451	0.950125	8.915076	

L + CQ 40°C	Week 2		100000				
>	5	1.11494		0.047251	-0.25037	0.561859	4.151875
>	50	27.97441		1.446761	0.620511	4.1736	
>	95	290.1013		2.46255	1.252613	17.89013	
L + CQ 40°C	Week 2		100000				
>	5	1.12695		0.051905	-0.24748	0.565618	4.316661
>	50	24.84314		1.395206	0.58843	3.87641	
>	95	274.8465		2.43909	1.238015	17.29876	
L + CQ 40°C	Week 2		100000				
>	5	1.11517		0.047341	-0.25032	0.561931	4.196374
>	50	27.11397		1.433193	0.612068	4.093246	
>	95	286.1662		2.456618	1.248922	17.73872	

Liposomes 40°C	Week 4		100000				
>	5	1.09831		0.040725	-0.25443	0.55663	6.033539
>	50	4.47996		0.651274	0.125497	1.335049	
>	95	89.59586		1.952288	0.935089	8.611701	
Liposomes 40°C	Week 4		100000				
>	5	1.1758		0.070333	-0.23601	0.580752	5.043086
>	50	5.52963		0.742696	0.182387	1.521903	
>	95	83.72123		1.922836	0.916761	8.255843	
Liposomes 40°C	Week 4		100000				
>	5	1.09895		0.040978	-0.25428	0.556831	5.702072
>	50	5.37272		0.730194	0.174607	1.494884	
>	95	97.56699		1.989303	0.958123	9.080767	

L + CQ 40°C	Week 4		100000				
>	5	1.10661		0.043995	-0.2524	0.559243	10.1227
>	50	4.84249		0.685069	0.146527	1.401286	
>	95	212.6018		2.327567	1.168617	14.74404	
L + CQ 40°C	Week 4		100000				
>	5	1.07908		0.033054	-0.25921	0.550545	10.78433
>	50	4.09124		0.611855	0.100968	1.261733	
>	95	199.1751		2.299235	1.150986	14.15749	
L + CQ 40°C	Week 4		100000				
>	5	1.10428		0.043079	-0.25297	0.55851	6.596971
>	50	10.36406		1.01553	0.352165	2.249912	
>	95	228.0322		2.357996	1.187552	15.40111	

Liposomes 40°C	Week 6		100000				
>	5	1.08498		0.035422	-0.25773	0.552416	6.185066
>	50	4.00309		0.602395	0.095081	1.244747	
>	95	83.64653		1.922448	0.91652	8.251258	
Liposomes 40°C	Week 6		100000				
>	5	1.09372		0.038906	-0.25557	0.555181	5.951149
>	50	4.23663		0.627021	0.110405	1.289451	
>	95	83.28251		1.920554	0.915342	8.228895	
Liposomes 40°C	Week 6		100000				
>	5	1.0578		0.024404	-0.26459	0.543763	18.4955
>	50	2.05728		0.313293	-0.08482	0.822583	
>	95	236.5794		2.373977	1.197497	15.75783	

L + CQ 40°C	Week 6		100000				
>	5	1.06348		0.026729	-0.26314	0.545578	8.943414
>	50	2.24297		0.350823	-0.06147	0.868028	
>	95	84.58443		1.92729	0.919534	8.308709	
L + CQ 40°C	Week 6		100000				
>	5	1.07388		0.030956	-0.26051	0.548892	8.698902
>	50	2.58024		0.41166	-0.02361	0.947089	
>	95	92.55371		1.966394	0.943867	8.787528	
L + CQ 40°C	Week 6		100000				
>	5	1.06994		0.029359	-0.26151	0.547638	8.619482
>	50	2.37992		0.376562	-0.04545	0.900638	
>	95	84.61655		1.927455	0.919636	8.310672	

Liposomes 40°C	Week 8		100000				
>	5	1.08185		0.034167	-0.25851	0.551424	6.096896
>	50	4.13892		0.616887	0.104099	1.270864	
>	95	84.43787		1.926537	0.919065	8.299748	
Liposomes 40°C	Week 8		100000				
>	5	1.08167		0.034095	-0.25856	0.551367	5.953143
>	50	4.40503		0.643949	0.120939	1.32111	
>	95	86.34856		1.936255	0.925112	8.416123	
Liposomes 40°C	Week 8		100000				
>	5	1.05313		0.022482	-0.26579	0.542268	19.59924
>	50	1.841		0.265054	-0.11484	0.767646	
>	95	232.4845		2.366394	1.192778	15.58755	

L + CQ 40°C	Week 8		100000				
>	5	1.06113		0.025769	-0.26374	0.544828	8.224612
>	50	2.18882		0.34021	-0.06807	0.854927	
>	95	72.92644		1.862885	0.879456	7.576271	
L + CQ 40°C	Week 8		100000				
>	5	1.06255		0.026349	-0.26338	0.545281	8.082067
>	50	2.29725		0.361208	-0.055	0.88104	
>	95	74.31796		1.871094	0.884564	7.665909	
L + CQ 40°C	Week 8		100000				
>	5	1.06919		0.029055	-0.2617	0.547399	8.091651
>	50	2.6159		0.417621	-0.0199	0.955213	
>	95	84.06058		1.924592	0.917855	8.276651	

Liposomes 40°C	Week 10		100000				
>	5	1.08131		0.03395	-0.25865	0.551252	5.97736
>	50	4.42855		0.646262	0.122378	1.325495	
>	95	87.30833		1.941056	0.928099	8.474213	
Liposomes 40°C	Week 10		100000				
>	5	1.08975		0.037327	-0.25655	0.553926	5.883562
>	50	4.38489		0.641959	0.1197	1.317348	
>	95	84.51762		1.926947	0.91932	8.304625	
Liposomes 40°C	Week 10		100000				
>	5	1.06666		0.028026	-0.26234	0.546593	11.13388
>	50	4.57786		0.660663	0.131339	1.35313	
>	95	233.075		2.367496	1.193463	15.61218	

L + CQ 40°C	Week 10		100000				
>	5	1.07573		0.031703	-0.26005	0.54948	10.20775
>	50	3.00766		0.478229	0.017815	1.041874	
>	95	136.3758		2.134737	1.048623	11.18467	
L + CQ 40°C	Week 10		100000				
>	5	1.05655		0.02389	-0.26491	0.543363	10.69923
>	50	1.91545		0.282271	-0.10413	0.786819	
>	95	95.51974		1.980093	0.952391	8.961723	
L + CQ 40°C	Week 10		100000				
>	5	1.05579		0.023578	-0.2651	0.54312	10.51236
>	50	1.8688		0.271563	-0.11079	0.774839	
>	95	90.88345		1.958485	0.938945	8.688506	

Liposomes 40°C	Week 12		100000				
>	5	1.33607		0.125829	-0.20148	0.628818	3.263831
>	50	37.93407		1.579029	0.702819	5.044505	
>	95	269.6177		2.430748	1.232824	17.09323	
Liposomes 40°C	Week 12		100000				
>	5	1.06626		0.027863	-0.26244	0.546465	8.273199
>	50	4.41251		0.644686	0.121397	1.322505	
>	95	142.3645		2.153402	1.060237	11.48782	
Liposomes 40°C	Week 12		100000				
>	5	1.07557		0.031639	-0.26009	0.54943	8.528457
>	50	5.35698		0.72892	0.173815	1.492157	
>	95	179.6092		2.254328	1.123042	13.27523	

L + CQ 40°C	Week 12		100000				
>	5	1.04588		0.019482	-0.26765	0.539942	9.408244
>	50	1.82225		0.260608	-0.11761	0.762772	
>	95	75.10432		1.875665	0.887408	7.716283	
L + CQ 40°C	Week 12		100000				
>	5	1.05284		0.022362	-0.26586	0.542175	9.426382
>	50	2.01759		0.304833	-0.09009	0.812671	
>	95	82.85723		1.91833	0.913958	8.202721	
L + CQ 40°C	Week 12		100000				
>	5	1.05032		0.021322	-0.26651	0.541367	10.33363
>	50	1.85773		0.268983	-0.11239	0.77198	
>	95	88.04639		1.944712	0.930374	8.51872	

ANNEXURE B

The experimental values

Table B1: pH of liposomes and chloroquine entrapped in liposomes (L + CQ) at 5°C during a time period of twelve weeks.

	Liposomes	Liposomes	Liposomes	L + CQ	L + CQ	L + CQ
Week 0	7.22	7.18	7.19	5.96	5.95	5.93
Week 1	7.24	7.19	7.19	6.03	5.99	5.98
Week 2	7.32	7.21	7.19	6.02	5.99	5.97
Week 4	7.29	7.15	7.15	6.00	5.97	5.95
Week 6	7.13	6.99	6.99	5.89	5.84	5.82
Week 8	7.27	7.17	7.14	5.99	5.94	5.93
Week 10	7.18	7.11	7.03	5.92	5.86	5.87
Week 12	6.99	7.12	7.07	6.01	5.88	5.88

Table B2: pH of liposomes and chloroquine entrapped in liposomes (L + CQ) at 25°C during a time period of twelve weeks.

	Liposomes	Liposomes	Liposomes	L + CQ	L + CQ	L + CQ
Week 0	7.22	7.18	7.19	5.96	5.95	5.93
Week 1	7.09	7.11	7.10	5.96	5.92	5.92
Week 2	7.10	7.11	7.12	5.95	5.91	5.9
Week 4	7.03	6.62	6.54	5.91	5.87	5.81
Week 6	6.60	6.02	5.48	5.85	5.81	5.05
Week 8	6.67	5.47	4.31	5.71	5.80	4.38
Week 10	6.59	4.93	3.91	5.32	5.50	3.93
Week 12	6.49	4.96	3.52	4.83	5.06	3.78

Table B3: pH of liposomes and chloroquine entrapped in liposomes (L + CQ) at 40°C during a time period of twelve weeks.

