PREPARATION AND CHARACTERISATION OF PHEROID VESICLES

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"Nothing has such power to broaden the mind as the ability to investigate systemically and truly all that comes under thy observation in life."

∞ ***Marcus Aurelius*** ∞

Dedicated to my parents

Dirk and Helena Uys

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ABSTRACT

Pheroid is a patented system comprising of a unique submicron emulsion type formulation. Pheroid vesicles consist mainly of plant and essential fatty acids and can entrap, transport and deliver pharmacologically active compounds and other useful molecules. The aim of this study was to show that a modulation of components and parameters is necessary to obtain the optimum formula to be used in pharmaceutical preparations.

Non-optimal or non-predictable stability properties of emulsions can be limiting for the applications of emulsions (Bjerregaard *et al.*, 2001:23). Careful consideration was given to the apparatus used during the processing along with the ratios of the various components added to the formulation and the storage conditions of the Pheroid vesicles.

A preliminary study was performed to optimize the most accurate processing parameters during emulsification. The effect of emulsification rate and time, the temperature of the aqueous phase, the number of days the water phase were gassed, the concentration of the surfactant, Cremophor® RH 40, used and the concentration of Vitamin F Ethyl Ester CLR added to the oil phase of the o/w emulsion has been studied. Quantification of the mean particle size, zeta potential, turbidity, pH and current values were used to characterize the emulsions. The samples were characterised after 1, 2, 3, 7, 14, 21 and 28 days of storage. The emulsions were also characterised with confocal laser scanning microscopy (CLSM) to measure the number and size and size distribution of the vesicles.

After determination of the processing variables influencing the emulsion stability an accelerated stability test was conducted on a final formula. In the present study, accelerated stability testing employing elevated temperatures and relative humidity were used with good accuracy to predict long-term stability of an o/w emulsion kept at both 5 and 25 °C with 60 % relative humidity and 40 °C with 75 % relative humidity. The results of the stability tests were presented in histograms of the physical properties 24 hours, 1 month, 2 months and 3 months after preparation of the emulsion.

It was concluded that Pheroid vesicles demonstrate much potential as a drug delivery system. The high stability of this formula allows its use in a wide variety of applications in the pharmaceutical industry.

Keywords: Pheroid; emulsion stability; particle size and size distribution; zeta potential; turbidity; pH; current; confocal laser scanning microscope (CLSM); accelerated stability testing.

UITTREKSEL

Pheroid is 'n gepatenteerde sisteem wat uit 'n unieke submikron emulsietipe formulering bestaan. Pheroidvesikels bestaan hoofsaaklik uit plant en essensiële vetsure en kan farmakologies aktiewe bestanddele en ander bruikbare molekules enkapsuleer, transporteer en aflewer. Die doel van die studie was om die optimum formule te verkry wat gebruik kan word in farmaseutiese preparate deur formuleringsparameters en komponente te verander.

Sub-optimale eienskappe van emulsies kan die gebruike van emulsies beperk (Bjerregaard *et al.*, 2001:23). Die apparatuur wat gebruik is gedurende prosessering, die verhouding van die verskillende komponente en die bewaringstoestande vir Pheroidvesikels is in hierdie studie geëvalueer.

'n Studie is uitgevoer om die mees akkurate prosesseringsparameters gedurende emulsifisering te optimaliseer. Die effek van emulsifikasietempo en -tyd, die waterfasetemperatuur, die aantal dae van waterfasevergassing, surfaktantkonsentrasie (Cremophor® RH 40) en Vitamien F etielester-CLR-konsentrasie, is ondersoek. Kwantifisering van die gemiddelde deeltjiegrootte, zeta-potensiaal, turbiditeit, pH- en stroomwaardes is, gebruik om die emulsie te karakteriseer. Evaluering van hierdie parameters is na 1, 2, 3, 7, 14, 21 en 28 dae van bewaring gedoen. Konfokale laserskanderingsmikroskopie (KLSM) is gebruik om die aantal, grootte en grootteverspreiding van die vesikels te bepaal.

Na optimalisering van die prosesseringsveranderlikes wat die emulsiestabiliteit beïnvloed is 'n versnelde stabiliteitstoets uitgevoer op 'n spesifieke formule. 'n Versnelde stabiliteitstudie by verhoogde temperature en relatiewe humiditeit (5 en 25 °C by 60 % relatiewe humiditeit en 40 °C by 75 % relatiewe humiditeit) is gebruik. Die resultate van die stabiliteitstoetse is grafies voorgestel met histogramme van die fisiese eienskappe 24 uur, 1 maand, 2 maande en 3 maande na bereiding van die emulsie.

Die gevolgtrekking van hierdie studie is dat Pheroidvesikels baie potensiaal het as 'n geneesmiddelaflewerings-sisteem. Die goeie stabiliteit van die formule maak 'n groot verskeidenheid van toepassings in die farmaseutiese industrie moontlik.

Sleutelwoorde: Pheroid; emulsie stabiliteit; deeltjiegrootte en grootte verspreiding; zeta-potensiaal; turbiditeit; pH; stroom; konfokale laserskanderingmikroskopie (KLSM), versnelde stabiliteitstoetsing.

INTRODUCTION AND AIM OF THE STUDY

In recent years colloidal particles, such as microparticles, nanospheres, emulsion particles, liposomes and mixed micelles, have been investigated as potential carrier systems for the delivery or targeting of drugs to specific sites in the body. Among them emulsion formulations have gained particular interest as a carrier of lipophilic drugs due to its biocompatibility and satisfactory long-term stability and because they can easily be manufactured on an industrial scale using proven technology (Buszello and Müller, 2000:192).

An obvious major consideration in the use of emulsions in drug delivery is that a given emulsion-drug formulation should exhibit clear benefits over and above those seen with the conventional formulations of the drug. Encouraging results with emulsion-drug carriers in the treatment or prevention of a wide spectrum of diseases in experimental animals and in humans indicate that emulsion based products for clinical and veterinary application may be forthcoming. These could include anticancer and antimicrobial therapy, vaccines and diagnostic imaging, artificial blood substitution and treatment of ophthalmic diseases (Buszello and Müller, 2000:191).

Research into the use of emulsions in drug delivery has led to remarkable improvements in the design of formulations for specialized tasks, in emulsion long-term stability and scaled-up production. Several emulsion preparations have already been licensed and a number of others are likely to follow soon. The future of emulsions as drug delivery systems appears to be secure (Buszello and Müller, 2000:191).

Pheroid technology is a system comprising of a unique submicron emulsion type formulation. Pheroids consist mainly of plant and essential fatty acids and can entrap, transport and deliver pharmacologically active compounds and other useful molecules. A few of the key advantages of the Pheroid system include an increase in delivery of active compounds, a decrease in time to onset of action, a reduction of minimal effective concentrations necessary, an increase in therapeutic efficacy, a reduction in cytotoxicity, penetration of most known barriers in the body and in cells, the ability to target treatment areas, lack of immunological response, the ability to transfer genes to cell nuclei and reduction of drug resistance.

This study aims to optimize the current known emulsion like formulation of the Pheroid vesicles. Specific obstacles that had to be overcome, in the small scale production of the product, include the selection of suitable apparatus to be used and

finding glassware suitable for the manufacturing and storage of the product. Other aspects that had to be investigated are the amount of each component to be used in the formulation along with processing variables such as mixing time and mixing rate, temperature of the aqueous phase of the emulsion and the number of days the aqueous phase had to be gassed. All of the above mentioned factors were investigated randomly to obtain the necessary stability data to determine the ultimate formulation that can be used to prepare stable Pheroid vesicles for future clinical applications.

The specific objectives of this study were to:

- Conduct a literature study on the formulation factors that influence the manufacturing process of emulsions.
- Conduct a literature study on accelerated stability testing for emulsion preparations.
- Optimize apparatus and instruments used for the manufacturing of the Pheroid vesicles.
- Develop analytical methods for characterizing Pheroid vesicles.
- Investigate different variables to obtain the optimum Pheroid formulation for future clinical use.
- Conduct an accelerated stability test on the optimal Pheroid formulation.

Chapter 1 will focus on the feasibility of Pheroid vesicles as a drug delivery system while chapter 2 will provide more information on the classification of Pheroid vesicles as an emulsion like system. Stability concerns which influence emulsion preparations will also be discussed. Chapter 3 described the general experimental design used in this study. This chapter also describes the preparation and characterisation of several Pheroid formulations from which the optimum formulation, exposed to accelerated stability testing, was chosen. All the results obtained with these studies are presented and discussed in chapter 4 and chapter 5.

CHAPTER 1

PHEROID VESICLES AS A DRUG DELIVERY SYSTEM

1.1 INTRODUCTION

General considerations for a drug carrier, in liquid form, are that it should be biocompatible, biodegradable, of fine and uniform particle size, have good stability, be suitable for targeting and be pharmaceutically acceptable. The choice of a particular emulsion system to be used for drug delivery is generally dependent on the route of administration, the drug characteristics and the effect required (Buszello & Müller, 2000:194).

Certain specificity in biodistribution may be achieved passively by control of the physicochemical properties of the injected or swallowed carrier system, such as particle size and dose, together with surface charge and surface characteristics (Buszello & Müller, 2000:194).

Pheroid/s (previously known as Emzaloid™) is an emulsion like system and is often confused with lipid-based delivery systems. Some of the similarities and differences between Pheroid and lipid-based delivery systems are described in section 1.2. A few of the key advantages of Pheroid, which include increased delivery of active compounds, decreased time to onset of action, reduction of minimal effective concentration, increased therapeutic efficacy, reduction in cytotoxicity, penetration of most known barriers in the body and in cells, ability to target treatment areas, lack of immunological response, ability to transfer genes to cell nuclei and reduction of drug resistance, are discussed in section 1.3.

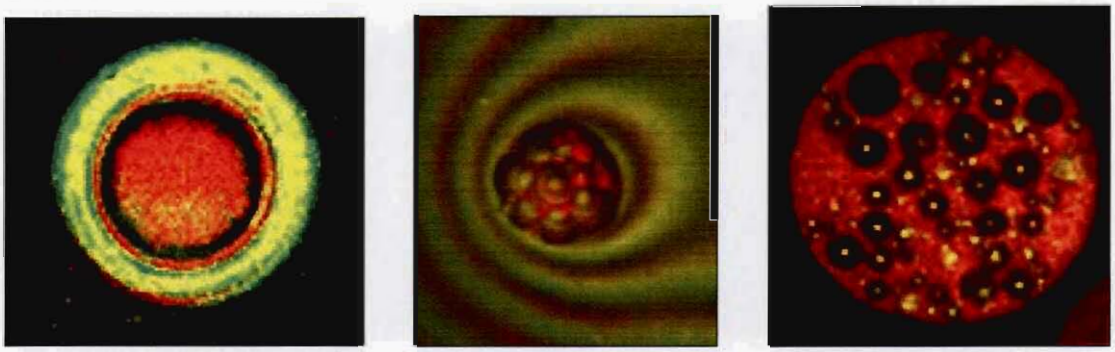
1.2 CLASSIFICATION OF THE PHEROID SYSTEM

A variety of Pheroid types can be formulated, depending on the composition and method of manufacturing. Figure 1.1 shows confocal laser scanning micrographs of various formulations of the Pheroid delivery system (Grobler, 2004:7). The three main types of Pheroids are:

- lipid-bilayer vesicles in both the nano- and micrometer size range,
- micro sponges and
- depots or reservoirs that contain pro-Pheroids.

Each type of Pheroid has a specific composition. The size and shape of the vesicles can be reproducibly controlled (typically between 0.5 - 1.5 μm), whereas that of the

micro sponges usually ranges between 1.5 - 5 µm. The sizes of depots or reservoirs are determined by the amount of pro-Pheroids contained in the reservoirs (Schlebusch, 2002:8). The micro sponges and depots support prolonged release according to a concentration gradient (Grobler, 2004:7).



(a) (b) (c)

Figure 1.1 *(a) A bilayer membrane vesicle containing Rifampicin. (b) The formation of small pro-Pheroids that are used in oral drug delivery. (c) A reservoir that contains multiple particles of coaltar (Grobler, 2004:5).*

The basic fundamentals of the Pheroid system show that the system differs substantially from conventional macromolecular carriers, such as liposomal delivery systems. Table 1.1 provides a comparison of the similarities, differences and key advantages of the Pheroid and other lipid-based or liposomal drug delivery systems (Grobler, 2004:7).

Table 1.1 *Similarities and differences of Pheroid and lipid-based delivery systems (Grobler, 2004:7).*

Pheroids	Other delivery systems
Consist mainly of essential fatty acids, a natural and essential ingredient of the body.	Contain a proportion of substances foreign to the body, e.g. artificial polymers, or egg phosphatidylcholine or lysolecithin.
Cytokine studies demonstrated no immune responses in man.	Some have shown to elicit immune responses.
Since it is comprised of fatty acids, an affinity exists between the Pheroids and cell membranes, to ensure penetration and delivery.	Binding and uptake mechanisms by mammalian cells have not been described for most other lipid-based delivery systems.
Since it is part of the natural biochemical pathways, the Pheroids causes no cytotoxicity.	Cytotoxicity and impaired cell integrity are common problems with substances that enter the body.
The Pheroids are polyphilic and is capable of entrapping drugs that have different solubilities as well as insoluble drugs.	Most delivery systems are either lipophilic or hydrophilic
Drug resistance is reduced or eliminated in all *in vitro* studies done. One possible mechanism is that the intracellular release of drugs occurs beyond the membrane zone, which contains drug efflux pumps found in drug resistant organisms.	Some delivery systems are prone to drug resistance or adverse immune responses. The composition of the systems generally prohibits active compounds to be released beyond drug resistant mechanisms related to drug efflux pump mechanisms.
The Pheroids protect the drug from metabolism, opsonization and inactivation in the plasma and other body fluids.	Some liposomal systems have been shown to act as protection against metabolism and opsonization.
Entrapment of active compounds in Pheroids reduces the volume of distribution and consequently increase target concentrations.	Liposomes encapsulating small molecule chemotherapeutic agents have been shown to reduce the volume of distribution.
Pheroids contain no cholesterol but the interior volume is nevertheless stably maintained.	Most if not all lipid-based delivery systems consist of phospholipids and cholesterol.

Table 1.1 *Similarities and differences of Pheroid and lipid-based delivery systems (Grobler, 2004:7).*

Pheroids	Other delivery systems
Pheroids, due to its composition, are able to inhibit the drug efflux mechanism in the intestinal lumen to enhance bioavailability.	Liposomal systems containing this feature have not been described.
Pheroids were shown to enhance the bioavailability of orally, topically and buccally administered entrapped actives.	Some liposomal systems have been shown to enhance absorption through biological barriers.
Entrapment in Pheroids changes the pharmacokinetics of active compounds.	Liposomes have similarly been shown to change the pharmcodynamics of active compounds
Entrapment efficiency in all compounds tested is very high (between 85 % and 100 %).	Due to charge and steric limitations of delivery systems, entrapment efficiencies can be problematic.
The type of Pheroid formulated for a specific active compound determines the loading capacity of that Pheroid.	The loading capacity of most lipid-based delivery systems is dependent on intra-membrane volume and is therefore limited.
Pheroids can be formulated as pro-Pheroids.	Liposomes can be formulated as pro-liposomes.
Micro-sponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other in the sponge spaces.	Combination treatments are problematic for most delivery systems.
Pheroids have entrapped peptides and antibodies and these Pheroids have been shown to interact with specific micro-domains on cells in culture and *in vivo.*	Antibody-containing liposomes for drug targeting have been described.

1.3 CHARACTERISTICS OF PHEROID/S IN THERAPEUTIC SYSTEMS

Pheroids are a system comprising of a unique submicron emulsion type formulation. It is a stable structure within a novel system that can be manipulated in terms of morphology, structure, size and function. Pheriods consist mainly of plant and essential fatty acids and can entrap, transport and deliver pharmacologically active compounds and other useful molecules. Depending upon the clinical indication, it can also act in synergism with such compounds or molecules, resulting in an enhancement of the therapeutic action (Grobler, 2004:5). Research confirmed the following key characteristics of this unique therapeutic system.

1.3.1 Reduction of minimum inhibitory concentration

Research has shown that, for certain antimicrobials, using as little as $1/40^{th}$ of the active entrapped compound, the formulation based on the Pheroid system was as effective as and sometimes more effective than the pure active. In practice, these characteristics would translate into reduction of patient side effects and cost savings in product formulation (Grobler, 2004:11).

1.3.2 Reduction in cytotoxicity

The Pheroid system has the potential to enhance normal cell integrity and minimize cellular damage that occurs as a result of exposure to harmful effects of active ingredients. Side effects of active ingredients are, in most instances, the result of cellular damage (Grobler, 2004:13).

1.3.3 Penetration of most known barriers such as cells, tissues and organisms

The Pheroid is capable of penetrating skin, keratinized tissue, intestinal epithelium, vascular walls, sub cellular organelles, sensitive and resistant parasites, bacteria and fungi. Research has not only shown effective penetration of these last organisms but also the capability of the Pheroid to deliver drugs to these organisms and destroy them (Grobler, 2004:13).

1.3.4 Increased delivery of active compounds

The percentage of active compound delivered was shown to be enhanced by *in vitro* and *in vivo* studies. Some results of membrane diffusion studies, which mimic the delivery of active compounds across specific membranes, are reflected in table 1.2. The compounds tested are used as an antifungal and antiviral respectively, and the

membrane used in this case was skin. "EMZ" indicates the Pheroid-entrapped product and "COM" a comparable commercial product (Grobler, 2004:11).

Table 1.2 *Release rates and percentage release per label claim for product tested (Grobler, 2004:11).*

Active Agent	% Active	Release Rate ($\mu g.cm^{-2}.h^{-1}$)	% Release
Acyclovir EMZ	0.500	69.153	0.121
Acyclovir COM	0.500	54.094	0.095
Miconazole Nitrate EMZ	2.000	389.924	6.816
Miconazole Nitrate COM	2.000	111.222	1.947

1.3.5 The pro-Pheroid concept

Grobler (2004:14) explains that although all Pheroid systems contain a small polyethylene glycol (PEG) component, the use of increased concentrations and larger polymers has led to the development of the pro-Pheroid. This has only been possible when the resultant formulation has been treated to stabilize the Pheroid once it is formed. Polyethylene glycol is a relatively non-reactive and non-toxic polymer that is frequently used in food and pharmaceutical products. Pro-Pheroid systems were designed to have significant advantages over other delivery systems. Polyethylene glycol (PEG) has been shown to contribute to the following aspects of drug administrations:

- increased bioavailability
- increased drug stability and extended circulating life
- lower toxicity
- enhanced drug solubility

PEG has been shown to render a protein therapeutically effective, where the unmodified form had not been effective (Grobler, 2004:14).

1.3.6 Decreased time to onset of action

According to Grobler (2004:10) initial research findings have repeatedly indicated that the Pheroid delivery system rapidly traverses most physiological barriers and delivers the active. An active delivered via the Pheroid has been shown to act significantly quicker than that same active delivered via a conventional approach, suggesting a potentially faster relief from target symptoms (figure 1.2).

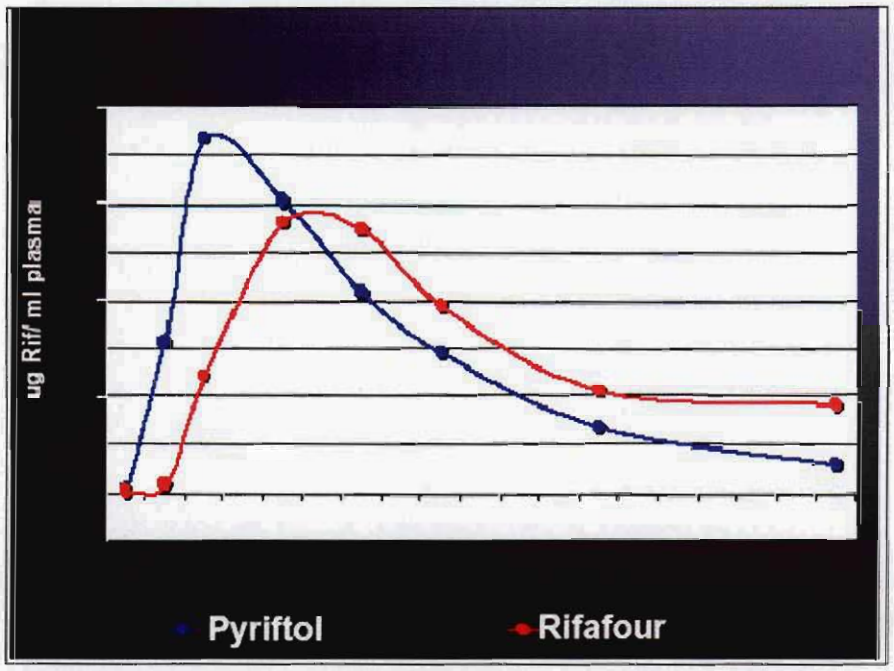


Figure 1.2 *The curves illustrate the average plasma levels of Rifampicin for 14 healthy volunteers after oral administration of combination anti-tuberculosis directly observed therapy short-term treatment (Grobler, 2004:10).*

Figure 1.2 shows that the time needed to achieve maximum plasma concentration were reduced by entrapment in Pheroid when compared to that of one of the preferred comparative products. Pyriftol contained only 60% of the amount of Rifampicin contained in the commercial product (Grobler, 2004:10).

1.3.7 Immunological responses

Some drugs, such as proteins or peptides, may induce an immunologic response or adverse intolerance reaction. Masking of the compounds by Pheroid reduces recognition by the patient's immune response, especially as essential fatty acids are immunological friendly. Frequency of dosing can be reduced without diminishing potency, or higher doses can be given to achieve a more powerful therapeutic impact (Grobler, 2004:14).

1.3.8 Increased therapeutic efficacy

In all cases tested, the formulation of an active compound in Pheroid increased the efficacy. Examples of enhancement of the action of anti-infective agents, as determined by zone inhibition are shown in Table 1.3 (Grobler, 2004:12).

Table 1.3 *Zone of Inhibition study: Five commercial anti-infective products against Pheroid-formulations of the same active compound (Grobler, 2004:12).*

Active Agent	EMZ / COM	Dose (mg/5ml)	S. Aureus	P. Aerugin	B. Cereus	E. Coli	A. Niger	C. Albicans
Cloxacillin	EMZ	125	30.74	23.96				
Cloxacillin	COM	125	29.45	19.78				
Erythromycin	EMZ	250	26.7		29.89			
Erythromycin	COM	250	25.84		27.78			
Ciprofloxacin	EMZ	250	33.05			35.78		
Ciprofloxacin	COM	250	30.14			33.4		
Cotrimoxazole	EMZ	240	13.95			24.64		
Cotrimoxazole	COM	240	11			22.83		
Itraconazole	EMZ	50					16.03	14.28
Itraconazole	COM	50					11.47	10.21
Control			9	9	9	9	9	9

All the above formulations were of one single Pheroid type. Reformulation of Ciprofloxacin and Erythromycin as sponges has since increased efficacy. As the above analysis does not reflect increased bioavailability, the *in vivo* results should further increase the efficacy of the Pheroid formulations (Grobler, 2004:13).

1.3.9 Ability to entrap and transfer genes to cell nuclei and expression of proteins

Initial experiments performed on the Pheroid delivery system demonstrated applicability for the technology in DNA vaccines and gene therapy. *In vitro* studies have shown entrapment of human and viral DNA of various lengths into Pheroids. Delivery of DNA fragments and vectors entrapped in Pheroids to mammalian cells has been observed. Reproducible expressions of appropriate proteins were observed after transfection of cells by Pheroid-entrapped genes (Grobler, 2004:15).

1.3.10 Reduction and suggested elimination of drug resistance

The Pheroid has been shown to reduce or completely eliminate drug resistance *in vitro*. Analysis of bacterial growth of multidrug resistance-TB has shown that formulations containing the standard antimicrobial, Rifampicin, entrapped in Pheroid,

obviated pre-existing drug resistance (figure 1.3). This benefit is attributable partly to the ingredients that make up the Pheroid and that shield the active from the targeted organism. The ability to potentially revive the effectiveness of antibiotics such as penicillin has widespread application in the healthcare industry. The scope of the Pheroid is of such unique nature that its application in the pharmaceutical field is almost limitless. Broad fields of applications that have been defined at this early stage include TB, Malaria, Cancer, AIDS and gene delivery (Grobler, 2004:15).

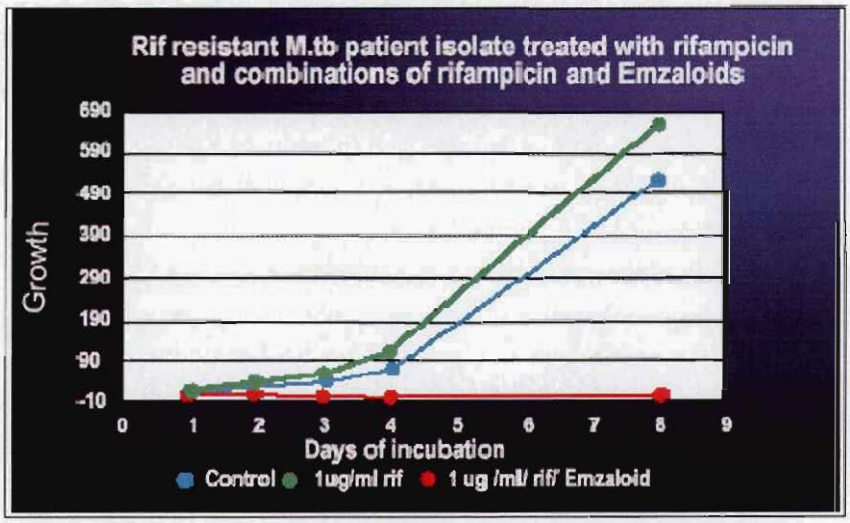


Figure 1.3 *The growth of resistant Mycobacteria isolated from a multiple drug resistant patient. The entrapment of Rifampicin in the Pheroid results in complete bactericidal activity, whereas free Rifampicin shows no growth inhibition (Grobler, 2004:16).*

The effect of Pheroid-entrapment on drug resistance was also tested on organisms other than Mycobacteria. For example, the effect of commercially available chloroquine versus Pheroid-entrapped chloroquine against resistant Malaria (Falciparum drug resistant reference strain W2) was preliminarily determined in the conventional manner. Using the Pheroid system, it would be possible to treat malaria with an existing, well-known inexpensive drug, but with greatly reduced cytotoxicity and lower incidence of side effects. Prophylactic treatment with previously effective drugs would then also be beneficial (Grobler, 2004:16).

1.4 CLINICAL APPLICATIONS OF THE PHEROID SYSTEM

1.4.1 Transdermal delivery

Whilst the delivery system is not limited to topical application, research has indicated that many medications can be administered topically instead of orally. Many side effects may occur in the digestive system. Figure 1.4 illustrates the enhancement of

efficacy of Pheroid–entrapment for transdermal delivery (Grobler, 2004:14).

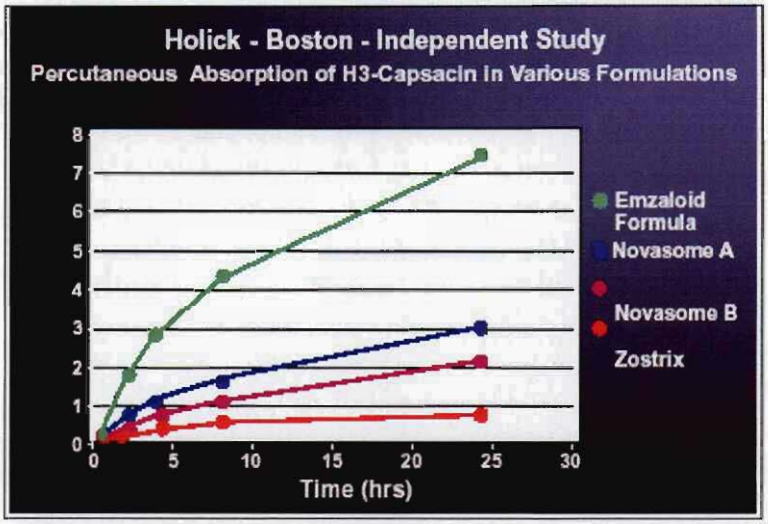


Figure 1.4 *Radioactive capsaicin was entrapped in Pheroid and used in a comparative membrane diffusion study with other commercial preparations. As is clear from the graphs, the penetration of the radio-labelled active compound is dramatically increased by entrapment in Pheroid. The study was performed independently by Prof Holick of Boston University School of Medicine (Grobler, 2004:15).*

In vitro transdermal efficacy studies of an oil/water emulsion in two separate Pheroid preparations containing the actives, coal tar and the non-steroidal anti inflammatory drug, diclofenac sodium, suggested that the oil/water base in formulations is a highly efficient transdermal vehicle able to transport a wide range of indication- specific actives to their site of action (Saunders *et al.*, 1999:99).

1.4.2 Therapy of tuberculosis

A cross-over bioequivalence study in 16 healthy volunteers, measured efficiency of a Pheroid delivery system in which rifampicin, pyrazinamide, isoniazid and ethambutol were entrapped (named Pyriftol) against a highly regarded anti-tuberculosis commercial product (Rifafour®-e200 form Aventis®), containing the same ingredients. Due to the anticipated increased absorption of all four actives in Pyriftol, as demonstrated by *in vitro* and *in vivo* studies, only 60 % of the dosages used in Rifafour® were used in the Pheroid formulation (Grobler, 2004:10).

The following conclusions were made from this study:

- The entrapment of antimicrobials into Pheroid led to an increase of absorption of the antimicrobials after oral administration, with a resultant dramatic increase in the plasma levels of these antimicrobials.

- The entrapment of the antimicrobials led to a much quicker absorption and cellular response, with T_{max} decreased by nearly half.
- The therapeutic concentrations were maintained for longer and the circulatory time of the drug extended, indicating that exposure of the bacteria to the antimicrobials was increased.
- The entrapment of the antimicrobials increased delivery of the antimicrobials to the target cells and led to a decrease in the minimum amount of antimicrobial necessary to kill the bacteria (minimum inhibitory concentration).
- A lower dosage can be used to abstain similar therapeutic concentrations.
- The decrease in side effects observed will result in better compliance, with less chance for the development of multi-drug resistance.
- The concentration of Pheroid in the blood cells suggested that the decline of concentration with time may reflect mobilization of the actives to the cell fraction (cellular reservoirs) rather than clearance (Grobler, 2004:19).

1.4.3 Preventive therapies with vaccines

Historically, vaccination is the only strategy that has led to the elimination of a viral disease, namely smallpox. An indirect relationship has been observed for vaccine immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor; hence there is an urgent need for effective vaccine adjuvants to enhance the immunogenicity and immunostimulatory properties of vaccines. Such adjuvants can be broadly separated into two classes, namely immunostimulatory or –modulatory adjuvants and vaccine delivery system (Grobler, 2004:20).

1.4.3.1 A virus-based vaccine: Rabies

The protection of animals afforded by Pheroid-based vaccines versus that by commercially available vaccines was investigated for rabies. Rabies is a viral zoonosis using carnivores as well as bat species as hosts. Each year at least 50 000 people die form rabies, more than 10 million receive post-exposure vaccination against this disease, whilst more than 2.5 billion people live in regions where rabies is endemic. Infection of humans from rabid animals is almost invariably fatal once signs of disease occur. Comparative animal studies were undertaken on different formulations of the inactivated virus formulated as a vaccine (Grobler, 2004:20). The Pheroid-adjuvanted rabies vaccine showed a 9 fold increase in antibody response in comparison to the unadjuvanted sample. This study was repeated in four similar

animal studies with similar results (Grobler, 2004:21).

1.4.3.2 A peptide-based vaccine: Hepatitis B

The Pheroid is *per se* an adjuvant as it is based on a novel micro-colloidal carrier system that was found to confer marked superiority in drug delivery over competitive products. There is of course greater potential in vaccines capable of inducing potentially relevant immune responses than in those that are not. Animal studies and laboratory measurements of immune responses investigated the efficiency of a peptide-based hepatitis vaccine (Grobler, 2004:21).

Non-recombinant hepatitis B vaccines are generally based on the use of one of the surface molecules of the virus and antigen. The induction of an antibody response was monitored in a mouse study, following the entrapment of this peptide in Pheroids. The study was executed by the SA State Vaccine Institute and the Department of Immunology, University of Cape Town (Grobler, 2004:21). The use of Pheroid technology led to a more than 10-fold increase in the efficacy of the peptide-based hepatitis B vaccine (Grobler, 2004:22).

1.4.4 Peptide drug delivery

Two recent studies were done involving the delivery of the peptide drug calcitonin making use of Pheroid technology. The Pheroid system, based on nano- and microtechnology, proved to be an important technology in the delivery of peptide and protein drugs. The most important advantages include increased delivery of active compounds, penetration of most known barriers in the body and cells and increased therapeutic efficiency (Strauss, 2005:96).

The results obtained in one study indicated that the quaternised chitosan derivative TMC, with its mucoadhesive properties and ability to open tight junctions, produced the highest absorption enhancement of salmon calcitonin orally. Pheroid micro sponges were also able to enhance the absorption of salmon calcitonin. This absorption enhancement with Pheroids was not as high as obtained with *N*-trimethyl chitosan chloride (TMC), but Pheroids also have the potential of enhancing peptide absorption (Strauss, 2005:96).

To enhance the nasal absorption of calcitonin, a peptide hormone, several absorption enhancers was considered for nasal administration with calcitonin *in vivo* rats. Pheroid micro sponges and Pheroid vesicles were prepared for this study and calcitonin was entrapped into Pheroid vesicles and Pheroid micro sponges. The

size and morphology of the Pheroid vesicles and Pheroid micro sponges were investigated before and after entrapment of calcitonin. TMC and *N*-trimethyl chitosan oligosaccharide (TMO) solutions containing calcitonin were also prepared and administered nasally to rats. These calcitonin formulations were administered nasally to male Sprague Dawley rats (250-350 g) at a dose of 10 IU/kg body-weight calcitonin. After blood samples were collected at different intervals over a period of 180 minutes, it was analyzed to determine the plasma concentrations and plasma calcium concentrations (Kotzé, 2005:115). The results of this study showed that both Pheroid vesicles and Pheroid micro sponges and TMC have the ability to enhance the nasal absorption of calcitonin, with a decrease in the plasma calcium levels and that TMC proved to be better in enhancing the absorption of calcitonin compared to the other preparations that was used (Kotzé, 2005:116).

1.5 STABILITY CONCERNS

Emulsion droplets are normally stabilized by enhancing the mechanical strength of the interfacial film formed around the oil droplets, by steric stabilization effects, and/or by the presence of charged surfactants which create an electrostatic barrier. The stabilizing factor of the latter is the electrostatic repulsion of similarly charged droplets. The emulsion stability can be considerably improved with the use of mixed emulsifying agents (Buszello & Müller, 2000:203).

Pheroid, due to its composition, is sterically stabilized without the disadvantages of increased size or decreased elasticity. Steric stabilization refers to colloidal stability. Other delivery systems generally need to be sterically stabilized. One example is pegylation of liposomes in the sterically stabilized liposomes. This generally leads to an increase in size and rigidity of the carrier. Transferosomes were developed in an attempt to obtain elastic liposomes. However, the manufacturing process is complicated by this development (Grobler, 2004:8). Large scale manufacturing of other delivery systems often shows low batch-to-batch reproducibility and, in some instances, problems with size control. Furthermore, the Pheroids showed *in vivo* stability during vaccination of animals and in initial phase I volunteer trials. Both product and *in vivo* chemical and physical instability are problems of some of the lipid-based delivery systems (Grobler, 2004:9).

1.5.1 Influence of particle size

Oil-in-water (o/w) emulsions are commonly formulated for parenteral and topical administration but also for the oral and ocular routes. Each route of administration

has to meet its own requirements of formulation, e.g. sterility for parenteral preparations and aesthetic attractiveness for topical products. Another interesting point for emulsions is the size of the droplets of oil dispersed in the water. The median size as well as the distribution of sizes is very important since they determine the safety of the preparation in the case of intravenous preparations or the release properties of the active ingredient in topical formulations (Roland, *et al.* 2003:85).

The biodistribution of colloidal systems can be related to various physiological processes as a function of particle size (Buszello & Müller, 2000:195). All Pheroid-based products currently on the market are topical products, supported by the results of various clinical trials. Further investigation is currently been done on the application of the Pheroid technology in oral and parenteral administration (Grobler, 2004:3).

Generally, particles smaller that 7 μm are retained by the phagocytotic mononuclear cells of the reticuloendothelial system (RES) in the liver, spleen and bone marrow. The smaller the particles the more likely they are to accumulate in the bone marrow (Buszello & Müller, 2000:195).

The Pheroid system passively targets the reticuloendothelial system (RES). Body distribution experiments show accumulation of the Pheroid in the spleen and liver. This distribution can however be changed to prevent phagocytosis by the incorporation of specific molecules in the Pheroid membrane (Grobler, 2004:8).

1.5.2 Effect of surface charge

Particle surface charge has marketed effects on the clearance and deposition of colloids. Clearly, the connection between phagocytosis, RES uptake, and surface charge is far from simple and concomitant changes in other surface properties may override any effects produced by variations in surface charge. Therefore, the difference in organ distribution cannot be attributed to surface charge alone. Other factors also need to be taken into account (Buszello & Müller, 2000:200).

Surface charge affects the phagocytosis of emulsion particles by leukocytes and has a subsequent impact on the type of opsonin. It has already been shown that emulsions with neutral surface charges are taken up more slowly by macrophages than those bearing charged surfaces (Buszello & Müller, 2000:201).

Typically, emulsion formulations reported in the literature are negatively charged. They are based on lecithin combined with nonionic or anionic emulsifiers (Buszello &

Müller, 2000:200). Negatively charged emulsions, as compared with neutral or positively charged ones, showed a faster rate of clearance and higher liver and spleen uptake, while positively charged colloids showed an initial accumulation in the lungs and subsequent relocation to liver and spleen (Buszello & Müller, 2000:201).

1.6 CONCLUSION

It is clear from the discussion above that Pheroid technology compares well with and even exceeds current delivery systems on the market to a great extend. The results obtained in previous studies to determine the effectiveness of this drug carrier system predicts great prospects for the future. With the help of this carrier system formulators will be able to design products with lower drug loading and fewer side effects that will aid in curing people with serious and life-threatening diseases.

The Pheroid system has already proved to be a formulation that is highly efficient as a transdermal vehicle able to transport a wide range of indication-specific actives to their site of action (Saunders, *et al.*, 1999:99).

Although the Pheroid vesicles can be considered to fall into the same general category as liposomes, noisomes or submicron emulsions, they differ both in constitution and preparation techniques and are thus regarded as unique (Saunders, *et al.*, 1999:105).

A further significant improvement in the treatment regime currently used for TB patients is possible with implementation of the Pheroid delivery system (Grobler, 2004:20). A few noted advantages with the use of the Pheroid system may include:

- A decrease of the treatment period of 6 months to 2 months or even less with a Pheroid formulation, which will result in reduced treatment cost and increased productivity.
- An increase in the intervals between dose administrations because of the quick absorption and maintained plasma levels.
- Increase in patient compliance.
- Decreased chances of developing multi-drug resistance.

Furthermore, reformulation of existing vaccines should save considerable development time and cost. The Pheroid therefore has an obvious dual role in vaccines, firstly as delivery system for disease specific antigens, and secondly as immuno-stimulatory adjuvant (Grobler, 2004:22).

Two studies recently performed to investigate the effectiveness of the Pheroid

system as an absorption enhancer for calcitonin delivered via the nasal and oral delivery routes, proved to be effective in decreasing plasma calcium levels and therefore showing promise to enhance the absorption of poorly absorbable protein and peptide drugs.

In Chapter 2 a general discussion of emulsions, with the emphasis on the Pheroid system as an emulsion like system is given. A detailed description of the components forming part of the Pheroid system and its stability will also be given.

CHAPTER 2

DISPERSE SYSTEMS: EMULSIONS

2.1 INTRODUCTION

Pharmaceutical dispersed systems such as suspensions and emulsions are among the most used dosage forms. They are utilized for various routes of administration – oral, topical, parenteral, mucosal and ophthalmic. These dosage forms present many significant advantages such as an easy dividing of the dosage form for pediatric and geriatric patients. Reduction of drug particle size and formulation in these dosage forms may also enhance the bioavailability of the active agents. Moreover, colloidal particles, such as microparticles, nanosperes, emulsions and liposomes, have been developed as promising carrier systems for the delivery or the targeting of drugs. Emulsions are also particularly attractive as a vehicle for the administration of poorly soluble drugs (Marti-mestres & Nielloud, 2000:3). The properties of the unique Pheroid system have been discussed in chapter 1.

2.2 DEFINITIONS AND CLASSIFICATION OF DISPERSE SYSTEMS

By definition a dispersion can be defined as a heterogeneous system in which one phase is dispersed (with some degree of uniformity) in a second phase. The state of the dispersed phase (gas, solid or liquid) in the dispersion medium defines the system as a foam, suspension or emulsion. Like wise, the particle size of the dispersed phase provides further classification (colloidal dispersion vs. suspension and microemulsion vs. macroemulsion). If the size of the dispersed particles is within the range of 10^{-9} m (1 nm) to about 10^{-6} m (1 μm) it is termed a colloidal system. However, the upper size limit is often extended to include emulsions and suspensions, which are very polydisperse systems in which the droplet size frequently exceeds 1 μm but which show many of the properties of colloidal systems (Attwood, 2002:70). These definitions, particularly the latter set, are somewhat arbitrary, since there is no specific particle size at which one type of system begins and the other ends. Furthermore, almost without exception, disperse systems are heterogenous in particle size. To complicate matters even further, many commercial disperse systems cannot (and should not) be categorized easily and must be classified as complex systems. If the difficulty in defining these complex systems were merely a matter of semantics, the issue would be trivial, but these complexities influence the physicochemical properties of the system which, in turn, determine

most of the properties with which formulators are concerned. Figure 2.1 illustrates the more common types of disperse systems (Weiner, 1996:1).

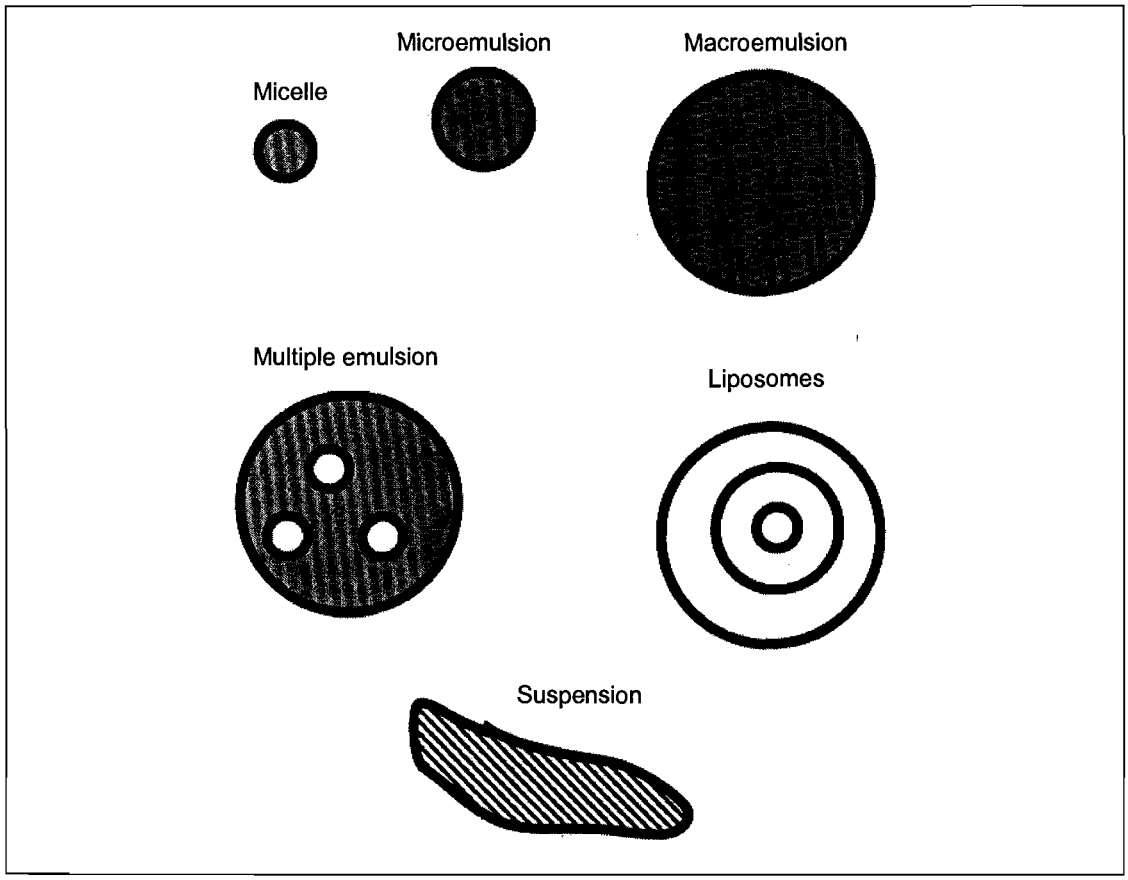


Figure 2.1 *Common types of disperse systems found in pharmaceutical formulations (Weiner, 1996:2).*

Emulsions are metastable colloids made from two immiscible fluids, one fluid being dispersed into the other, in the presence of surface active agents. Emulsions are in principle made out of two immiscible phases for which the surface tension is therefore non-zero, and may in principle involve other hydrophilic-like or lipophilic-like fluids in the presence of suitable surface active species, each phase being possible comprised of numerous components (Pays *et al.*, 2002:175).

According to Friberg *et al.* (1996:54) an emulsion is formed when two immiscible liquids (usually oil and water) are mechanically agitated. During agitation, both liquids tend to form droplets, but when the agitation ceases, the droplets separate into two phases. If a stabilizing compound, an emulsifier, is added to the two immiscible liquids, one phase usually becomes continuous and the other one remains in droplet form for a prolonged time. Droplets are formed by both phases during agitation and the continuous phase is actually obtained because its droplets are unstable. When water and oil are stirred together, both oil droplets in water and

water droplets in oil are formed continuously, and the final result, an oil-in-water (o/w) emulsion, is obtained because the water droplets coalesce with one another much faster than the oil droplets. When a sufficiently large number of water droplets have coalesced, they will form a continuous phase surrounding the oil droplets. This continuous phase is also called the external phase; it surrounds the dispersed (internal) phase (figure 2.2).

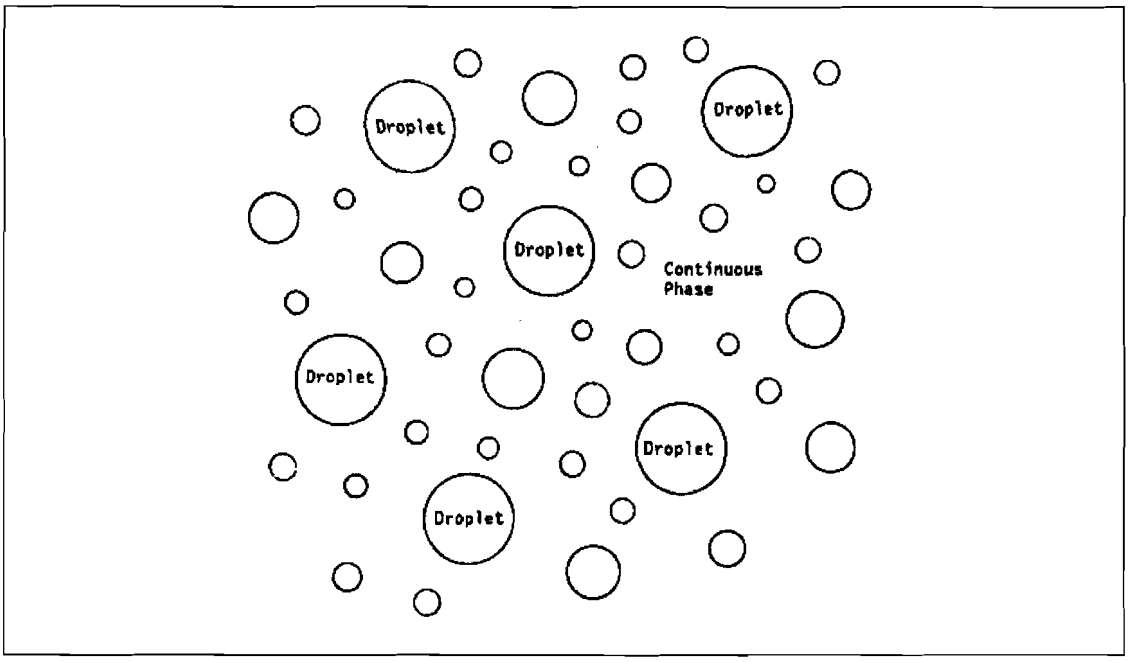


Figure 2.2 *The majority of emulsions consist of one liquid dispersed in another in the form of macroscopic droplets (Friberg et al., 1996:54).*

The disperse system under investigation in this study is that of an oil-in-water emulsion consisting of oil droplets dispersed in water. In the next sections the properties of o/w emulsions will be discussed in more detail with the emphasis on the components of the Pheroid system.

2.3 FORMULATION AND PREPARATION OF EMULSIONS

2.3.1 Importance of formulation

Emulsion formulation requires tedious study in order to check the most important parameters to obtain stable emulsions. Optimizing a process implies determination of the experimental conditions giving optimal performance (Prinderre *et al.*, 1998:73).

The inability of emulsion theory to predict the composition of appropriate emulsion systems is mitigated to some extent by the development of optimization techniques that facilitate product development. The formulation of any product, even by a trial

and error approach, involves an optimization process: goals are defined, evaluation procedures are selected, initial compositions are defined, products are prepared and evaluated appropriately and the prospective formulation then modified until acceptable data are obtained. Presumably, a series of logical steps is taken by the scientist who controls the variables until a satisfactory product results. Nonetheless, in the absence of a mathematically or statistically rigorous approach to optimization, this satisfactory product is but a provisionally satisfactory product; it is not necessarily the optimal formulation. Subsequent experience with the less than optimal formulation during scale-up or processing or in the marketplace often demonstrates the formulation's suboptimal character whether by instability, poor performance or lack of acceptance by the consumer (Block, 1996:74).

2.3.2 Components of emulsions

2.3.2.1 Oil phase

In many instances the oil phase of an emulsion is the active agent, and therefore its concentration in the product is predetermined (Billany, 2002:343). Some of the common ingredients used to prepare emulsions are soybean oil, hydrocarbon oil, corn oil, and various derivatives of polyoxyethylene castor oil acting as the emulsifying agent within the formulation. According to Grobler (2004:5) Pheroid vesicles consists mainly of plant and essential fatty acids and can entrap, transport and deliver pharmacologically active compounds and other useful molecules. The oil phase of the Pheroid system contains unsaturated fatty acids such as combinations of linoleic, linolenic and oleic acid, a nonionic surfactant namely Cremophor® RH 40 and DL-α-Tocopherol as antioxidant. The properties of these compounds are discussed below.

2.3.2.1.1 Unsaturated fatty acids

Essential fatty acids are necessary for various cell functions but cannot be manufactured by human cells. It therefore has to be ingested. The Western diet has been shown to be limited in its supply of these basic lipid molecules. Some of the functions of these components of the Pheroid system are maintenance of membrane integrity of cells, energy homeostasis, modulation of the immune system through amongst others the prostaglandins / leukotrins and some regulatory aspects of programmed cell death (Grobler, 2004:5).

Fatty acids fill two major roles in the body:

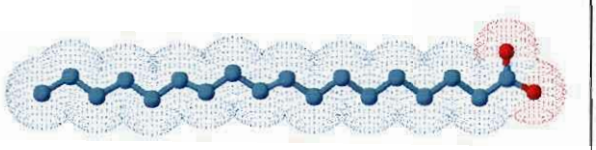
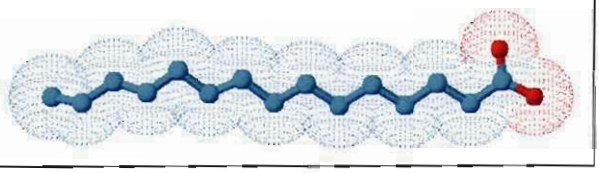
- They serve as the components of more complex membrane lipids.
- They act as the major components of stored fat in the form of triacylglycerols.

Fatty acids are long-chain hydrocarbon molecules containing a carboxylic acid moiety at one end. The numbering of carbons in fatty acids begins with the carbon of the carboxylate group. At physiological pH, the carboxyl group is readily ionized, rendering a negative charge onto fatty acids in bodily fluids (King, 2003).

According to King (2003) fatty acids that contain no carbon-carbon double bonds are termed saturated fatty acids; those that contain double bonds are unsaturated fatty acids. The numeric designations used for fatty acids come from the number of carbon atoms, followed by the number of sites of unsaturation (eg, palmitic acid is a 16-carbon fatty acid with no unsaturation and is designated by 16:0). The site of unsaturation in a fatty acid is indicated by the symbol "D" and the number of the first carbon of the double bond (e.g. palmitoleic acid is a 16-carbon fatty acid with one site of unsaturation between carbons 9 and 10, and is designated by $16:1^{D9}$). Saturated fatty acids of less than eight carbon atoms are liquids at physiological temperature, whereas those containing more than ten are solids. The presence of double bonds in fatty acids significantly lowers the melting point relative to a saturated fatty acid (King, 2003).

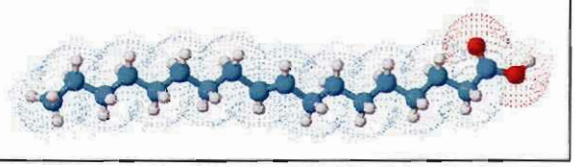
The majority of body fatty acids are acquired in the diet. However, the lipid biosynthetic capacity of the body (fatty acid synthase and other fatty acid modifying enzymes) can supply the body with all the various fatty acid structures needed. Two key exceptions to this are the highly unsaturated fatty acids know as linoleic acid and linolenic acid, containing unsaturation sites beyond carbons 9 and 10 (table 2.1).

Table 2.1 *Physiologically relevant fatty acids (King, 2003).*

Numerical Symbol	Common Name	Structure
$18:2^{D9,12}$	Linoleic acid	
$18:3^{D9,12,15}$	Linolenic acid	

Oleic acid also forms an integral part, along with the above mentioned fatty acids, of the Vitamin F Ethyl Ester CLR used in this study. It is further believed that oleic acid in the Pheroid formulation may also play a role in enhancing transdermal penetration by temporarily disrupting the packed structure of the intercellular lipids because of the incorporation of its kinked structure (Saunders *et al.*, 1999,106). According to Clark (2004), oleic acid is a typical mono-unsaturated acid (table 2.2).

Table 2.2 *A typical mono-unsaturated acid (Clark, 2004).*

Numerical Symbol	Common name	Structure
$18:1^{D9}$	Oleic acid	

2.3.2.1.2 Surfactants

Surface–active agents, or surfactants, are molecules distinguished by the presence of both a polar and a nonpolar region. Surface-active agent is the general term that includes detergent, dispersing agent, emulsifying agent, foaming agent, penetrating agent and wetting agent. In the pharmaceutical field surfactants are used especially as emulsifiers, solubilizers and wetting agents (Marti-mestres & Nielloud, 2000:2).

2.3.2.1.2.1 Functionality within formulation

When emulsion droplets collide, they can either bounce away or coalesce into larger droplets, ultimately leading to the destruction of the emulsion. The latter event will result in a reduction of interfacial free energy and, unless barriers are placed in the

way, will occur with each collision. Lin (1979:167) describes this mechanism as advantageously since it is used by introducing materials (emulsifiers) into the formulation that concentrate at the oil-water droplet interface and present barriers to droplet coalescence. The principle mechanism by which emulsifiers stabilize emulsions is not a reduction of the interfacial free energy of the system, but involves the introduction of a mechanical barrier to delay the ultimate destruction of the system. Although the concentration of surface active emulsifier is greater at the oil-water interface than in either of the bulk phases, most of the emulsifier molecules are in the water phase (hydrophilic emulsifier), or in the oil phase (hydrophobic emulsifier), and not at the emulsion droplet interface. A reduction of the interfacial free energy probably does help somewhat in the ease of preparing the emulsion (since energy needs to be added to the system to prepare the product), but it is not a major factor for long-term stability. Finally, proper orientation of the molecules at the interface (polar groups directed toward the water phase and nonpolar groups directed toward the oil phase) further reduces interfacial free energy. It is extremely important for the formulator to keep in mind that, throughout the processing of the formulation (whether by simple mixing with a stirring rod or the use of high-energy shear equipment), the emulsifier molecules are continuously partitioning between the bulk phases and the interface, and are continually changing their orientation at the interface (Weiner, 1996:5).

According to Weiner (1996:4) another important method that nature uses to reduce interfacial free energy is to vary the composition of the interface to make it rich in surface active material, and poor in highly polar compounds (e.g., water; a surface active agent or surfactant containing at least one prominent polar group and one prominent nonpolar group).

An emulsion containing only oil and water, with no added stabilizer, shows extremely fast flocculation and coalescence. Hence, the emulsion must be made more stable by addition of at least one substance. These added substances, the stabilizers, act to slow the flocculation and coalescence of the droplets by preventing their movement through the increased viscosity of the continuous phase, or by protection of the droplets through the establishment of some form of energy barrier between them (Friberg *et al.*, 1996:65).

Only the addition of a suitable emulsifier enables that a fine dispersity after production could be maintained during storage and coalescence could be prevented (Lindenstruth *et al.*, 2004:187). According to Lee *et al.* (2005:486) the stability of a

colloidal dispersion is closely related to the charged conditions on the surfaces of the dispersed entities. The surface of a drop in an emulsion system is usually charged, which arises from the dissociation of the surfactant molecules.

2.3.2.1.2.2 Nonionic surfactants

Nonionic surfactants differ from ionic surfactants in the absence of charge on the molecule. They are generally less irritating than anionic or cationic surfactants. They are compatible with other types of surfactants, but they may diminish the antimicrobial activity of some preservatives. The characteristics of nonionic surfactants are essentially dependent on the proportions of hydrophilic or hydrophobic groups in the molecule (figure 2.3). The hydrophilic part contains polyoxyethylene, polyoxypropylene or polyol derivatives and the hydroxyl group. The hydrophobic part includes saturated or unsaturated fatty acids or fatty alcohols. By varying the number of hydrophilic groups or the length of the lipophilic chain, compounds are obtained with a wide range of hydrophilic-lipophilic balance (HLB) values. According to Attwood & Florence, (1983:481) this empirical scale is useful to classify nonionic surfactants and to select surfactant mixtures for emulsification of particular oils. Lipophilic surfactants (0 < HLB > 10) are known for their antifoaming, water-in-oil emulsifying or wetting properties. Hydrophilic surfactants (10 < HLB > 20) have generally oil-in-water emulsifying or solubilizing properties (Marti-Mestres *et al.*, 2000:7).

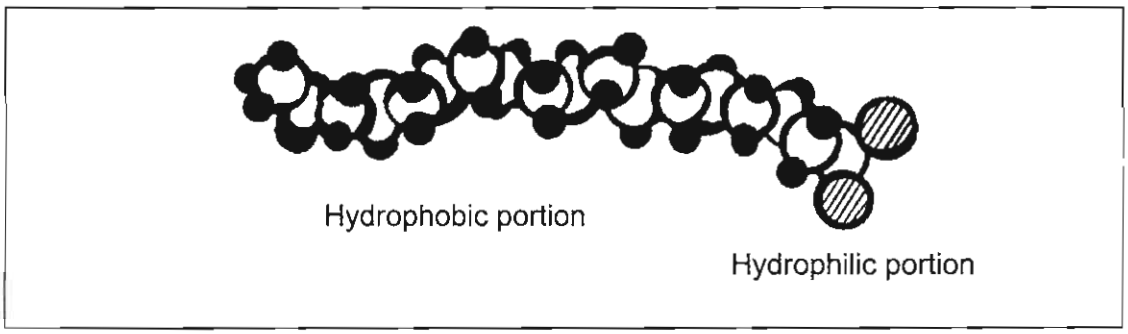


Figure 2.3 *An emulsifier contains a hydrophobic portion (hydrocarbon) and a hydrophilic portion (polar) (Friberg et al., 1996:58).*

Surface energy or surface tension arises due to attractive forces between similar molecules which are greater than between different ones. Hence, the forces on the molecule at the interface are directed toward its own phase (figure 2.4).

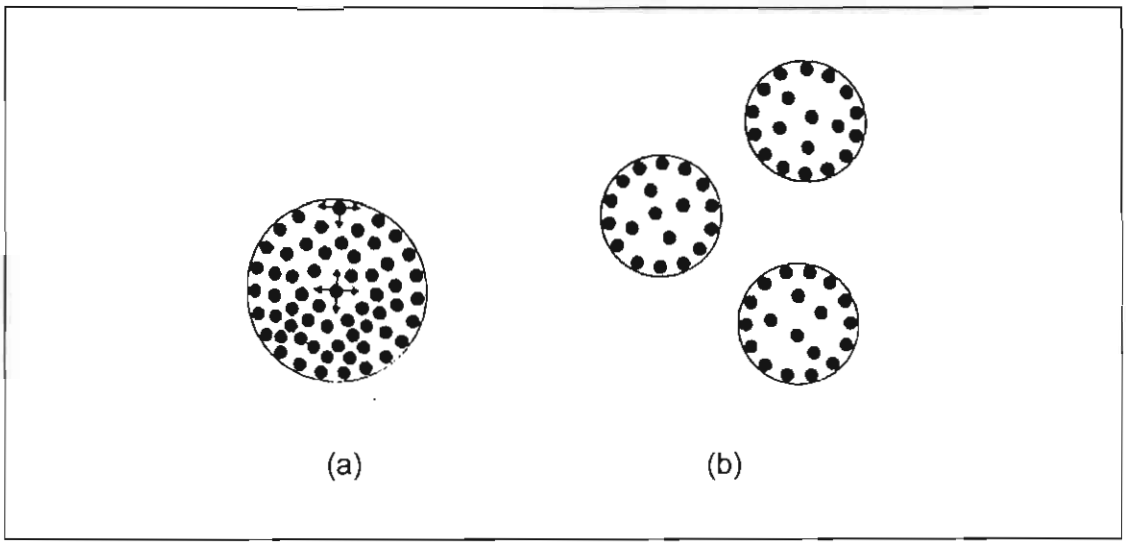


Figure 2.4 *A molecule at the surface is exposed (a) to a resultant force inward while the forces in a molecule interior cancel each other. Bringing more molecules to the surface by forming more droplets (b) means a greater number of molecules at the surface, and energy must be added to bring these molecules there. This is the surface fee energy or surface tension (Friberg et al., 1996:57).*

In the interior of the phase, the forces are equal in all directions and cancel each other. This means that energy must be added to bring a molecule form the interior to the surface. If the total surface of the phase is not changed, no energy is spent in bringing one molecule to the surface, since another molecule must leave the surface; consequently, as much energy is gained as lost. An increase in the total surface, on the other hand (e.g. by forming droplets), means that energy must be added because molecules must be brought to the new surface (figure 2.4) (Friberg *et al.*, 1996:57). Schick (1966:1) found that over many years, nonionic surfactants have become more and more important in the pharmaceutical field because of their ability to solubilize poorly soluble substances and because of their low toxicity.

Cremophor® RH 40 is a polyoxyethylene castor oil derivative known to have a HLB value in the range of 14-16 and is the surfactant used in the Pheroid system.

2.3.2.1.2.3 Polyoxyethelene Esters and Ethers

Surface-active compounds are characterized by having two distinct regions in their chemical structure, termed hydrophilic (water-liking) and hydrophobic (water hating). The existence of two such regions in a molecule is referred to as amphipathy and the molecules are consequently often referred to as amphipathic molecules. The hydrophobic portions are usually saturated or unsaturated hydrocarbon chains or, less commonly, heterocyclic or aromatic ring systems. The hydrophilic regions can

be anionic, cationic, or nonionic. Surfactants are generally classified according to the nature of the hydrophilic group (Attwood, 2002:86).

In polyoxy 40 hydrogenated castor oil (Cremophor® RH 40); approximately 75% of the components of the mixture are hydrophobic. These comprise mainly of fatty acid esters of glycerol polyethylene glycol and fatty acid esters of polyethylene glycol. The hydrophilic portion consists of polyethylene glycols and glycerol ethoxylates (Yu, 2003:474).

2.3.2.1.3 Anti-oxidants

The efficiency of an antioxidant in a product will depend on many factors, including its compatibility with other ingredients, its o/w partition coefficient, the extent of its solubilization within micelles of the emulgent, and its sorption on to the container and its closure. It must be realized, therefore, that the choice of antioxidant and the concentration at which it is to be used can only be determined by testing its effectiveness in the final product and in the package in which the product is to be sold (Billany, 2002:351).

Before including an antioxidant in emulsion formulations, it is essential to ensure that its use is not restricted in whichever country it is desired to sell the product (Billany, 2002:351).

Many of the oils and fats used in emulsion formulation are of animal or vegetable origin and can be susceptible to oxidation by atmospheric oxygen or by the action of microorganisms (Billany, 2002:354).

The resulting rancidity is manifested by the formation of degradation products of unpleasant odour and taste. These problems can also occur with certain emulsifying agents, such as wool fat or wool alcohols. Oxidation of microbiological origin is controlled by the use of antimicrobial preservatives, and atmospheric oxidation by the use of reducing agents or, more usually, antioxidants (Billany, 2002:355). In the Pheroid system, DL-α-Tocopherol is the anti-oxidant used to protect against atmospheric oxidation.

2.3.2.2 Aqueous phase

The common practice in formulating emulsions is to dissolve or disperse lyophilic components in the corresponding phases before emulsification is initiated. Thus, oil-soluble or dispersible ingredients are incorporated in the oil and water-soluble or dispersible ingredients in the water phase (Block, 1996:76). Normally water is used

to prepare o/w emulsions but in the case of the Pheroid system the continuous phase is dinitrous oxide satured water.

2.3.2.2.1 Dinitrous oxide saturated water

Laughing gas is nitrous oxide, N_2O (more properly called dinitrogen oxide). It is a colorless gas with a sweet odour and taste. Inhalation leads to disorientation, euphoria, numbness, loss of motor coordination, dizziness and ultimately a loss of consciousness (Senese, 2005).

Formal charge considerations suggest that the most important resonance structure is as depicted in figure 2.5 (Senese, 2005).

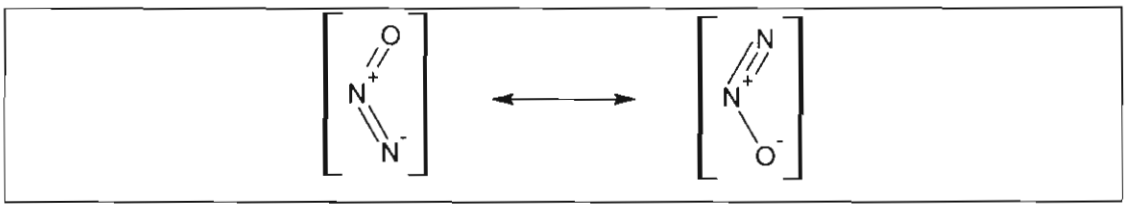


Figure 2.5 *Resonance structure of dinitrogen oxide (Senese, 2005).*

The molecule is not strongly polar, despite the large electro negativity difference between nitrogen and oxygen. The resonance structures again can be used to explain why the negative formal charge is concentrated on the terminal nitrogen in the structure at the left. The oxygen bears a negative formal charge in the other structure. Each structure is polar, but the dipole moments point in opposite directions. The dipole moment is expected to be small due to cancellation of the contributions from both structures (Senese, 2005).

It is hypothesized by Saunders *et al.* (1999:105) that the presence of nitrous oxide in the Pheroid formulation may enhance both intercellular lipid fluidity, as well as the fluidity of the Pheroid particles.

The system nitrous oxide and water are an interesting system. First, it exhibits liquid-liquid immiscibility, which extends from about 12 to 36 °C. At temperatures greater than 36.6 °C, the three-phase critical end point, the second liquid does not form. Secondly, the system forms a hydrate. Hydrates are solid, ice like crystals that form at conditions where a solid would otherwise not be expected (Jaffer, 1993:324). At 20 °C and 2 atm one liter of the gas dissolves in 1.5 liters of water (Budavari, 2001:1191).

2.3.3 Dispersion equipment used in the preparation of emulsions

Various types of equipment are available to effect droplet dispersion and emulsification either in the laboratory or in production. The choice of emulsification equipment is usually dictated by the application of the resulting emulsions and by the throughput required. This equipment, whether simple or complex, serves to break up or disperse the internal phase into the external phase so that the droplet size of the resulting emulsion is sufficiently small to prevent coalescence and resulting instability (Block, 1996:77).

According to Billany (2002:357) the choice of suitable equipment for the emulsification process depends mainly on the intensity of shearing required to produce this optimum particle size. Other considerations, however, include the volume and viscosity of the emulsion and the interfacial tension between the oil and the water. The presence of surfactants, which will reduce interfacial tension, will aid the process of emulsification as well as promoting emulsion stability.

In many cases simple blending of the oil and water phases with a suitable emulgent system may be sufficient to produce satisfactory emulsions. Further processing using a homogenizer can also be carried out to reduce globule size still further. The initial blending may be accomplished on a small scale by the use of a pestle and mortar or by using a mixer fitted with an impeller type of agitator (Billany, 2002:357).

2.3.3.1 Turbine mixer

A more intense rate of shearing can be achieved using a turbine mixer such as the Silverson® mixer-homogenizer. In this type of machine the short, vertical or angled rotor blades are enclosed within a stationary perforate ring and connected by a central rod to a motor. The liquids are therefore subjected to intense shearing, caused initially by the rotating blades, and then by the forced discharge through the perforated ring. Homogenizers are often used after initial mixing to enable smaller globules sizes to be produced (Billany, 2002:357).

Emulsions can be formed from immiscible liquids by employing different methods such as stirring by high-pressure homogenizers and ultrasonification. Extensional flow is created, which first elongates the droplets and then breaks them into smaller ones, by shear forces as shown in figure 2.6 (Baloch, 2005:804).

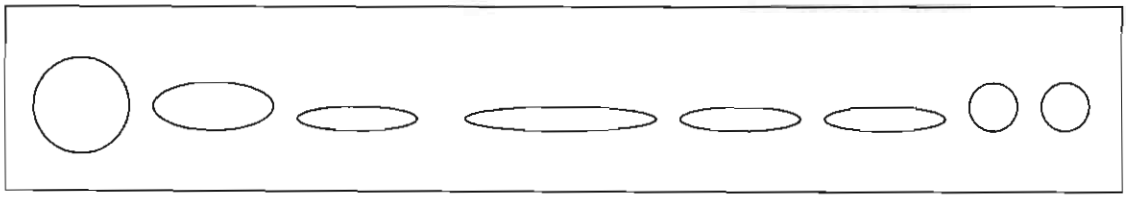


Figure 2.6 *A schematic presentation of droplet deformation and breaking up with increase in flow rate (Baloch, 2005:805).*

The overall impact will therefore depend upon the applied shear forces generating shear rate. If the shear rate is above the critical value, emulsion will be formed, otherwise de-emulsification will take place. This process can be presented as shown in figure 2.7 (Baloch, 2005:805).

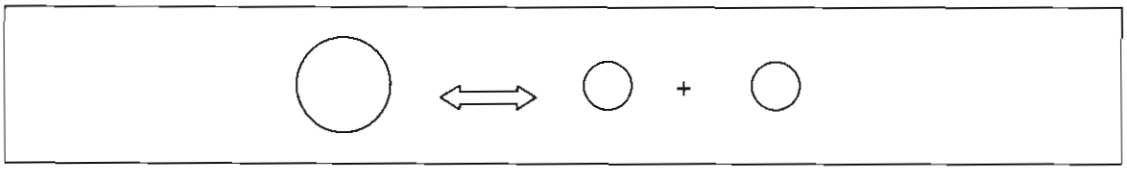


Figure 2.7 *A schematic representation of the emulsification and de-emulsification process (Baloch, 2005:805).*

2.3.4 Influence of processing on emulsion properties

In processing emulsions, the initial location of the emulsifiers (i.e., whether in one bulk phase or another), the method of incorporation of the phases, the rates of addition, the temperature of each phase, and the rate of cooling after mixing of the phases have considerable effects on the droplet size distribution, viscosity and stability of the final emulsion (Block, 1996:75).

The usual practice in preparing emulsions is to add the internal phase to the external phase, while subjecting the system to shear or fracture, so that the internal phase is dispersed within the external phase. For example, in the preparation of o/w emulsions, the internal phase (the oil phase) is usually added to the water phase (Block, 1996:75).

Often the various phases are heated, prior to emulsification, to temperatures about 5 to 10 °C above the melting point of the highest melting ingredient (e.g. waxes or fats). The elevated temperatures are then maintained as the phases are combined. This practice minimizes the likelihood of premature or inappropriate solidification or crystallization of high melting point components during admixture of the phases. It also facilitates processing due to the lower viscosities at higher temperatures (Block, 1996:76).

After the emulsion has formed at an elevated temperature, the rate of cooling is extremely important in determining the final texture and consistency of the emulsion (Block, 1996:76).

2.3.4.1 Droplet size

Initially, most methods of emulsification mechanically induce deformation of the liquid-liquid interface such that droplets are formed. These droplets are then disrupted and dispersed further. The greater the number of droplets formed, the greater the likelihood of droplet collision and coalescence. Walstra (1983:70) hypothesized that the number of droplets of a given size that can be formed per unit time is a function of the phase volume ratio, Φ, and of the mechanical factors associated with the specific type of emulsification equipment employed and whether laminar flow, turbulent flow or cavitation is involved.

It has already been explained that the smaller the globules of the disperse phase, the slower will be the rate of creaming in an emulsion. The size of these globules can also affect the viscosity of the product, and in general it has been found that the best emulsions with respect to physical stability and texture exhibit a mean globule diameter of between 0.5 and 2.5 μm (Billany, 2002:357).

Since emulsification is generally a more or less random stirring process in which the breaking and coalescence steps are in dynamic equilibrium, the resulting emulsion is a polydispersed system in which small and big drops coexist. The outcome of this equilibrium depends on a large number of factors that can influence the two antagonistic steps. Generally speaking, a lower tension or an increase in stirring energy and duration are expected to increase the break up, while a higher fluid viscosity would slow it down, and temperature would increase the coalescence rate (Salager, 2000:76).

2.3.4.2 Viscosity

The viscosity of a fluid may be described simply as its resistance to flow or movement. The definition of viscosity was put in a quantitative basis by Newton, who was first to realize that the rate of flow (γ) was directly related to the applied stress (σ): the constant of proportionality is the coefficient of dynamic viscosity, (η), more usually referred to simply as the viscosity. Simple fluids which obey the relationship are referred to as Newtonian fluids and those which do not are known as non-Newtonian (Marriott, 2002:42).

As Newton's law can be expressed as:

$$\sigma = \eta\gamma \tag{2.1}$$

then

$$\eta = \frac{\sigma}{\gamma} \tag{2.2}$$

and η will have units of $N.m^{-2}.s^{-1}$. Thus by reference to equation 2.1 it can be seen that a Newtonian fluid of viscosity 1 $N.m^{-2}.s^{-1}$ would produce a velocity of 1 $m.s^{-1}$ for a cube of a 1 m dimension with an applied force of 1 N. Because the name for the derived unit of force per unit area in the SI system is the pascal (Pa), then viscosity should be referred to in $Pa.s^{-1}$. It is common to use the submultiple $mPa.s^{-1}$, and the viscosity of water at 20 °C is virtually 1 $mPa.s^{-1}$ (Marriott, 2002:42).

However, most pharmaceutical fluids do not follow this law because the viscosity of the fluid varies with the rate of shear. The reason for these deviations is that the fluids concerned are not simple fluids such as water and syrup, but are disperse or colloidal systems, including emulsions, suspensions and gels (Marriott, 2002:49).

Disperse systems show a wide range of rheological properties, depending on the nature of the dispersed particles they contain and on the composition of the dispersion media. The rheology of dispersed systems is among the most important of their physical properties, which influences not only the physical stability of the systems, but often also profoundly affects the performance features, their quality and their utility (Radebaugh, 1996:207).

Because nearly all but the most dilute of medicinal emulsions exhibit non-Newtonian behavior, their rheological characteristics have a marked effect on their usefulness. The fluid emulsions are usually pseudoplastic (Marriott, 2002:58).