	Liposomes	Liposomes	Liposomes	L + CQ	L + CQ	L + CQ
Week 0	7.22	7.18	7.19	5.96	5.95	5.93
Week 1	7.03	7.05	7.04	5.92	5.91	5.90
Week 2	6.98	6.99	7.00	5.92	5.9	5.90
Week 4	6.51	6.58	6.18	6.00	6.00	5.98
Week 6	6.03	6.03	5.13	5.86	5.85	5.83
Week 8	6.05	6.03	4.84	5.82	5.80	5.77
Week 10	5.86	5.82	4.66	5.63	5.60	5.59
Week 12	5.60	5.53	4.53	5.52	5.51	5.47

Table B4: Comparison between pH and entrapment efficiency (EE %) of chloroquine entrapped in liposomes (L + CQ) at 5°C during a time period of twelve weeks.

	pH	pH	pH	EE %	EE %	EE %
Week 0	5.96	5.95	5.93	13.97291	15.61741	14.06965
Week 1	6.03	5.99	5.98	15.85925	14.65006	16.10109
Week 2	6.02	5.99	5.97	23.83990	26.06481	13.15067
Week 4	6.00	5.97	5.95	25.24256	25.09746	25.09746
Week 6	5.89	5.84	5.82	14.16638	14.79516	13.73108
Week 8	5.99	5.94	5.93	14.89190	15.42394	13.63434
Week 10	5.92	5.86	5.87	12.57025	15.32721	13.29577
Week 12	6.01	5.88	5.88	13.73108	13.15067	17.21354

Table B5: Comparison between pH and entrapment efficiency (EE %) of chloroquine entrapped in liposomes (L + CQ) at 25°C during a time period of twelve weeks.

	pH	pH	pH	EE %	EE %	EE %
Week 0	5.96	5.95	5.93	13.97291	15.61741	14.06965
Week 1	5.96	5.92	5.92	16.19782	16.68150	14.55333
Week 2	5.95	5.91	5.90	19.68029	6.18573	10.00677
Week 4	5.91	5.87	5.81	27.70931	25.43603	26.06481
Week 6	5.85	5.81	5.05	15.13374	16.14946	16.72987
Week 8	5.71	5.80	4.38	14.89190	15.76252	16.53640
Week 10	5.32	5.50	3.93	14.50496	15.23047	7.92697
Week 12	4.83	5.06	3.78	16.43966	17.02007	7.54002

Table B6: Comparison between pH and entrapment efficiency (EE %) of chloroquine entrapped in liposomes (L + CQ) at 40°C during a time period of twelve weeks.

	pH	pH	pH	EE %	EE %	EE %
Week 0	5.96	5.95	5.93	13.97291	15.61741	14.06965
Week 1	5.92	5.91	5.90	13.39250	14.02128	16.24619
Week 2	5.92	5.90	5.90	16.34293	14.35985	14.79516
Week 4	6.00	6.00	5.98	26.54849	26.45175	25.53277
Week 6	5.86	5.85	5.83	15.37557	16.24619	15.95599
Week 8	5.82	5.80	5.77	17.40701	18.56783	19.19662
Week 10	5.63	5.60	5.59	18.42273	18.71294	19.29335
Week 12	5.52	5.51	5.47	19.34172	19.72866	19.77703

Table B7: Span values (μm) of liposomes and chloroquine entrapped in liposomes (L + CQ) at 5°C during a time period of twelve weeks.

	Liposomes	Liposomes	Liposomes	L + CQ	L + CQ	L + CQ
Week 0	5.777835	8.816706	8.149220	2.821196	2.998893	3.06566
Week 1	10.559100	9.993933	10.025050	4.890745	3.554696	4.093119
Week 2	4.687642	9.063390	9.629034	4.724959	4.45416	3.915796
Week 4	5.021017	7.144677	7.095772	12.005420	11.4039	10.79564
Week 6	5.509030	7.811690	7.004709	17.495730	14.76481	13.43379
Week 8	7.976492	6.472506	7.236117	16.338620	15.71391	16.09204
Week 10	8.644492	6.251157	6.799746	14.086140	14.61017	14.01853
Week 12	3.162600	3.468079	3.331708	9.953386	13.52852	14.47429

Table B8: Span values (μm) of liposomes and chloroquine entrapped in liposomes (L + CQ) at 25°C during a time period of twelve weeks.

	Liposomes	Liposomes	Liposomes	L + CQ	L + CQ	L + CQ
Week 0	5.777835	8.816706	8.14922	2.821196	2.998893	3.06566
Week 1	9.821814	9.374307	6.511107	4.607371	4.344923	3.919789
Week 2	9.41664	9.380329	9.172966	10.31564	3.842626	3.193735
Week 4	7.00632	6.007558	6.230396	12.17272	12.12536	8.860975
Week 6	6.293763	6.324007	5.760502	8.75817	9.41387	6.904671
Week 8	6.406993	6.375216	8.842836	7.995629	7.655775	6.78993
Week 10	6.074246	7.770127	13.59345	7.197298	6.570047	8.336899
Week 12	3.481986	5.362027	27.30857	12.24961	10.05945	7.395948

Table B9: Span values (μm) of liposomes and chloroquine entrapped in liposomes (L + CQ) at 40°C during a time period of twelve weeks.

	Liposomes	Liposomes	Liposomes	L + CQ	L + CQ	L + CQ
Week 0	5.777835	8.816706	8.14922	2.821196	2.998893	3.06566
Week 1	6.150559	7.048568	10.11489	3.176889	4.620883	3.738626
Week 2	7.09733	6.613044	6.752656	4.151875	4.316661	4.196374
Week 4	6.033539	5.043087	5.702072	10.1227	10.78433	6.596971
Week 6	6.185066	5.951149	18.4955	8.943414	8.698902	8.619482
Week 8	6.096896	5.953143	19.59924	8.224611	8.082067	8.091651
Week 10	5.97736	5.883563	11.13388	10.20775	10.69923	10.51236
Week 12	3.263831	8.273199	8.528457	9.408244	9.426382	10.33363

Table B10: Comparison between entrapment efficiency (EE %) and span (μm) of various chloroquine entrapped in liposomes (L + CQ) formulations.

Concentrations	EE %	EE %	EE %	Span	Span	Span
[0.2%]	5.367594	2.465538	2.707376	6.785271	7.255331	7.033139
[0.3%]	13.85651	11.67997	12.88916	9.715219	9.459318	8.985413
[0.4%]	9.576179	10.36215	10.60399	9.583877	8.623961	8.25886
[0.5%] S	12.47352	12.66699	12.28005	9.389999	9.403961	9.721296
[0.5%] N/S	13.44087	13.3925	13.58597	14.50727	15.90013	16.06531

Table B11: Geometric means of fluorescence (%) of the control group and five liposome concentrations within red blood cells (RBC) and infected red blood cells (iRBC) obtained from the ROS assay.

	RBC	RBC	iRBC	iRBC
Control	181.0743	224.2518	175.3681	255.0902
0% liposomes	23.05142	32.81105	37.91401	38.73172
25% liposomes	33.35772	34.83782	35.7845	35.69185
50% liposomes	35.05450	34.99845	35.10313	34.46044
75% liposomes	35.79222	35.04837	37.49235	35.54529
100% liposomes	40.15578	40.70264	42.92395	38.90315

Table B12: Geometric means of fluorescence (%) of the control group, chloroquine [0.5 %] and five chloroquine entrapped in liposome concentrations (L + CQ) within red blood cells (RBC) and infected red blood cells (iRBC) obtained from the ROS assay.

	RBC	RBC	iRBC	iRBC
Control	181.074	224.252	175.368	255.09
Chloroquine	26.7984	30.1063	41.0494	40.2765
0% L + CQ	38.9564	41.9307	36.0637	36.1223
25% L + CQ	37.2665	39.9658	37.7993	39.7867
50% L + CQ	40.3053	46.085	38.8659	40.8361
75% L + CQ	44.4545	41.8566	41.3727	41.1259
100% L + CQ	43.0454	44.4771	52.6408	47.5095

Table B13: Comparison between the ROS (%), in terms of the control group's ROS percentage, of liposomes and chloroquine (CQ) entrapped in liposomes at five different concentrations within red blood cells (RBC).

	Liposomes	Liposomes	CQ entrapped in liposomes	CQ entrapped in liposomes
Control	89.3474	110.6525	89.3474	110.6525
0%	11.37426	16.18995	19.22225	20.68984
25%	16.45969	17.19002	18.38838	19.72033
50%	17.29693	17.26928	19.88782	22.73969
75%	17.66095	17.29391	21.93515	20.6533
100%	19.81406	20.08389	21.23989	21.94634

Table B14: Comparison between the ROS (%), in terms of the control group's ROS percentage, of liposomes and chloroquine (CQ) entrapped in liposomes at five different concentrations within infected red blood cells (iRBC).

	Liposomes	Liposomes	CQ entrapped in liposomes	CQ entrapped in liposomes
Control	81.4797	118.5203	81.4797	118.5203
0%	17.61565	17.99557	16.75595	16.78318
25%	16.62623	16.58319	17.56235	18.48572
50%	16.30965	16.01105	18.05791	18.97331
75%	17.41974	16.51509	19.22262	19.10798
100%	19.94337	18.07522	24.45802	22.07392

Table B15: Geometric means of fluorescence (%) of the control group and five liposome concentrations within red blood cells (RBC) and infected red blood cells (iRBC) obtained from the LP assay.

	RBC	RBC	iRBC	iRBC
Control	0.11828	0.12353	0.2726	0.33032
0% liposomes	0.11024	0.10972	0.12654	0.13415
25% liposomes	0.29017	0.3577	0.38064	0.42755
50% liposomes	0.59037	0.39613	0.43575	0.42396
75% liposomes	0.43288	0.39339	0.45491	0.45023
100% liposomes	0.32867	0.43336	0.35019	0.46298

Table B16: Geometric means of fluorescence (%) of the control group, chloroquine [0.5 %] and five chloroquine entrapped in liposomes concentrations (L + CQ) within red blood cells (RBC) and infected red blood cells (iRBC) obtained from the LP assay.

	RBC	RBC	iRBC	iRBC
Control	0.11828	0.12353	0.2726	0.33032
Chloroquine	0.58326	0.52737	0.59512	0.58837
0% L + CQ	0.46667	0.36216	0.34739	0.46576
25% L + CQ	0.36331	0.33473	0.36545	0.35652
50% L + CQ	0.45878	0.46476	0.55285	0.5495
75% L + CQ	0.58164	0.50833	0.56908	0.54426
100% L + CQ	0.56784	0.61981	0.48473	0.62529

Table B17: Comparison between the LP (%), in terms of the control group's LP percentage, of liposomes and chloroquine (CQ) entrapped in liposomes at five different concentrations within red blood cells (RBC).

	Liposomes	Liposomes	CQ entrapped in liposomes	CQ entrapped in liposomes
Control	-2.170946	2.170959	-2.170946	2.170959
0 %	-9.625242	-10.14554	74.1052	66.63082
25 %	58.35208	65.83959	66.73658	63.89655
50 %	79.52988	69.49213	73.65834	73.99709
75 %	72.08214	69.28017	79.22275	76.22619
100 %	63.23108	72.11357	78.71774	80.50211

Table B18: Comparison between the LP (%), in terms of the control group's LP percentage, of liposomes and chloroquine (CQ) entrapped in liposomes at five different concentrations within infected red blood cells (iRBC).

	Liposomes	Liposomes	CQ entrapped in liposomes	CQ entrapped in liposomes
Control	-9.573277	9.573277	-9.573277	9.573277
0 %	-136.0453	-122.6632	14.01752	35.86859
25 %	21.52698	30.13765	18.26689	16.21837
50 %	31.45308	29.5459	45.97142	45.6422
75 %	34.3395	33.65695	47.51266	45.11853
100 %	14.70333	35.48428	38.37823	52.23081

Table B19: Hemolysis of red blood cells (RBC) containing different concentrations of liposomes and chloroquine (CQ) entrapped in liposomes.

Percentage	Liposomes	Liposomes	CQ entrapped in liposome	CQ entrapped in liposomes
0	0.000342372	0.000342372	-1.588983	1.588983
25	-24.68186	-23.41068	0.95339	3.072034
50	-4.554742	-29.55474	-4.661017	5.29661
75	-6.35559	-29.23695	-9.957627	-6.991525
100	-4.131013	4.979156	-5.508474	-4.449152

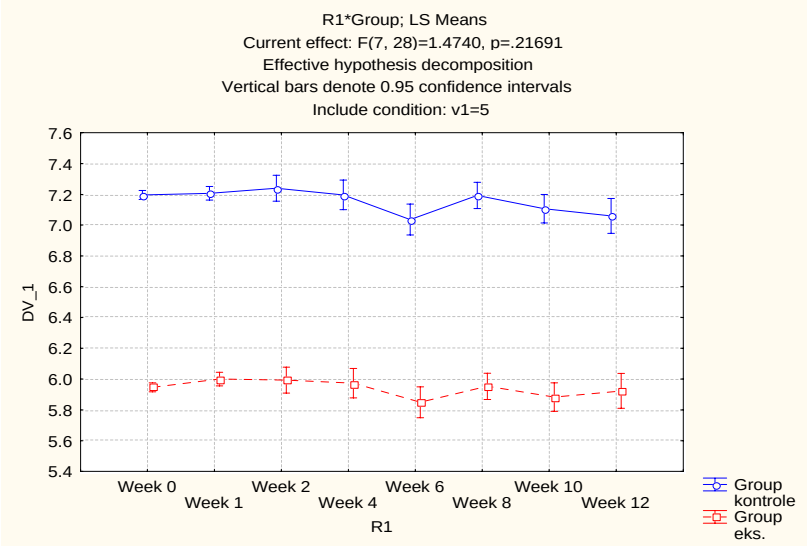
Table B20: Hemolysis of red blood cells (RBC) containing different concentrations of liposomes and chloroquine (CQ) entrapped in liposomes (after normalization and transformation of the data presented in table B19).

Percentage	Liposomes	Liposomes	CQ entrapped in liposomes	CQ entrapped in liposomes
	0.000342372	0.000342372	-1.588983	1.588983
1.39794	-24.68186	-23.41068	0.95339	3.072034
1.69897	-4.554742	-29.55474	-4.661017	5.29661
1.875061	-6.35559	-29.23695	-9.957627	-6.991525
2	-4.131013	4.979156	-5.508474	-4.449152

ANNEXURE C

Data analyzed with STATISTICA

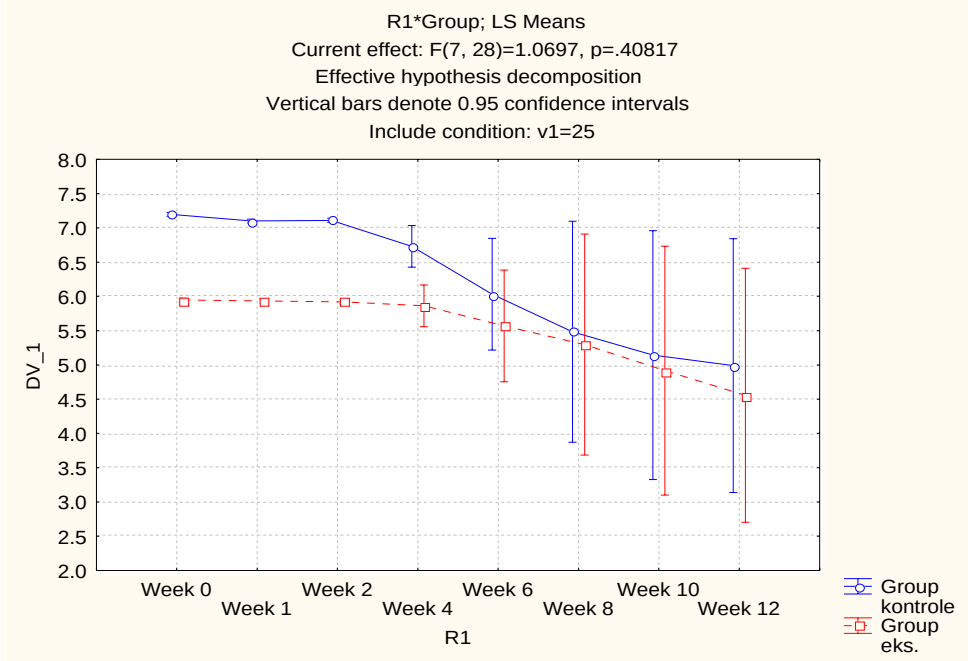
pH data



Effect	SS	Degr. o Freedor	MS	F	p
Repeated Measures Analysis of Variance (Repeated measures vir pH by Sigma-restricted parameterization Effective hypothesis decomposition Include condition: v1=5					
Intercep	2057.7	1	2057.7	166900	0.0000
Group	17.69	1	17.69	1434.	0.0000
Error	0.04	4	0.01		
R1	0.16	7	0.02	16.:	0.0000
R1*Grou	0.01	7	0.00	1.5	0.2169
Error	0.04	28	0.00		

Variable	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Temp=5, Group=kontrole T-test for Dependent Samples (Repeated measures vir pH by 3 temperature) Marked differences are significant at p < .05000										
Week 0	7.196667	0.020817								
Week 12	7.060000	0.065574	3	0.136667	0.086217	2.745563	2	0.111003	-0.077508	0.350841

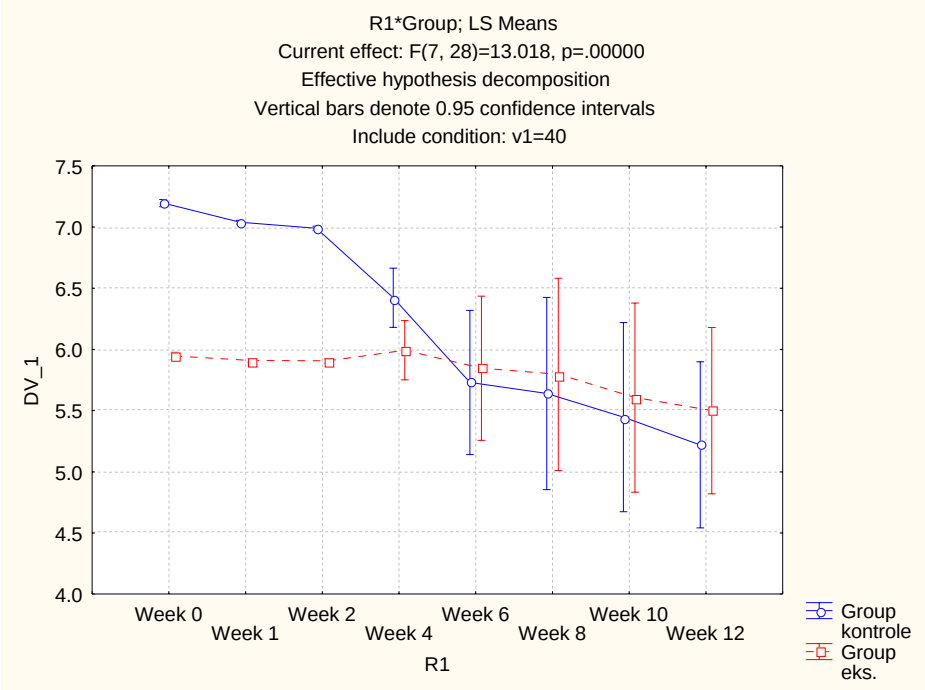
Variable	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Temp=5, Group=eks. T-test for Dependent Samples (Repeated measures vir pH by 3 temperature) Marked differences are significant at p < .05000										
Week 0	5.946667	0.015275								
Week 12	5.923333	0.075056	3	0.023333	0.064291	0.628619	2	0.593819	-0.136374	0.183041



Effect	Repeated Measures Analysis of Variance (Repeated r Sigma-restricted parameterization Effective hypothesis decomposition Include condition: v1=25				
	SS	Degr. of Freedom	MS	F	p
Intercept	1649.356	1	1649.356	838.8474	0.000008
Group	6.271	1	6.271	3.1895	0.148655
Error	7.865	4	1.966		
R1	21.908	7	3.130	11.1706	0.000001
R1*Group	2.098	7	0.300	1.0697	0.408173
Error	7.845	28	0.280		

Variable	Temp=25, Group=kontrole T-test for Dependent Samples (Repeated measures vir pH by 3 temperature) Marked differences are significant at p < .05000									
	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Week 0	7.196667	0.020817								
Week 12	4.990000	1.485227	3	2.206667	1.470045	2.599960	2	0.121545	-1.44513	5.858462

Variable	Temp=25, Group=eks. T-test for Dependent Samples (Repeated measures vir pH by 3 temperature) Marked differences are significant at p < .05000									
	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Week 0	5.946667	0.015275								
Week 12	4.556667	0.682373	3	1.390000	0.669029	3.598574	2	0.069291	-0.271961	3.051961