Pseudoplastic flow is also seen in emulsions containing macromolecules. Since macromolecules tend to show greater entanglement at rest than when subjected to shear, the viscosity is initially high and decreases, nonlinearly, as the shear rate increases. At high shear rates, the macromolecules align themselves and squeeze out water thereby lowering the viscosity of the system (Radebaugh, 1996:206).

According to results obtained in an experiment done by Prindere *et al.* (1998:75) mixing time has a strong effect on viscosity, viscosity decreases when mixing time increases. Viscosity principally depends on mixing time; when the emulsion is mixed

for 20 minutes, droplets are well dispersed, giving higher stability.

2.4 EVALUATION OF EMULSION PROPERTIES

Block (1996:87) notes that physicochemical changes in emulsion components are often accompanied by corresponding changes in emulsion properties; chemical instability generally requires analytical verification. Definitions of product stability based solely upon physicochemical or chemical determinations are arbitrary to the extent that (a) laboratory test conditions may not duplicate the actual storage environment during the course of a product's shelf life; and (b) such tests may not mirror changes in the consumer's perception of product performance. According to Moskowitz (1984:371) the latter issue, psychophysical aspects of product evaluation, is now the subject of renewed scrutiny.

2.4.1 Influence of processing on emulsion stability and characteristics

2.4.1.1 Introduction and definitions

In practice, formulators consider a macroemulsion to be stable if there is no evidence of coalescence or creaming under ambient conditions, or when frozen and thawed repeatedly, or upon exposure to moderately elevated temperatures (e.g., 40 to 50 °C) for various time intervals. Failure under any of these conditions may be allowable in specific instances (e.g., elevated temperature stability is of no concern with a heat-sensitive drug). For that matter, creaming or flocculation that can be reversed by simple shaking may not be a deterrent to product development or marketing (Block, 1996:94).

According to Friberg *et al.* (1996:62), a stable emulsion is more easily described from a practical point of view: it is an emulsion that does not change with time.

Nonetheless, at present, there is general agreement on at least four aspects of emulsion product stability testing:

- freshly prepared, unequilibrated emulsions are not the appropriate subjects of an emulsion product stability testing protocol: only emulsion products that have had an opportunity to "rest" or equilibrate (e.g. , for 24 or 48 hours) after manufacturing should be employed;
- whenever possible, comparisons should be made between the "test" emulsion product and a similar "reference" emulsion product with known stability characteristics and shelf life;
- storage and stress conditions should be comparable to those the emulsion

product is likely to encounter during its commercial lifetime, i.e. in the course of shipping, handling, storage and use; and

- each emulsion product necessitates the development of explicit stability criteria to be employed in conjunction with product-specific stability testing methods and protocols (Block, 1996:94).

Industrial practice, for most pharmaceutical emulsions, involves storage at 5, 25, 40 and 50 °C. Stability at 5 and 40 °C for 3 months is considered the minimal criteria for accelerated stability testing performed. Unfortunately, "real-time" ambient temperature testing of emulsion products is not a reasonable alternative. As Rieger (1982:28) has noted, real-time testing for periods up to or exceeding 2 years has been described as intolerable to formulators or manufacturers.

2.4.1.2 Instability

The first step for solving stability problems of disperse systems is to define clearly the type or types of stability of concern. Categorizing stability as either physical or chemical is not sufficient. The different groups of people involved with the product's development, production, analysis, marketing, etc. must have a clear and precise reference frame for stability. For example, with emulsions, various types of stability problems can occur (Weiner, 1996:10).

For emulsions, higher temperatures will dramatically alter the nature of the interfacial film, especially if nonionic emulsifiers are used. The principle mechanism for stability in these systems is the hydration of the polyoxyethelene groups of the emulsifier molecules (Weiner, 1996:12).

The various types of emulsion instabilities require different testing procedures and have different degrees of reliability for making shelf life predictions. Figure 2.8 diagrammatically depicts the various types of emulsion instability (Weiner, 1996:10).

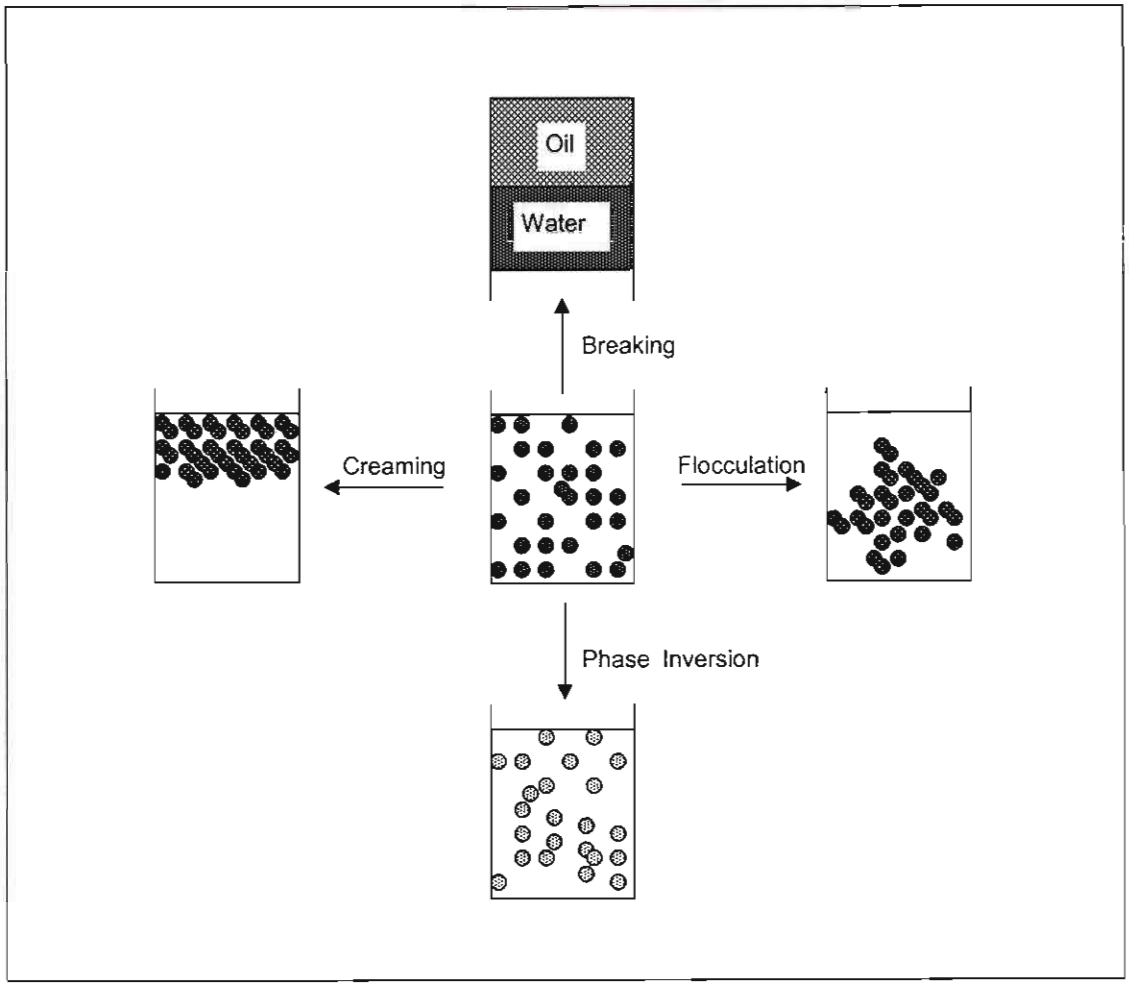


Figure 2.8 *Diagrammatic representations of four different types of physical stability problems seen with emulsion formulations (Weiner, 1996:11).*

Friberg *et al.* (1996:61) noted that the first process namely flocculation, is when two droplets become attached to each other, but are still separated by a thin film of the liquid (figure 2.9).

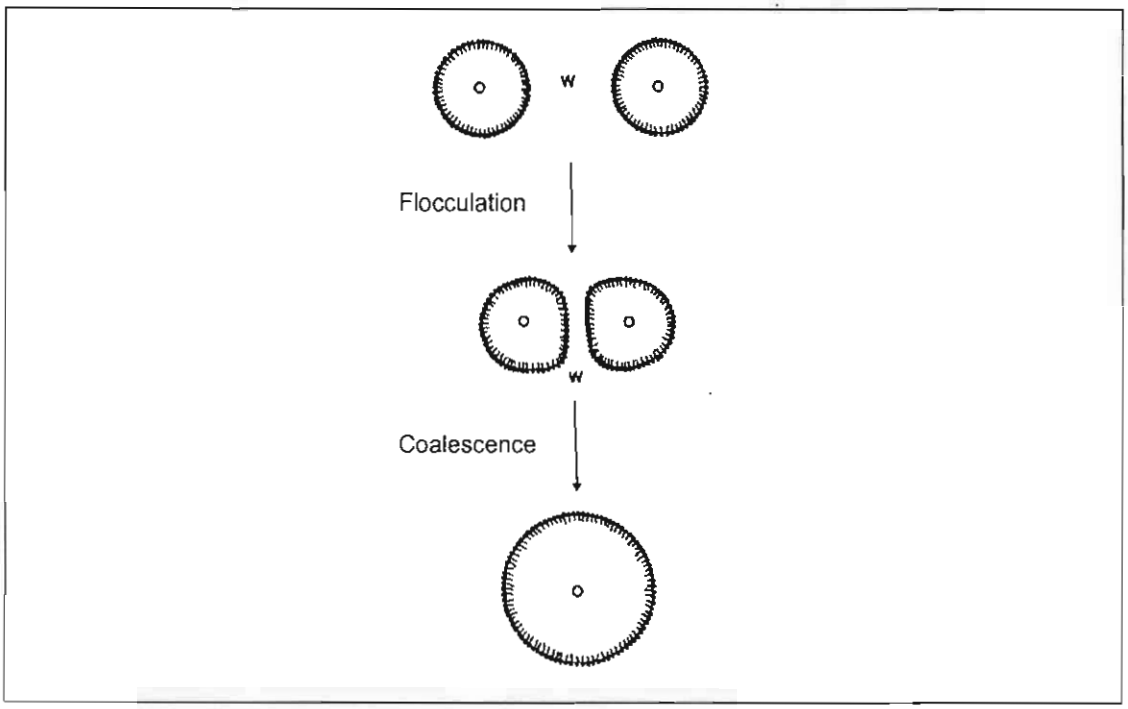
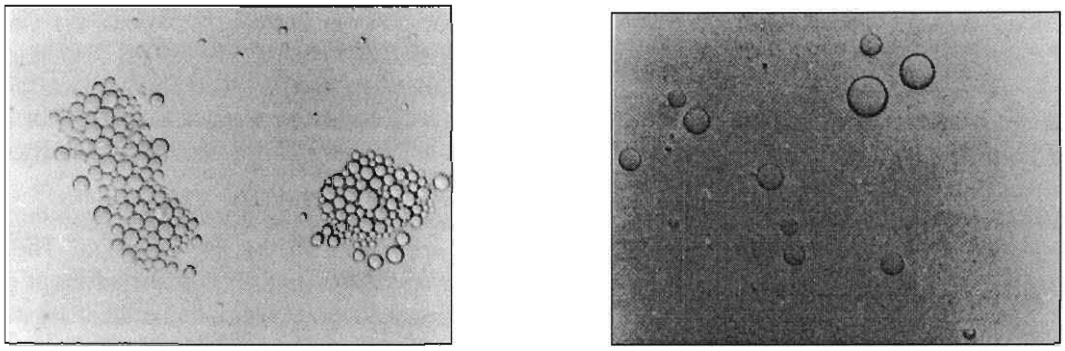


Figure 2.9 *In flocculation, two droplets become attached to each other, separated by a thin film, whereas in coalescence, the thin film is disrupted and the droplets are united (Friberg et al., 1996:60).*

When more droplets are added, an aggregate is formed in which the individual droplets cluster, but retain the thin liquid films between them (figure 2.10a), the emulsifier molecules remain at the surface of the individual droplets during this process, as indicated in figure 2.9. In the final step, coalescence occurs when the thin liquid film between the two droplets is removed, and a single large droplet is formed. This process, when continued, leads to larger and larger droplets. The coalescing emulsion is characterized by a wide distribution of droplet sizes, but no clusters are present (figure 2.10b) (Friberg *et al.*, 1996:61).

The large droplets cream or sediment much faster than the original small ones. Hence, a droplet of 10 times the radius will move 100 times faster and, as a consequence, droplets are collected on top (creaming) or at the bottom (sedimentation). In fact, these layers are more concentrated emulsions, and the closeness of the droplets result in enhanced flocculation and coalescence. As a consequence, the final state of phase separation is approached faster owing to the flocculation and coalescence (Friberg et al., 1996:62).



(a) (b)

Figure 2.10 *In a flocculated system (a), aggregates of droplets are present. In a coalesced system (b), a wide variety of droplet size is found, but no aggregates (Friberg et al., 1996:62).*

According to Attwood (2002:97) the separation of an emulsion into its constituent phases is termed cracking or breaking. It follows that any agent that will destroy the interfacial film will crack the emulsion. Some of the factors that cause an emulsion to crack are:

- The addition of a chemical that is incompatible with the emulsifying agent, thus destroying its emulsifying ability.
- Bacterial growth: protein materials and nonionic surface-active agents are excellent media for bacterial growth.
- Temperature change: protein emulsifying agents may be denatured and the solubility characteristics of nonionic emulsifying agents change with a rise in temperature, heating above 70 °C destroys most emulsions. Freezing will also crack an emulsion; this may be due to the ice formed and disrupting the interfacial film around the droplets.

Various other types of stability problems can also occur, such as phase inversion, changes in rheological characteristics (as a result of creaming, coalescence or other factors), various changes in physical properties owing to water evaporation, flocculation of the droplets, microbial contamination and chemical decomposition, to name a few.

The final sign of an unstable emulsion is easy to observe. When an emulsion starts to separate, typically an oil layer appears on top, and an aqueous layer appears on the bottom (figure 2.11). This separation is the final state of an unstable emulsion. It may take months or years to develop, and the detection of earlier phenomena is necessary to remedy the situation in time. This means that attention must be focused on the initial mechanism in the many processes involved in the

destabilization of an emulsion (Friberg *et al.*, 1996:61).

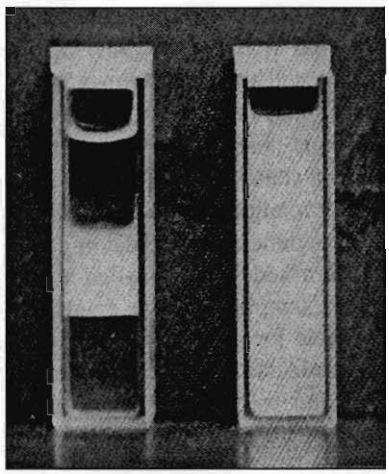


Figure 2.11 *In destabilization (left), an oil layer or an aqueous layer appears on top and bottom; a stable emulsion (right) shows no layers (Friberg et al., 1996:61).*

2.4.2 Accelerated stability tests

All medicinal products decompose with time. Paradoxically, when this decomposition is being assessed the skilled formulator becomes a victim of his own expertise, as a good formulation will take a long time to decompose. Instabilities in modern formulations are often detectable only after considerable storage periods under normal conditions. To assess the stability of a formulated product it is usual to expose it to "high stress", i.e. conditions of temperature, humidity and light intensity that are known from experience to be likely causes of breakdown. High stress conditions enhance the deterioration of the product and therefore reduce the time required for testing. This enables more data to be gathered in a shorter time, which in turn will allow unsatisfactory formulations to be eliminated early in a study and will also reduce the time for a successful product to reach the market. It must be emphasized that extrapolations to "normal" storage conditions must be made with care, and that the formulator must be sure such extrapolations are valid. It is advisable therefore to run concurrently a batch under expected normal conditions to confirm later that these assumptions are valid (Pugh, 2002:109).

The objectives of such accelerated tests may be defined as:

- The rapid detection of deterioration in different initial formulations of the same product. This is of use in selecting the best formulation from a series of possible choices.
- The prediction of shelf-life, which is the time a product will remain satisfactory when stored under expected or directed storage conditions.

- The provision of a rapid means of quality control, which ensures that no unexpected change has occurred in the stored product.

Good formulations will invariably break down more slowly than poor ones. Even though no absolute conclusions can be drawn about their predicted stability under normal storage from data obtained under stress conditions, such tests allow formulations to be optimized relatively quickly. When the perceived optimal formulation is decided on, attempts can be made to predict its likely stability at proposed storage conditions (Pugh, 2002:109).

The stability of an emulsion depends on several key parameters such as particle size and zeta potential. These parameters are greatly affected by the choice, concentration, and placement of the surfactant, on one hand and by manufacturing technique and water / oil ratio, on the other hand (Hsu & Nacu, 2003:374).

2.4.2.1 Particle size determining

Studies on the effect of time on particle size distribution are perhaps the single most important test to evaluate emulsion stability. Deterioration of the rheological properties of an emulsion on aging is largely due to changes in particle size. However, since most emulsions contain a heterogeneous distribution of particles, one must be extremely careful in analyzing the droplet size distribution of an emulsion. These systems change their size distributions with time to produce a more diffuse distribution to increase the average droplet diameter (Weiner, 1996:12).

The best evaluation of an emulsion's stability is probably to determine its particle size distribution. An increasing number of huge droplets, giving the long "tail" of the distribution towards large sizes, are a bad sign indeed. Such an emulsion invariably becomes unstable with time. An even and narrow size distribution is not a guarantee for stability, but such an emulsion may be stable (Friberg *et al.*, 1996:74).

Prinderre (1998:74) mentions that emulsion stability is estimated by the average size of the droplets and the variation of emulsion viscosity. Friberg & Goldsmith (1968:85) described that the smaller the emulsion droplet size and the smaller the viscosity variations, the better the stability of the system.

If the mean globule size increases with time (coupled with a decrease in globule numbers), it can be assumed that coalescence is the cause. It is therefore possible to compare the rates of coalescence for a variety of emulsion formulations by this method (Billany, 2002:355).

It is said that an emulsion is stable when it does not change its properties in 3 years and that it is unstable if it has completely separated after a few minutes. Anything in-between these extremes require a quantitive measurement of the emulsion evolution with time (Salager, 2000:88).

2.4.2.2 Zeta potential measurement

Roland *et al.* (2003:87) notes that if a stable emulsion is to be produced, an emulsifying agent must be added to the oil and water. It acts as a barrier to alter the rate of coalescence of droplets or creates an interfacial film which can produce repulsive electrostatic forces between approaching droplets. Although this repulsive electrostatic potential at the emulsion interface can be calculated, it cannot be measured directly. However, a related quantity called the zeta potential can be determined.

The zeta potential (ζ) is defined as the difference in potential between the surface of the tightly bound layer of ions on the particle surface and the electro neutral region of the solution. When the zeta potential is relatively high (> 25 mV) the repulsive forces exceed the attractive London forces. The particles are dispersed and the system is deflocculated. On the other hand, when the zeta potential is low (< 25 mV), the attractive forces exceed the repulsive forces, and the particles come together leading to flocculation (Roland *et al.*, 2003:87).

The particles in a colloidal suspension or emulsion usually carry an electrical charge. The charge is more often negative than positive and it may arise in a number of ways. Sometimes the surface of the particles contains chemical groups that can ionize to produce a charged surface. Sometimes the surface itself preferentially adsorbs ions of one sign of charge in preference to charges of the opposite sign. In other cases there may be deliberately added chemical compounds that preferentially adsorb on the particle surface to generate the charge. The amount of charge on the particle surface is an important particle characteristic because it determines many of the properties of the suspension or emulsion. It is important to realize that the charge on the surface of each particle is counterbalanced by charges (ions) of opposite sign in the surrounding solution. The emulsion is neutral overall and also on a scale somewhat larger than the particle themselves. Because of the thermal motions of the solvent molecules and ions, this countercharge is spread in a diffuse layer which stretches out for some distance (of nanometer order) from the particle surface (figure 2.12) (Colloidal Dynamics, 2005).

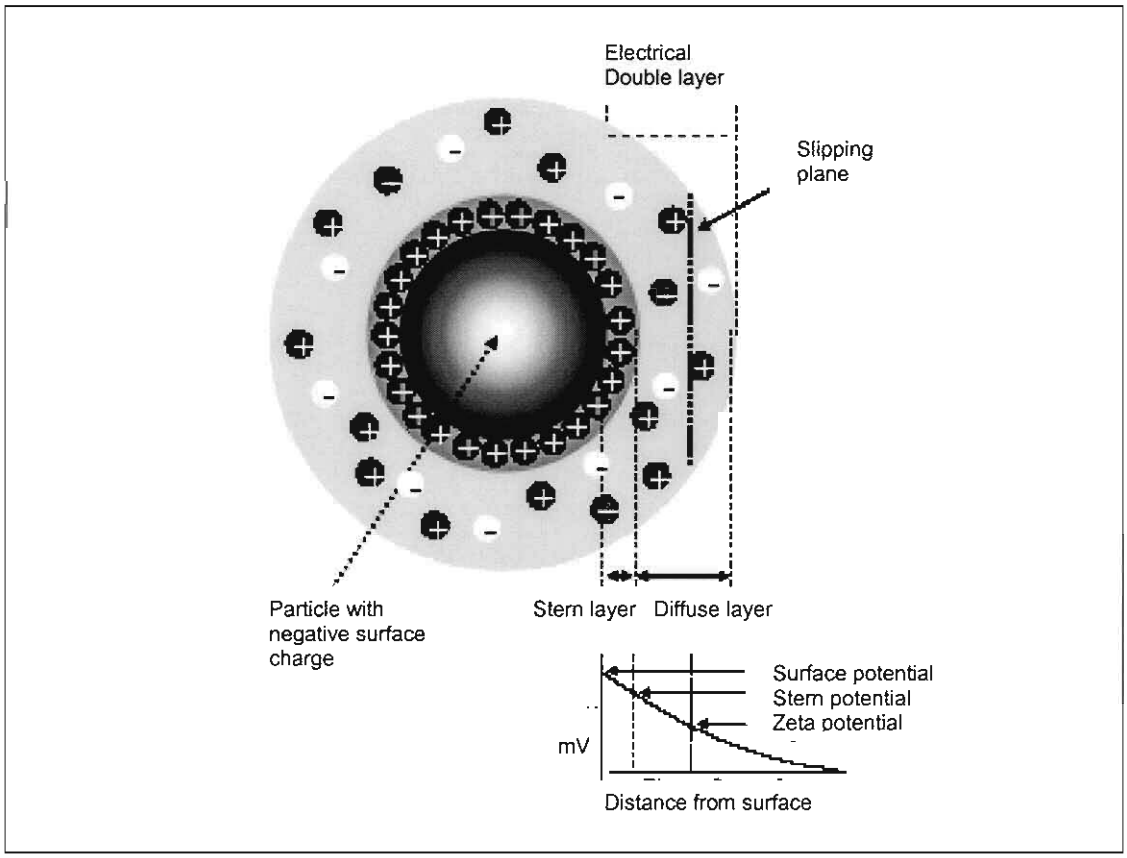


Figure 2.12 *Schematic representation of the distribution of ions around a charged particle in solution (Colloidal Dynamics, 2005).*

The oppositely charged ions (called counter-ions) tend to congregate around the particle and very few negatively charged co-ions can get close to the surface because of the repulsion from the charges on the particle. Farther away from the particle the co-ions suffer less repulsion and eventually, at distance of at most a few tens of nanometers, the numbers of cationic and anionic charges are evenly balanced (Colloidal Dynamics, 2005).

There are various ways to measure the particle charge but it must be recognized that the different methods do not always measure the same quantity. One of the most effective methods is to apply an electric field to the emulsion and to measure how fast the particles move as a result. That process is called electrophoresis. The bigger the charge they carry, the faster the particles will move (Colloidal Dynamics, 2005).

It turns out; however, that in such an experiment one does not usually observe all of the particle charge. The electric field pulls the particle in one direction but it will also be pulling the counter ions in the opposite direction. Some of the counter ions will move with the particle (those within the dotted circle in figure 2.12) so the measured

charge will be a nett charge taking that effect into account (Colloidal Dynamics, 2005).

The electrostatic potential near the particle surface changes very quickly (and linearly) from its value at the surface through the first layer of counter ions and then changes more or less exponentially through the diffuse layer. The junction between the bound charges and the diffuse layer is again marked by the broken line. That surface which separates the bound charge from the diffuse charge around the particle, marks where the solution and the particle move in opposite directions when an external field is applied. It is called the surface or the shear or the slip surface. The electrostatic potential on that surface is called the zeta potential and it is that potential which is measured, when one measures the velocity of the particles in a dual current electric field. The velocity ($m.s^{-1}$) for an unit field strength (1 Volt per meter) is called the electrophorectic mobility, and is given the symbol μ_E which denotes the unit for zeta potential (ζ) (Colloidal Dynamics, 2005).

At first it would seem to be a distinct disadvantage of this method that it only measures a part of the potential on the particle. But in fact that turns out to be an advantage. When the charge is measured in this way it reflects more realistically what one particle "sees" as it approaches another particle and that is what determines the properties of the emulsion. If the repulsion between approaching particles is large enough they will bounce away from one another and that will keep the particles in a state of dispersion. If the repulsive force is not strong enough, the particles will come together and may stick in a permanent doublet. Then other particles may come along and also be caught in the growing aggregate. The emulsion is then unstable and the aggregates will quickly settle out from the surrounding medium. If one is relying on the electric charge alone to keep the system in a disperse state then the zeta potential usually needs to be kept above 25 mV (positive or negative) (Colloidal Dynamics, 2005).

2.4.2.3 Turbidity measurement

When a beam of light is passed through a colloidal solution some of the light may be absorbed (when light of certain wavelengths is selectively absorbed a color is produced), some is scattered and the remainder is transmitted undisturbed through the sample. Because of the scattered light the solution appears turbid (Attwood, 2002:75).

Scattering largely determines the "turbidity", "cloudiness", "opacity" or "lightness" of

an emulsion. The degree of light scattering by an emulsion depends on the concentration, size and refractive index of any particles present. Emulsion lightness increased with increasing droplet concentration (0-20 % w/w), decreasing droplet size (30-0.2 μm) and decreasing dye concentration (Chanamai & McClements, 2001:83).

The phenomena of aggregation, coalescence and Ostwald ripening are all reflected in the particle size distribution of the emulsion. As a first approximation, the appearance (i.e. color and opacity) of the emulsion may be indicative of the size of the dispersed phase: if the particle size is greater than 1 μm, the emulsion tends to be milky white; if the size of the dispersed phase decreases, the emulsion becomes less opaque (table 2.3). An emulsion's color and opacity are the result of the absorption, scattering, reflection and refraction of light. A reduction in the heterogeneity of the particle size distribution of an emulsion – toward a more monodisperse system – and a decrease in the difference between the refractive indices of the phases would tend to reduce the opacity and color of the emulsion. The direct or indirect measurement of particle size or particle size distributions, as a function of time, can help to characterize emulsion stability (Block, 1996:88).

Table 2.3 *Emulsion appearances as a function of size of the dispersed phase (Block, 1996:88).*

Particle size (μm)	Appearance
> 1	White
0.1 – 1	Blue-white
0.05 - 0.1	Opalescent, semitransparent
< 0.5	Transparent

The turbidity ratio gives the information about the particle size distribution (PSD). The changes in PSD under conditions of simultaneous flocculation and creaming for various times showed that the PSD shifts to large particle sizes, owing to flocculation of the small particles initially. Since the stability to flocculation increases exponentially with increasing particle size, the smaller particles flocculate quickly to form relatively stable larger flocks. If the prepared emulsions are relatively stable, shifts in PSD would occur slowly. From this point of view, it is possible to evaluate the emulsion stability by measurement of the change of PSD with time and the turbidity method can be a useful one, because the turbidity measurements are rapid and simple (Song, 2000:213).

It is known that the maximum turbidity is attained when the drop size is in the 2 - 5 μm range, and that it decreases when the droplets become smaller or larger (Salager, 2000:77).

2.4.2.4 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a powerful technique that permits direct visualization of penetration pathways in unfixed skin and other biological samples. The use of a laser as an energy source enables the microscope to be used as an optical knife that can optically section a sample at varying depths; in addition, dynamic changes can also be visualized in real time. This is achieved by labeling the sample with a fluorescent marker, a molecule that enters an excited state during laser interaction and emits light of a specific wavelength (Saunders *et al.*, 1999:100).

Although microscopy in general are not used frequently as a quantitative method to determine particle sizes, it may give very useful information about size and other changes of oil droplets, especially when advanced techniques such as CLSM are used.

2.4.2.5 Viscosity measurement

Suspension and emulsions are non-Newtonian fluids. From a practical point of view, having to deal with non-Newtonian fluids means that viscosity is not enough to describe their response to deformation. This property no longer depends on temperature alone but on many other factors such as shear rate, shear stress, strain, time and measuring geometry (Briceno, 2000:561).

Under shear conditions, the flow response is now called apparent viscosity and it is defined in equation 2.3

$$\eta = \frac{\tau}{\gamma} \tag{2.3}$$

where η is apparent viscosity and τ is the shear stress whilst γ represents the shear rate. Equation 2.3 does not imply a linear relationship between shear stress and shear rate. It only asserts that a given shear rate will bring about a given shear stress (or the other way around) to calculate viscosity for this given shear rate. The idea is illustrated in figure 2.13, which is a flow curve corresponding to a shear-thinning fluid. It can be seen that the slope of the curve flow decreases as shear rate progresses; hence η diminishes (Briceno, 2000:561).

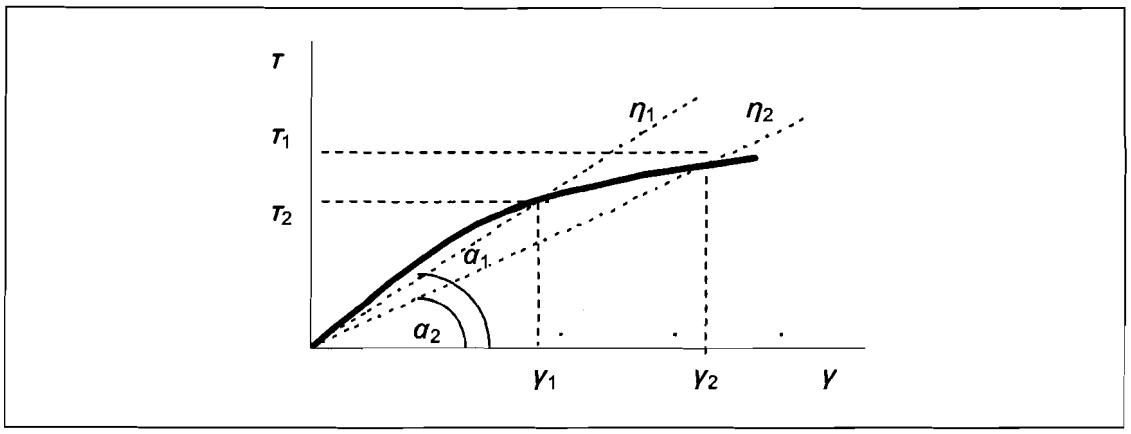


Figure 2.13 *Schematic flow curve of a non-Newtonian fluid exhibiting shear thinning behavior (Briceno, 2000:562).*

Particle interactions are the main basis of non-Newtonian behavior and the extent of these interactions will dictate how complex the flow behavior is. Most suspensions and emulsions display a shear-thinning viscosity and this is the most common behavior of non-Newtonian fluids. An alternative classification for shear thinning is pseudo plasticity; however, this flow category is rather limited to the so-called power law fluids (Briceno, 2000:562).

2.5 CONCLUSION

Of the various pharmaceutical dosage forms, liquid disperse systems are the most complex. The method of manufacturing, formulation approach, component material selection, and the effect of environmental factors, such as temperature, profoundly affects the variability in the product's bioavailability, stability characteristics and a host of other variables. These parameters must be known first, if a production method with a high probability of success is to be tried. It is necessary to establish a formulation approach for the resolution of problems in drug delivery, which also meets the criteria of the consumer or patient.

The formulator has the responsibility to anticipate, as far as possible, which parameters are likely to affect the product and which parameters can be controlled. The emulsion system is prone to instabilities broad about by activities performed during the manufacturing of the emulsion preparation. Special care has to be given to the constituents that form part of the emulsion formulation as each of them has an influence on the properties of the emulsion system. Special attention should be paid to the way the samples are handled to ensure that the resting state is not disturbed that may alter the stability properties.

Accelerated tests can be performed on the chosen formula to assist in determining

the short-term stability and the usefulness of the preparation as a drug delivery system. The appropriate storage conditions, to ensure the integrity of the product, can be determined through exposing formulas to conditions likely to cause breakdown.

Studies on the effect of time on particle size distribution, the zeta potential, viscosity changes and turbidity values of the chosen formula will provide the formulator with enough criteria to judge the long-term stability of the prepared formula. Along with the use of confocal laser scanning microscopy, a judgment can be made about the functionality of the formula.

CHAPTER 3

PREPARATION AND CHARACTERISATION OF PHEROID VESICLES: EXPERIMENTAL PROCEDURES

3.1 INTRODUCTION

This chapter describes the experimental procedures followed to prepare Pheroid vesicles. An explanation of the different tests performed to characterize the vesicles is given and the apparatus used are also described.

The particles in a colloidal suspension or emulsion are seldom all of the same size and they often have varying shapes. Describing the size and shape is therefore a significant problem. Emulsion droplets can usually be assumed to be spherical (as long as the distances between the droplets are large enough) (Colloidal Dynamics, 1999:2).

The preparation of the sample before it is added to the measurement system can be critical. Over half the problems encountered when measuring a sample are caused by bad sample preparation. If the sample is sticking together, dissolving, floating on the surface or if the analyst has failed to get a representative sample, an incorrect result will be obtained (Malvern Instruments Ltd., 1997:109). There are many techniques available to ensure that the sample is prepared successfully. Once a suitable dispersion technique for your sample has been found the procedure can be standardized so that comparisons can be made between different formulations (Malvern Instruments Ltd., 1997:109).

3.2 MATERIALS

The materials used for preparation of Pheroid vesicles are given in table 3.1 (Annexure A).

Table 3.1 *Materials used in the preparation of Pheroid vesicles.*

Raw materials	Lot number	Manufacturer/ Distributor
Vitamin F Ethyl Ester CLR	3046007	Chemisches Laboratorium, Dr. Kurt Richter GmbH, Germany
Cremophor® RH 40	50369256 PO	BASF Aktiengesellschaft, Ludwigshafen, Germany
DL-α-Tocopherol	UT 0412 0072	Chernpure (Pty) Ltd., Germiston, South Africa
Medicinal Nitrous Oxide	82612	Afrox, Klerksdorp, South Africa

Distilled and deionized water, prepared in our laboratory, was used for the preparation of all solutions and emulsions.

3.3 BASIC METHOD OF PREPARATION FOR PHEROID VESICLES

An oil-in-water emulsion (o/w) was prepared with a Heidolph Diax 600 homogenizer (Labotec (Pty) Ltd., Johannesburg). In a 50 ml glass beaker the non-ionic surfactant, Cremophor® RH 40, was preheated on a hotplate until transparent and then added to the oil phase consisting of Vitamin F Ethyl Ester CLR and the anti-oxidant, DL-α-Tocopherol. This mixture (4 % w/v) was stirred before heating it in a microwave (LG Electronics S.A. (Pty) Ltd., Isando, South Africa) for approximately two minutes or until transparent, on the 900 Watt setting. Care should be taken not to overheat the sample as oxidation may take place and discoloring to a yellowish color occurs.

The water phase (96 %), gassed with nitrous oxide for 4 days, were weighed in an Erlenmeyer flask and heated to ± 75 °C on a hotplate. As soon as the same temperature was reached for both mixtures the oil phase was added to the water phase and the mixture was homogenized at 13500 revolutions per minute (rpm) for 120 seconds.

The emulsion was transferred to an amber glass container, to protect it from light, and sealed under inert atmosphere (nitrogen) to protect the emulsion from oxidation. Lastly the emulsion was placed on a GFL shaker (GFL Gesellschaft für Labortechnik mbH, Germany) until room temperature (25 ± 2 °C) was reached and then stored in a refrigerator at 6 °C for 24 hours before different tests for emulsion characteristics were performed (section 3.4).

In part this study investigates the effect of varying the conditions under which Pheroid vesicles are prepared. The effect of varying the concentration of Vitamin F Ethyl Ester CLR and Cremophor® RH 40 were also investigated. These procedures are

described in section 3.5 and the obtained results are presented in chapter 4. From these results an optimum preparation procedure and Pheroid component concentration were selected for accelerated stability investigation (section 3.6) and the results of this study are presented in chapter 5.

3.4 PHYSICAL CHARACTERISATION OF PHEROID VESICLES

3.4.1 Droplet size

Scientists have for centuries tried to predict the way particles scatter and absorb light. There are many theories and models that the modern particle analyst can use (Malvern Instruments, Ltd., 1997:42). One of the simplest theories used is the Fruanhofer model. This model can predict the scattering pattern that is created when a solid, opaque disc of a known size is passed through a laser beam. This model is fine for a lot of particles but it does not describe the scattering exactly. Very few particles are disc shaped and a lot of particles are transparent. The Mie theory was developed to predict the way light is scattered by spherical particles and deals with the way light passes through, or is adsorbed by, the particle. This theory is more accurate but it does assume that specific information about the particle such as its refractive index and its absorption is known (Malvern Instruments, Ltd., 1997:42).

The key fact about these theories is that if you know the size of the particle and other details about its structure you can accurately predict the way it will scatter light. Each size of particle will have its own characteristic scattering pattern, like a fingerprint that is unlike another size of particle (Malvern Instruments, Ltd., 1997:42).

The Malvern Mastersizer works backwards form the above theories by using the Mastersizer's optical unit to capture the actual scattering pattern from the field of particles. Then, by using the theories above, it can predict the size of particles that created that pattern (Malvern Instruments, Ltd., 1997:42).

3.4.1.1 Apparatus and experimental conditions

Particle size analysis was performed by the method of laser diffraction using a Malvern Mastersizer Micro (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The apparatus was switched on half an hour before starting the measurements to allow the laser beam to warm up and stabilize. The emulsion samples were mixed moderately to ensure uniform distribution of droplets before addition to the Mastersizer cell for measurement. Care was taken to eliminate bubbles in the dispersion fluid since they are also detected and may cause variation in the data

obtained.