Effect	Repeated Measures Analysis of Variance (Repeated measu Sigma-restricted parameterization Effective hypothesis decomposition Include condition: v1=40				
	SS	Degr. of Freedom	MS	F	p
Intercept	1734.967	1	1734.967	3800.862	0.000000
Group	1.896	1	1.896	4.154	0.111185
Error	1.826	4	0.456		
R1	9.321	7	1.332	26.297	0.000000
R1*Group	4.614	7	0.659	13.018	0.000000
Error	1.418	28	0.051		

Variable	Temp=40, Group=kontrole T-test for Dependent Samples (Repeated measures vir pH by 3 temperature) Marked differences are significant at p < .05000									
	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Week 0	7.196667	0.020817								
Week 12	5.220000	0.598582	3	1.976667	0.591974	5.783508	2	0.028619	0.506121	3.447212

Variable	Temp=40, Group=eks. T-test for Dependent Samples (Repeated measures vir pH by 3 temperature) Marked differences are significant at p < .05000									
	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Week 0	5.946667	0.015275								
Week 12	5.500000	0.026458	3	0.446667	0.011547	67.00000	2	0.000223	0.417982	0.475351

Correlation between pH and EE %

All Groups
Spearman Rank Order Correlations (Spearman rangorde korrelasies vir pH en EE by 3 temp'e)
MD pairwise deleted
Marked correlations are significant at p <.05000

Variable	pH	EE
pH	1.000000	0.001352
EE	0.001352	1.000000

Temp=5
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

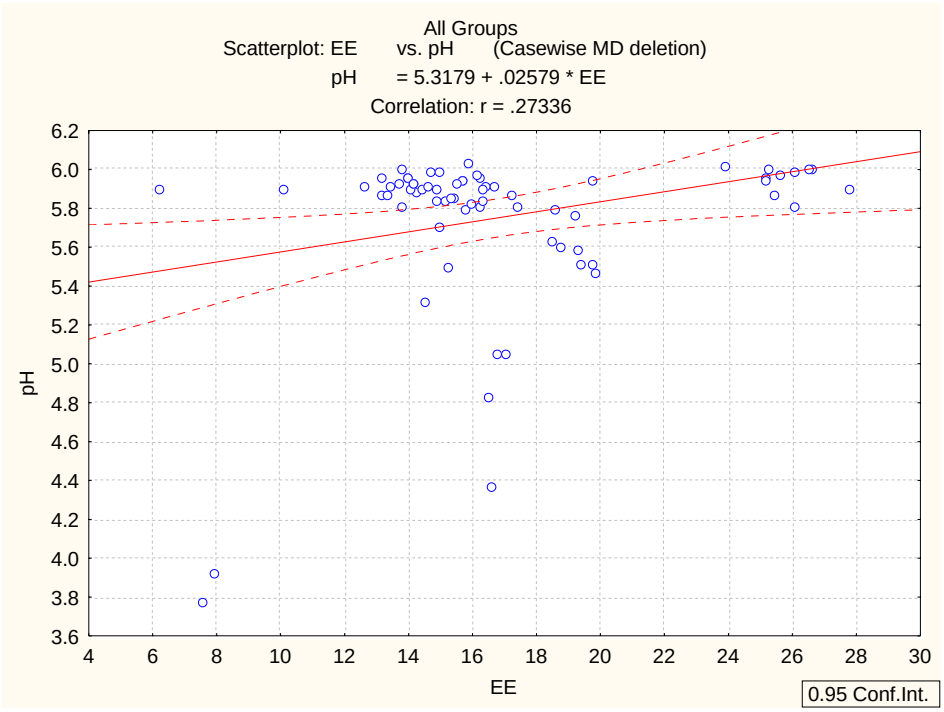
Variable	pH	EE
pH	1.000000	0.433646
EE	0.433646	1.000000

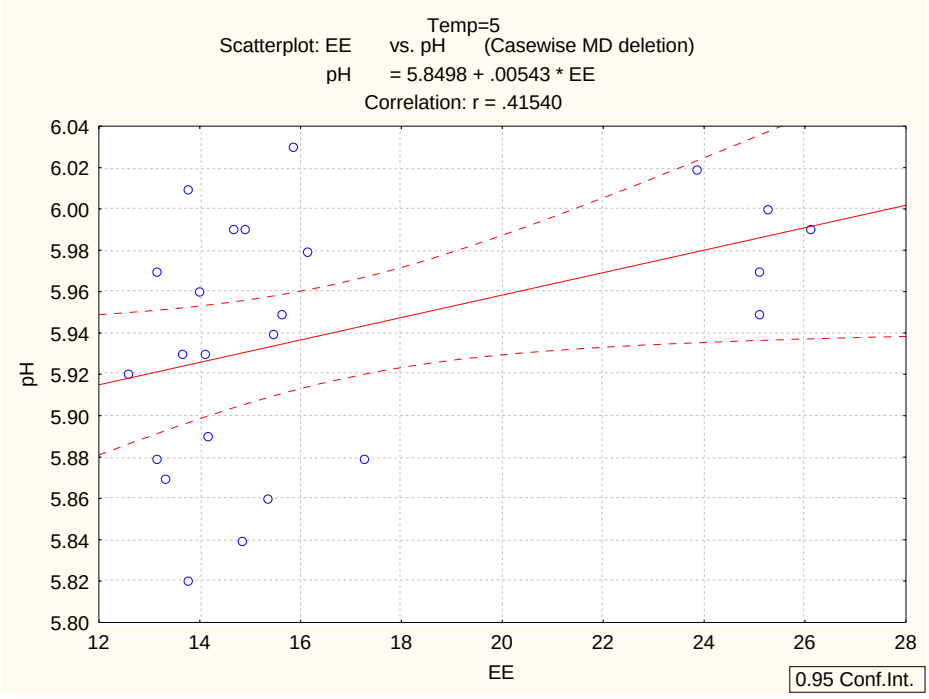
Temp=25
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

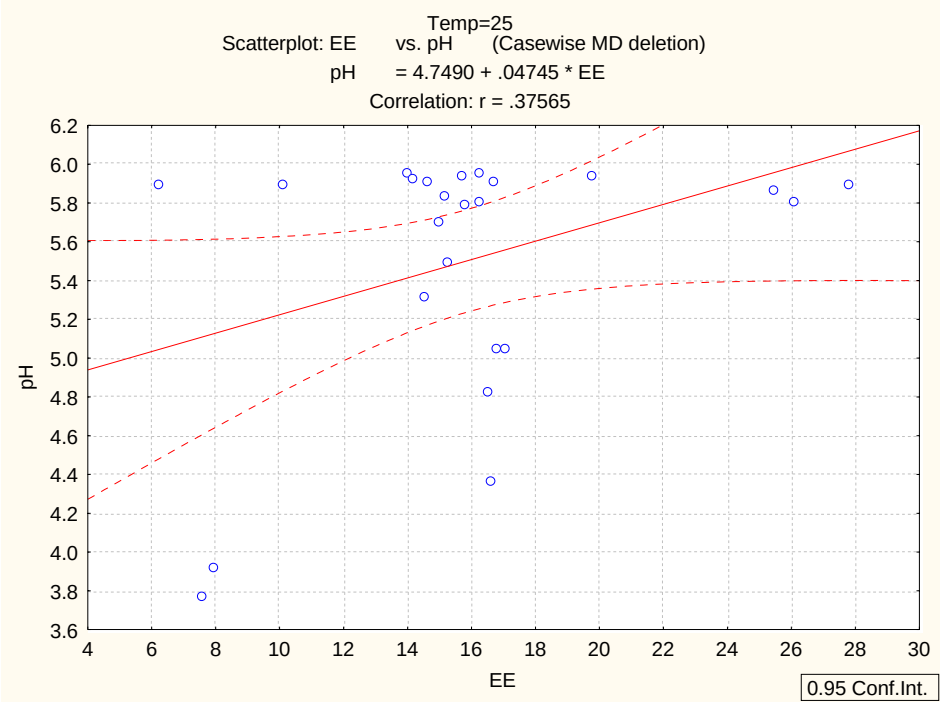
Variable	pH	EE
pH	1.000000	0.045702
EE	0.045702	1.000000

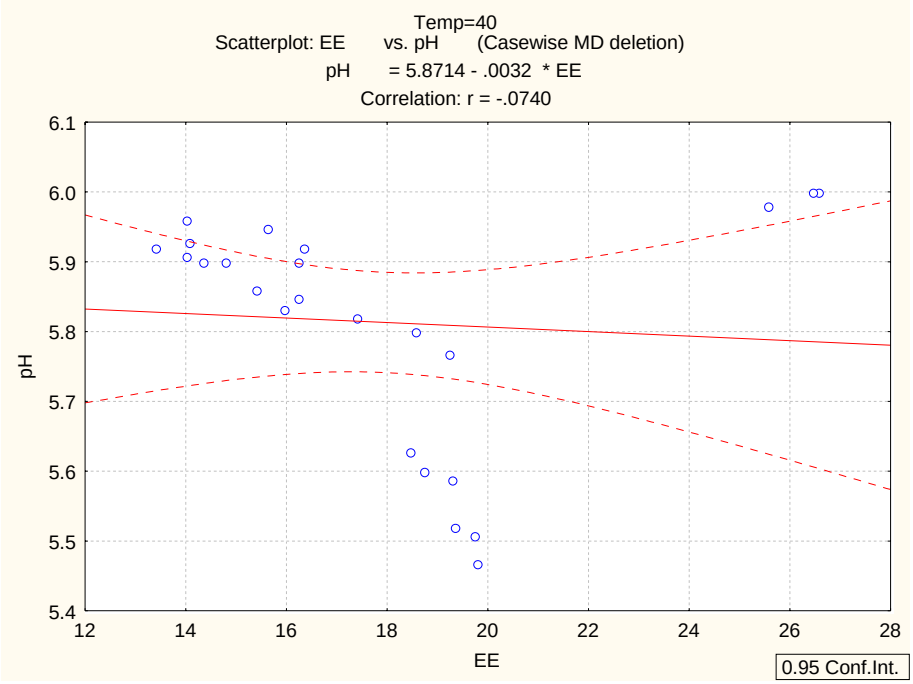
Temp=40
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

Variable	pH	EE
pH	1.000000	-0.267799
EE	-0.267799	1.000000

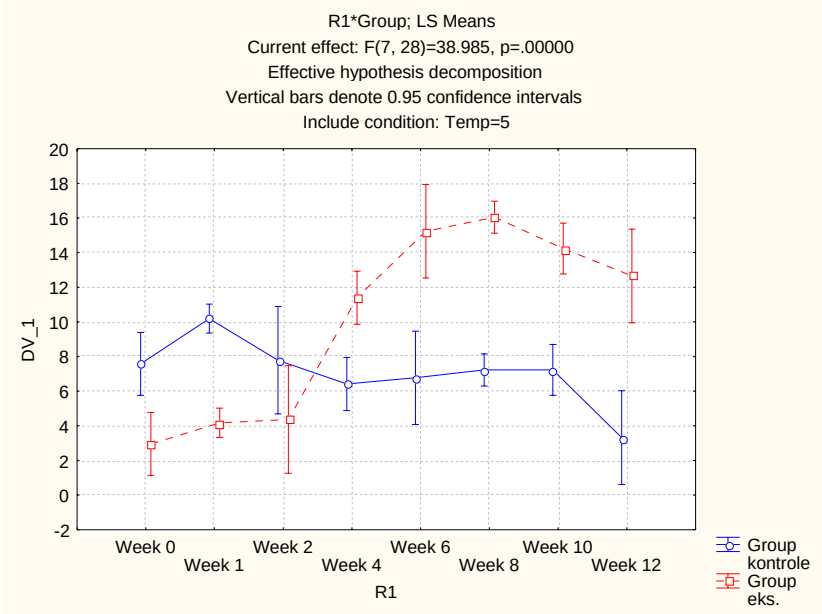

All Groups
Scatterplot: EE vs. pH (Casewise MD deletion)
pH = 5.3179 + .02579 * EE
Correlation: r = .27336
pH
EE
0.95 Conf.Int.


Temp=5
Scatterplot: EE vs. pH (Casewise MD deletion)
pH = 5.8498 + .00543 * EE
Correlation: r = .41540
pH
EE
0.95 Conf.Int.


Temp=25
Scatterplot: EE vs. pH (Casewise MD deletion)
pH = 4.7490 + .04745 * EE
Correlation: r = .37565
pH
EE
0.95 Conf.Int.


Temp=40
Scatterplot: EE vs. pH (Casewise MD deletion)
pH = 5.8714 - .0032 * EE
Correlation: r = -.0740
pH
EE
0.95 Conf.Int.

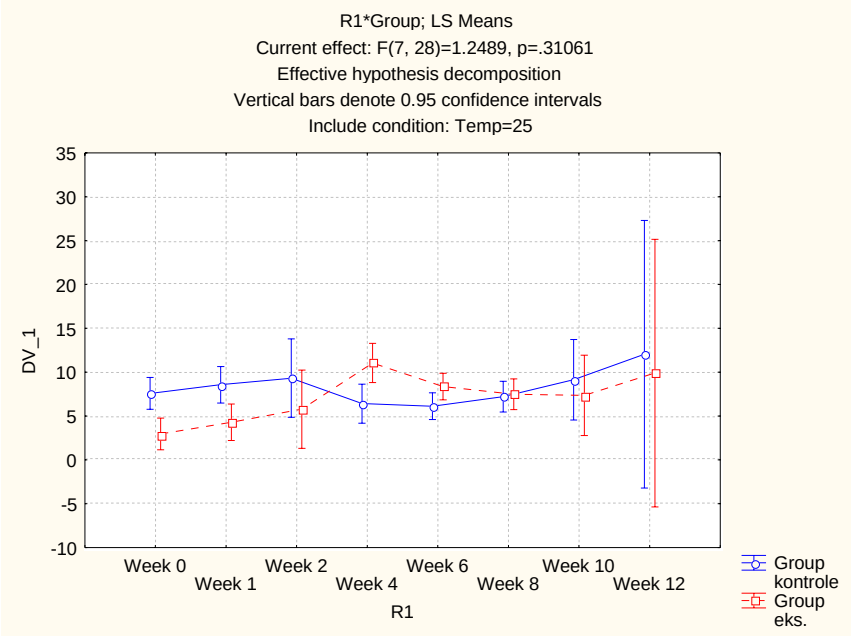
Span data



Effect	Repeated Measures Analysis of Variance (Repeated measures vir Span by 3 temperature) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: Temp=5				
	SS	Degr. of Freedom	MS	F	p
Intercept	3551.212	1	3551.212	2604.633	0.000001
Group	112.861	1	112.861	82.778	0.000809
Error	5.454	4	1.363		
R1	236.824	7	33.832	20.228	0.000000
R1*Group	456.425	7	65.204	38.985	0.000000
Error	46.831	28	1.673		

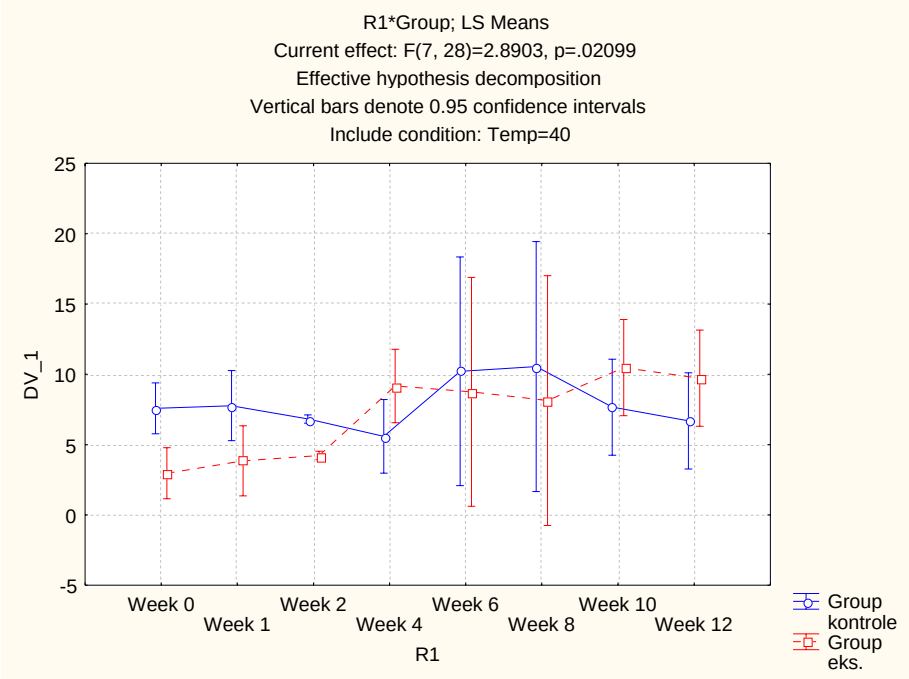
Variable	Group=kontrole, Temp=5 T-test for Dependent Samples (Repeated measures vir Span by 3 temperature) Marked differences are significant at p < .05000									
	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Week 0	7.581254	1.597067								
Week 12	3.320796	0.153032	3	4.260458	1.449341	5.091506	2	0.036478	0.660095	7.860821

Variable	Group=eks., Temp=5 T-test for Dependent Samples (Repeated measures vir Span by 3 temperature) Marked differences are significant at p < .05000									
	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Week 0	2.96192	0.126357								
Week 12	12.65207	2.384486	3	-9.69015	2.258435	-7.43162	2	0.017629	-15.3004	-4.07989



Effect	SS	Degr. of Freedom	MS	F	p
Repeated Measures Analysis of Variance (Repeated r Sigma-restricted parameterization Effective hypothesis decomposition Include condition: Temp=25					
Intercept	2865.281	1	2865.281	127.5183	0.000350
Group	15.942	1	15.942	0.7095	0.447026
Error	89.878	4	22.470		
R1	119.851	7	17.122	1.3148	0.280020
R1*Group	113.841	7	16.263	1.2489	0.310614
Error	364.618	28	13.022		

Variable	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Group=kontrole, Temp=25 T-test for Dependent Samples (Repeated measures vir Span by 3 temperature) Marked differences are significant at p < .05000										
Week 0	7.58125	1.59707								
Week 12	12.05086	13.24696	3	-4.46961	12.73488	-0.607904	2	0.605086	-36.1048	27.16558
Group=eks., Temp=25 T-test for Dependent Samples (Repeated measures vir Span by 3 temperature) Marked differences are significant at p < .05000										
Week 0	2.961916	0.126357								
Week 12	9.901669	2.430675	3	-6.93975	2.551209	-4.71149	2	0.042217	-13.2773	-0.602199



Effect	SS	Degr. of Freedom	MS	F	p
Repeated Measures Analysis of Variance (Repeated r Sigma-restricted parameterization Effective hypothesis decomposition Include condition: Temp=40					
Intercept	2707.198	1	2707.198	85.15856	0.000766
Group	5.881	1	5.881	0.18501	0.689265
Error	127.160	4	31.790		
R1	132.212	7	18.887	3.29038	0.011115
R1*Group	116.136	7	16.591	2.89030	0.020985
Error	160.725	28	5.740		

Variable	Mean	Std.Dv.	N	Diff.	Std.Dv. Diff.	t	df	p	Confidence -95.000%	Confidence +95.000%
Group=kontrole, Temp=40 T-test for Dependent Samples (Repeated measures vir Span by 3 temperature) Marked differences are significant at p < .05000										
Week 0	7.581254	1.597067								
Week 12	6.688496	2.968591	3	0.892758	1.477902	1.046282	2	0.405244	-2.77855	4.564069
Group=eks., Temp=40 T-test for Dependent Samples (Repeated measures vir Span by 3 temperature) Marked differences are significant at p < .05000										
Week 0	2.961916	0.126357								
Week 12	9.722752	0.529114	3	-6.76084	0.446378	-26.2336	2	0.001450	-7.86970	-5.65197

Correlation between span (µm) and EE %

All Groups
Spearman Rank Order Correlations (Spearman rangorde korrelasies vir Span en EE)
MD pairwise deleted
Marked correlations are significant at p <.05000