3.4.1.2 Method

A dispersion of 2 ml of the sample was added to 800 ml of distilled water at a pump speed of 2500 rpm until an obscuration rate of 10 – 20 % was obtained. Background and samples were measured for 12 seconds, with a delay of 20 seconds between measurements. The parameters that were used to analyze the droplet size distribution were defined by the presentation code 4NHD. Optical properties of the sample were defined as follow: refractive index 1.4564 for the emulsion droplets and 1.3300 for the dispersion medium used (distilled water). The absorbance value of the emulsion droplets was 0.1000 (similar to the particles of olive oil in water in the Malvern software). This setting was maintained for all evaluations. Each sample was measured in duplicate, 24 hours after preparation of the emulsion.

3.4.2 Zeta potential

To determine zeta potential in an o/w emulsion consideration must be given to the physical properties of the sample such as its particle size and sample concentration. Each type of sample material has its own ideal range of sample concentration for optimal measurements:

- If the sample concentration is too low, there may not be enough light scattered to make a measurement.
- If the sample is too concentrated, then light scattered by one particle will itself be scattered by another (this is known as multiple scattering).
- The upper limit of the concentration is also governed by the point at which the concentration no longer allows the sample to freely diffuse, due to particle interactions.

Whenever possible, the sample concentration should be selected such that the sample develops a slightly milky appearance – or in more technical terms, gets slightly turbid. If such a concentration cannot be selected easily various concentrations of the sample should be measured to detect and then avoid concentration dependant effects. However, these effects do not appear at concentrations below 0.1 % by volume. On the other hand particle interactions may occur at sample concentrations larger than 1 % by volume – particle interactions will influence the zeta potential results (Malvern Instruments, Ltd., 2004:97).

3.4.2.1 Apparatus and experimental conditions

The zeta potential was measured with a Malvern Zetasizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The technique is very sensitive to dirt or dust in the sample and therefore great care is required in sample preparation. Preparation of samples was carried out in a laminar flow cabinet to ensure minimization of dust contamination. Great care should be taken to remove all visible bubbles in the preparation before injecting it into the apparatus since they interfere with the measurement procedure.

Table 3.2 shows values of the zeta potential and counter rates obtained from standard solutions with concentrations ranging from 0.005 - 2% v/v.

Table 3.2 *Zeta potential and counter rate values obtained with a concentration range of Pheroid vesicles diluted with Water-for-Injection®.*

Pheroid concentration (% v/v)	Average zeta potential (mV)	Standard deviation on the average Zeta potential (±)	Average count rate (Kcps.10^{-2})	Standard deviation on the average count rate (±)
0.005	-35.9	1.3	32.62	305.1
0.010	-34.3	1.1	42.38	283.0
0.050	-35.7	1.1	39.73	188.3
0.100	-30.0	1.6	46.56	240.2
0.200	-26.2	0.7	52.98	129.6
0.400	-21.6	1.0	57.84	65.3
0.600	-17.0	1.9	59.56	58.8
0.800	-19.4	1.9	59.48	71.7
1.000	-16.9	1.5	59.94	30.2
1.500	-10.7	2.8	60.72	31.2
2.000	-9.6	2.0	60.83	38.0

From these values it was concluded that 10 μl Pheroid vesicles will be diluted to 10 ml with Water-for-Injection® (Adcock Ingram, Critical Care, Johannesburg) and used for further experimental work. This solution showed a stable zeta potential value of -30 mV, with a low standard deviation of 1.6.

3.4.2.2 Method

A Pheroid sample (10 μl) was extemporaneously diluted to 10 ml with Water-for-Injection® and was injected into the apparatus leaving a delay of 20 seconds before starting the measurement to allow stabilization of the counter rate.

Each sample consisted of ± 5 ml and was analyzed twice, each analysis consisting of ten replicates. The apparatus was rinsed after each measurement done with 5 ml Ethanol Absolute (Merck Chemicals (Pty) Ltd., Johannesburg) and 10 ml Water-for-Injection®. All of the zeta potential measurements were made 24 hours after preparation of the emulsion.

3.4.3 Optical characterization / Turbidity

There is a relationship between the total suspended solids in the liquid and the light intensity due to particle scatter. This relationship can be determined through the development of a working curve for each specific sample. This relationship exists up to a transition point where the rate of scatter intensity no longer increases with more undissolved particles. This point is the maximum limit of the optical design of a turbidimeter. Light which is detected by the photocell that is not caused by the scattering of light by suspended particles is called "stray light". The lower limit of the optical design of a turbidimeter is dependent on the amount of stray light. Causes of stray light include reflections, scattering by dust, scratches or fingerprints on the sample cell or imperfections of the glass (Omega Engineering Inc., 2001).

3.4.3.1 Apparatus and experimental conditions

The Hach model 2100P Portable Turbidimeter (Hach Company, Colorado, U.S.A.) was used to measure the turbidity values of Pheroid samples. It was first calibrated with Formazin Primary Standard before any further measurements were made. The measurement unit for turbidity is the NTU (Nephelometric Turbidity Unit).

A concentration range was measured to obtain a turbidity range. Each measurement was made in duplicate and an average value obtained to plot on a standard curve (figure 3.1). Measurement for sample data was chosen to fall between the limits of the standard curve, thus 5 μl sample was diluted to 20 ml with Water-for-Injection®. The samples were prepared in a laminar flow cabinet and care was taken to clean the cell from any fingerprints on the surface of the cylindrical sample cell.

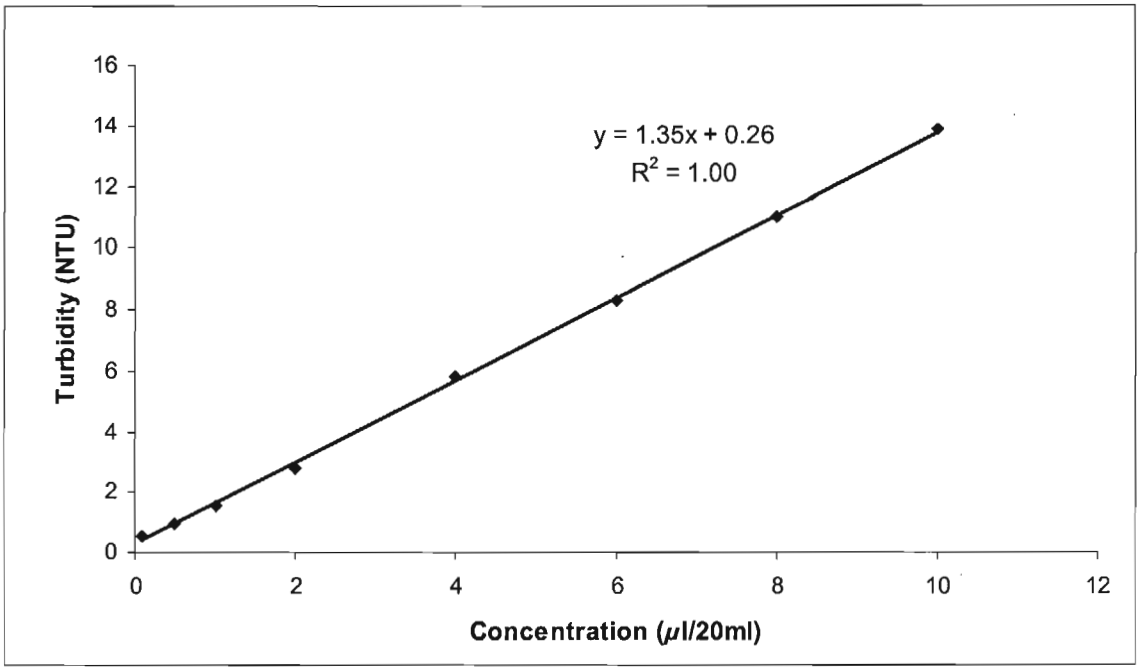


Figure 3.1 *A standard curve of turbidity values plotted against Pheroid concentration.*

3.4.3.2 Method

The measurement of the turbidity of the sample was measured relative to that of a reference cell containing Water-for-Injection® used to prepare the samples. After dilution of the samples approximately 15 ml of the diluted sample was transferred to a cylindrical sample cell. Measurement was done using the automatic range selection of the turbidimeter. Turbidity measurements were made in duplicate and 24 hours after the preparation of the emulsion.

3.4.4 Confocal laser scanning microscopy

To aid with visualizing of the formed Pheroid vesicles, a confocal laser scanning microscope (CLSM) was used. The advantage of the use of this analytical method over and above normal light microscopy is the ability to be able to view the fluorescent stained vesicles at varying depths. This is achieved by labeling the sample with a fluorescent marker, a molecule that enters an excited state during laser exposure (excitation) that emits photons at a specific wavelength (emission). The laser used as an energy source enables the microscope to act as an optical knife.

3.4.4.1 Apparatus and experimental conditions

The microstructure of the Pheroid vesicles was determined by a confocal laser scanning microscope (CLSM) (Nikon PCM 2000 with digital camera DMX 1200, The

Netherlands). A He/Ne laser was used with an objective of 60 x, with an emission of 568 nm. 100 μl of the Pheroid vesicles were stained with 2 μl Nile Red (Molecular Probes, Inc., U.S.A.) for 5-10 minutes. Afterwards 20 μl of the stained Pheroid vesicles were placed on a glass slide and covered with a glass cover slip. Excessive Pheroid vesicles were removed by squeezing the cover slide on tissue paper.

3.4.4.2 Method

The pinhole of the CLSM was covered with immersion oil before the glass slide was placed on the microscope. The microstructure of the Pheroid vesicles was investigated in such a way that only the fluorescent wavelength band could reach the detector system. A normal image followed by a zoom image of the Pheroid vesicles was captured to characterisize the structure of the vesicles and to establish if oil residues are present in the formulation. Images were captured 24 hours after preparation of the emulsion.

3.4.5 pH and conductivity values

The pH of a solution may be considered in terms of a numeric scale having values form 0 to 14, which expresses in a quantitive way the degree of acidity (7 to 0) and alkalinity (7 to 14). The value 7 at which the hydrogen and hydroxyl ion concentrations are about equal at room temperature is referred to as the neutral point, or neutrality (Martin, 1993:150).

3.4.5.1 Apparatus and experimental conditions

A WTW, Level 1 inoLab® pH-meter (Merck (Pty) Ltd., Johannesburg) was used for the determination of the pH and current values of the Pheroid vesicles at room temperature (25 ± 2 °C).

The pH-meter was calibrated by means of the Auto Cal Tec Method using pH 4.00 and pH 10.00 buffer solutions (Merck Chemicals (Pty) Ltd., Johannesburg). A Sen Tix® HW glass probe was used for pH measurements of the oil-in-water emulsion. After each measurement the probe was thoroughly rinsed with distilled water and stored afterwards in a potassium chloride (KCl) solution (Merck Chemicals (Pty) Ltd., Johannesburg).

3.4.5.2 Method

Approximately 10 ml of the sample was placed in a 50 ml glass beaker for the measurement procedure. Each sample was analyzed in duplicate and an average

value determined. All of the measurements were done 24 hours after preparation of the emulsion.

3.4.6 Viscosity

The viscosity of a fluid may be described simply as its resistance to flow or movement. A proper understanding of the rheological properties of pharmaceutical materials is essential to the preparation, development, evaluation and performance of pharmaceutical dosage forms (Marriott, 2002:41).

3.4.6.1 Apparatus and experimental conditions

The shear thinning and thixotropic properties of the emulsion were determined by rotational viscometry using a Brookfield® DV-II+ viscometer (Brookfield Engineering Laboratories, Inc., Middleboro, U.S.A.) following the standard test methods for rheological properties of non-Newtonian materials.

3.4.6.2 Method

The runs were performed at 25.0 ± 0.1 °C with a small sample adapter, a spindle 18 and a chamber SC-4-18. The sample volume used was 13 ml. The hysteresis loop was obtained by recording the stress values by increasing the viscometer speed stepwise from 0 rpm to 100 rpm.

The rheological study was carried out 24 hours after the preparation of the emulsion. Unfortunately due to the high percentage of water in the preparation (around 96 %); low viscosity values were obtained and no relevant data could be obtained to determine the type of flow or to make any assumptions concerning the stability of the emulsion preparation under investigation. It was decided that for the remainder of the study no further viscosity measurements would be performed on the formulations prepared.

3.5 EVALUATION OF PROCEDURE AND CONCENTRATION DEPENDANT EFFECTS ON PHEROID STABILITY

The basic method for preparing Pheroid vesicles was given in section 3.3. Several factors may have an influence on the physical characteristics and therefore the physical stability of the vesicles. This may also negatively impact on the clinical efficacy of the Pheroid vesicles. Changing the composition of the Pheroid vesicles, by changing either the water, Vitamin F Ethyl Ester CLR or Cremophor® RH 40 concentration was not yet investigated previously. The effect of procedural changes

such as varying mixing rates and temperature may also impact on the physical stability and clinical efficacy of the Pheroid vesicles. Evaluation of clinical efficacy do not form part of this study but this investigation is a first attempt to investigate composition and procedural changes in the method of manufacturing of Pheroid vesicles. The effect of these factors on the physical characteristics and stability may provide useful information for future studies or when changes in apparatus are necessary. The sections below give an explanation of the different factors which will be investigated.

3.5.1 Mixing rates

The samples were prepared according to the basic procedure explained in section 3.3. During this experiment, everything was kept constant except the emulsification rate. Three samples were prepared by varying the mixing rates to 8000 rpm, 13500 rpm and 24000 rpm. Each of the 100 ml samples prepared consisted of 2.80 g Vitamin F Ethyl Ester CLR, 1.00 g Cremophor® RH 40 and 0.20 g DL-α-Tocopherol as the oil phase and 96.00 g of milli Q water, saturated with nitrous oxide for four days, as the water phase. The manufacturing sheet is presented in annexure B.1.1.

3.5.2 Mixing times

The samples were prepared according to the basic procedure explained in section 3.3. During this experiment, everything was kept constant except the emulsification time. Three samples were prepared, with a fixed mixing rate of 13500 rpm, varying the mixing times to 60 seconds, 120 seconds and 300 seconds. Each of the 100 ml samples prepared consisted of 2.80 g Vitamin F Ethyl Ester CLR, 1.00 g Cremophor® RH 40 and 0.20 g DL-α-Tocopherol as the oil phase and 96.00 g of milli Q water, saturated with nitrous oxide for four days, as the water phase. The manufacturing sheet is presented in annexure B.1.2.

3.5.3 Water phase temperatures

The samples were prepared according to the basic procedure explained in section 3.3. During this experiment, everything was kept constant except the water phase temperature. Five samples were prepared, with a fixed mixing rate of 13500 rpm and a mixing time of 120 seconds. The temperature of the water phase was 55, 65, 75, 85 or 95 °C. Each of the 100 ml samples prepared consisted of 2.80 g Vitamin F Ethyl Ester CLR, 1.00 g Cremophor® RH 40 and 0.20 g DL-α-Tocopherol as the oil phase and 96.00 g of milli Q water, saturated with nitrous oxide for four days, as the

water phase. The manufacturing sheet is presented in annexure B.1.3.

3.5.4 Number of days gassed

The samples were prepared according to the basic procedure explained in section 3.3. During this experiment, everything was kept constant except the number of days the water was gassed. Four samples were prepared, with a fixed mixing rate of 13500 rpm and a mixing time of 120 seconds. The water phase temperature was 75 °C. Each of the 100 ml samples prepared consisted of 2.80 g Vitamin F Ethyl Ester CLR, 1.00 g Cremophor® RH 40 and 0.20 g DL-α-Tocopherol as the oil phase and 96.00 g of milli Q water, gassed for 0, 1, 3 or 4 days, as the water phase. The manufacturing sheet is presented in annexure B.1.4.

3.5.5 Cremophor® RH 40 concentration

The samples were prepared according to the basic procedure explained in section 3.3. During this experiment, everything was kept constant except the concentration range of the Cremophor® RH 40. Five samples were prepared, with a fixed mixing rate of 13500 rpm and a mixing time of 120 seconds. The water phase temperature was 75 °C. Each of the 100 ml samples prepared consisted of 2.80 g Vitamin F Ethyl Ester CLR, varying concentrations (0.25, 0.50, 1.00, 2.00 and 4.00 g) of Cremophor® RH 40 and 0.20 g DL-α-Tocopherol as the oil phase and varying concentrations (to keep the total volume at 100 ml) of the milli Q water, 4 days saturated with nitrous oxide, as the water phase. The manufacturing sheet is represented in annexure B.1.5.

3.5.6 Vitamin F Ethyl Ester CLR concentration

The samples were prepared according to the basic procedure explained in section 3.3. During this experiment, everything was kept constant except the concentration range of the Vitamin F Ethyl Ester CLR. Six samples were prepared, with a fixed mixing rate of 13500 rpm and a mixing time of 120 seconds. The water phase temperature was 75 °C. Each of the 100 ml samples prepared consisted of varying concentrations (1.00, 2.00, 2.50, 2.80, 3.25 and 3.50 g) of Vitamin F Ethyl Ester CLR, 1.00 g of Cremophor® RH 40 and 0.20 g DL-α-Tocopherol as the oil phase and varying concentrations (to keep the total volume at 100 ml) of the milli Q water, 4 days saturated with nitrous oxide, as the water phase. The manufacturing sheet is represented in annexure B.1.6.

3.6 ACCELERATED STABILITY TEST

Based on the results obtained by the experiments described in section 3.5 a final formula was prepared. This formula shows the optimum ratio of components used to prepare the Pheroid vesicles along with the preparation procedures that has to be adhered to for optimal characteristics and stability. The formula was kept at various temperatures and humidity's throughout a 3 month period to determine the stability of the formulation. It is considered important for future projects to know the stability features of this formulation and to be able to amend the formulation process if necessary.

The final formulation was prepared according to the basic procedure explained in section 3.3. It consisted of 2.80 g of Vitamin F Ethyl Ester CLR, 1.00 g of Cremophor® RH 40, 0.20 g of DL-α-Tocopherol and 96.00 g of distilled water saturated with nitrous oxide for four days. The water phase was heated to 75 °C before adding the heated oil phase and homogenizing it for 120 seconds at a mixing rate of 13500 rpm. The formulation were transferred to amber glass bottles and sealed under an inert atmosphere (nitrogen). All of the formulations were stored either in a refrigerator at 5 °C or thermally stressed in thermostatic controlled heat cupboards at 25 °C with 60 % relative humidity or at 40 °C with 75 % relative humidity. After 1 month, 2 months and 3 months of storage at the various temperatures, samples were removed and examined for any visual changes before submitting it to the stability tests described in section 3.4. To determine if there were any changes a control sample were prepared and tested after 24 hours of storage at 6 °C. The manufacturing sheet is represented in annexure B.2. The results of this study are presented in chapter 5.

3.7 CONCLUSION

Finding the right procedure for the sample preparation of each measurement method performed is of great importance if consistent data values are to be retrieved. Each method is sensitive towards the concentration of the sample used in the apparatus. Specific dilution concentrations were determined through standard curves drawn for the Pheroid vesicles. It is important to know the limits of the apparatus used in measurements to ensure that relevant data is obtained.

Care was taken to keep the experimental conditions constant and to calibrate the apparatus used before measurements were done. All of the glassware used were of good standard and cleaned before proceeding to use them in measurements

performed. The standard of the experimental work done can be directly related to the earnestness with which the samples are prepared and tested afterwards.

CHAPTER 4

EFFECT OF PREPARATION VARIABLES ON THE PHYSICAL PROPERTIES OF PHEROID VESICLES

4.1 INTRODUCTION

Trial-and-error methodology is rampant in emulsion formulation because, all too often, the expected results are not obtained. The difficulty experienced by formulators is not entirely a reflection of the complex physicochemical interactions of the emulsion components. Emulsion processing also exerts considerable influence on the outcome (Block, 1996:78).

To formulate Pheroid vesicles (an oil-in-water emulsion) with the optimum particle size and stability features, the influence of variables in the formulation process were investigated to determine their contribution in the final product. All of the components in the formulation and the processing variables were randomly investigated and samples of each formulation were exposed to tests to determine their stability and physical characteristics (chapter 3).

The influence of each variable will be determined by the effect thereof on particle size, zeta potential, turbidity, visual appearance by CLSM and pH and current of the emulsion evaluated. The interpretation of the results obtained from these evaluations provided some insight into the choice of experimental procedures and excipient concentrations that were employed in formulating an optimal Pheroid preparation for accelerated stability testing.

4.2 INFLUENCE OF MIXING VARIABLES

Optimization of the manufacturing process must consider the degree of shear required to produce a product with the appropriate particle size distribution. The formulator should keep in mind that it is possible to adjust mixing equipment to satisfy product specifications, although it may be at cost of increased energy requirements, lengthier processing time, or poorer performance (Block, 1996:80).

4.2.1 Mixing rate

4.2.1.1 Introduction

The influence of variable speeds of the homogenizer (Heidolph Diax 600), used to prepare the Pheroid vesicles, was investigated. It was done to investigate the

influence of the increased shear-rate and shear-stress (8000 rpm, 13500 rpm and 24000 rpm) on the particle size distribution and on overall stability of the formulations. It was also done to investigate the possibility of over processing the emulsion system and the influence there of. The results for all the stability parameters were obtained 1, 2, 3, 7, 14, 21 and 28 days after preparation of the samples, with exception of the pH and current values that were only measured 1 day after preparation and the confocal laser scanning microscopy (CLSM) images that were taken on the 1st day after preparation and thereafter again after 12 weeks of storage. All of the samples were withdrawn from the same sample bottle that was kept throughout the study period at ± 6 °C. Results are presented in Annexure C.1.1.

4.2.1.2 Particle size analysis

The particle size distribution of the three samples was determined according to the method described in 3.4.1.2. Of each measurement conducted the D(v, 0.5); which represents the volume size of the particles at which 50 % of the sample is smaller and 50 % is larger than this size, also known as the mass median diameter (MMD); was used to plot a graph against the different mixing rates and the different sampling periods (figure 4.1). The mean particle size of the samples decreased with an increase in the mixing rate. In general, a lower tension or an increase in stirring energy and duration are expected to increase the break up of particles. The sample prepared at a mixing rate of 8000 rpm was characterised by a relative stable mean particle size between 1.630 - 1.720 μm over the 28 day sampling period. The mean particle size remained more or less stable over the first three days of sampling after which it slightly decreased (Annexure C.1.1). This phenomenon may be due to further self-emulsification in the preparation that breaks the larger droplets into two or more smaller droplets. A mixing rate of 13500 rpm resulted in mean particle sizes between 1.060 - 1.120 μm. The sample that was prepared at a mixing rate of 24000 rpm showed the smallest mean particle size (0.810 - 0.850 μm). The mean particle sizes remained stable throughout the four weeks of measurements for the samples prepared at 13500 rpm and 24000 rpm. This experiment clearly shows that a higher mixing rate produces smaller particles.

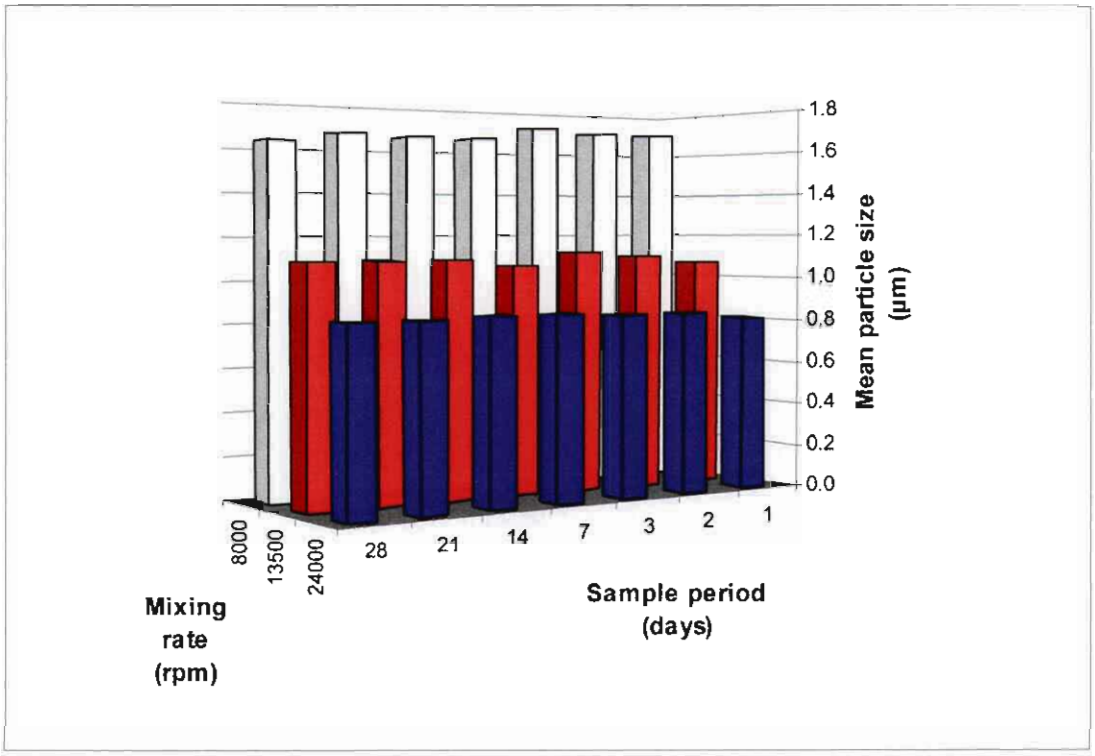


Figure 4.1 *The influence of different mixing rates on the mass median diameter (MMD) of Pheroid vesicles.*

4.2.1.3 Zeta potential

The zeta potential values of the three samples were obtained according to the method described in 3.4.2.2. Of each measurement conducted the average zeta potential value obtained from the 10 replicates measured was used to plot a graph against the different mixing rates and the sampling periods used (figure 4.2).

From figure 4.2 and Annexure C.1.1 it is evident that the zeta potential ranges between -23.80 mV and -35.80 mV for the sample prepared at 8000 rpm, between -24.40 mV and -34.40 mV for the sample prepared at 13500 rpm and between -26.00 mV and -35.70 mV for the sample prepared at 24000 rpm. In general, figure 4.2 indicates that a slight increase in zeta potential occurred after manufacturing up to 7 days where after the zeta potential remains relative consistent for the remainder of the test interval for all three samples. No major differences between samples, prepared at different mixing rates, were observed over the test period. In general a zeta potential above 20 - 25 mV is considered sufficient in an emulsion system to provide steric repulsion between droplets to prevent creaming of the droplets. The data described in this experiment suggest that different mixing rates did not influence the zeta potential of the Pheroid vesicles to a major extend and that sufficient values were obtained to ensure droplet repulsion and that mixing rate is a more important

factor in obtaining different particle size distributions.

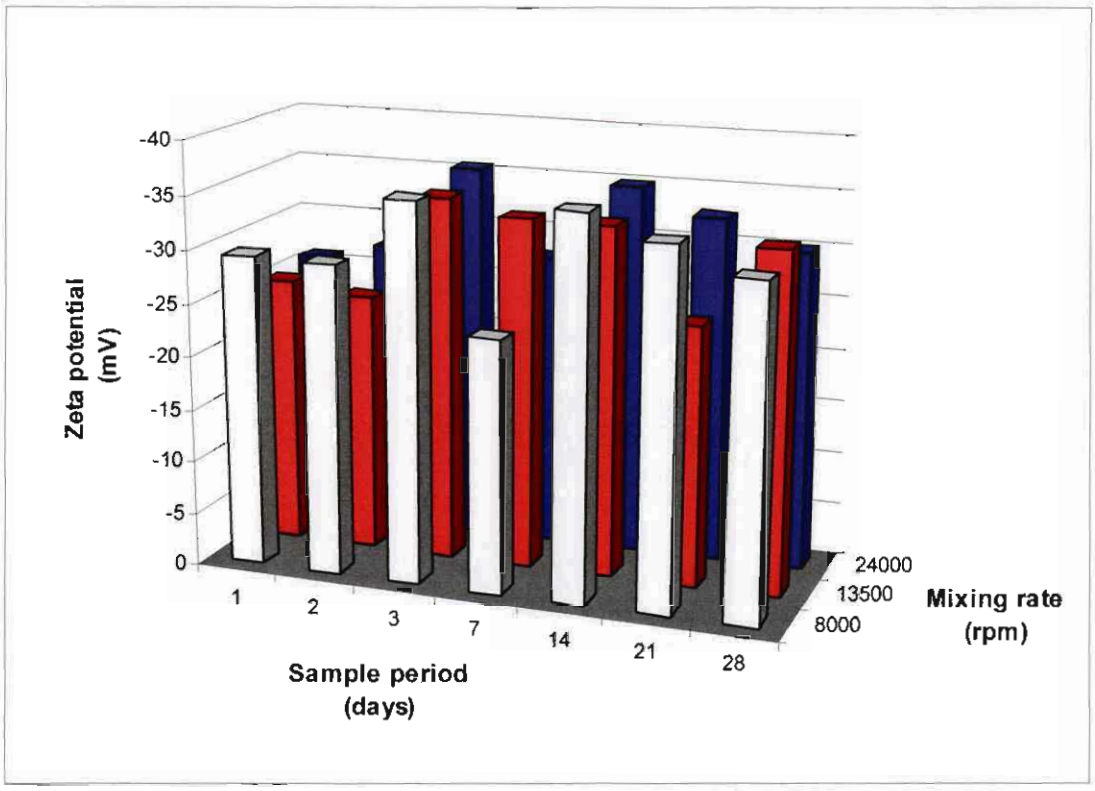


Figure 4.2 *The influence of different mixing rates on the zeta potential of Pheroid vesicles.*

4.2.1.4 Turbidity

The turbidity values of the three samples were obtained according to the method described in 3.4.3.2. Of each measurement conducted the turbidity value used to plot against the different mixing rates over the sampling period, was obtained through deduction of the average value of the reference (Water-for-Injection®), from the average turbidity values measured (figure 4.3).

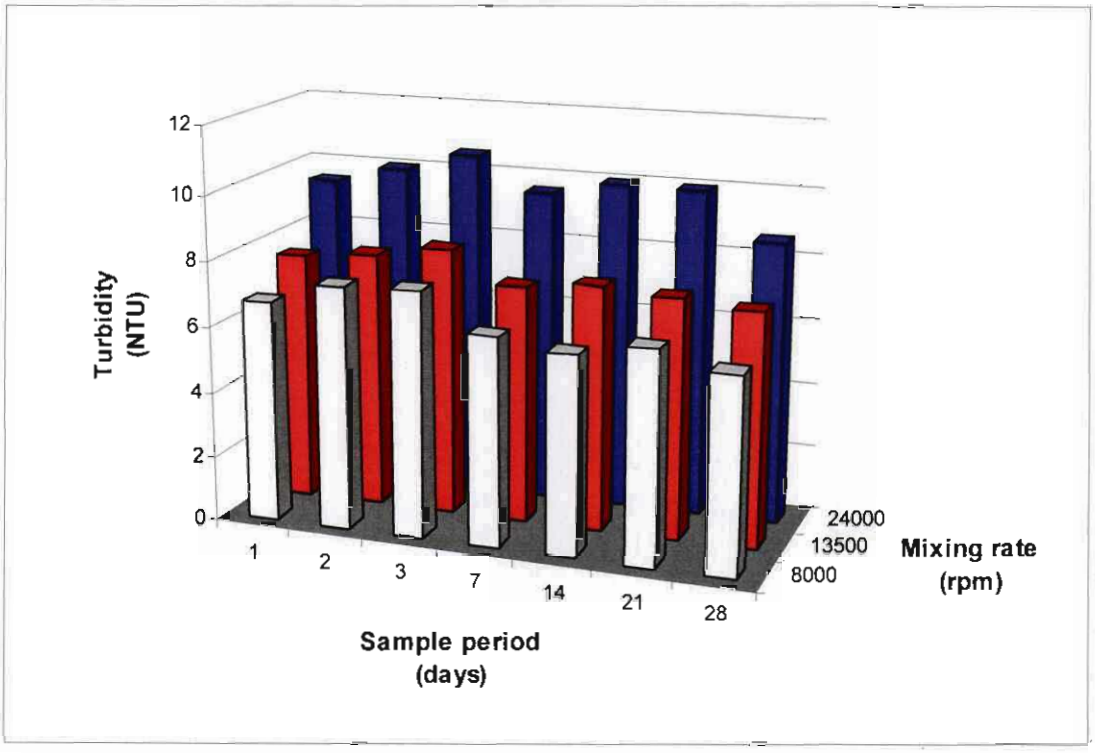


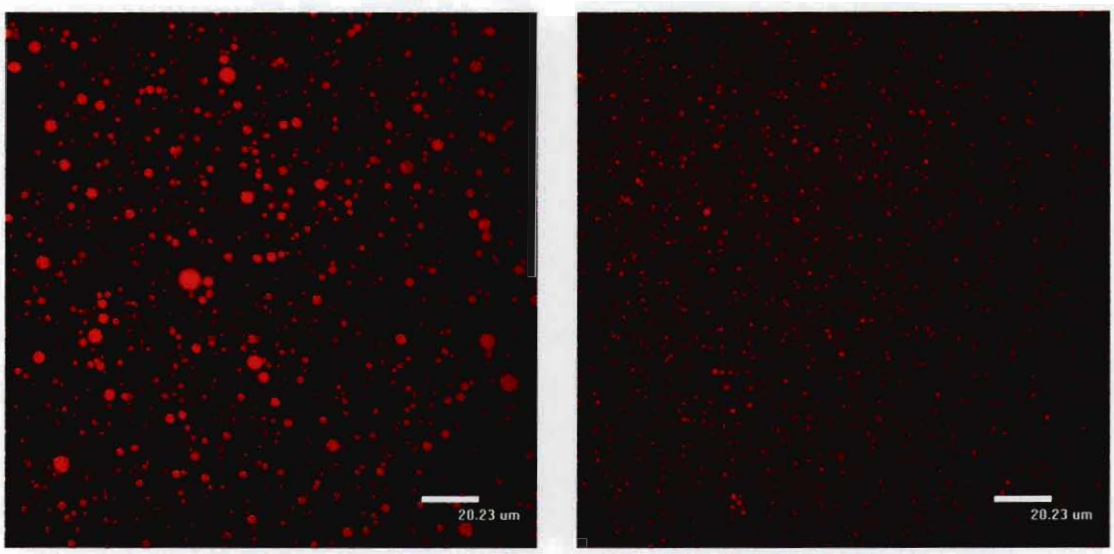
Figure 4.3 *The influence of different mixing rates on the turbidity of Pheroid vesicles.*

The turbidity values obtained are found to be the direct inverse of the mean particle size values obtained. A higher turbidity value is linked to a smaller particle size value as can be seen for the sample with the smallest particle size (24000 rpm). The rest of the values show the same tendency with the sample prepared at 13500 rpm showing the second highest turbidity values and the sample prepared at 8000 rpm the lowest turbidity values. The tendency of the samples to keep on dividing their particles over the first three days of sampling, followed by a slight increase in particle size on the 7th day of sampling can clearly be observed. Over the rest of the period very small variation in the data could be observed. Higher turbidity values can be explained by the increase in smaller particles or more particles whilst lower turbidity values can be explained by bigger or less particles scattered by the light source of the turbidimeter. These results are in agreement with the results obtained with the particle size analysis.

4.2.1.5 CLSM

Confocal images of the three samples were obtained according to the method described in 3.4.4.2. Figure 4.4 depict typical confocal laser scanning microscope (CLSM) micrographs of the prepared Pheroid vesicles. The micrographs confirm the data obtained with the particle size analysis. A decrease in particle size is observed between the preparation made at 8000 rpm and the preparation made at 24000 rpm.

The scale bar can be used to quantify the size of the vesicles. CLSM micrographs obtained after a storage period of 12 weeks showed similar results.



(a) (b)

Figure 4.4 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles prepared at 8000 rpm and (b) Pheroid vesicles prepared at 24000 rpm.*

4.2.1.6 pH and current values

The pH and current values (Annexure C.1.1) of the three samples were obtained according to the method described in 3.4.5.2. The data values showed a slight lowering in the pH value's of the samples with an increase in mixing rates. The sample prepared at 8000 rpm had the highest pH value of 6.54. The lowest pH value of 6.24 was found for the sample prepared at 24000 rpm while a pH value of 6.37 was found for the sample prepared at 13500 rpm.

The current values measured showed the inverse of the pH values obtained. The lowest value of 20.8 mV was found for the sample prepared at 8000 rpm and values of 39.2 and 46.2 mV were obtained for the samples prepared at 13500 rpm and 24000 rpm respectively.

4.2.1.7 Conclusion

It is difficult to determine the optimum mixing speed by evaluation of only a single parameter. Therefore the need arise to combine the above described parameters to determine the optimum mixing rate. Data combinations were made with data values obtained for the mean particles sizes (P) of the vesicles and the zeta potential values (Z) and turbidity (T) measurements for each sample prepared (Annexure C.1.1).

The best possible formulation will be one with a small mean particle size, a high zeta potential and a high turbidity. All of the data combinations chosen took this into account in an effort to select the best possible formulation.

The best graphic indication of the influence of mixing speed was obtained when the turbidity and mean particle size parameters were combined. The results depicted in figure 4.5 indicate that the best possible values are obtained with the sample prepared at 24000 rpm. These values showed the highest turbidity and the smallest mean particle size that can be obtained by varying the mixing rate.