Variable	EE	Span
EE	1.000000	0.782143
Span	0.782143	1.000000

kons.=[0.2%]
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

Variable	EE	Span
EE	1.00000	-1.00000
Span	-1.00000	1.00000

kons.=[0.3%]
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

Variable	EE	Span
EE	1.000000	0.500000
Span	0.500000	1.000000

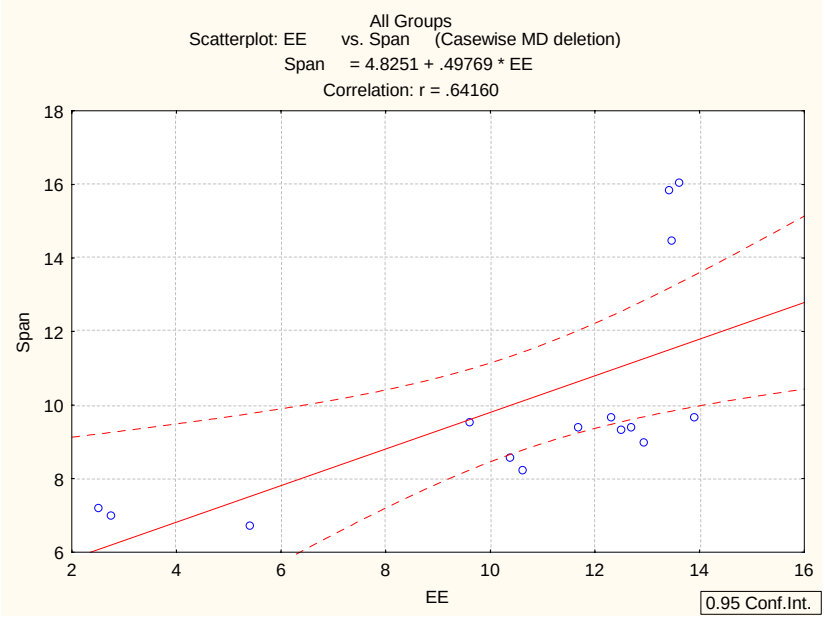
kons.=[0.4%]
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

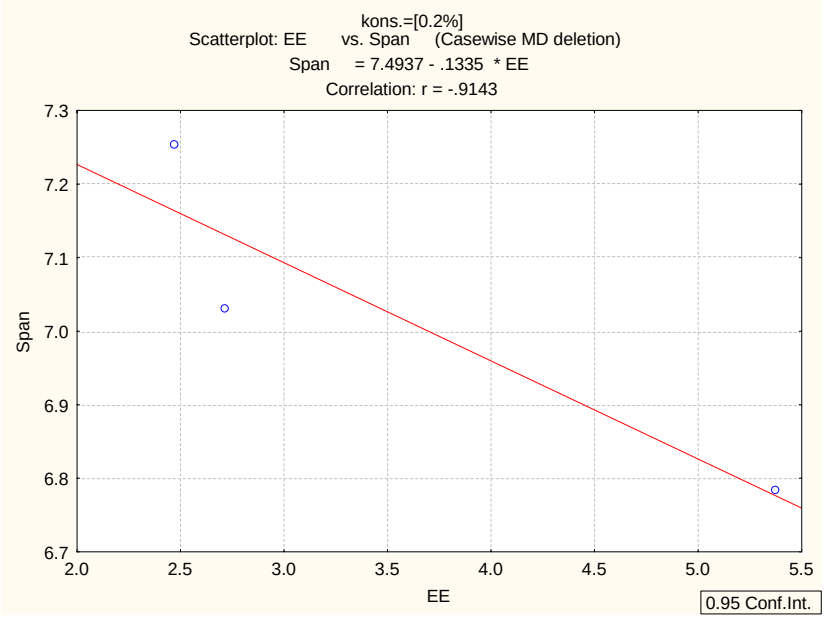
Variable	EE	Span
EE	1.00000	-1.00000
Span	-1.00000	1.00000

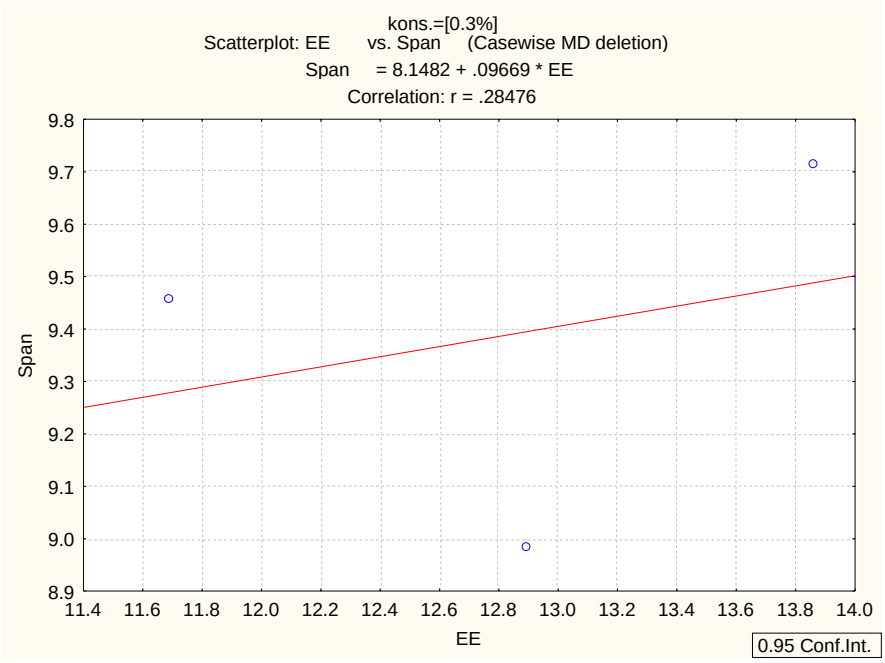
kons.=[0.5%] S
Spearman Rank Order Correlations (Spearman rangord
MD pairwise deleted
Marked correlations are significant at p <.05000

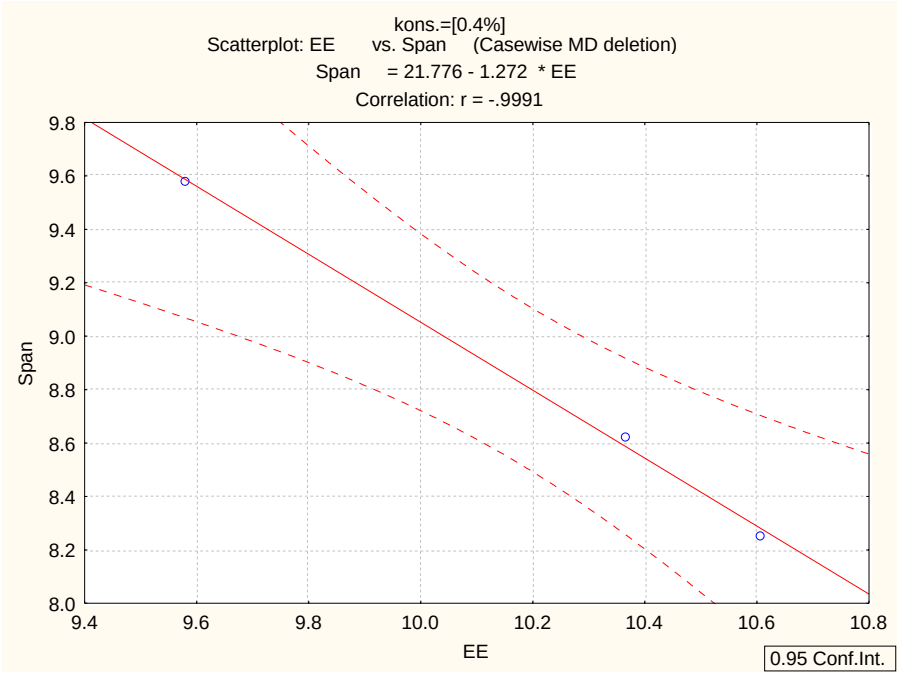
Variable	EE	Span
EE	1.000000	-0.500000
Span	-0.500000	1.000000

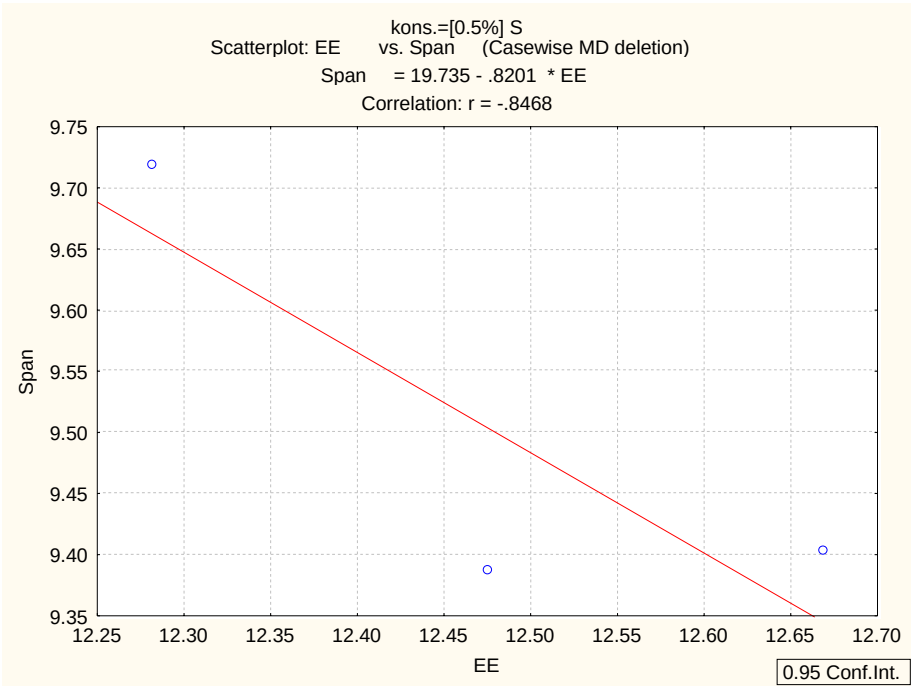
	kons.=[0.5%] N/S Spearman Rank Order Correlations (Spearman rangord MD pairwise deleted Marked correlations are significant at p <.05000	
Variable	EE	Span
EE	1.000000	0.500000
Span	0.500000	1.000000

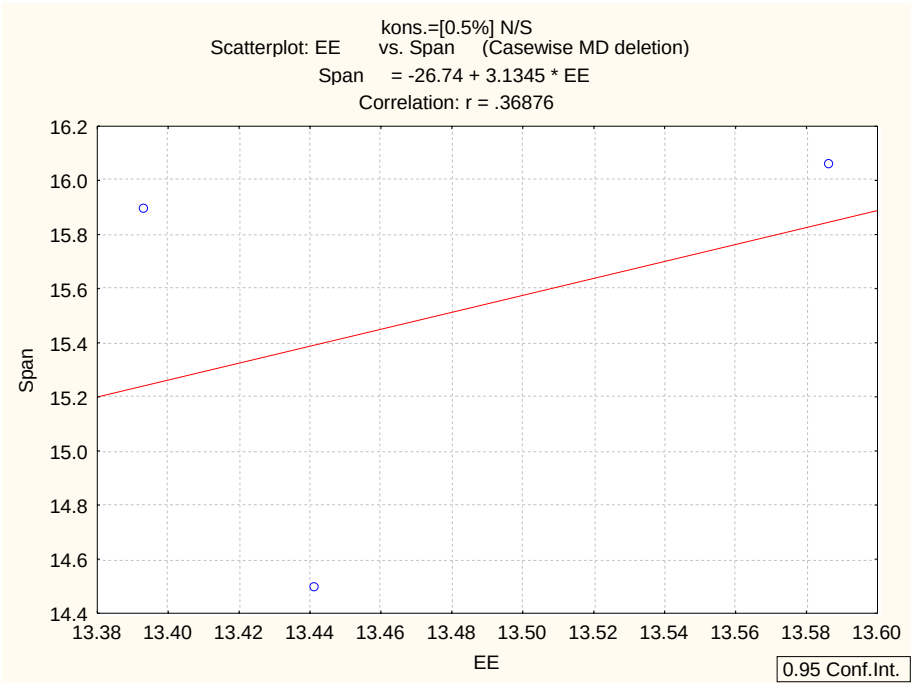





kons.=[0.3%]
Scatterplot: EE vs. Span (Casewise MD deletion)
Span = 8.1482 + .09669 * EE
Correlation: r = .28476
Span
EE
0.95 Conf.Int.

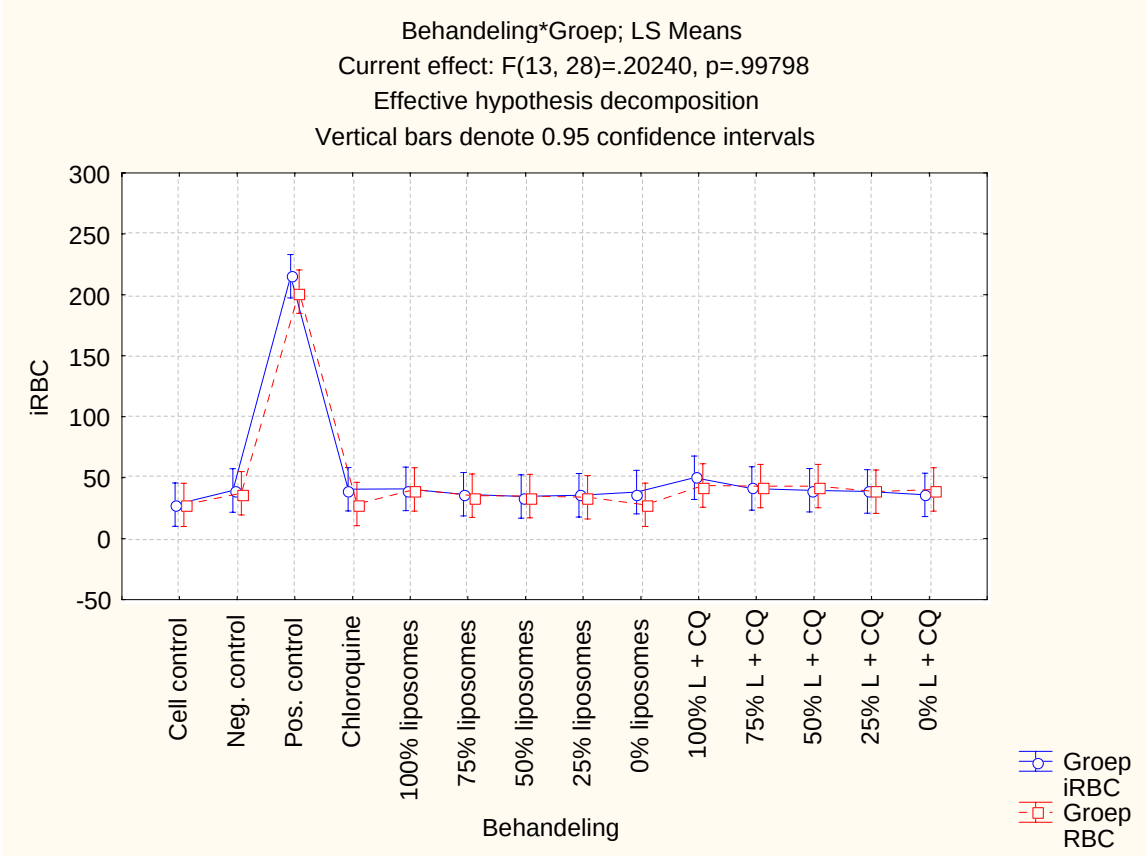

kons.=[0.4%]
Scatterplot: EE vs. Span (Casewise MD deletion)
Span = 21.776 - 1.272 * EE
Correlation: r = -.9991
Span
EE
0.95 Conf.Int.


kons.=[0.5%] S
Scatterplot: EE vs. Span (Casewise MD deletion)
Span = 19.735 - .8201 * EE
Correlation: r = -.8468
Span
EE
0.95 Conf.Int.


kons.=[0.5%] N/S
Scatterplot: EE vs. Span (Casewise MD deletion)
Span = -26.74 + 3.1345 * EE
Correlation: r = .36876
Span
EE
0.95 Conf.Int.

ROS data

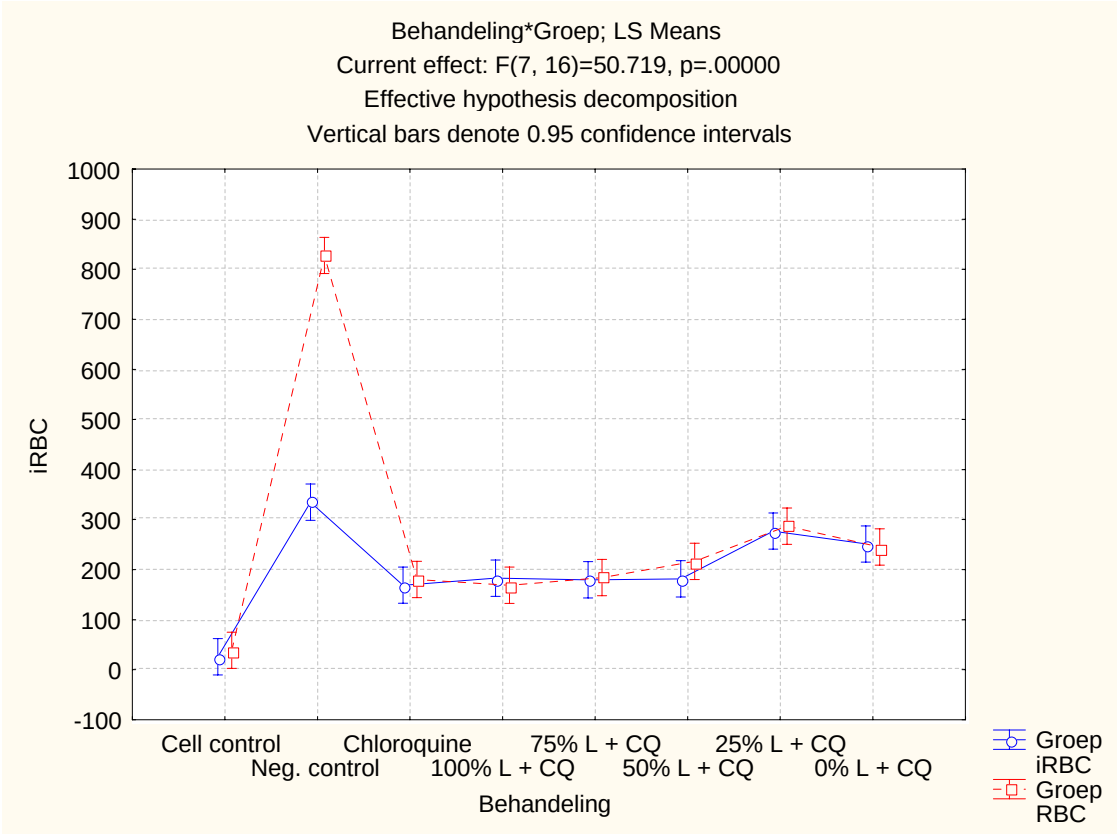
Effect	Univariate Tests of Significance for iRBC (2 way ANOVA tussen iRBC en RBC en d Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. (Freedo	MS	F	p
Intercept	13884:	1	13884:	918.91	0.0000
Behandeling	11021(	13	8478	56.11	0.0000
Groep	100	1	100.	0.662	0.4227
Behandeling*(	397	13	30.	0.202	0.9979
Error	4230	28	151.		