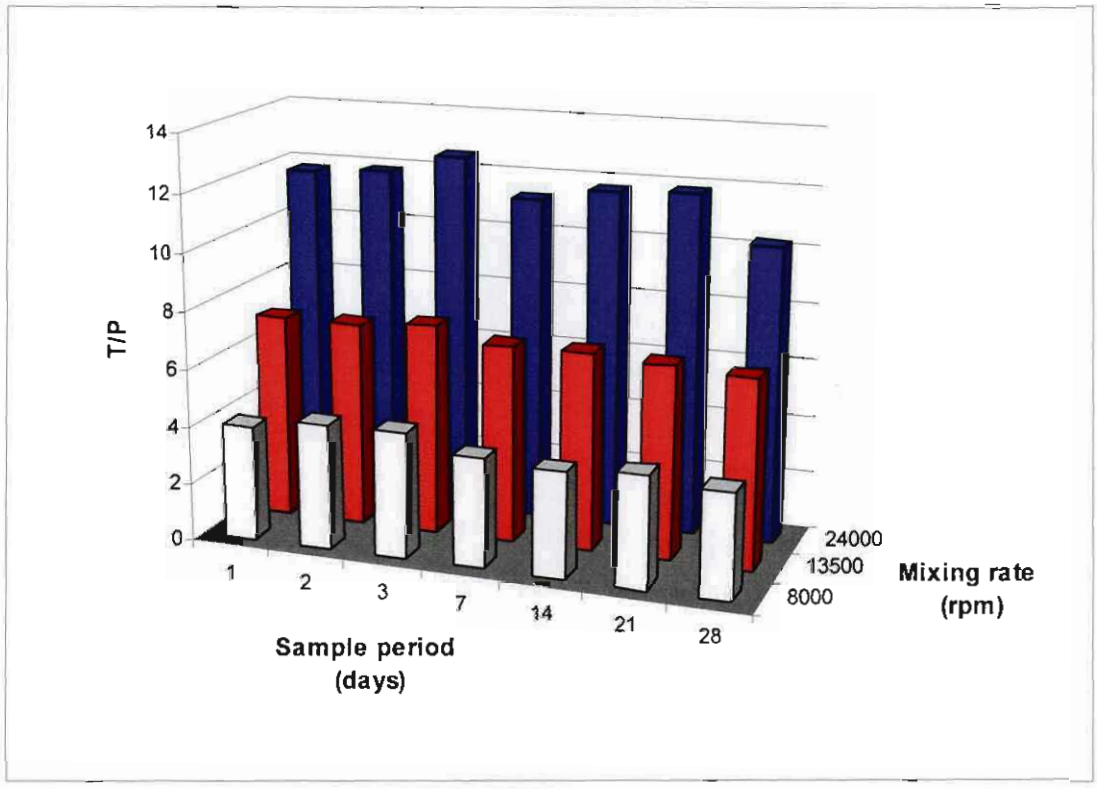


Figure 4.5 *The influence of different mixing rates on the combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

The same trends were observed with other parameter combinations such as TZ and ZP, although not as clear as in the graph of T/P (figure 4.5). The reason for this may be found in the fact that no clear trends were observed in the zeta potential data between different mixing speeds.

It became clear from all the data obtained for the three different mixing rates used to prepare samples of the Pheroid vesicles, that vesicles prepared at 24000 rpm provided the best formulation. However the mixing rate chosen for the final formulation to be put on accelerated stability and for further investigation was 13500 rpm. This mixing rate also proved to give small particle sizes that were stable

throughout the 28 day sampling period with a high zeta potential and intermediate turbidity values. The reason for choosing 13500 rpm was to ensure that less of the nitrous oxide was lost during the mixing procedure that could prove to be fatal in the working mechanism of the Pheroid vesicles that greatly depends on nitrous oxide for delivery of active substances. Further experimentation done will confirm the influence of processing variables on the nitrous oxide content of the formula under investigation.

4.2.2 Mixing time

4.2.2.1 Introduction

According to Block (1996:79) the duration of processing can affect emulsion stability. Repeated processing or cycling resulted in a decrease in average particle size and in narrowing of the particle size distribution, hence improving the emulsion quality. The influence of variation in the mixing time of the components in the formulation were evaluated by preparing three samples with mixing times of 60, 120 and 300 seconds at 13500 rpm. The influence of this variable on the stability and characteristics of the Pheroid vesicles are described below. Results are summarized in Annexure C.1.2.

4.2.2.2 Particle size analysis

Figure 4.6 showed that the mean particle size varied between 7.250 - 7.710 μm for the sample prepared with 60 seconds of mixing time. The individual analysis reports shows a bimodal presentation (data not shown) which Salager (2000:80) explains can be related to the mixing of two emulsions or to improper mixing. The preparation mixed for 120 seconds showed a mean particle size between 3.040 - 3.190 μm and the preparation mixed for 300 seconds had a mean particle size between 1.740 - 1.850 μm. No major deviation was found in the mean particle size of the three samples measured over the 28 day time period. From this results it is clear that mixing times of either 120 seconds or 300 seconds are sufficient to form Pheroid vesicles below 5 µm and that a mixing time of 60 seconds are not suitable for preparing Pheroid vesicles with a mean particle size below 5 µm.

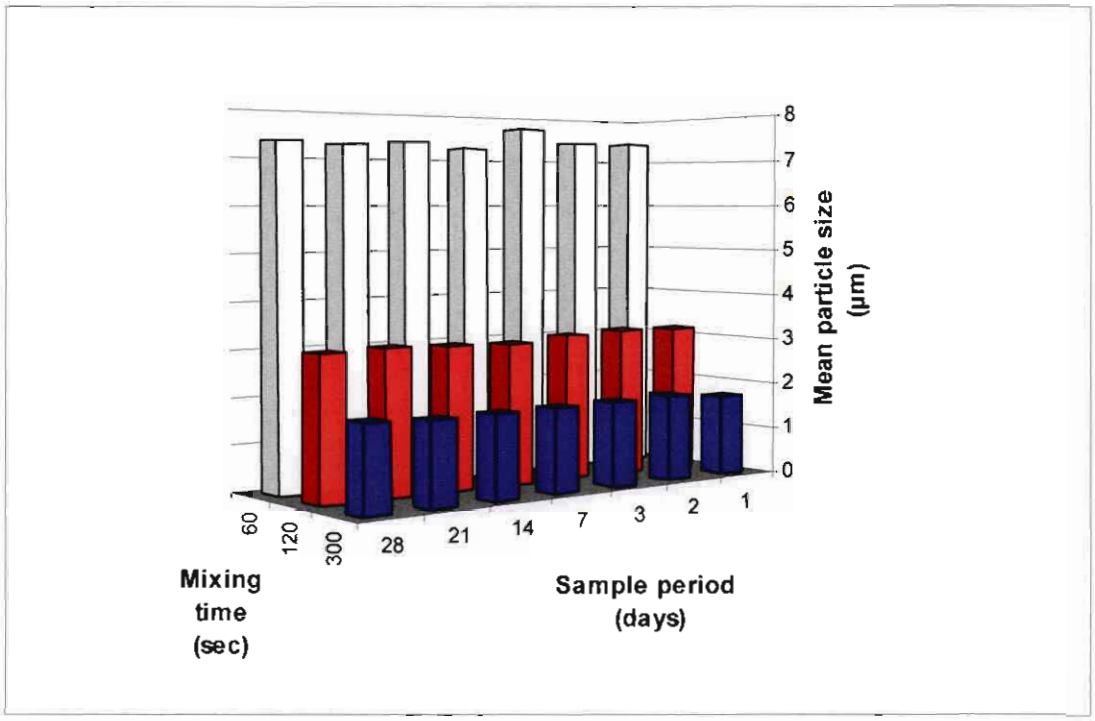


Figure 4.6 *The influence of different mixing times on the mass median diameter (MMD) of Pheroid vesicles.*

4.2.2.3 Zeta potential

As was observed for the effect of mixing rates (figure 4.2) only a general trend was observed for the zeta potential between the samples prepared with different mixing times (figure 4.7). From figure 4.7 and Annexure C.1.2 it is evident that the zeta potential range between -26.15 mV and -40.70 mV for the preparation mixed for 60 seconds, between -21.45 mV and -33.85 mV for the preparation mixed for 120 seconds and between -18.95 mV and -35.90 mV for the preparation mixed for 300 seconds. In general a slight increase in the zeta potential is observed after manufacturing of all three samples up to 7 - 14 days where after the zeta potential values remain relative consistent for the rest of the test period (28 days). However all the values obtained is considered to be acceptable zeta potential values within emulsion systems. As with the results obtained with mixing rates (4.2.1.3) it is clear that particle size is an important parameter when studying the effect of different mixing times and that the mixing times selected in this experiment did not influence the zeta potential to a great extend. However the interaction between particle size and zeta potential together may influence overall stability over extended time periods and under stress conditions more drastically as observed in this experiment.

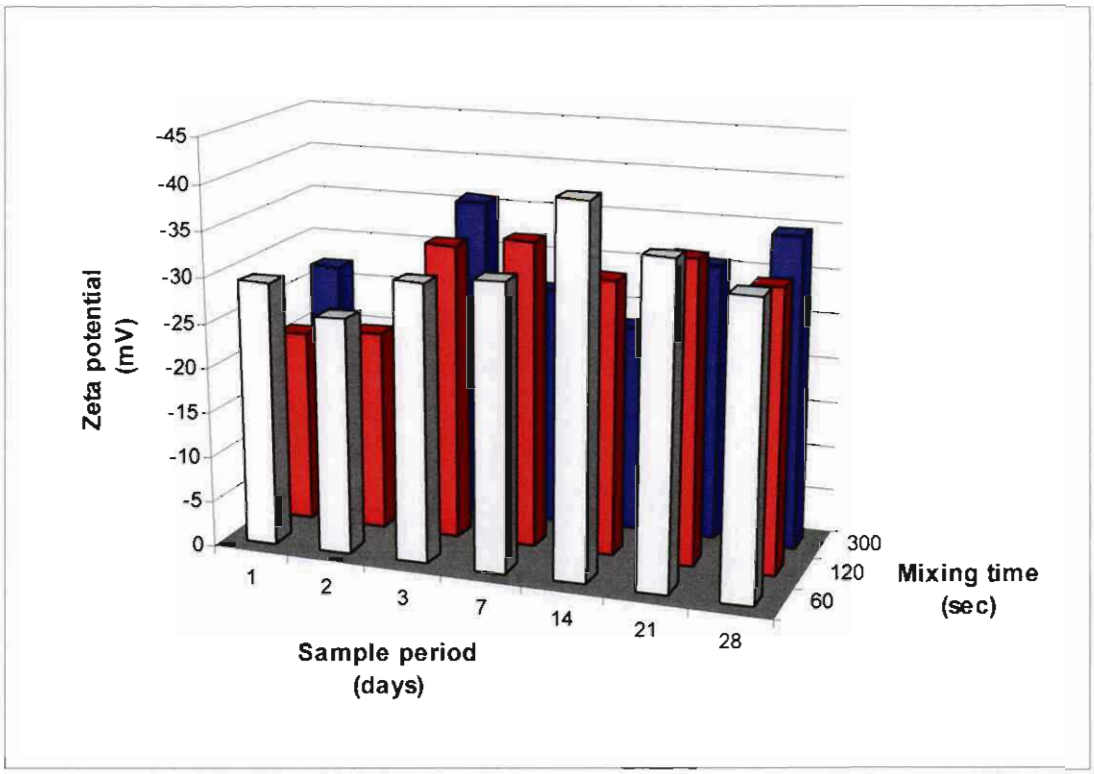


Figure 4.7 *The influence of different mixing times on the zeta potential of Pheroid vesicles.*

4.2.2.4 Turbidity

Figure 4.8 show the results obtained for the turbidity measurements. It was found that the particles with the smallest particle size (300 seconds mixing time) did not show the highest turbidity values as expected. The preparation mixed for 60 seconds showed the highest turbidity value throughout the 28 day period with the exception of day 7 where there was a reduction in the turbidity value. No clear explanation for the obtained results could be given at this stage and further experimentation is needed to clarify this results.

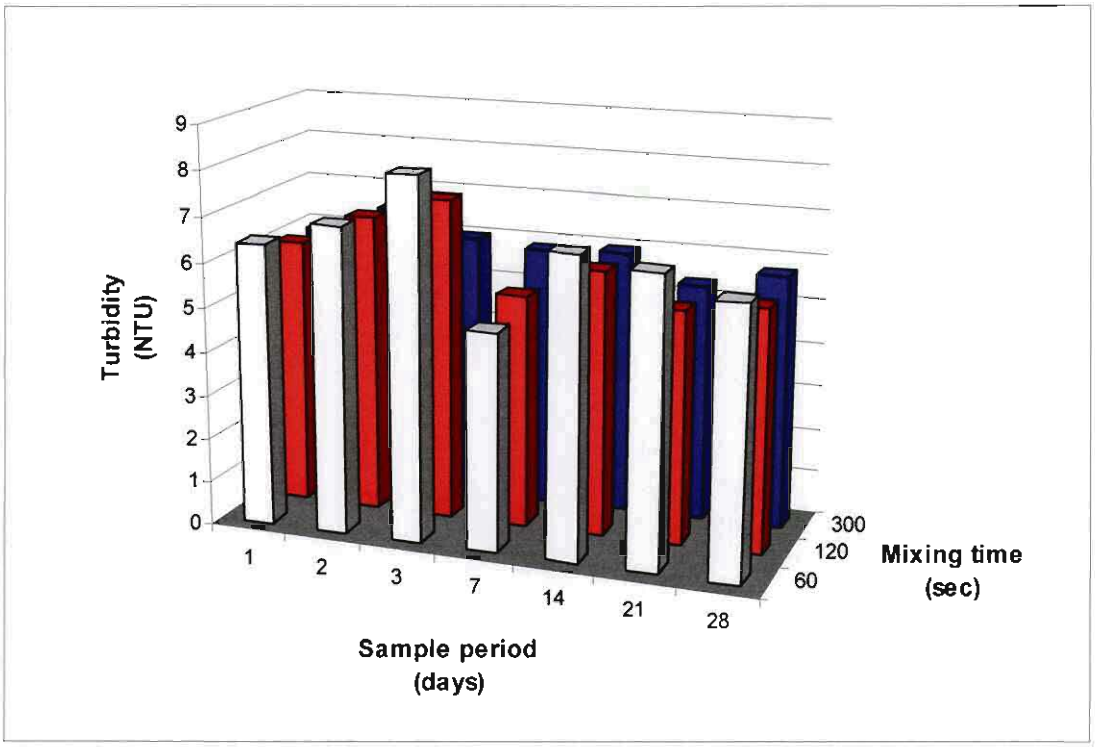
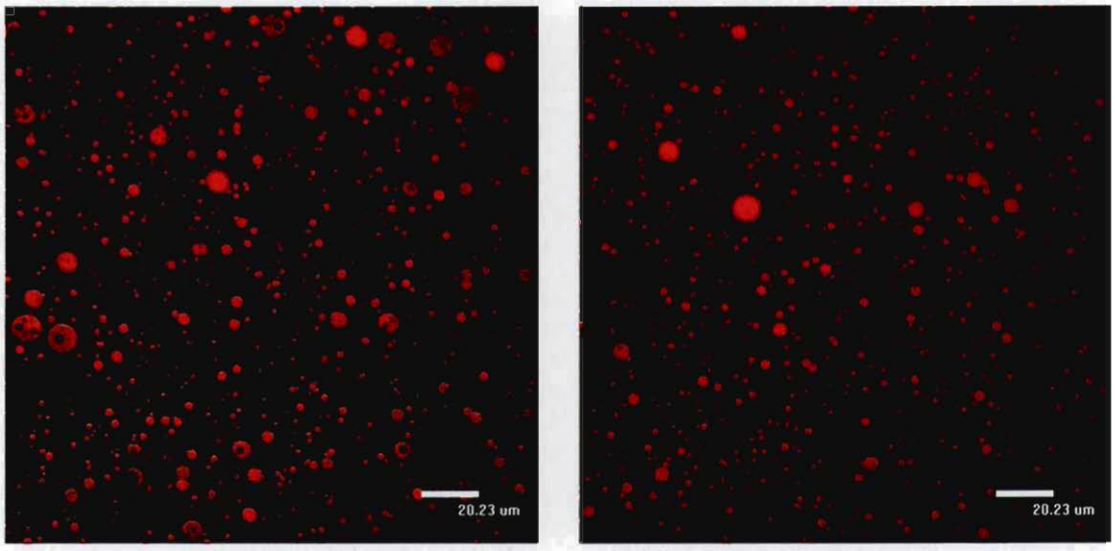


Figure 4.8 *The influence of different mixing times on the turbidity of Pheroid vesicles.*

4.2.2.5 CLSM

The influence of different mixing times on the size of the Pheroid vesicles can be seen in figure 4.9. In general smaller vesicles were obtained at longer mixing times. An interesting feature commonly known as depots is evident in the preparation mixed for 60 seconds. One large droplet consisting out of a number of smaller droplets within it. This might be due to the fact that the emulsion has not been exposed to enough shear forces to fully emulsify the oil phase in the water phase. The preparation mixed for 300 seconds contained Pheroid vesicles that are uniform in size and fully emulsified.



(a) (b)

Figure 4.9 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles mixed for 60 seconds and (b) Pheroid vesicles mixed for 300 seconds.*

4.2.2.6 pH and current values

The pH values for the three preparations were between 6.02 and 6.50. The preparation mixed for 60 seconds had a pH of 6.02 whilst the preparation mixed for 120 and 300 seconds had pH values of 6.50 and 6.42 respectively.

The current values increased as the mixing time decreased. The preparation mixed for 60 seconds measured 55.0 mV, whilst the preparation mixed for 120 seconds had a current of 40.0 mV. The preparation mixed for 300 seconds had a current value of 35.4 mV.

4.2.2.7 Conclusion

The turbidity and mean particle size values of the prepared samples give the best data combination to determine the optimum mixing time (figure 4.10). The best formulation of the three samples is the preparation mixed for 300 seconds which shows the largest value for this data combination set. The preparation mixed for 60 seconds show the least promising combination data values.

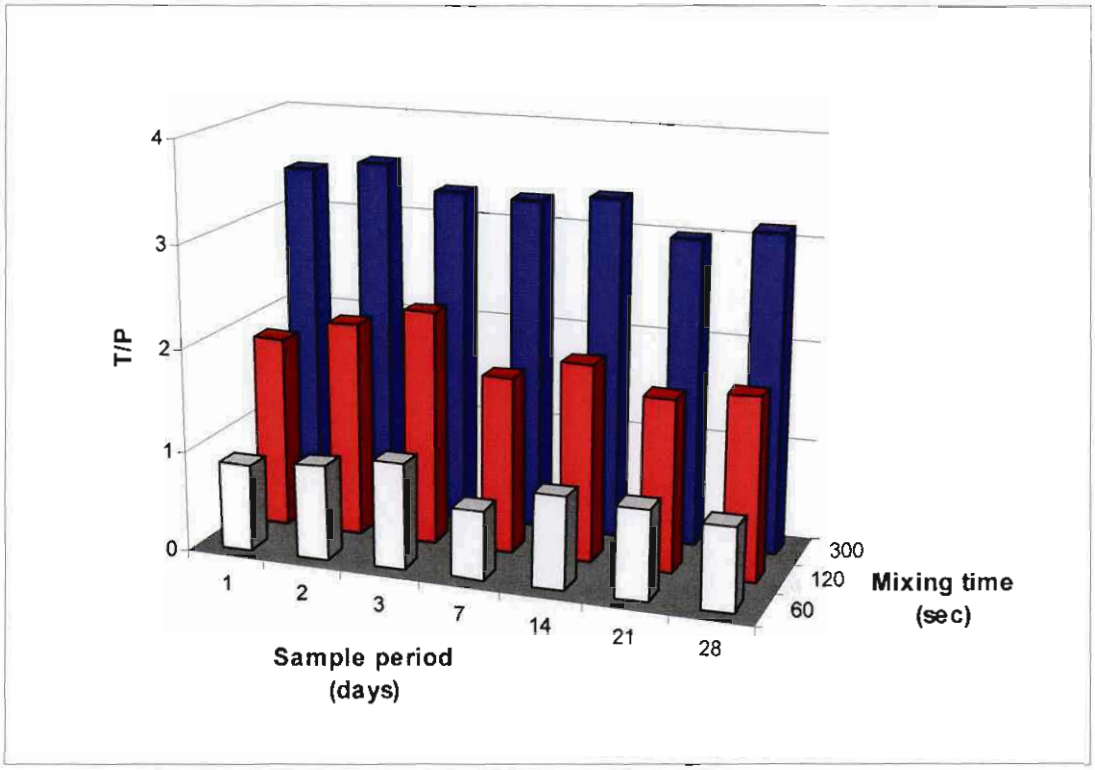


Figure 4.10 *The influence of different mixing times on the combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

The best mixing time used to prepare the Pheroid vesicles seems to be 300 seconds. It gave the smallest particle sizes but the zeta potential measurement showed some variation while the turbidity values were the lowest of the three samples. In contrast to this the preparation mixed for 120 seconds gave intermediate mean particles size values with acceptable zeta potential values of above -25.0 mV and turbidity values within 1 NTU of the preparation mixed for 60 seconds. The preparation mixed for 60 seconds showed the highest zeta potential and turbidity values but unfortunately the high mean particle sizes around 8.00 µm makes it less useful as an option for the final formula. The final decision to use a mixing time of 120 seconds came down to the decision of how many gas can be afforded to be lost in the preparation process. The preparation mixed for 120 seconds showed acceptable characteristics without the necessity to expose the formula to a further 180 seconds of shearing.

4.3 INFLUENCE OF THE WATER PHASE TEMPERATURE

4.3.1 Introduction

It is common practice in emulsion formulation to heat the water phase to 75 ± 1 °C before adding it to the heated oil phase (Nielloud, *et al.*, 2003:611; Prinderre, *et al.*, 1997:74; Carlotti, *et al.*, 1992:246). Five samples, prepared at five different

temperatures by varying the temperature with 10 °C, from 55 °C to 95 °C, were prepared and characterised for changes in the stability of the Pheroid vesicles. A mixing rate of 13500 rpm for 120 seconds was used to prepare the samples. The obtained results are given in Annexure C.1.3.

4.3.2 Particle size analysis

An increase in the temperature of the nitrous oxide satured water phase of the formulation, before adding the heated oil phase, resulted in a decrease of the mean particle sizes of the samples. From Figure 4.11 and Annexure C.1.3 it is evident that the sample prepared at a temperature of 55 °C was characterised by a relative stable mean particle size around 1.550 - 1.610 µm over the 28 day sampling period. A temperature increase to 65 °C resulted in a mean particle size between 1.410 - 1.520 µm whilst the 75 °C temperature setting, resulted in a mean particle size between 1.370 - 1.470 µm. The sample that was prepared at a water phase temperature of 85 °C showed a further reduction in mean particle size to 1.240 - 1.320 µm and the highest temperature (95 °C) had the smallest mean particle size (1.220 - 1.320 µm). The reduction in mean particle size with an increase in the temperature of the water phase may be related to the loss of nitrous oxide from the water although further experimentation should be done to confirm this.

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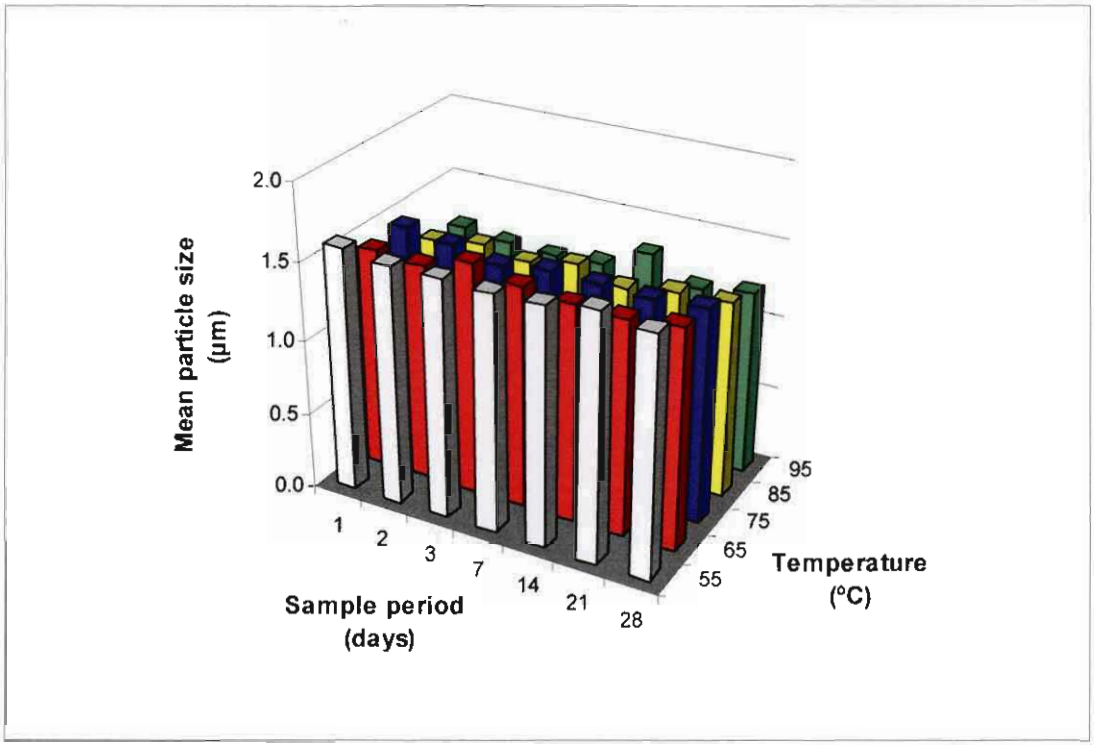


Figure 4.11 *The influence of different water phase temperatures on the mass median diameter (MMD) of Pheroid vesicles.*

4.3.3 Zeta potential

As was observed for the effect of different mixing rates (figure 4.2) and different mixing times (figure 4.7) only a general trend was observed in the zeta potential values between the samples prepared with different water phase temperatures (figure 4.12). From figure 4.12 and Annexure C.1.3 it is evident that the zeta potential range between -28.85 mV and -46.05 mV for the preparation heated to 55 °C and between -22.35 mV and -49.05 mV for the preparation heated to 65 °C. A further temperature increase to 75 °C resulted in zeta potential values between -23.65 mV and -40.75 mV and for the sample heated to 85 °C the zeta potential range between -22.50 mV and -37.65 mV whilst the zeta potential values for the sample prepared at the highest temperature (95 °C) varied between -21.55 mV and -37.55 mV. In general figure 4.12 indicate that the zeta potential remained relative consistent after manufacturing until the last day of sampling (28th day) where a general increase in stability of the lower temperature ranges were found (55, 65 and 75 °C). The preparation which showed the most fluctuation over the 28 day period was the sample prepared at 95 °C. This confirms the results obtained with the particle size analysis, that an increase in the temperature to which the water phase is heated, may result in a further loss of the nitrous oxide which forms an integral part of

the Pheroid vesicles.

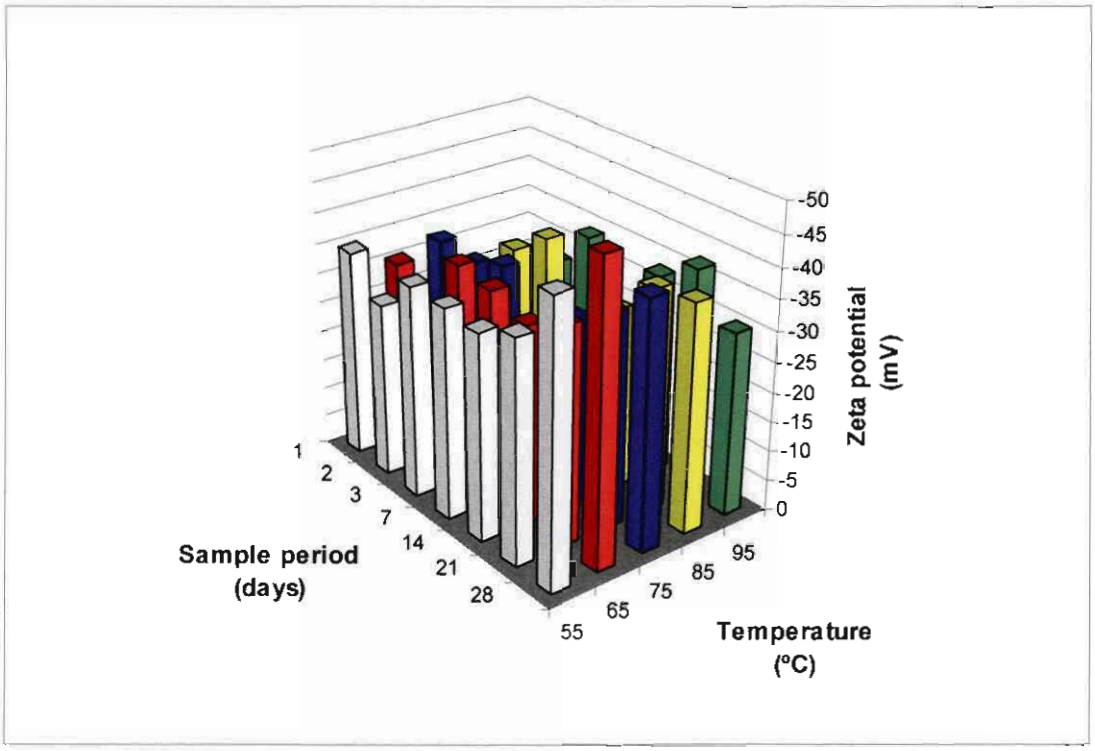


Figure 4.12 *The influence of different water phase temperatures on the zeta potential of Pheroid vesicles.*

4.3.4 Turbidity

The results obtained for the turbidity measurements for the five samples prepared at different water phase temperatures are depicted in figure 4.13. The turbidity values obtained are the direct inverse of the mean particle size values obtained (figure 4.11). A higher turbidity value is linked to a smaller particle size value as can be seen for the sample with the smallest particles (95 °C). The small deviation in data between the different temperatures and the sampling period used, suggest that the water phase temperature does not influence the stability and characteristics of the Pheroid vesicles to the same extend as the previous parameters investigated (mixing rate and mixing time).

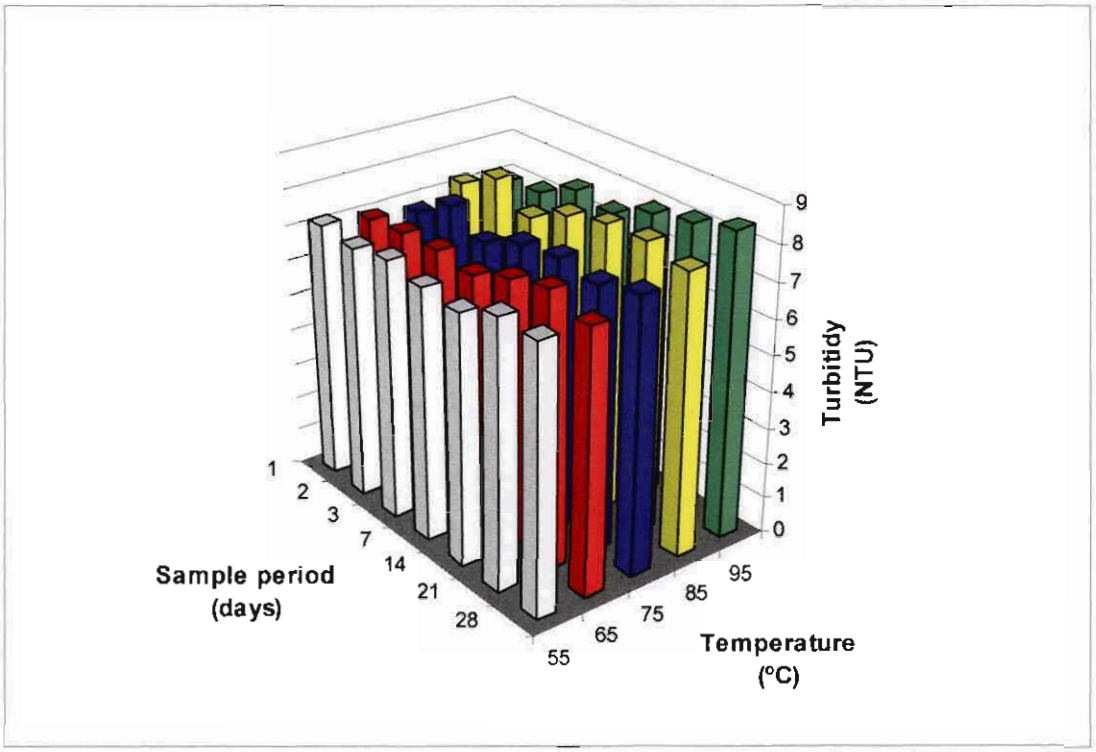
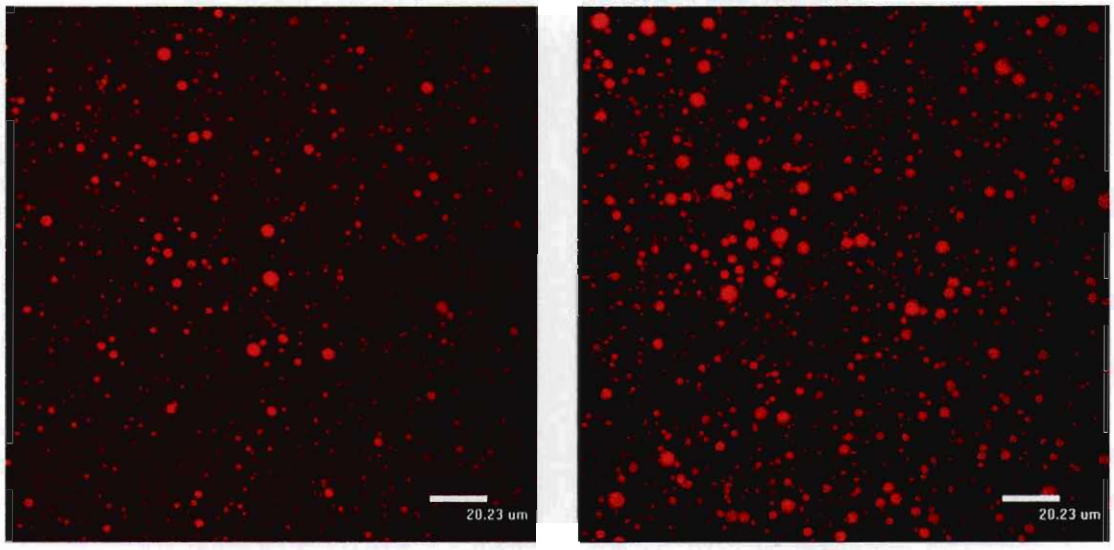


Figure 4.13 *The influence of different water phase temperatures on the turbidity of Pheroid vesicles.*

4.3.5 CLSM

Figure 4.14 contains micrographs of Pheroid vesicles for the lowest (55 °C) and the highest temperature (95 °C) used to prepare the Pheroid vesicles. The temperature to which the water phase is heated before emulsification plays an important role in the uniformity of an emulsion system. It appears that the sample prepared at 55 °C have a more even particle size distribution opposed to the sample prepared at 95 °C. From the results obtained with the particle size analysis (section 4.3.2) it were expected that the smallest particle sizes would be found in the preparation prepared at a water phase temperature of 95 °C. The micrograph obtained of the sample prepared at 95 °C seems to contain larger Pheroid vesicles than the preparation prepared at 55 °C. However, this may be due to sampling effects as it is generally accepted that particle size determination with laser diffraction (Malvern Mastersizer) is a very accurate and quantitive method. These results stress the importance of future experimental work to establish the effect of the nitrous oxide that is lost during the heating of the water on the structure and size of the Pheroid vesicles and of the sampling procedures followed while determining different parameters of the Pheroid vesicles.



(a) (b)

Figure 4.14 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles prepared at 55 °C and (b) Pheroid vesicles prepared at 95 °C.*

4.3.6 pH and current values

In general the pH values measured for the five samples prepared at different water phase temperatures seem to decrease as the temperature increases. The Pheroid vesicles still maintained a slightly acidic pH with the highest pH value of 6.48 measured for the sample prepared at 55 °C and the lowest pH value of 5.81 for the sample prepared at 95 °C. The sample prepared at 85 °C had a pH value of 6.06 while the sample prepared at 65 °C had a pH of 5.97 and the sample prepared at 75 °C a pH of 5.95.

The current values measured showed the inverse of the pH values obtained. The current values increased as the water phase temperatures increased; starting at 27.55 mV for the sample prepared at 55 °C and 56.80 mV for the sample prepared at 65 °C. The sample prepared at a water phase temperature of 75 °C had a current value of 56.85 mV whilst values of 57.15 mV and 65.20 mV were obtained for samples prepared at 85 °C and 95 °C respectively.

4.3.7 Conclusion

The best graphic indication of the influence of water phase temperatures was obtained when the turbidity and mean particle size parameters were combined (figure 4.15). Taking into account that the best possible formulation will be one with a small mean particle size, a high zeta potential and a high turbidity, the results depicted in figure 4.15 indicate that the best possible values are obtained with the sample

prepared at 95 °C, even though a lot of fluctuation in the data is found over the 28 day period.

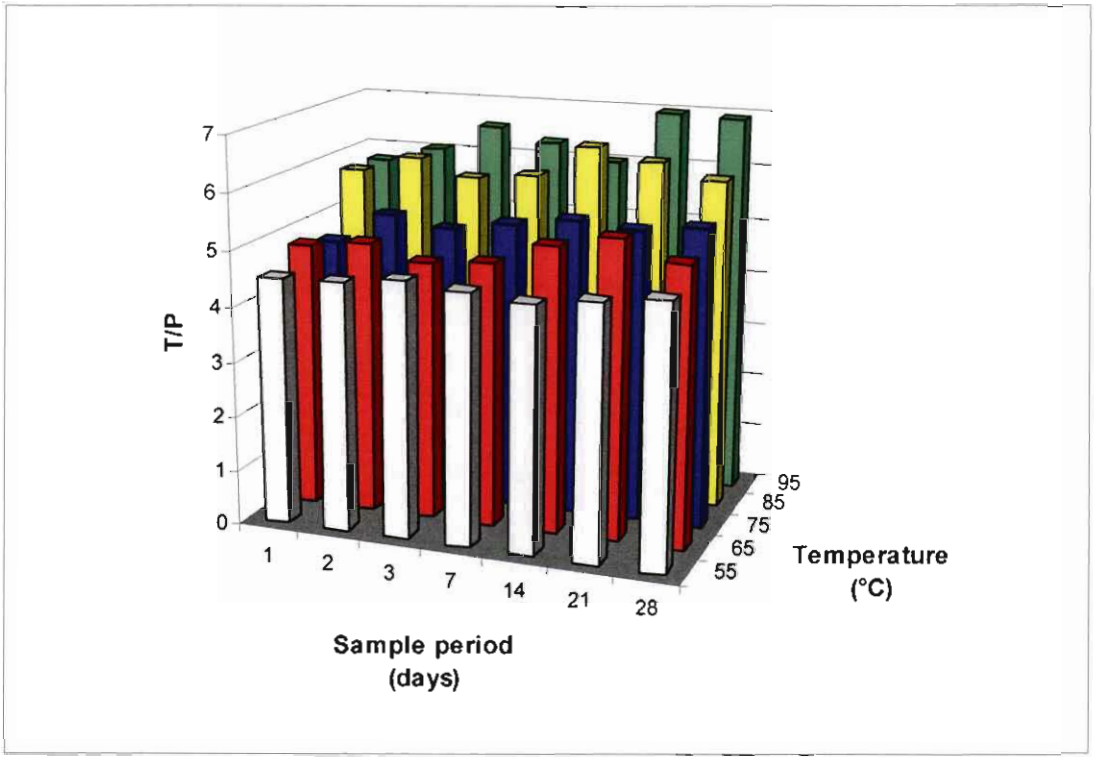


Figure 4.15 *The influence of different water phase temperatures on the combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

Since no clear trends were observed with the zeta potential, graphs containing this parameter is not shown. The water phase temperature during emulsification is considered important because the effect of the loss of nitrous oxide during heating on the characteristics of the Pheroid vesicles is not yet known. For this reason it was decided that a water phase temperature of 75 °C will be used in all further experiments. The reason for rather choosing 75 °C above the preparation heated to 95 °C also rests upon the knowledge of this being the temperature most often used during emulsification (4.3.1).