Depend.: iRBC	Multiple Comparisons p values (2-tailed); iRBC (2 way ANOVA tussen iRBC en RBC en die 3 kc Independent (grouping) variable: Behandeling Kruskal-Wallis test: H (13, N= 56) =44.07331 p =.0000								
	Cell cont R:4.250	Neg. cont R:28.75(	Pos. cont R:54.50(	Chloroqui R:22.00(	100% liposo R:37.500	75% liposor R:18.250	50% liposor R:12.750	25% liposor R:14.000	0% R
Cell control		1.0000	0.0011	1.0000	0.3582	1.0000	1.0000	1.0000	
Neg. control	1.0000		1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	
Pos. control	0.0011	1.0000		0.4396	1.0000	0.1520	0.0267	0.0405	
Chloroquine	1.0000	1.0000	0.4396		1.0000	1.0000	1.0000	1.0000	
100% liposo	0.3582	1.0000	1.0000	1.0000		1.0000	1.0000	1.0000	
75% liposom	1.0000	1.0000	0.1520	1.0000	1.0000		1.0000	1.0000	
50% liposom	1.0000	1.0000	0.0267	1.0000	1.0000	1.0000		1.0000	
25% liposom	1.0000	1.0000	0.0405	1.0000	1.0000	1.0000	1.0000		
0% liposome	1.0000	1.0000	0.0655	1.0000	1.0000	1.0000	1.0000	1.0000	
100% L + C(	0.0072	1.0000	1.0000	1.0000	1.0000	0.5739	0.1215	0.1761	
75% L + CQ	0.0476	1.0000	1.0000	1.0000	1.0000	1.0000	0.5739	0.7931	
50% L + CQ	0.2352	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	
25% L + CQ	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	
0% L + CQ	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

LP data

Effect	Univariate Tests of Significance for iRBC (2 way Anova tussen verskillende kons. iRBC en RBc en die 2 kontroles LP Assay) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	1758934	1	1758934	3011.917	0.000000
Behandeling	697692	7	99670	170.671	0.000000
Groep	37299	1	37299	63.869	0.000001
Behandeling*Groep	207336	7	29619	50.719	0.000000
Error	9344	16	584		

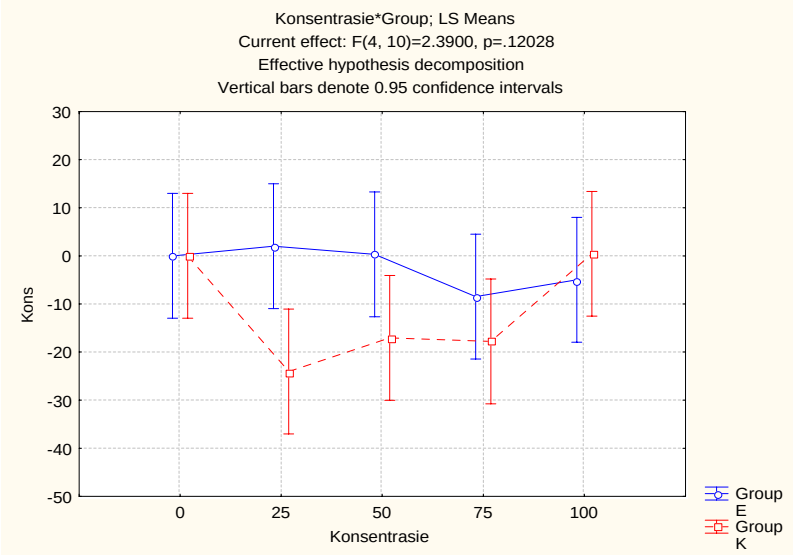


Tukey HSD test; variable iRBC (2 way ANOVA tussen iRBC en RBC en die 2kontroles.sta)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 1069.3, df = 14.000

Cell No.	Behandeling	Groep	{1} 26.068	{2} 38.909	{3} 334.79	{4} 827.47	{5} 250.78	{6} 267.50	{7} 220.97	{8} 242.61	{9} 232.68	{10} 210.91	{11} 248.30	{12} 313.65	{13} 767.85	{14} 909.27
1	Cell control	iRBC		1.000000	0.000188	0.000185	0.000542	0.000325	0.001849	0.000725	0.001092	0.002993	0.000589	0.000195	0.000185	0.000185
2	Cell control	RBC	1.000000		0.000191	0.000185	0.000879	0.000469	0.003434	0.001238	0.001949	0.005702	0.000971	0.000206	0.000185	0.000185
3	Neg. control	iRBC	0.000188	0.000191		0.000185	0.430038	0.715698	0.115742	0.312256	0.202286	0.069861	0.391881	0.999978	0.000185	0.000185
4	Neg. control	RBC	0.000185	0.000185	0.000185		0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.834604	0.465575
5	100% liposomes	iRBC	0.000542	0.000879	0.430038	0.000185		0.999999	0.999151	1.000000	0.999996	0.988289	1.000000	0.787144	0.000185	0.000185
6	100% liposomes	RBC	0.000325	0.000469	0.715698	0.000185	0.999999		0.962404	0.999867	0.996317	0.873859	0.999993	0.964566	0.000185	0.000185
7	75% liposomes	iRBC	0.001849	0.003434	0.115742	0.000185	0.999151	0.962404		0.999971	1.000000	1.000000	0.999645	0.305866	0.000185	0.000185
8	75% liposomes	RBC	0.000725	0.001238	0.312256	0.000185	1.000000	0.999867	0.999971		1.000000	0.998460	1.000000	0.651079	0.000185	0.000185
9	50% liposomes	iRBC	0.001092	0.001949	0.202286	0.000185	0.999996	0.996317	1.000000	1.000000		0.999969	0.999999	0.479277	0.000185	0.000185
10	50% liposomes	RBC	0.002993	0.005702	0.069861	0.000185	0.988289	0.873859	1.000000	0.998460	0.999969		0.993126	0.196583	0.000185	0.000185
11	25% liposomes	iRBC	0.000589	0.000971	0.391881	0.000185	1.000000	0.999993	0.999645	1.000000	0.999999	0.993126		0.747949	0.000185	0.000185
12	25% liposomes	RBC	0.000195	0.000206	0.999978	0.000185	0.787144	0.964566	0.305866	0.651079	0.479277	0.196583	0.747949		0.000185	0.000185
13	0% liposomes	iRBC	0.000185	0.000185	0.000185	0.834604	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185		0.028096
14	0% liposomes	RBC	0.000185	0.000185	0.000185	0.465575	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.000185	0.028096	

Hemolysis data

Effect	Univariate Tests of Significance for Kons (Repeated measures hemolise) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	968.7136	1	968.7136	14.27907	0.003609
Konsentrasie	507.7924	4	126.9481	1.87125	0.192158
Group	448.4042	1	448.4042	6.60959	0.027847
Konsentrasie*Group	648.5565	4	162.1391	2.38997	0.120282
Error	678.4150	10	67.8415		



Depend.: Kons	Group=E Multiple Comparisons p values (2-tailed); Kons (Repeated measures hemolise) Independent (grouping) variable: Konsentrasie Kruskal-Wallis test: H (4, N= 10) =6.327273 p =.1760				
	0 R:7.0000	25 R:8.0000	50 R:7.0000	75 R:1.5000	100 R:4.0000
0		1.000000	1.000000	0.692799	1.000000
25	1.000000		1.000000	0.318029	1.000000
50	1.000000	1.000000		0.692799	1.000000
75	0.692799	0.318029	0.692799		1.000000
100	1.000000	1.000000	1.000000	1.000000	

Depend.: Kons	Group=K Multiple Comparisons p values (2-tailed); Kons (Repeated measures hemolise) Independent (grouping) variable: Konsentrasie Kruskal-Wallis test: H (4, N= 10) =6.585366 p =.1595				
	0 R:8.5000	25 R:3.5000	50 R:3.5000	75 R:3.5000	100 R:8.5000
0		0.986476	0.986476	0.986476	1.000000
25	0.986476		1.000000	1.000000	0.986476
50	0.986476	1.000000		1.000000	0.986476
75	0.986476	1.000000	1.000000		0.986476
100	1.000000	0.986476	0.986476	0.986476	

ANNEXURE D

Certificate of analysis and the ethics approval

Ipca Laboratories Limited

FACTORY & OFFICE : 69 A/B/90/91, INDUSTRIAL ESTATE, POLOGROUND, INDORE - 452 003 (M.P.)

Tel. : (0731) 2421163 / 2421317, 2422081
Telefax : (91-731) 2422082
E-mail : ipcalib@ipca.co.in

QUALITY DIVISION
CERTIFICATE OF ANALYSIS

NAME OF THE PRODUCT	:	CHLOROQUINE PHOSPHATE BP			
BATCH NO.	:	8234C1RJB	A.R. NO.	:	B/81600 – J
BATCH SIZE	:	776.00 Kgs.	DT. OF RECEIPT	:	20/11/2008
MFG. DATE	:	NOV. 2008	DT. OF ANALYSIS	:	20/11/2008
EXP. DATE	:	OCT. 2013			

PROTOCOL OF TESTS	SPECIFICATIONS	RESULTS
CHARACTERS	A white or almost white, crystalline powder, hygroscopic, freely soluble in water, very slightly soluble in alcohol and in methanol.	Conforms
MELTING POINT	Between 193°C and 198°C.	197°C
IDENTIFICATION	A) Conforms by UV.	Conforms
	B) Infrared absorption spectrum of sample and standard are concordant.	Conforms
	C) The picrate derivative melts at 206°C to 209°C.	208°C
	D) Gives reaction (b) of Phosphates.	Conforms
APPEARANCE OF SOLUTION	A 10% solution is clear and not more intensely coloured than reference solution BY_5 or GY_5.	Conforms
pH	pH of a 10% w/v solution is 3.8 to 4.3.	4.21
RELATED SUBSTANCES (By TLC)	Any Related Substance : NMT 1.0% NMT one Related Substance : NMT 0.5%	< 1.0% < 0.5%
HEAVY METALS	NMT 20 ppm	< 20ppm
LOSS ON DRYING (at 105°C)	NMT 2.0% w/w	1.18%
ASSAY	98.5% -101.0% (on dried basis)	99.54%

REMARKS : The sample **CONFORMS** with respect to **BP** specification.

(ANALYST)
Date : 24/11/2008

REPRESENTED BY
D B Fine Chemicals (Pty) Limited
P.O. Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS 1995 2005

(MANAGER QUALITY CONTROL)
Date : 24/11/2008

Regd. Off. : 48, Kandivli Industrial Estate, Kandivli (West), Mumbai - 400 067. Phone : 6647 4444
Corporate Office : 142-48, Kandivli Industrial Estate, Kandivli (West), Mumbai-400 067. Phone : 6647 4747

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Research Ethics Committee
North-West University
Potchefstroom Campus
Box116

15 April 2008

Dear Ms Botha

FINAL RESPONSE: ETHICS APPLICATION: NWU-0008-08-S5

The abovementioned application has reference.

We have received satisfacory answers to all the questions posed by the Ethics panel and has therefore found the ethical aspects to be in order.

PROF. J. DU PLESSIS
DIRECTOR

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