4.4 INFLUENCE OF THE NUMBER OF DAYS GASSED

4.4.1 Introduction

In Chapter 1 Saunders *et al.* (1999:105) hypothesized that the presence of nitrous oxide in the formulation may enhance both intercellular lipid fluidity, as well as the fluidity of the Pheroid vesicles. It does not per say tell us enough about the influence of this component on the stability of the Pheroid vesicles but it is believed that the nitrous oxide forms an integral part of the structure of the Pheroid vesicles and

therefore is a very important component to evaluate. To help understand the influence of the gassing period on the stability and characteristics of the Pheroid vesicles, a number of days from 0 days to 4 days, were chosen as the period to which the milli Q water would be exposed to the nitrous oxide gas. A mixing rate of 13500 rpm for 120 seconds with a water phase temperature of 75 °C was used to prepare the samples. The obtained results are given in Annexure C.1.4.

4.4.2 Particle size analysis

From figure 4.16 and Annexure C.1.4 it is evident that an increase in the number of days the water was gassed resulted in a slight increase in the mean particle size of the Pheroid vesicles. The sample prepared without gas had a mean particle size between 1.130 - 1.260 μm and the sample gassed for 1 day showed a mean particle size between 1.150 - 1.200 μm over the 28 day sampling period. The mean particle size of the sample gassed for 3 days varied between 1.180 - 1.240 μm whilst the sample gassed for 4 days had the highest mean particle size (1.320 - 1.410 μm). The mean particle size of the sample prepared without gas and the sample gassed for 1 day remained stable throughout the four weeks of measurements. The samples prepared with a 3 day and 4 day gassing period showed an increase in mean particle size on the 7th day of sampling after which the mean particle size remained stable until the 28th day of sampling. These results confirm the particle size analysis for samples prepared at different water phase temperatures (4.3.2), where an increase in temperature were believed to be the cause for a loss of nitrous oxide thus resulting in a smaller mean particle size of the Pheroid vesicles.

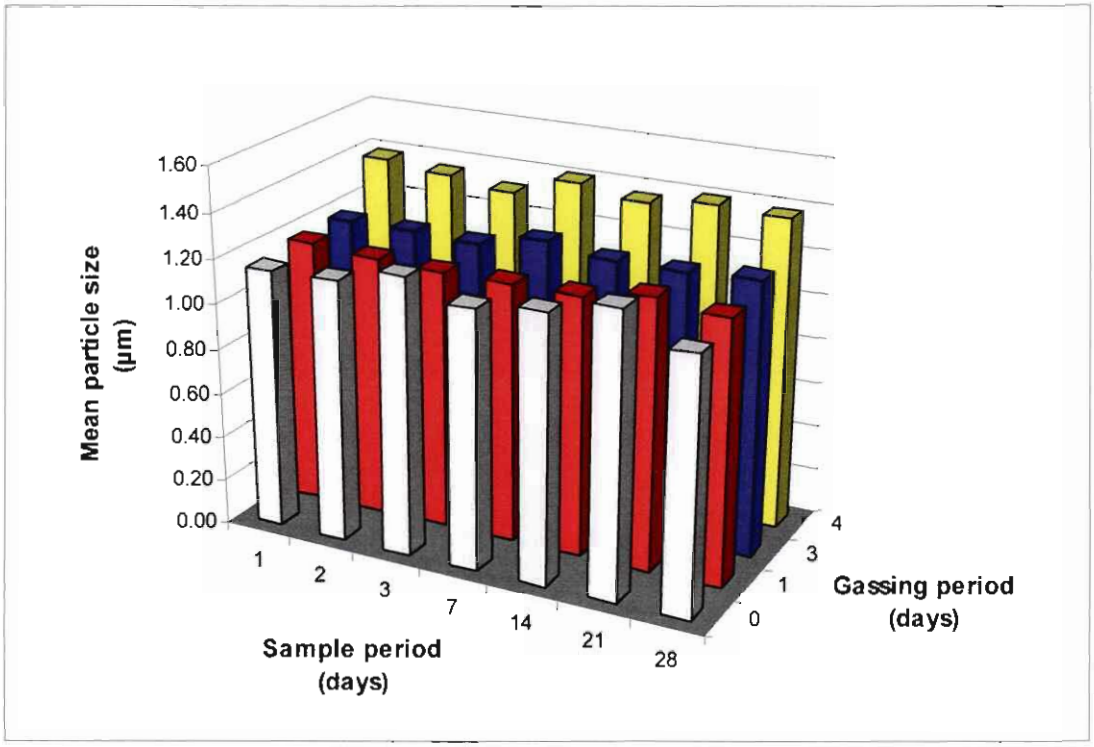


Figure 4.16 *The influence of different number of days gassed on the mass median diameter (MMD) of Pheroid vesicles.*

4.4.3 Zeta potential

From Figure 4.17 and Annexure C.1.4 only a general trend in zeta potential values was observed for the samples prepared with different number of days gassed. The zeta potential of the preparation without nitrous oxide was between -31.35 mV and -54.10 mV and the zeta potential of the preparation with a gassing period of 1 day was between -32.85 mV and -51.00 mV. The 3 day gassing period resulted in zeta potential values between -31.90 mV and -39.30 mV whilst a 4 day gassing period resulted in zeta potential values between -24.15 mV and -39.15 mV. In general figure 4.17 indicate that the zeta potential remained relative consistent after manufacturing until the 21st day where a relative high increase in the zeta potential of the 0 day and 1 day gassing preparations were found. This may be attributed towards the method used to analyze the zeta potential since the values obtained on the 28th day of sampling correlated with measurements performed earlier for these two preparations (0 day and 1 day gassing).

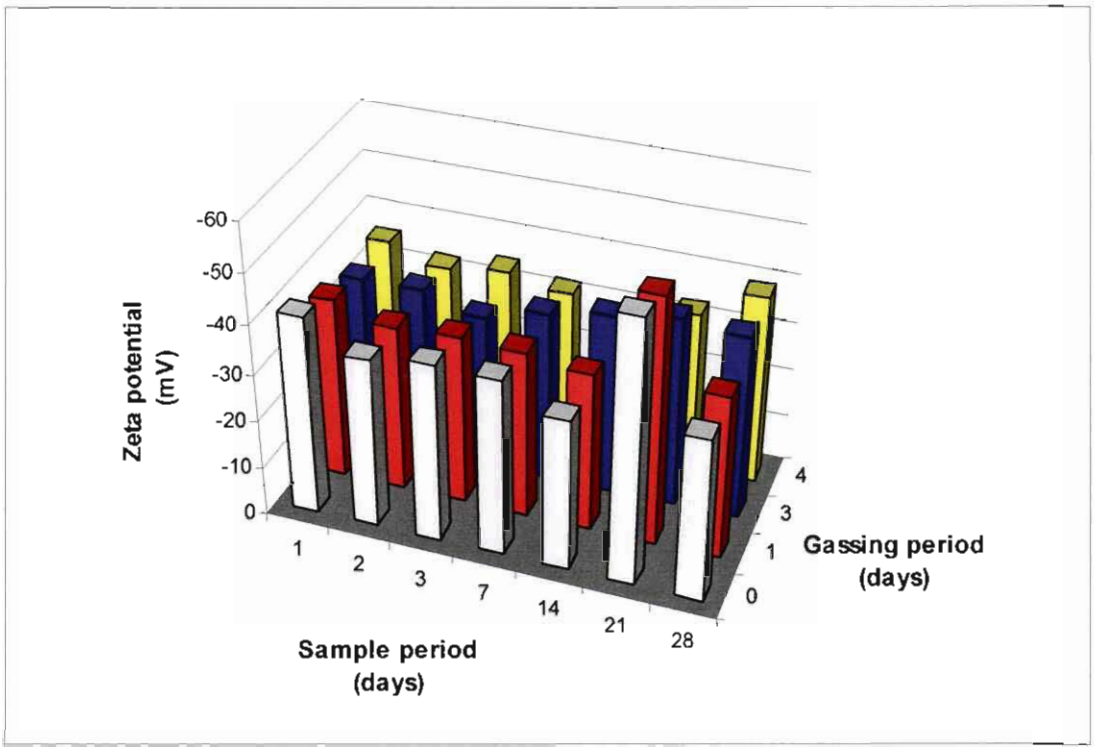


Figure 4.17 *The influence of different number of days gassed on the zeta potential of Pheroid vesicles.*

4.4.4 Turbidity

The results obtained for the turbidity measurements of the samples prepared at different gassing periods are depicted in figure 4.18. The preparation with the largest mean particle size (4 days gassing period) had the lowest turbidity values because the larger particles refracted less of the light source it is exposed to during measurement. The turbidity values of the preparations with 0 days, 1 day and 3 days of gassing varied between 8.19 - 9.09 NTU over the 28 day sampling period with the 3 days gassing period having the highest turbidity of all the preparations under investigations.

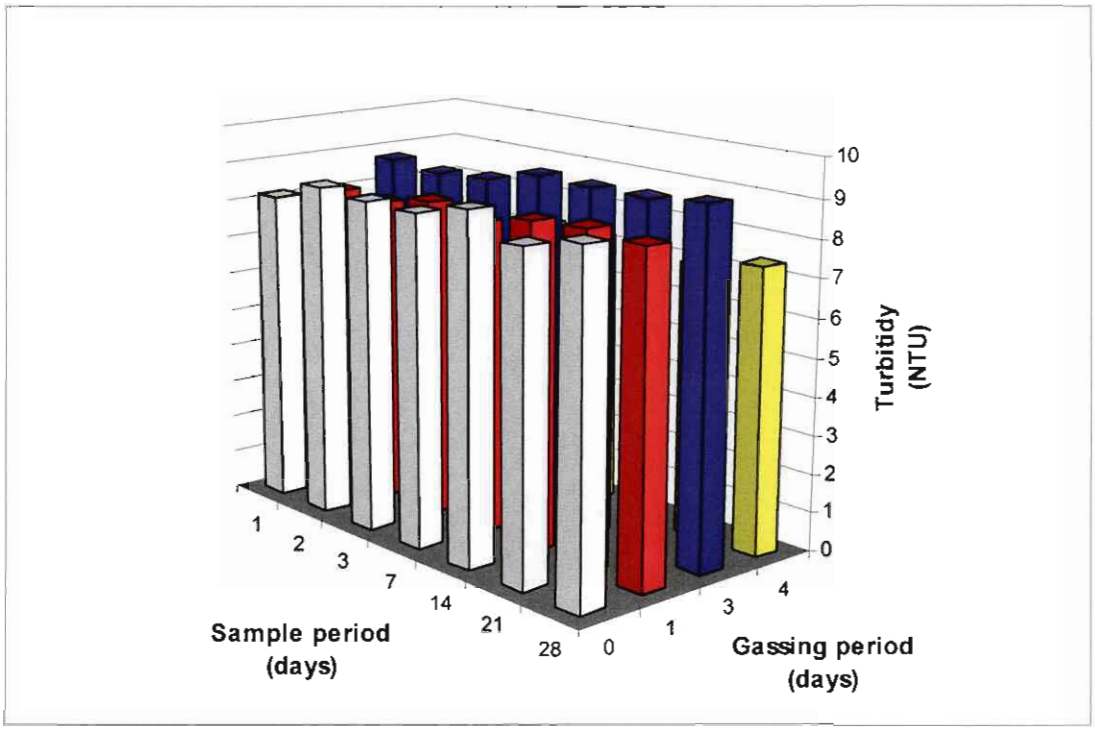
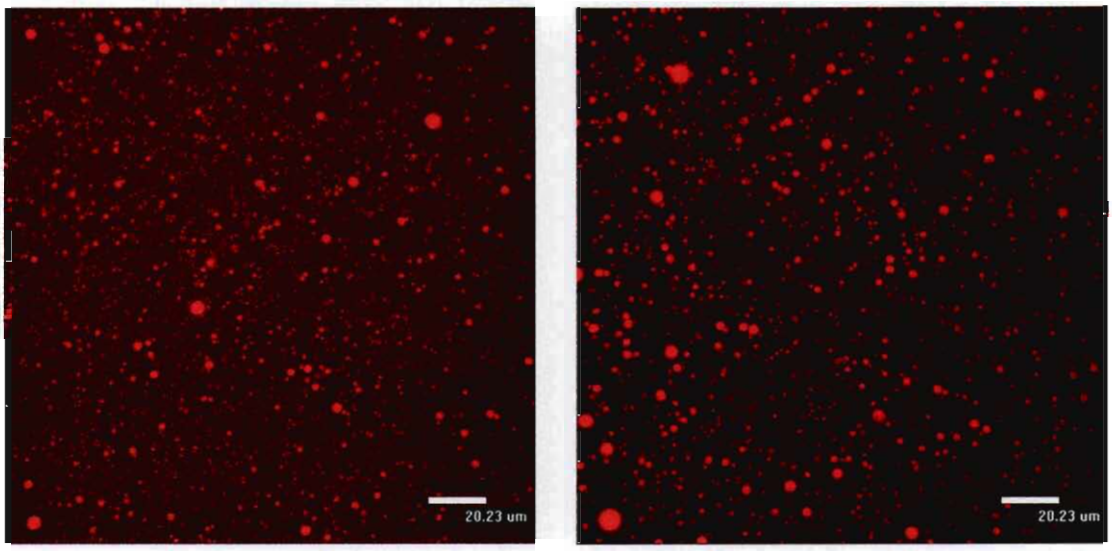


Figure 4.18 *The influence of different number of days gassed on the turbidity of Pheroid vesicles.*

4.4.5 CLSM

Figure 4.19 depicts micrographs of the Pheroid vesicles prepared without nitrous oxide and with a gassing period of 4 days. It can be seen that without the added gas phase the vesicles that formed is small with only a few larger droplets present. Addition of nitrous oxide gas resulted in an increase in the particle size of the vesicles. This may indicate that the nitrous oxide indeed form part of the structure of the Pheroid vesicles. As mentioned in 4.4.1 the nitrous oxide is believed to form an integral part of this unique delivery system. It distinguishes the Pheroid vesicles from any other emulsion type system. Further experimental work must be done to determine the interaction of the nitrous oxide with other components used in the preparation of Pheroid vesicles.



(a) **(b)**

Figure 4.19 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles prepared with 0 days of gassing and (b) Pheroid vesicles prepared with 4 days of gassing.*

4.4.6 pH and current values

The effect of the amount of gas on the pH values measured show no clear trend. The pH values ranged from 7.38 for the sample without any nitrous oxide gas to 5.56 for the 1 day gassed sample, 6.11 for the 3 days gassed sample and 8.60 for the 4 days gassed sample.

It was found that the current values measured for these samples switched from negative to positive values when nitrous oxide gas was introduced into the formula of the Pheroid vesicles. The preparation made without any nitrous oxide had a negative current value of -22.95 mV. The other three samples measured 82.10 mV for the preparation with a 1 day gassing period, 51.90 mV for the preparation with 3 days gassing period and 90.60 mV for the preparation with 4 days gassing period. The variation in the data may be related to excess nitrous oxide present in the formulations that causes steric repulsions between the Pheroid vesicles.

4.4.7 Conclusion

The turbidity and mean particle size values of the prepared samples give the best data combination to determine the optimum gassing period (figure 4.20). The sample prepared without any nitrous oxide showed a lot of fluctuation during the period of sampling. The best formulation of the four samples seems to be the preparation gassed for 3 days which shows the largest values for this specific data combination

set. The least promising preparation seems to be the preparation gassed for 4 days.

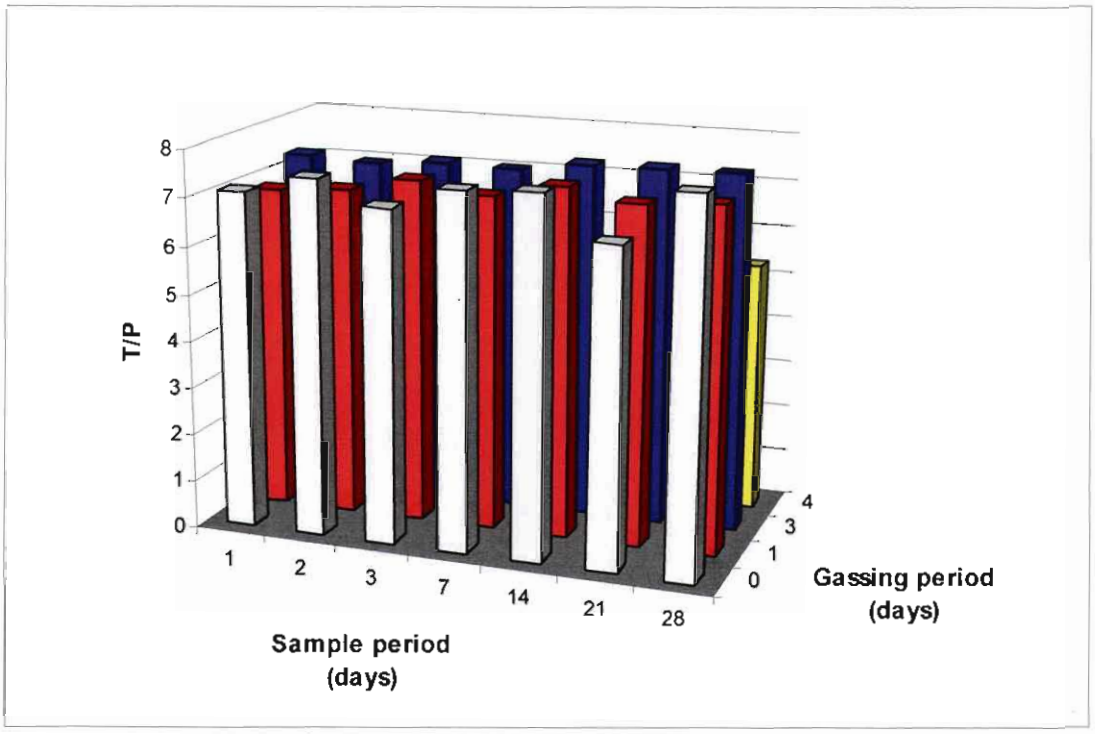


Figure 4.20 *The influence of different number of days gassed on the combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

Since there was no quantitive method used to assess how much gas is present in each preparation and to determine after which amount of days the milli Q water was saturated with the nitrous oxide, it was decided to use the 4 days gassing period as the amount of time necessary to prepare the water phase. Results of particle size analysis described earlier (4.2.1.2) showed that the Pheroid vesicles are dynamic and throughout the time of storage further dividing of the vesicles still occurs. An excess amount of nitrous oxide in the formulation is thus considered advantageous.

4.5 INFLUENCE OF THE CREMOPHOR® RH 40 CONCENTRATION

4.5.1 Introduction

Emulsion components could be grouped into the oil component, the water phase and the surfactants which are necessary to build a stable interfacial film and reduce the interfacial tension between the two non-miscible phases. Only the addition of a suitable emulsifier enables that a fine dispersity after production could be maintained during storage and that coalescence could be prevented (Lindenstruth and Müller, 2004:187). The influence of a variation in the surfactant concentration was investigated by preparing Pheroid vesicles with concentrations of 0.25 g, 0.50 g, 1.00 g, 2.00 g and 4.00 g of Cremophor® RH 40 at a mixing rate of 13500 rpm for 120

seconds. The water phase was gassed for a period of 4 days. The influence of Cremophor® RH 40 concentration on the stability and characteristics of the Pheroid vesicles are described below. Results are summarized in Annexure C.1.5.

4.5.2 Particle size analysis

From Figure 4.21 and Annexure C.1.5 it is evident that the sample prepared with 0.25 g of Cremophor® RH 40 was characterised by a relative stable mean particle size around 3.040 - 3.200 µm over the 28 day sampling period. An increase in Cremophor® RH 40 concentration to 0.50 g resulted in Pheroid vesicles with mean particle sizes between 3.620 - 3.940 µm. The smallest mean particle size (1.190 - 1.370 µm) were measured for the sample with 1.00 g of Cremophor® RH 40. A further increase to 2.00 g of Cremophor® RH 40 resulted in mean particle sizes between 1.450 -1.890 µm whilst mean particle sizes between 1.290 - 45.77 µm were measured for the sample with 4.00 g of Cremophor® RH 40 present in the formulation. It is evident from the mean particle size analysis of the samples that an increase in the Cremophor® RH 40 concentration resulted in an optimum particle size at 1.00 g of the surfactant present in the formulation. Quantities less than this ratio resulted in an increase in the mean particle size of the samples. This increase in mean particle sizes is related to the amount of emulsifier not being enough to ensure a fine dispersion of particles after production. Quantities above this ratio gave mean particle size values that varied over the 28 day sampling period. The sample containing 4.00 g of Cremophor® RH 40 showed the most variation in data values with a mean particle size of 45.77 μm measured on the 28th day of sampling. Figure 4.21 was constructed with the deletion of this abnormal high value. The individual analysis reports (not shown) of the 4.00 g sample shows the presence of a population of larger particles with 42.08 % of the particles with sizes between 35.56 μm and 258.95 μm. This increase in the mean particle size of the formula containing 4.00 g of Cremophor® RH 40 is contributed towards an excess of the surfactant being present in the formula in the form of large particles.

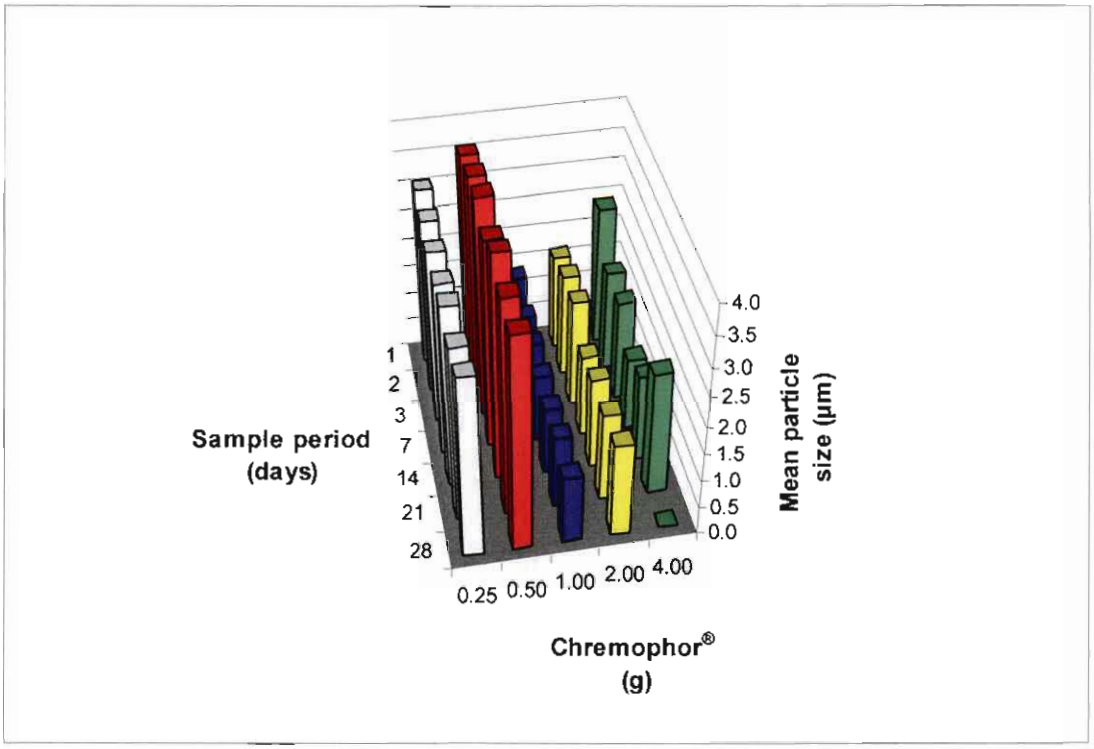


Figure 4.21 *The influence of different Cremophor® RH 40 concentrations on the mass median diameter (MMD) of Pheroid vesicles.*

4.5.3 Zeta potential

From Figure 4.22 and Annexure C.1.5 it is clear that only a general trend in zeta potential values can be observed for the samples prepared with different concentrations of surfactant present in the formula. The zeta potential of the preparation with 0.25 g of Cremophor® RH 40 was between -34.45 mV and -43.05 mV and the preparation with 0.50 g of Cremophor® RH 40 was between -31.90 mV and -38.30 mV. An increase in the amount of surfactant to 1.00 g resulted in zeta potential values between -29.85 mV and -36.05 mV. The zeta potential of the preparation with 2.00 g of Cremophor® RH 40 was between -28.80 mV and -38.10 mV whilst the preparation with 4.00 g of Cremophor® RH 40 was between -20.90 mV and -31.10 mV. In general the lowest zeta potential values were observed on the 3rd day after manufacturing where after the zeta potential values remained relative consistent for the rest of the test period (28 days).

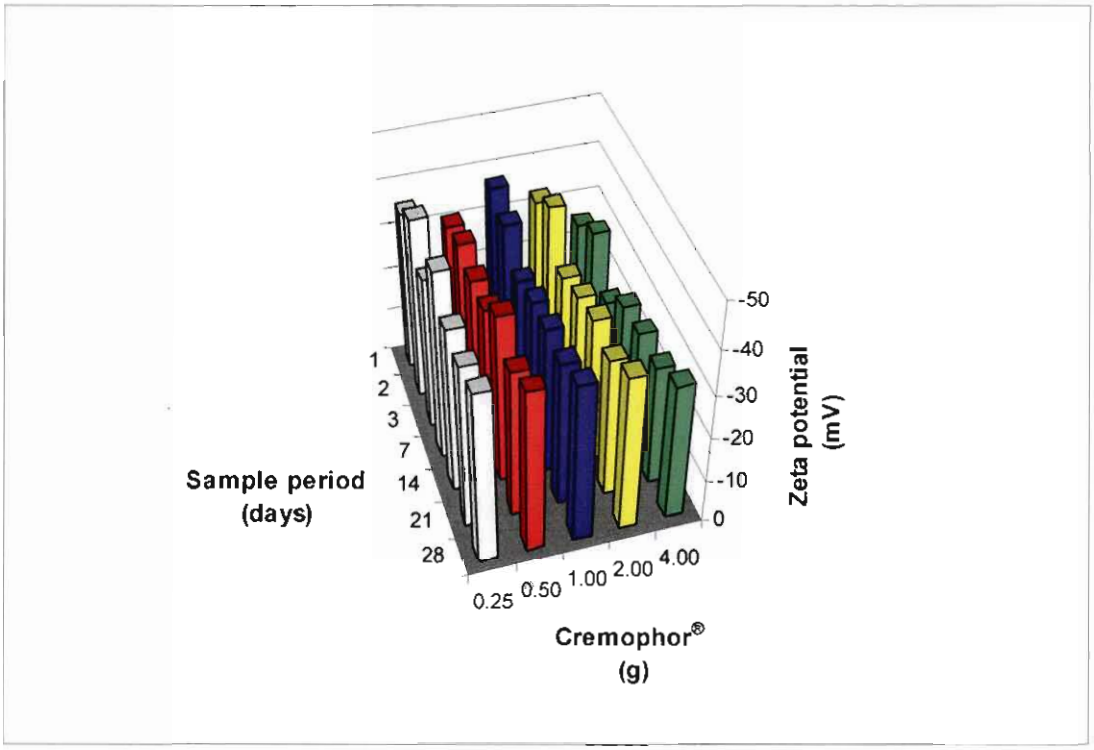


Figure 4.22 *The influence of different Cremophor® RH 40 concentrations on the zeta potential of Pheroid vesicles.*

4.5.4 Turbidity

The results obtained for the turbidity measurements of the samples prepared with different concentrations of Cremophor® RH 40 are depicted in figure 4.23. The high turbidity values obtained for the preparation containing 1.00 g of Cremophor® RH 40 is in agreement with the results of the particle size analysis (4.5.2). The samples prepared with lesser amounts of the surfactant (0.25 g and 0.50 g) gave intermediate high turbidity values. The samples with higher amounts of Cremophor® RH 40 present in the formula (2.00 g and 4.00 g) gave very low turbidity values which are typical of samples with either a few vesicles formed or vesicles of larger proportion.

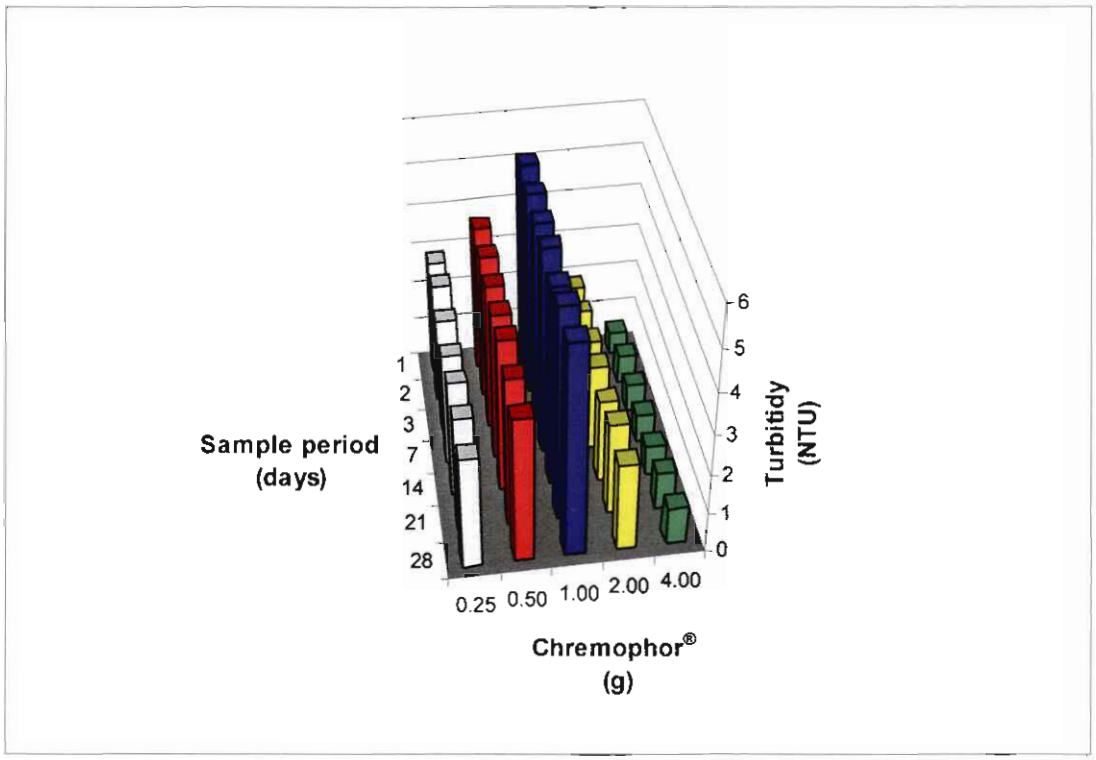
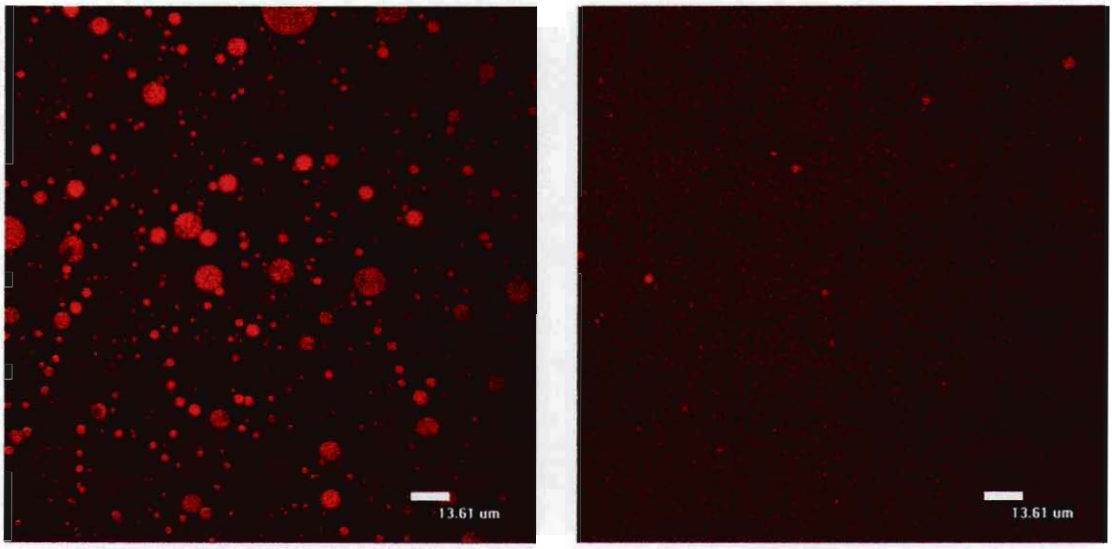


Figure 4.23 *The influence of different Cremophor® RH 40 concentrations on the turbidity of Pheroid vesicles.*

4.5.5 CLSM

Figure 4.24 depicted micrographs of the Pheroid vesicles prepared with 0.25 g and 4.00 g of Cremophor® RH 40 present in the oil phase of the formula. The micrograph of the preparation with 0.25 g of Cremophor® RH 40 contains depots of excess oil still present in the formulation. This confirms that the addition of a suitable amount of emulsifier is necessary to enable a fine dispersion of oil in water droplets after production. The preparation containing 0.50 g of Cremophor® RH 40 also contained depots of the oil phase (micrograph not shown). The Pheroid vesicles prepared with 1.00 g of Cremophor® RH 40 resulted in a preparation with small and evenly distributed vesicles (micrograph not shown). With higher quantities of Cremophor® RH 40 (2.00 g and 4.00 g) only a few small vesicles were formed. This confirms the low turbidity values obtained for these preparations (4.5.4).



(a) (b)

Figure 4.24 *Confocal laser scanning microscopy (CLSM) migrographs of (a) Pheroid vesicles prepared with 0.25 g Cremophor® RH 40 and (b) Pheroid vesicles prepared with 4.00 g Cremophor® RH 40.*

4.5.6 pH and current values

In general the pH values measured for the five samples prepared with different concentrations of Cremophor® RH 40 increased with an increase in the amount of surfactant. The preparation with 0.25 g of Cremophor® RH 40 had the lowest pH value of 5.30 whilst the preparation with 0.50 g of Cremophor® RH 40 had a pH value of 5.48. The highest pH value (6.81) was measured for the preparation with 1.00 g of Cremophor® RH 40. The preparations with 2.00 g and 4.00 g of surfactant present in the formula had intermediate pH values of 6.47 and 6.37 respectively.

The current values varied with different concentrations of surfactant used to prepare the samples. Current values of 94.40 mV and 84.70 mV were measured for the preparations with 0.25 g and 0.50 g of Cremophor® RH 40. The lowest current value of 7.90 mV was measured for the sample with 1.00 g Cremophor® RH 40. Higher concentrations of Cremophor® RH 40 resulted in values of 27.80 mV for the 2.00 g sample and 33.80 mV for the 4.00 g sample.

4.5.7 Conclusion

The turbidity and mean particle size values of the prepared samples give the best data combination to determine the optimum concentration for Cremophor® RH 40 (figure 4.25). The best formulation of the five samples is the preparation with 1.00 g of Cremophor® RH 40 which shows the largest value for this data combination set.

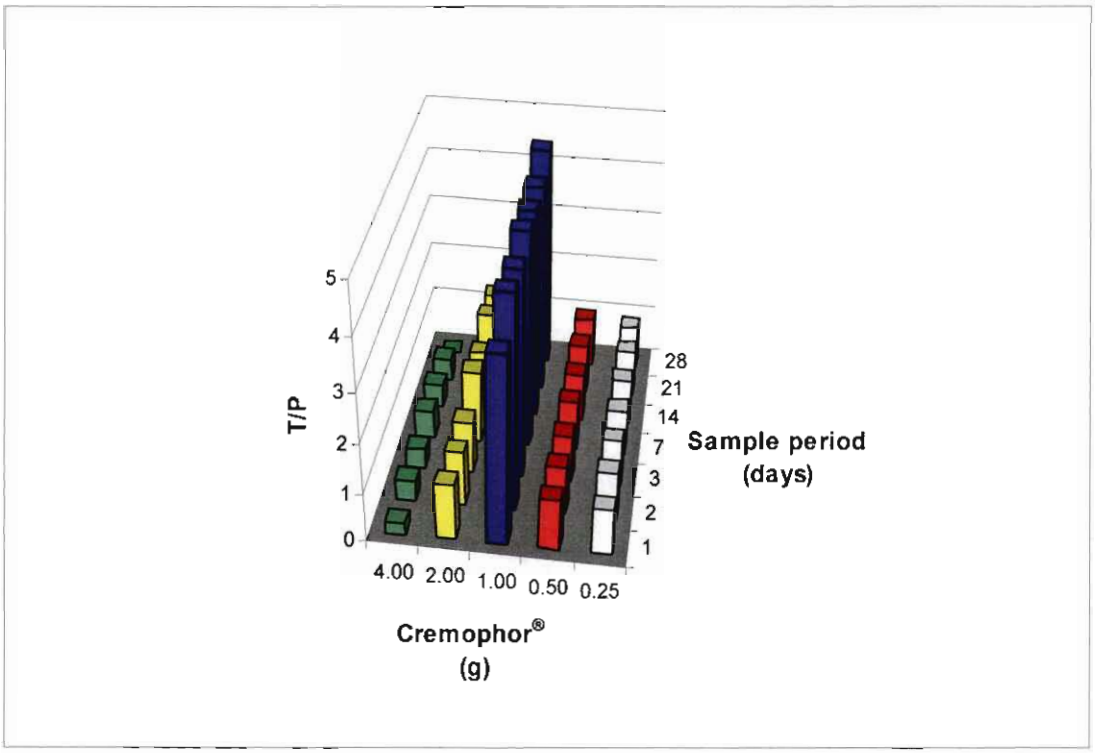


Figure 4.25 *The influence of different Cremophor® RH 40 concentrations on the combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

The preparation with 4.00 g of Cremophor® RH 40 shows the least promising combination of data values. It is clear from the analysis perfomed that the preparation with 1.00 g of Cremophor® RH 40 is suitable to prepare Pheroid vesicles with small mean particle sizes, high turbidity and stable zeta potential values. The lower concentrations of Cremophor® RH 40 used (0.25 g and 0.50 g) were unable to fully emulsify the system and thus resulted in vesicles with larger mean particle sizes and an excess amount of oil phase still present in the formulation. The higher concentrations of Cremophor® RH 40 (2.00 g and 4.00 g) produced formulas with a few small vesicles along with a portion of the Cremophor® RH 40 being present as larger particles in the formula.

4.6 INFLUENCE OF THE VITAMIN F ETHYL ESTER CLR CONCENTRATION

4.6.1 Introduction

O/w emulsions as disperse systems contain oil droplets in a continuous water phase and their droplet size and distribution are important criteria which describe the formulation quality. To prevent signs of instability an appropriate production process and the right composition of oil and surfactant phase has to be chosen (Lindenstruth and Müller, 2004:187).

The influence of variation in the oil phase concentration used was investigated by preparing a sample range with 1.00 g, 2.00 g, 2.50 g, 2.80 g, 3.25 g and 3.50 g of Vitamin F Ethyl Ester CLR along with 1.00 g of Cremophor® RH 40. A mixing rate of 13500 rpm for 120 seconds was used and the water phase was gassed for a period of 4 days. The influence of this variable on the stability and characteristics of the Pheroid vesicles are described below. Results are summarized in Annexure C.1.6.

4.6.2 Particle size analysis

The data of the particle size analysis for the different Vitamin F Ethyl Ester CLR concentrations is represented in figure 4.26 with exception of the 1.00 g Vitamin F Ethyl Ester CLR sample which was left out due to the large particle sizes obtained on the 7th day and 21st day of sampling. Over the 28 days after manufacturing the preparation with 1.00 g of Vitamin F Ethyl Ester CLR had mean particle size values between 1.040 - 29.350 μm. The individual analysis reports on the 7th day of sampling (report not shown) shows a bimodal distribution of particles with 54.57 % of the particles with sizes between 35.56 μm and 258.95 μm. This increase in the mean particle size of the preparation may be related to an excess of the oil phase not forming part of the Pheroid vesicles structure. The preparation with 2.00 g of Vitamin F Ethyl Ester CLR gave mean particle size values of between 1.050 - 1.270 μm whilst the preparation with 2.50 g of Vitamin F Ethyl Ester CLR had mean particle size values between 1.020 - 1.940 μm. Increasing the Vitamin F Ethyl Ester CLR concentration to 2.80 g resulted in mean particle size values between 1.080 - 1.400 μm. A further increase in the Vitamin F Ethyl Ester CLR concentration to 3.25 g and 3.50 g gave mean particle sizes between 1.540 - 1.700 and 1.960 - 2.060 μm respectively.

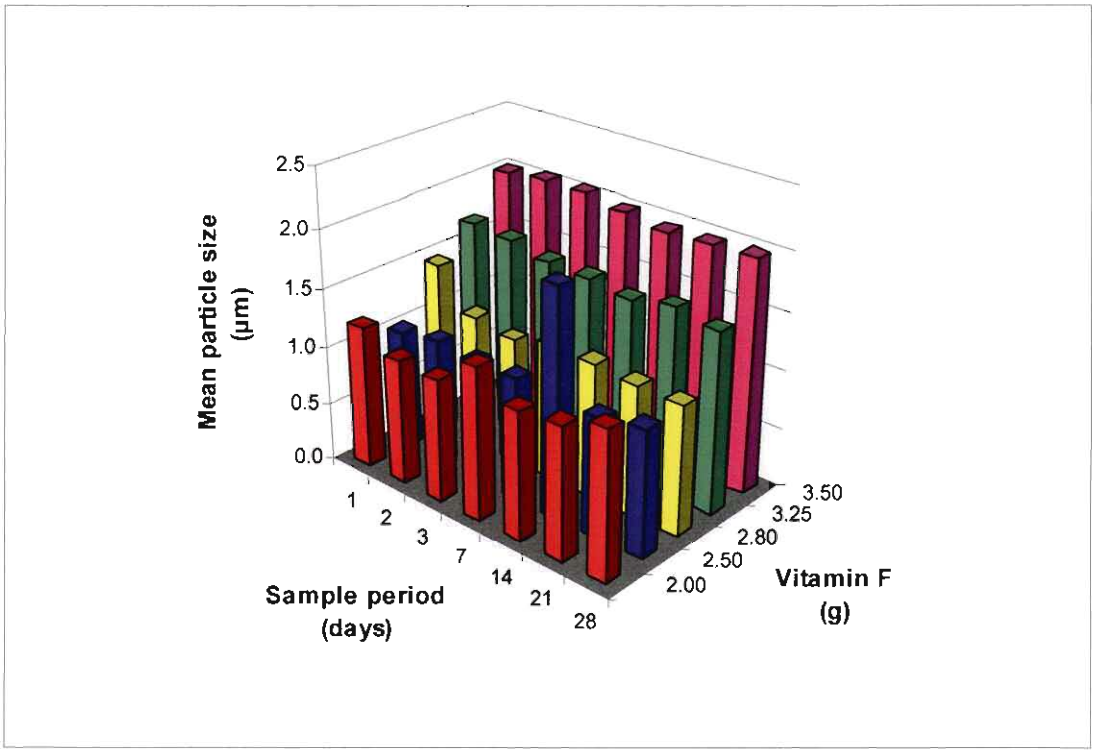


Figure 4.26 *The influence of different Vitamin F Ethyl Ester CLR concentrations on the mass median diameter (MMD) of Pheroid vesicles.*

4.6.3 Zeta potential

From Figure 4.27 and Annexure C.1.6 it is clear that only a general trend in zeta potential values was observed for the samples prepared with different concentrations of Vitamin F Ethyl Ester CLR. The zeta potential of the preparation with 1.00 g of Vitamin F Ethyl Ester CLR varied between -21.70 mV and -45.55 mV. Zeta potential values for the preparation with 2.00 g of Vitamin F Ethyl Ester CLR varied between -25.70 mV and -40.20 mV whilst the preparation with 2.50 g of Vitamin F Ethyl Ester CLR had zeta potential values between -23.60 mV and -37.95 mV. A promising sample proved to be the preparation with 2.80 g of Vitamin F Ethyl Ester CLR with values between -32.45 mV and -43.20 mV. After a slight decrease in zeta potential on the 7th day of sampling it was marked by an increase in stability until the 28th day of sampling. The preparation containing 3.25 g of Vitamin F Ethyl Ester CLR gives zeta potential values between -30.85 mV and -46.55 mV. The preparation with 3.50 g of Vitamin F Ethyl Ester CLR gives relative stable zeta potential values throughout the 28 days of sampling, varying between -32.90 mV and -39.45 mV.

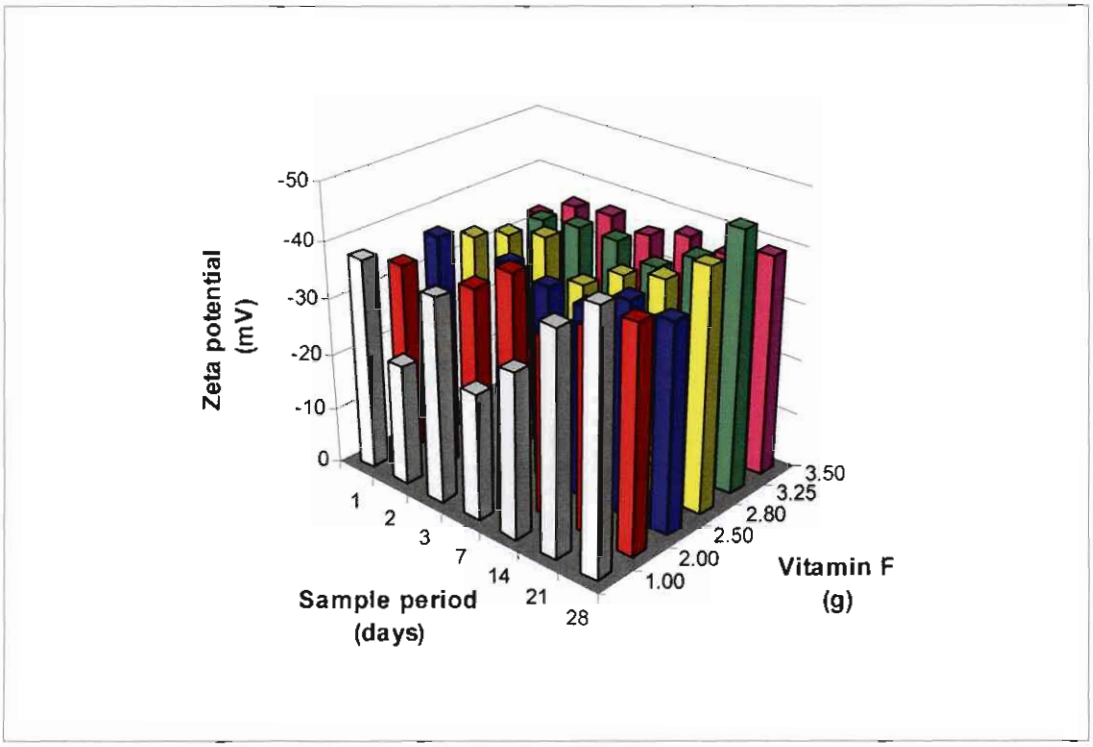


Figure 4.27 *The influence of different Vitamin F Ethyl Ester CLR concentrations on the zeta potential of Pheroid vesicles.*

4.6.4 Turbidity

The turbidity values measured for each sample prepared are depicted in figure 4.28. Overall the turbidity increased with an increase in the amount of Vitamin F Ethyl Ester CLR added to the preparation up to the 2.80 g Vitamin F Ethyl Ester CLR sample before a decrease in the turbidity was detected. The preparation with 1.00 g of Vitamin F Ethyl Ester CLR show very low turbidity values proving that this formula forms very few particles that can reflect the light source with a great part of the formula remaining as the oil phase.

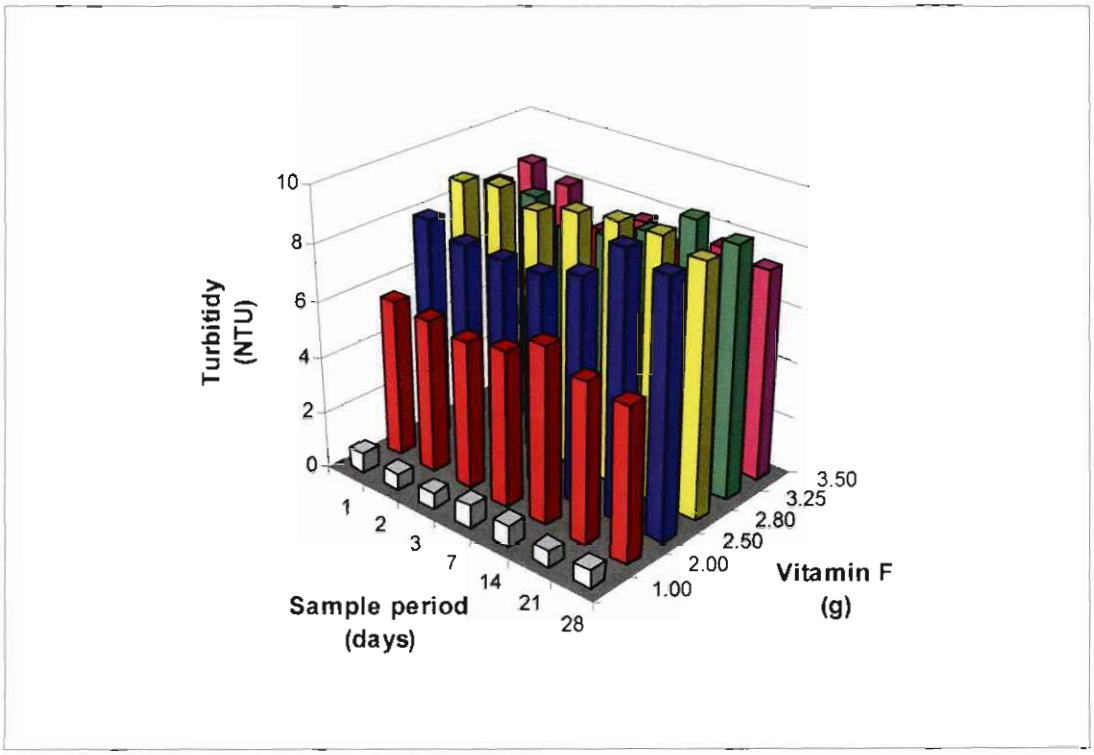
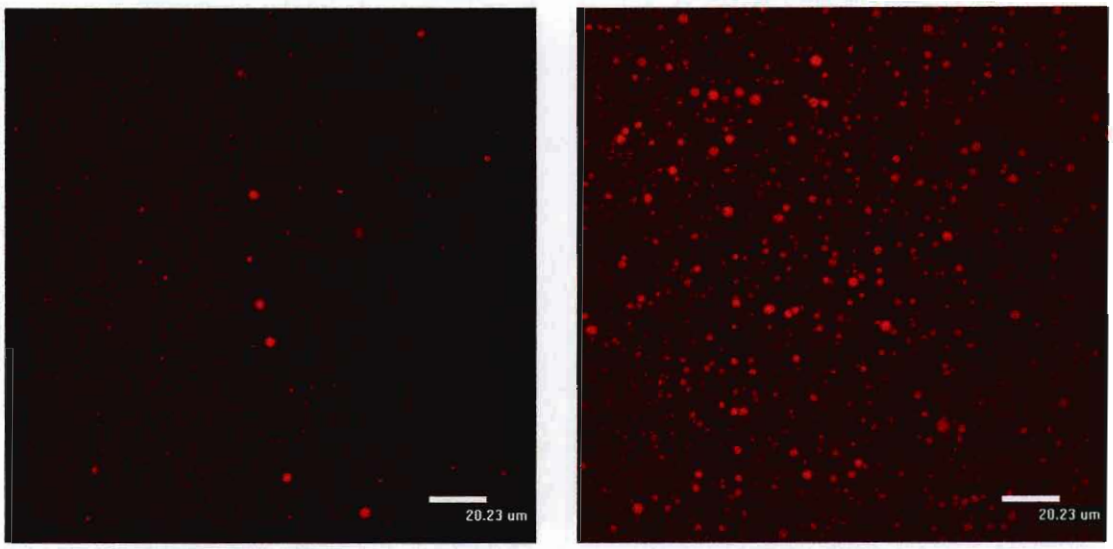


Figure 4.28 *The influence of different Vitamin F Ethyl Ester CLR concentrations on the turbidity of Pheroid vesicles.*

4.6.5 CLSM

Figure 4.30 contains micrographs of the preparations with 1.00 g and 3.50 g of Vitamin F Ethyl Ester CLR. The micrograph of the preparation with 1.00 g of Vitamin F Ethyl Ester CLR shows the small amount of Pheroid vesicles present in the preparation. The micrographs (not shown) of the preparations with 2.00 g and 2.50 g of Vitamin F Ethyl Ester CLR showed an increase in the amount of particles. The preparation with 2.80 g of Vitamin F Ethyl Ester CLR resulted in small and uniform sized Pheroid vesicles. The image of the preparation containing 3.50 g of Vitamin F Ethyl Ester CLR confirms the particle size analysis which indicated an increase in the mean particle size with an increase in the concentration of the Vitamin F Ethyl Ester CLR added.



(a) **(b)**

Figure 4.29 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles prepared with 1.00 g Vitamin F Ethyl Ester CLR and (b) Pheroid vesicles prepared with 3.50 g Vitamin F Ethyl Ester CLR.*

4.6.6 pH and current values

In general the pH values changed from slightly basic to slightly acidic with an increase in the amount of Vitamin F Ethyl Ester CLR added. A pH value of 8.33 was measured for the sample prepared with the lowest amount of Vitamin F Ethyl Ester CLR (1.00 g). The preparation with 2.00 g of Vitamin F Ethyl Ester CLR had a pH value of 7.04 whilst the preparation with 2.50 g of Vitamin F Ethyl Ester CLR has a pH value of 6.78. A further increase in the concentration of the Vitamin F Ethyl Ester CLR to 2.80 g resulted in a pH of 6.44. The preparations with 3.25 g and 3.50 g of Vitamin F Ethyl Ester CLR have pH values of 6.53 and 6.23 respectively.

The current values obtained varied from negative to positive with an increase in the concentration of the Vitamin F Ethyl Ester CLR. The preparation with 1.00 g and 2.00 g of Vitamin F Ethyl Ester CLR had current values of -76.15 mV and -2.35 mV respectively. Positive current values of 12.45 mV and 31.75 mV were measured for the preparations with 2.50 g and 2.80 g of Vitamin F Ethyl Ester CLR. The preparations with the highest amount of Vitamin F Ethyl Ester CLR (3.25 g and 3.50 g) gave current values of 26.45 mV and 43.95 mV respectively.

4.6.7 Conclusion

The best graphic indication of the influence of the concentration of the Vitamin F Ethyl Ester CLR, which forms part of the oil phase of the Pheroid vesicles, was

obtained when the turbidity and mean particle size parameters were combined (figure 4.30).

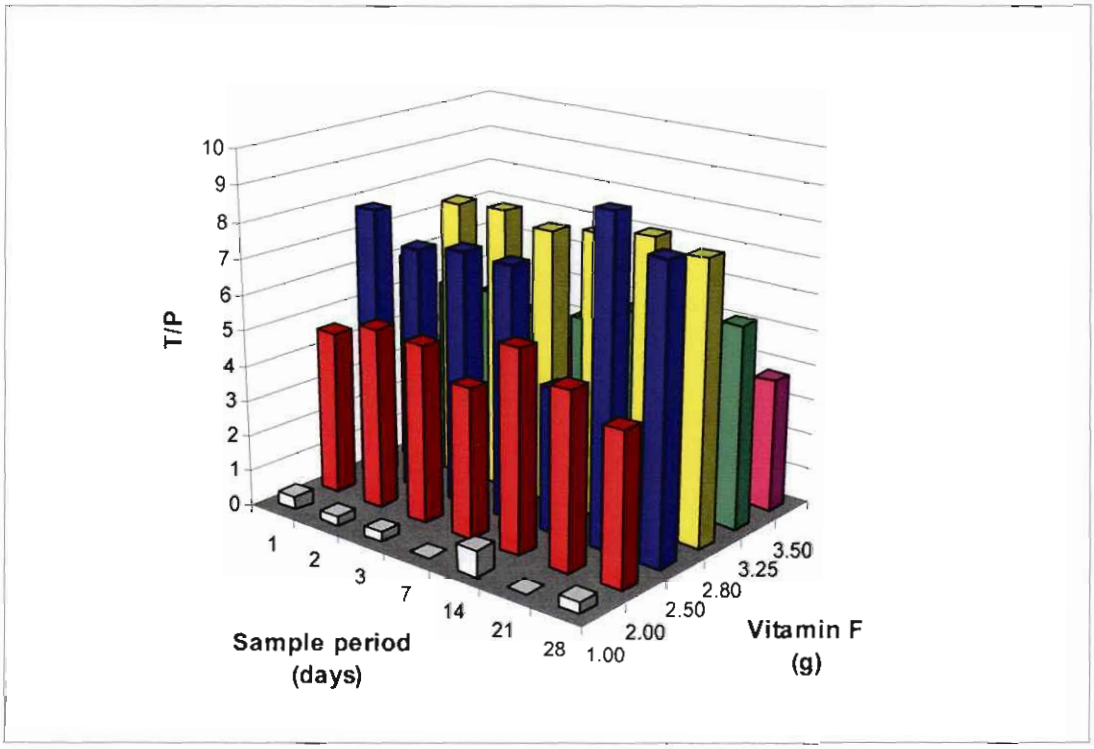


Figure 4.30 *The influence of different Vitamin F Ethyl Ester CLR concentrations on the combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

The preparation with the least promising characteristics was obtained with a concentration of 1.00 g of Vitamin F Ethyl Ester CLR in combination with 1.00 g of Cremophor® RH 40. The low concentration of Vitamin F Ethyl Ester CLR formed only a few vesicles with a percentage of the oil phase still left in the formulation. The preparation with the most stable values obtained over the 28 day sampling period is the preparation with 2.80 g of Vitamin F Ethyl Ester CLR present in the formula. The analysis performed for this preparation resulted in small mean particle size values, high turbidity and stable zeta potential values. Concentrations below or above 2.80 g of Vitamin F Ethyl Ester CLR gave data values that varied over the 28 day sampling period.

4.7 STATISTICAL ANALYSIS

Statistical analysis was not performed on the obtained data since the aim of this study was to establish general guidelines for the manufacturing of Pheroid vesicles with suitable characteristics for further clinical experimentation. Further studies will focus on designing experiments for advanced statistical analysis.

4.8 CONCLUSION

Each variable within the formulation process were randomly investigated to determine the influence of changes, within the formulation, on the stability and characteristics of the Pheroid vesicles. Variables under investigation included the mixing rate and mixing time used to homogenize the emulsion system along with the temperature to which the water phase is heated. Variables that forms a part of the composition of the Pheroid vesicles includes the amount of nitrous oxide in the water phase and the concentrations of the components within the oil phase of the formulation.

A mixing rate of 13500 rpm for 120 seconds resulted in Pheroid vesicles with acceptable particle sizes and stable zeta potential values. The decision to use 75 °C as the temperature to which the water phase needs to be heated before adding the oil phase is based upon the knowledge that emulsification occurs at ± 1 °C of this temperature and that it would ensure that less of the nitrous oxide would evaporate out of the water, compared to higher temperatures. The optimum number of days of gassing seems to be 4 days within our experimental setup. The amount of Cremophor® RH 40 and Vitamin F Ethyl Ester CLR to be used in the formula of the Pheroid vesicles was also investigated. The optimum ratio proved to be 2.80 g of Vitamin F Ethyl Ester CLR in combination with 1.00 g of Cremophor® RH 40. At these amounts of each component the formed vesicles were small and without any excess oil or surfactant left in the formula. The optimal formula chosen for future experimental work is again given in chapter 5 and results of an accelerated stability test on this formula is also given in chapter 5.

CHAPTER 5

ACCELERATED STABILITY TESTING OF THE PHEROID VESICLES

5.1 INTRODUCTION

Non-optimal or non-predictable stability properties of emulsions can be limiting for the application of emulsions. Therefore, it is of general interest to predict stability behavior of emulsions (Bjerregaard *et al.*, 2001:23). Prediction of long-term stability of emulsions is of major importance in formulation work. Some accelerated stability tests have been suggested for the prediction of long-term stability such as centrifugation and storage at elevated temperatures. However, elevated temperatures can change the viscosity of liquid phases and can result in solubility and partitioning of molecules between the oil and aqueous phases. This may effectuate a dramatical change in the nature of surfactants. O/w emulsions stabilized with non-ionic surfactants tend to invert to w/o emulsions at temperatures above the phase inversion temperature (PIT) as the surfactant molecules dehydrate and become more lipophilic (Bjerregaard *et al.*, 2001:24).

An increase in temperature causes increases in the rate of chemical reactions. The products are therefore stored at temperatures higher than room temperature. The nature of the product often determines the temperature range covered in the accelerated test. Samples are removed at various time intervals and the extent of decomposition is determined by analysis. Sensitive analytical methods should be used in all stability tests of this nature, as small changes may be detected after very short storage periods (Pugh, 2002:111).

Storage of the product in atmospheres of high humidity will accelerate decomposition that result from hydrolysis. Marked acceleration will be obtained if the "naked" product (i.e. not enclosed in a container) is subjected to these tests, which usually indicate the minimum humidity tolerated by the product without undue decomposition, and are therefore useful in determining the degree of protection that should be afforded by a container (Pugh, 2002:112).

5.2 OPTIMAL PHEROID FORMULATION

From the results of chapter 4 it was concluded that the optimal formulation of Pheroid vesicles contains 2.80 g of Vitamin F Ethyl Ester CLR, 1.00 g of Cremophor® RH 40 and 0.20 g of DL-α-Tocopherol in the oil phase along with 96.00 g of water saturated

with nitrous oxide for 4 days. The water phase was heated to 75 °C before emulsifying the system at a mixing rate of 13500 rpm for 120 seconds.

5.3 ACCELERATION STABILITY TEST

The Pheroid formulation (5.2) prepared for accelerated stability testing was kept under the storage conditions described in table 5.1 throughout a 3 month sampling period.

Table 5.1 *Accelerated stability test conditions*

Storage conditions	Storage period
5 °C	1, 2 and 3 months
25 °C and 60 % Relative Humidity	1, 2 and 3 months
40 °C and 75 % Relative Humidity	1, 2 and 3 months

The Pheroid formulation was prepared according to the conditions described in chapter 4. The samples were stored in 100ml amber glass bottles at the various temperatures and humidity's before conducting particle size analysis, zeta potential analysis, turbidity measurements, confocal laser scanning microscopy and pH and current measurements on the pre-determined sampling periods (table 5.1). A "test" sample was prepared and kept at ± 6 °C for a period of 24 hours before baseline characterisation of the Pheroid vesicles were done. The values obtained for the "test" sample were summarized on the graphs as time t = 0 and compared to the results obtained during the accelerated stability test.

5.4 RESULTS AND DISCUSSION

5.4.1 Particle size analysis

The data values for particle size analysis for the samples on accelerated stability are depicted in figure 5.1. The particle size data of the three samples were obtained according to the method described in 3.4.1.2. Annexure C.2 summarises the results obtained.

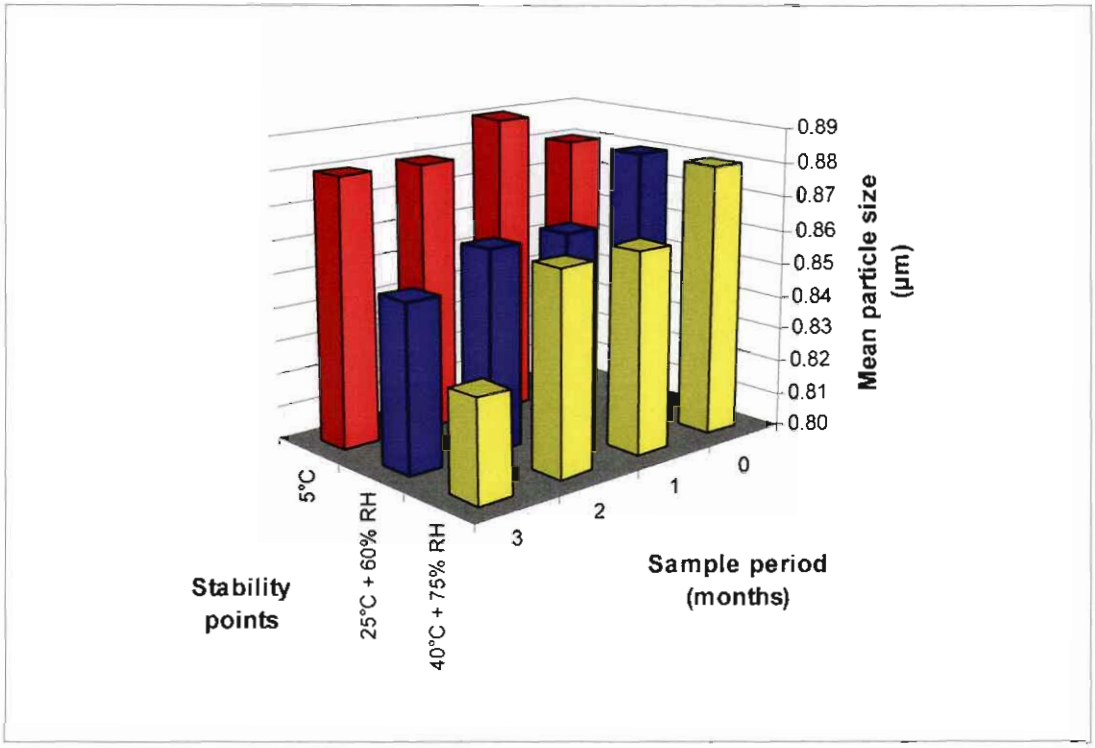


Figure 5.1 *The mass median diameter (MMD) of the Pheroid vesicles.*

The particle size analysis performed at time t = 0, measured after 24 hours of storage at ± 6 °C, showed vesicles with a mean particle size of 0.880 µm. This value slightly increased to 0.890 µm for the sample stored at 5 °C after 1 month of storage and thereafter the mean particle size remained at 0.880 µm for the following two months. The sample stored at 25 °C with 60 % relative humidity showed a reduction in the mean particle size from 0.880 µm to 0.860 µm after the 1st and 2nd month of storage and to 0.850 µm after the 3rd month of storage. The sample kept at 40 °C with 75 % relative humidity showed the same trend with a reduction in mean particle size from 0.880 µm to 0.860 µm after the 1st and 2nd month of storage and to 0.830 µm after the 3rd month of storage. This results are considered positive for stability since this slight reduction in the mean particle size of the samples occurs throughout the storage period and because larger droplets cream or sediment much faster than the original small ones.

5.4.2 Zeta potential

Figure 5.2 depicted the zeta potential values for the three formulations under investigation. The zeta potential values of the three samples were assessed according to the method described in 3.4.2.2 and the results are summarised in Annexure C.2.

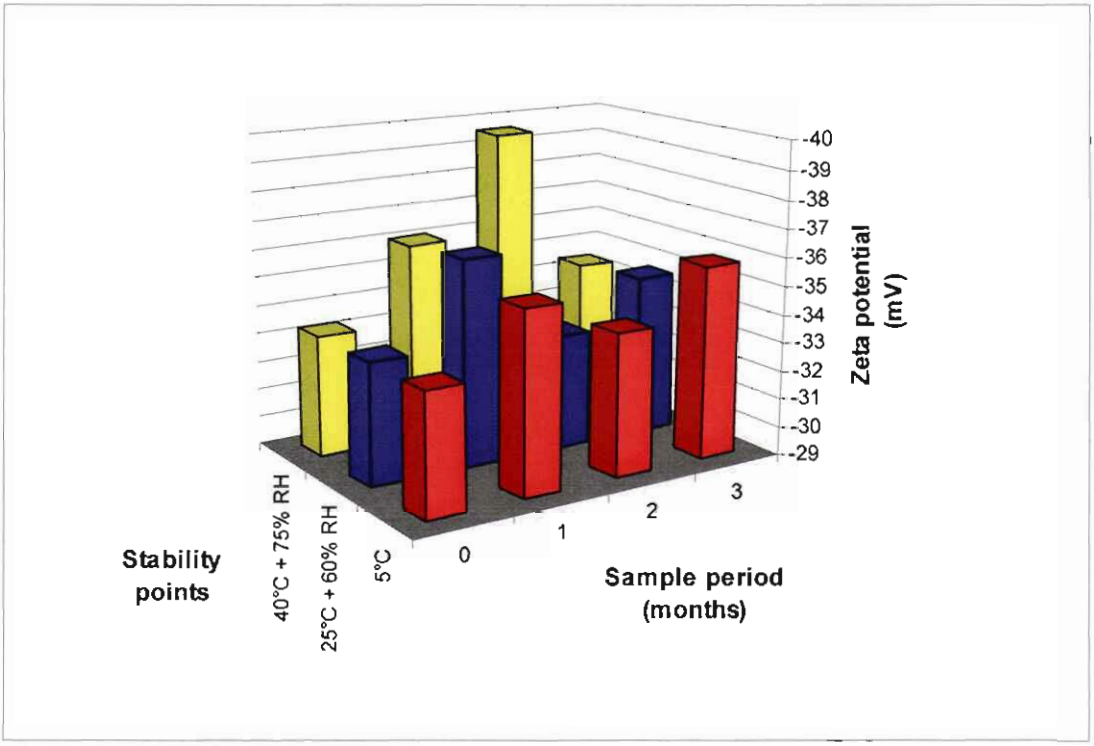


Figure 5.2 *The zeta potential values of the Pheroid vesicles.*

In general the samples had a stable zeta potential of -33.31 mV 24 hours after manufacturing. After 1 month of storage at the various temperatures all of the zeta potential values increased to -35.40 mV for the sample kept at 5 °C, -36.25 mV for the sample kept at 25 °C (60 % RH) and -36.05 mV for the sample kept at 40 °C (75 % RH). After the second month of sampling both the samples kept at 5 °C and 25 °C showed a slight reduction in zeta potential to -34.05 mV and -33.05 mV whilst the highest zeta potential of -39.60 mV were measured for the sample kept at 40 °C. After 3 months of storage the zeta potential of the sample kept at 5 °C increased to -38.80 mV whilst the sample kept at 25 °C has a zeta potential of -34.65 mV. The sample kept at 40 °C had a lower zeta potential of -34.40 mV after the 3rd month of storage. Overall the zeta potential of the samples stayed below -30 mV and this is considered as stable and useful for future formulation.

5.4.3 Turbidity

The turbidity values of the three samples were obtained according to the method described in 3.4.3.2. The data values of the turbidity values are depicted in figure 5.3. Results obtained are summarised in Annexure C.2.

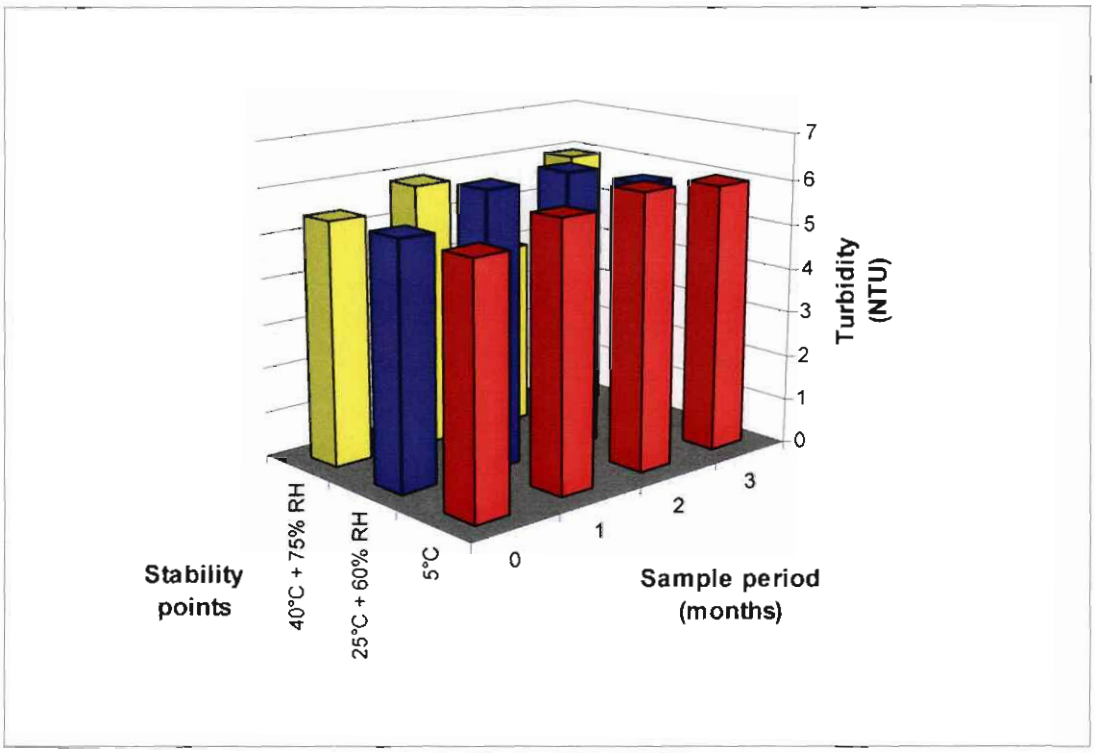
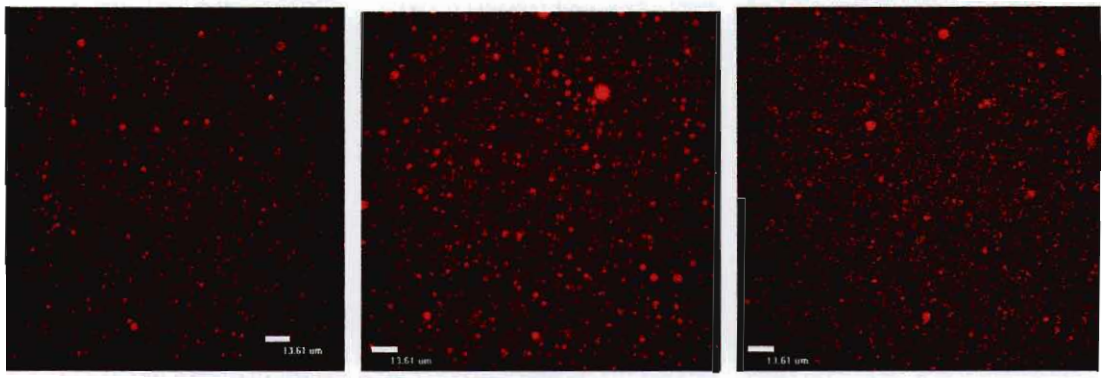


Figure 5.3 *The turbidity values of the Pheroid vesicles.*

The "test" sample gave a turbidity value of 5.440 NTU. This value increased after the first month of storage to 5.900 for both the samples kept at 5 °C and 40 °C and to 6.135 for the sample kept at 25 °C. After 2 months of storage the turbidity increased for the samples kept at 5 °C and 25 °C to 6.120 and 6.220 whilst the lowest turbidity value, during the entire test period, were obtained for the sample kept at 40 °C (4.140). This might be due to slight variation in the sampling method since the particle size analysis performed earlier (5.2.2) showed no increase in the droplet size of the sample kept at 40 °C. The values obtained after the 3rd month of storage were 5.980 for the sample kept at 5 °C, 5.690 for the sample kept at 25 °C and 6.000 for the sample kept at 40 °C.

5.4.4 CLSM

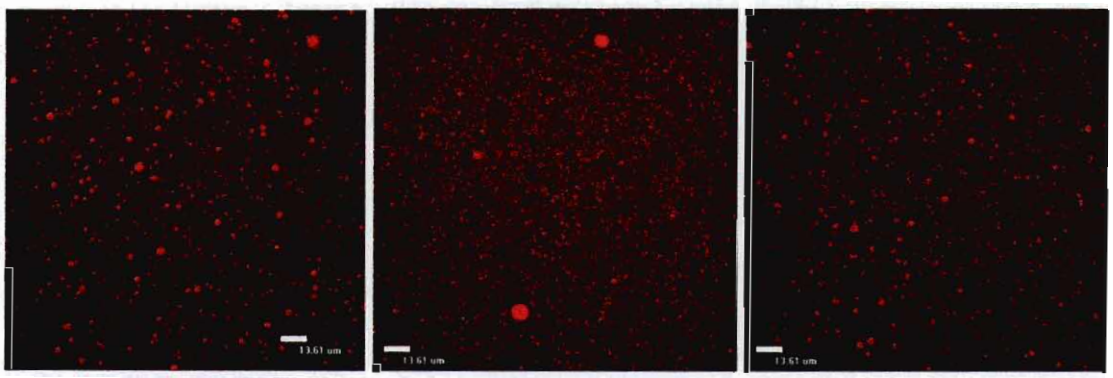
Confocal images of the three samples were obtained according to the method described in 3.4.4.2. Figure 5.4 show micrographs of the sample kept at 5 °C for a 3 month storage period. The vesicles were small with a visual increase in the number of vesicles after 2 months and 3 months of storage.



(a) (b) (c)

Figure 5.4 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles stored at 5 °C for 1 month (b) Pheroid vesicles stored at 5 °C for 2 months and (c) Pheroid vesicles stored at 5 °C for 3 months.*

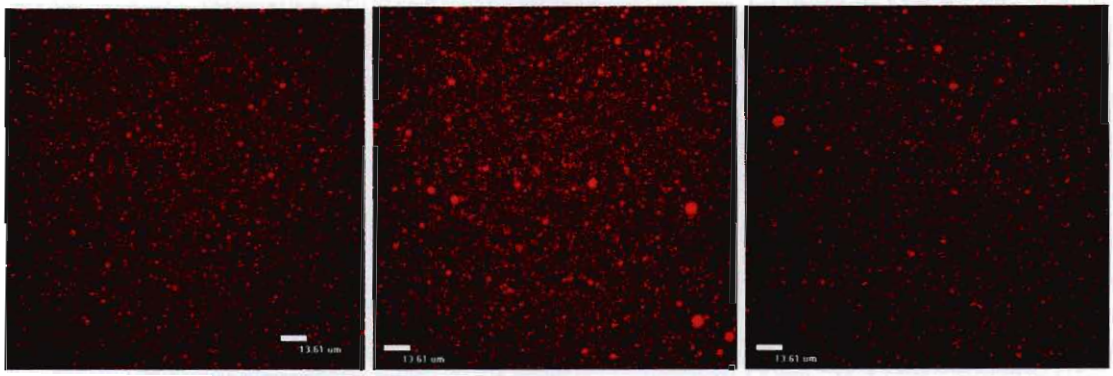
Figure 5.5 show micrographs of the sample kept at 25 °C (60 % RH). The images confirm the decrease in the mean particle size from 0.880 µm at time t = 0 to 0.860 µm after the 1st and 2nd month of storage and to 0.850 µm after the 3rd month of storage. The micrograph of the sample after 2 months of storage at 25 °C also confirmed the obtained highest turbidity value of 6.220 for this sample.



(a) (b) (c)

Figure 5.5 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles stored at 25 °C (60 % RH) for 1 month (b) Pheroid vesicles stored at 25 °C (60 % RH) for 2 months and (c) Pheroid vesicles stored at 25 °C (60 % RH) for 3 months.*

Figure 5.6 show micrographs of the sample kept at 40 °C (75 % RH). The micrograph of the sample after 2 months of storage confirms the low turbidity value obtained with a number of larger droplets visually present in this sample. The micrograph of the sample after the 3rd month of storage confirms the small mean particle size obtained for this sample.



(a) (b) (c)

Figure 5.6 *Confocal laser scanning microscopy (CLSM) micrographs of (a) Pheroid vesicles stored at 40 °C (75 % RH) for 1 month (b) Pheroid vesicles stored at 40 °C (75 % RH) for 2 months and (c) Pheroid vesicles stored at 40 °C (75 % RH) for 3 months.*

5.4.5 pH and current values

The pH and current values of the samples were obtained according to the method described in 3.4.5.2. In general the pH values of all the samples were slightly acidic with the exception of the sample kept at 40 °C which has a strong acidic pH value of 2.860 after 2 months of storage. This sample also has a very high current value of 231.65 mV. The rest of the current values of the samples varied over the period and with no clear trend it was decided to ignore these values in the final assessment of stability.

5.4.6 Conclusion

A combination of parameters was used to assist with the interpretation of the results obtained during the accelerated stability test. Figure 5.7 uses a combination of mean particle size values and the turbidity values. The best possible data set will be represented by a small mean particle size and a high turbidity value. The sample kept at 25 °C with 60 % relative humidity for 3 months gave the highest value for this data set, of the three storage conditions, even though the other two storage conditions compared well with this.

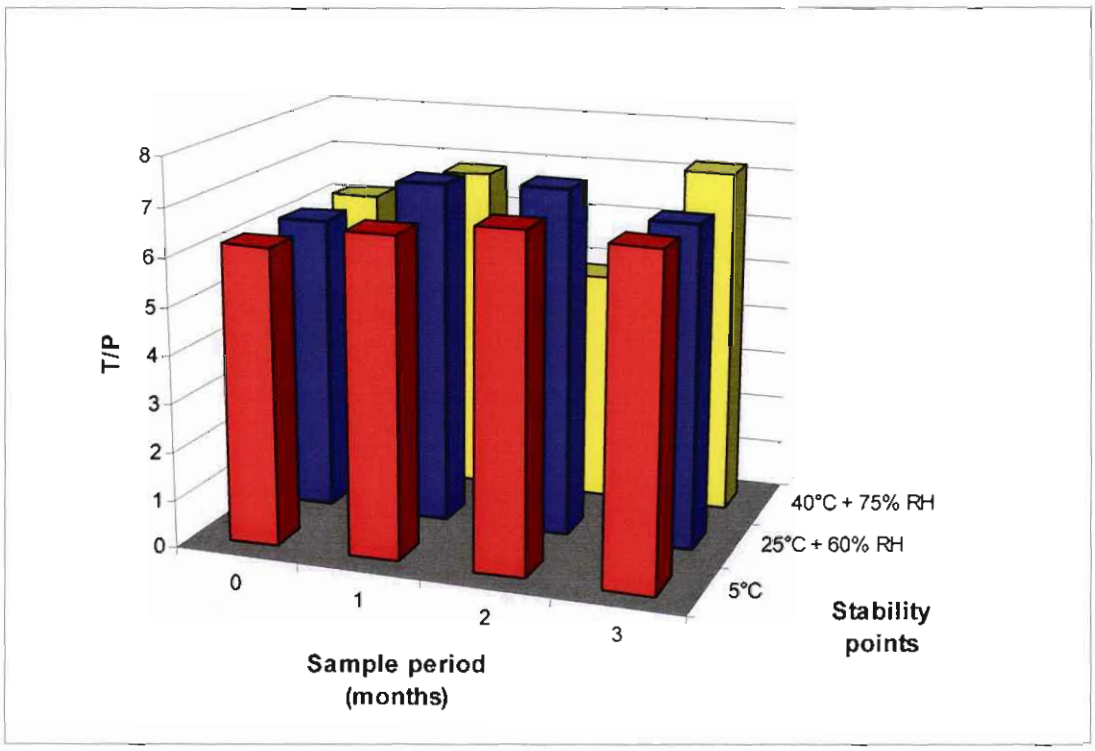


Figure 5.7 *The combined effect of turbidity (T) and mean particle size (P) of the Pheroid vesicles.*

Figure 5.8 contains another combination of parameters (turbidity and zeta potential). The most stable data set were obtained for the sample kept at 5 °C. The other two samples compared well with the sample kept at 5 °C, with the sample kept at 25 °C having the highest value overall.

Figure 5.9 contains a combination of data values of zeta potential and the mean particle size of the samples. For this scenario the highest values were obtained for the sample kept at 40 °C with the sample kept at 5 °C giving the most stable values over the 3 months of storage.

Although this particular way of comparing data may not be sufficient it provides valuable information for future studies on stability parameters for Pheroid formulations.

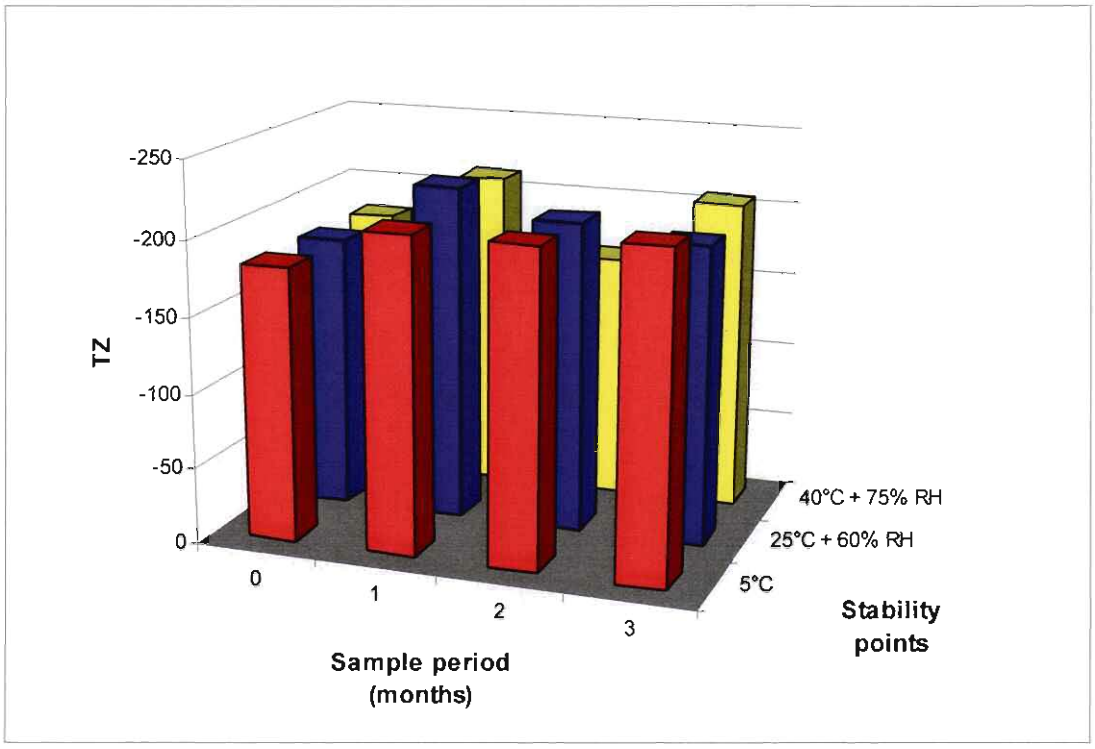


Figure 5.8 *The combined effect of turbidity (T) and zeta potential (Z) of the Pheroid vesicles.*

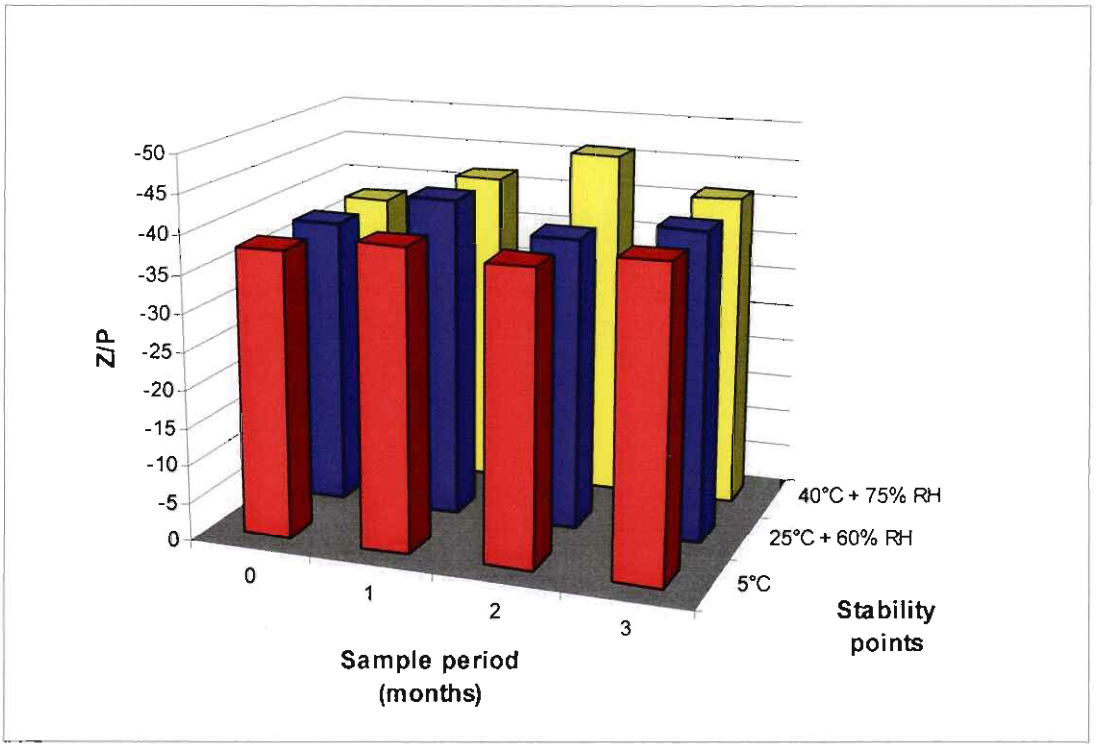


Figure 5.9 *The combined effect of zeta potential (Z) and mean particle size (P) of the Pheroid vesicles.*

5.5 INSTABILITIES DETECTED

Visual observation of the samples indicated creaming of the samples. Over the sampling period the degree of creaming increased from only the sample kept at 40 °C with a layer of cream after the first month of storage to excessive layers of creaming on all three samples after three months of storage.

Billany (2002:353) explains that a stable emulsion is one in which the dispersed globules retain their initial character and remain uniformly distributed throughout the continuous phase. Creaming occurs with the separation of an emulsion into two regions, one of which is richer in the disperse phase than the other. A simple example is the creaming of milk, when fat globules slowly rise to the top of the product. He further notes that this is not a serious instability problem as a uniform dispersion can be re-obtained simply by shaking or decanting of the emulsion. It is, however, undesirable because of the increased likelihood of coalescence of the droplets, owing to their close proximity to each other. A creamed emulsion is also inelegant and, if the emulsion is not shaken adequately, there is a risk of the patient obtaining an incorrect dosage.

5.6 CONCLUSION

A particular Pheroid formulation was exposed to a formal 3 month accelerated stability test to determine the stability of the Pheroid vesicles. The reason to expose the formulation to stress conditions is based upon the limited time in which the formulation need to be assessed to determine its functionality as a possible delivery system with advances over other lipid-based delivery systems. To assess the stability of the formulated product it was exposed to conditions of temperature and humidity that are known from experience to be likely causes of breakdown. High stress conditions enhance the deterioration of the product and therefore reduce the time required for testing.

The formulation was exposed to conditions varying from 5 °C to 25 °C with 60 % relative humidity and 40 °C with 75 % relative humidity. The concern that the higher temperature would influence the stability of the formulation prepared with a nonionic emulsifier, making use of hydrogen bonds to stabilize the droplets within the water phase, was dismissed by the data values obtained. The analysis methods used to assess the stability of the formulation were carefully chosen and used throughout this study. A formulation prepared and tested after 24 hours of storage were used as a standard against which the obtained stability data was compared to.

Three main objectives of accelerated stability tests were identified in Chapter 1. One was to assist in choosing the best possible formulation from a series of choices. Furthermore the prediction of the shelf-life of the product and ensuring quality control of formulations need to be performed. The data obtained during this study proved the usefulness of the Pheroid vesicles as a stable delivery system. In general the mean particle size of the formula prepared was below 1 μm, with a stable and high zeta potential value. The turbidity values confirmed the particle size values obtained and confocal images confirmed the uniform dispersion of the vesicles formed. The results of this study also indicate that Pheroid preparations, used in experimentation over a period of time, should be kept at 5 °C to maintain its initial stable physical characteristics.

CHAPTER 6

SUMMARY AND FUTURE PROSPECTS

Emulsion formulations have become well known as a potential carrier system for the delivery or targeting of drugs to specific sites in the body. The biotechnological approach of the Pheroid drug delivery system, which forms a part of submicron emulsion type formulations, are based on the ability to entrap drugs with high efficiency and deliver these with remarkable speed to target sites in the body.

Emulsion formulations have gained particular interest as a carrier system of drugs due to their biocompatibility and satisfactory long-term stability and the fact that they can easily be manufactured on an industrial scale using proven technology. The aim of this study was to prove that the Pheroid system also comply to these criteria and to determine an optimum formula for future projects.

The influences of specific variables changed within the processing of the Pheroid vesicles were examined. With an alternation of the mixing rate (8000, 13500 and 24000 rpm) used to prepare the Pheroid vesicles the following effects on the physical properties of the Pheroid vesicles were found. The mean particle size of the samples decreased with an increase in the mixing rate. It is believed that the stability of the formulation increased with a reduction in the mean particle size of the vesicles resulting in less coalescence of droplets which may lead to physical instabilities. All three samples prepared showed stable zeta potential values throughout the sampling period. The turbidity values were the inverse of the mean particles size with the highest turbidity measured for the highest mixing rate employed. Micrographs showed that the smallest and most evenly distributed vesicles were obtained with the highest mixing rate (24000 rpm) used to prepare the samples.

Different mixing times (60, 120 and 300 seconds) used to prepare the samples resulted in changes of the characteristics of the Pheroid vesicles. Firstly the mean particle size of the vesicles drastically decreased as the emulsification time increased. The zeta potential values varied considerably over the 28 day testing period. The highest turbidity values were obtained for the sample with the shortest time of emulsification. Micrographs showed depots of oil droplets at the shorter interval of mixing (60 seconds), probably due to not enough shearing taking place between the oil phase and the water phase.

Emulsification is known to take place at a certain temperature for the oil phase as well as the water phase. Stable vesicles seemed to form at a water phase temperature of 75 ± 1 °C. At this temperature the particles were small and evenly distributed throughout the testing period. All of the samples had acceptable zeta potential values. The turbidity values increased with an increase in water phase temperature, proving that an increase in water phase temperature resulted in the formation of smaller vesicles. This was confirmed by micrographs of the samples prepared.

An important component of the Pheroid vesicles proved to be the nitrous oxide gas used to saturate the water phase with. It was proved that an increase in the mean particle size of the vesicles could be detected with an alteration of the amount of days the water was gassed. It was concluded that 4 days of gassing would be used for any future studies to compensate for the loss of gas during the processing of the Pheroid vesicles and during handling and storage of the samples.

The addition of a suitable surfactant enables that a fine dispersity after production could be maintained during storage. The concentration of the surfactant to be added to the formula was investigated. It was found that an optimum amount of emulsifier (1.00 g) resulted in Pheroid vesicles with a small mean particle size that have a stable zeta potential with high turbidity values. Micrographs showed that lower concentrations of Cremophor® RH 40 resulted in a large amount of the oil phase still being left in the formulation. An increase in the amount of Cremophor® RH 40 resulted in the formation of a few small vesicles.

Another important part of the oil phase of the Pheroid vesicles is the Vitamin F Ethyl Ester CLR added that contains the essential fatty acids necessary for various cell functions in the human body. Different samples varied in mean particle sizes with the optimum ratio (2.80 g) of Vitamin F Ethyl Ester resulting in a small variation in size distribution and a stable zeta potential of above 30 mV.

Non-optimal or non-predictable stability properties of emulsions can be limiting for the application of emulsions. Therefore it was decided to perform a formal accelerated stability test on a specific formula. Samples were exposed to various temperatures with varying relative humidity values for a period of 3 months. The obtained results showed that the specific formula had acceptable stability even under stressed conditions and that this formula is suitable for further clinical experimentation and development.

Recommendations for future studies are:

- Large scale production of the Pheroid vesicles.
- The use of different methods of preparation for Pheroid vesicles to obtain different morphological characteristics.
- Reduction in the size of the Pheroid vesicles to obtain nano-vesicles.
- Inclusion of combinations of surfactants to improve the physical stability of the vesicles formed.
- Quantification of the amount of gas necessary to obtain stable vesicles.
- Determining the functionality of each component within the chemical structure of the vesicles formed.
- Facing the challenges involved in resolving creaming during storage of the samples along with possible chemical changes occurring within the formulations composition.

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LIST OF FIGURES

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ANNEXURES

ANNEXURE A

CERTIFICATE OF ANALYSIS OF CREMOPHOR® RH 40, VITAMIN F ETHYL ESTER CLR AND DL-α-TOCOPHEROL

Annexure A.1.1 *Certificate of analysis of Cremophor® RH 40.*

BASF Aktiengesellschaft

BASF

Certificate of analysis

	FAX NO 002727112542461
BASF South Africa (PTY) Ltd	
852 Sixteen Road	28.05.2002
2801	GKA/M320
1685	Dr.Leyendecker
MIDRAND	0621-60-45308
	CERTIFICATE NO 560
	PAGE 1 OF 4

INSPECTION CERTIFICATE 3.1 B ACCORDING TO EN 10204

Cremophor* RH 40	ARTICLE NO 50044005
	ORDER A003213814 000011
60kg Metal-Drum, removable head	DELIVERY 3080012532 000010
YOUR ORDER	LOT 730671.
6030039332	LOT/QTY 1080.000 KG
	TOTAL 1080.000 KG

Verseifungszahl / Saponification Value	50 mg	KOH/g
Hydroxylzahl / Hydroxyl Value	74 mg	KOH/g
Saeurezahl / Acid Value	0.1 mg	KOH/g
Iodzahl / Iodine Value	0.8 g	Iodine/100g
Wasser / Water (Karl- Fischer- Titration)	1.1	g/100g
Schwermetalle / Heavy Metals	max. 10	mg/kg
pH-Wert / pH-Value (100 g/L in Wasser / in water)	6.4	
Aussehen / Appearance weisse bis gelbl., weiche oder fliess.Paste white to yellowish,soft or flowing paste	Entspricht /	conforms
Geruch / Odour gering / faint	Entspricht /	conforms
Aussehen der waessrigen Loesung 10% / Appearance of aqueous solution 10%	Entspricht /	conforms
Erstarrungstemperatur / Congealing temp.	18	Grad C
Alkalinitaet / Alcalinity	Entspricht /	conforms
Arsen / Arsenic	max. 2	mg/kg

Annexure A.1.2 *Certificate of analysis of Cremophor® RH 40.*

BASF Aktiengesellschaft

BASF

Certificate of analysis

FAX NO 002727112542461

BASF South Africa (PTY) Ltd

852 Sixteen Road
2801
1685
MIDRAND

28.05.2002
GKA/M320
Dr.Leyendecker
0621-60-45308
CERTIFICATE NO 560
PAGE 2 OF 4

INSPECTION CERTIFICATE 3.1 B ACCORDING TO EN 10204

Cremophor* RH 40	ARTICLE NO	50044005
	ORDER	A003213814 000011
60kg Metal-Drum, removable head	DELIVERY	3080012532 000010
YOUR ORDER	LOT	730671.
6030039332	LOT/QTY	1080.000 KG
	TOTAL	1080.000 KG

Identitaet / Identification (USP / NF)	Test A	Entspricht / conforms
Identitaet / Identification (USP / NF)	Test B	Entspricht / conforms
Ethylenoxid / Ethylene Oxide (CGC)		<1 mg/kg
Dioxan / dioxane (CGC)		<10 mg/kg
Gesamtkeimzahl (aerobe Keime, Bakterien + Pilze) / Total viable aerobic count (aerobic bacteria + fungi)		<30 1/g
Escherichia Coli		0 1/g
Enterobakterien und andere gramnegative Bakterien / enterobacteria and certain other gram-negative bacteria		<1 1/g
Staphylococcus aureus		0 1/g
Pseudomonas aeruginosa		0 1/g
Mikrobiologischer Status / Microbiological Quality (Ph.Eur., Kat.2 + 3A)		Entspricht / conforms
Fluechtige organische Verunreinigungen / Organic Volatile Impurities (USP)		Entspricht / conforms

Annexure A.1.3 *Certificate of analysis of Cremophor® RH 40.*

BASF Aktiengesellschaft

BASF

Certificate of analysis

FAX NO 002727112542461

BASF South Africa (PTY) Ltd

852 Sixteen Road
2801
1685
MIDRAND

28.05.2002
GKA/M320
Dr.Leyendecker
0621-60-45308
CERTIFICATE NO 560
PAGE 3 OF 4

INSPECTION CERTIFICATE 3.1 B ACCORDING TO EN 10204

Cremophor* RH 40

60kg Metal-Drum, removable head
YOUR ORDER
6030039332

ARTICLE NO	50044005
ORDER	A003213814 000011
DELIVERY	3080012532 000010
LOT	730671.
LOT/QTY	1080.000 KG
TOTAL	1080.000 KG

Asche / Total ash 600 Grad C (Ph.Eur.)	0.17	g/100g
Identitaet / Identification (Ph.Eur., Test C)	Entspricht /	conforms
Identitaet / Identification (Ph.Eur., Test D)	Entspricht /	conforms
Sulfatasche / residue in ignition (USP / NF)	max. 0.2	g/100g
Restloesemittel / residual solvents (Ph.Eur., class 2)	Entspricht /	conforms
Restloesemittel / residual solvents (Ph.Eur., class 3)	Entspricht /	conforms

Nur die Restloesemittel Ethylenglykol und 1,4-Dioxan der Klasse 2 und Restloesemittel der Klasse 3 des Ph.Eur. koennen enthalten sein. Die Konzentrationen der Klasse 2 liegen unterhalb der im Ph.Eur., Kapitel 5.4 genannten Grenzwerte und Klasse 3 liegt unterhalb 0,5 %.

Only class 2 solvents ethylene glycol and 1,4-dioxane and class 3 solvents of Ph.Eur. are likely to be present. The concentrations of class 2 solvents are below the limits given in Ph.Eur., chapter 5.4. and class 3 solvents are below 0.5 %.

Das Produkt erfuellt die Anforderungen folgender Monographien:
Macrogol-Glycerolhydroxystearat des Ph.Eur.4.Edition 2002
Polyoxyl 40 Hydrogenated Castor Oil des USP 25/NF 20.

Annexure A.1.4 *Certificate of analysis of Cremophor® RH 40.*

BASF Aktiengesellschaft

BASF

Certificate of analysis

FAX NO 002727112542461

BASF South Africa (PTY) Ltd

852 Sixteen Road
2801
1685
MIDRAND

28.05.2002
GKA/M320
Dr.Leyendecker
0621-60-45308
CERTIFICATE NO 560
PAGE 4 OF 4

INSPECTION CERTIFICATE 3.1 B ACCORDING TO EN 10204

Cremophor* RH 40

50kg Metal-Drum, removable head
YOUR ORDER
6030039332

ARTICLE NO	50044005
ORDER	A003213814 000011
DELIVERY	3080012532 000010
LOT	730671
LOT/QTY	1080.000 KG
TOTAL	1080.000 KG

The product meets the specifications of the monographs:
Macrogol-Glycerolhydroxystearat of Ph.Eur.4.Edition 2002
Polyoxyl 40 Hydrogenated Castor Oil of the USP 25/NF 20.

QS-Referenz-Nr. / QC-Reference-No.	02C02902
Analysiert am / Analyzed on	13.05.2002
Mindestens haltbar bis / Best before	04.2004

BASF Aktiengesellschaft

GKA Analytik

Qualitaetskontrolle / Quality Control

gez. / sig. Dr.Leyendecker

Dieses Abnahmepruefzeugnis wurde maschinell erstellt und ist ohne Unterschrift gueltig.

This Certificate of Analysis has been produced electronically and is valid without signature.

Annexure A.2 *Certificate of analysis of Vitamin F Ethyl Ester CLR.*

for fine cosmetics · since 1926

CLR · Postfach 41 04 80 · 12114 Berlin · Germany
Chemimpo South Africa
(PTY) LTD.
275 Oak Avenue Randburg
2125 Randburg

Chemisches Laboratorium Dr. Kurt Richter GmbH
Bennigsenstraße 25
12159 Berlin
Germany
Tel +49 (30) 85 10 26-0
Fax +49 (30) 85 10 26-85
info@clr-berlin.com
www.clr-berlin.com

Certificate of Analysis
Vitamin F Ethylester CLR

Article	523	Customer	30135
Lot	3046007	Order No.	17003

Remark J001731

	Method	Specification	Result	Unit
General Parameters				
Refractive index (nD20)	CLR-pa001lch	1,4570 - 1,4590	1,4584	
Density (20°C)	CLR-pa002lch	0,8770 - 0,8800	0,8785	g/ml
Acid value	CLR-pa014lch	0,0 - 0,8	0,4	
Iodine value (Hanus)	CLR-pa018lch	145,0 - 155,0	154,0	
Fatty acid distribution				
< C18 + C18-0	CLR-pa042lch	11,0 - 15,0	11,6	%
C18-1	CLR-pa042lch	18,0 - 24,0	21,4	%
C18-2	CLR-pa042lch	31,0 - 37,0	35,5	%
C18-3	CLR-pa042lch	26,0 - 34,0	30,5	%
> C18	CLR-pa042lch	1,0 - 3,0	1,0	%
Microbiological tests				
Colony forming units			< 100 / ml	
Pathogenic germes			Not detectable	

12159 Berlin, 04.11.2004

W. Reinhold
(Director Quality Assurance)

The above data are the results of our quality control. They do not release the customer from carrying out his own quality check upon receipt of goods and are not intended to guarantee a certain property of the material or its suitability for a particular purpose.
This certificate of analysis is an automated printout and therefore bears no signature.

2004/9952

Annexure A.3 *Certificate of analysis of DL-α-Tocopherol.*

DL-A-TOCOPHEROL

DSM

CERTIFICATE OF ANALYSIS

Productcode : 0410276
Lot No. : UT04120072
Analysis No. : 03418157

Test	Result	Limits / Specifications	Dimension / Units
Appearance	clear viscous oil		
Colour	slightly yellow		
Identity	corresponds		
Refractive index, 589nm, 20°C	1.506	1.503 to 1.507	
Density at 20 °C	0.950	0.947 to 0.951	g/ml
Absorbance in ethanol			
- at about 292 nm	74.6	72.0 to 76.0	
- at about 255 nm	7.6	6.0 to 8.0	
Optical rotation	-0.00	-0.01 to +0.01	°
Acid value	0.1	max. 2.0	
Sulphated ash	<0.1		%
Heavy metals	<10		ppm
Lead	<2		ppm
Arsenic	<3		ppm
Mercury	<1		ppm
Organic volatile impurities	meets USP requirements		
Assay	99.2	97.0 to 102.0	%

This lot was analysed and released by our authorized Quality Control Department and was found to meet the specifications as given above.
The product meets all requirements of the following valid compendia when tested accordingly:
USP, FCC, Ph. Eur.

DSM Nutritional Products Ltd
The Quality Assurance Manager

Bruno Mueller

DSM Nutritional Products Ltd
Branch Site Sisseln
Quality Management
CH-4334 Sisseln

Date of issue : 17-Dec-2004

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ANNEXURE B

PHEROID MANUFACTURING SHEETS

Annexure B.1.1 *Manufacturing sheet of Pheroid vesicles produced at different mixing rates.*

Pheroid Manufacturing

Name of product: Mixing rate
Date of manufacture: 2005-08-16

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1. 2.81 2. 2.80 3. 2.81	CE	
Cremophor	50369256PO	1.00	1. 1.00 2. 1.00 3. 1.00	CE	
N_2O water	4 Days	96.00	1. 96.00 2. 96.01 3. 96.01	CE	
Vit E	UT03100045	0.20	1. 0.20 2. 0.21 3. 0.22	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 2 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.
2) 120 seconds at 13500 rpm.
3) 120 seconds at 24000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
Three
Mixing rate (16/08/05)

Prepared by: Charlene	Sign.	Date: 2005/08/16
Checked by: Jannie	Sign.	Date: 2005/08/16

Annexure B.1.2 *Manufacturing sheet of Pheroid vesicles produced at different mixing times.*

Pheroid Manufacturing

Name of product: Mixing time
Date of manufacture: 2005-08-16

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1. 2.81 2. 2.81 3. 2.80	CE	
Cremophor	50369256PO	1.00	1. 1.00 2. 1.00 3. 1.00	CE	
N_2O water	4 Days	96.00	1. 96.00 2. 96.01 3. 96.00	CE	
Vit E	UT03100045	0.20	1. 0.20 2. 0.21 3. 0.20	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 2 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 60 seconds at 8000 rpm.
2) 120 seconds at 8000 rpm.
3) 300 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
Three
Mixing time (16/08/05)

Prepared by: Charlene	Sign.	Date: 2005/08/16
Checked by: Jannie	Sign.	Date: 2005/08/16

Annexure B.1.3 *Manufacturing sheet of Pheroid vesicles produced with different water phase temperatures.*

Pheroid Manufacturing

Name of product: Water phase temperature
Date of manufacture: 2005-08-30

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1) 2.83 2) 2.81 3) 2.80 4) 2.82 5) 2.82	CE	
Cremophor	50369256PO	1.00	1) 1.00 2) 1.00 3) 1.00 4) 1.00 5) 1.00	CE	
N_2O water	4 Days	96.00	1) 96.00 2) 96.00 3) 96.00 4) 96.00 5) 96.00	CE	
Vit E	UT03100045	0.20	1) 0.20 2) 0.22 3) 0.22 4.)0.21 5.)0.20	CE	

Method of manufacture:

Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 2:30 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till 1) 55 °C 2) 65 °C
3) 75 °C 4) 85 °C
5) 95 °C

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:

Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
Five
Temperature (30/08/05)

Prepared by: Charlene	Sign.	Date: 2005/08/30
Checked by: Jannie	Sign.	Date: 2005/08/30

Annexure B.1.4.1 *Manufacturing sheet of Pheroid vesicles produced with different gassing periods of the water phase.*

Pheroid Manufacturing

Name of product: Gas period of 0 days and 1 day
Date of manufacture: 2005-10-04

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1. 2.82 2. 2.81	CE	
Cremophor	50369256PO	1.00	1. 1.01 2. 1.00	CE	
N_2O water	0 Days + 1Day	96.00	1. 96.01 2. 96.01	CE	
Vit E	UT03100045	0.20	1. 0.19 2. 0.21	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 1:40 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
One
Gas period (04/10/05)

Prepared by: Charlene	Sign.	Date: 2005/10/04
Checked by: Jannie	Sign.	Date: 2005/10/04

Annexure B.1.4.2 *Manufacturing sheet of Pheroid vesicles produced with different gassing periods of the water phase.*

Pheroid Manufacturing

Name of product: Gas period of 3 days
Date of manufacture: 2005-10-10

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1. 2.80	CE	
Cremophor	50369256PO	1.00	1. 1.01	CE	
N_2O water	3 Days	96.00	1. 96.00	CE	
Vit E	UT03100045	0.20	1. 0.22	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 1:40 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
One
Gas period (10/10/05)

Prepared by: Charlene	Sign.	Date: 2005/10/10
Checked by: Jannie	Sign.	Date: 2005/10/10

Annexure B.1.4.3 *Manufacturing sheet of Pheroid vesicles produced with different gassing periods of the water phase.*

Pheroid Manufacturing

Name of product: Gas period of 4 days
Date of manufacture: 2005-10-11

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1. 2.80	CE	
Cremophor	50369256PO	1.00	1. 1.00	CE	
N_2O water	4 Days	96.00	1. 96.00	CE	
Vit E	UT03100045	0.20	1. 0.20	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 1:30 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
One
Gas period (11/10/05)

Prepared by: Charlene	Sign.	Date: 2005/10/11
Checked by: Jannie	Sign.	Date: 2005/10/11

Annexure B.1.5 *Manufacturing sheet of Pheroid vesicles produced with different Cremophor® RH 40 concentrations.*

Pheroid Manufacturing

Name of product: Cremophor® concentration
Date of manufacture: 2005-11-22

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1) 2.82 2) 2.81 3) 2.81 4) 2.80 5) 2.80	CE	
Cremophor	50369256PO	1) 0.25 2) 0.50 3) 1.00 4) 2.00 5) 4.00	1) 0.25 2) 0.50 3) 1.01 4) 2.01 5) 4.01	CE	
N_2O water	4 Days	1) 96.75 2) 96.50 3) 96.00 4) 95.00 5) 93.00	1) 96.75 2) 96.50 3) 96.00 4) 95.00 5) 93.00	CE	
Vit E	UT03100045	0.20	1) 0.20 2) 0.21 3) 0.19 4.)0.19 5.)0.19	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 2:30 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till 1) 75 °C
Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
Five
Cremophor® (22/11/05)

Prepared by: Charlene	Sign.	Date: 2005/11/22
Checked by: Jannie	Sign.	Date: 2005/11/22

Annexure B.1.6.1 *Manufacturing sheet of Pheroid vesicles produced with different Vitamin F Ethyl Ester CLR concentrations.*

Pheroid Manufacturing

Name of product: Vitamin F concentration
Date of manufacture: 2005-10-18

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	1. 1.00 2. 2.00 3. 2.50	1. 1.00 2. 2.01 3. 2.50	CE	
Cremophor	50369256PO	1.00	1. 1.00 2. 1.00 3. 1.00	CE	
N_2O water	4 Days	1. 97.80 2. 96.80 3. 96.30	1. 97.80 2. 96.80 3. 96.30	CE	
Vit E	UT03100045	0.20	1. 0.20 2. 0.18 3. 0.20	CE	

Method of manufacture:

Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 1:20 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.
2) 120 seconds at 8000 rpm.
3) 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:

Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
Three
Vitamin F (18/10/05)

Prepared by: Charlene	Sign.	Date: 2005/10/18
Checked by: Jannie	Sign.	Date: 2005/10/18

Annexure B.1.6.2 *Manufacturing sheet of Pheroid vesicles produced with different Vitamin F Ethyl Ester CLR concentrations.*

Pheroid Manufacturing

Name of product: Vitamin F concentration
Date of manufacture: 2005-10-24

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	1. 2.80 2. 3.25 3. 3.50	1. 2.80 2. 3.25 3. 3.50	CE	
Cremophor	50369256PO	1.00	1. 1.00 2. 1.00 3. 1.00	CE	
N_2O water	4 Days	1. 96.00 2. 95.55 3. 95.30	1. 95.99 2. 95.55 3. 95.31	CE	
Vit E	UT03100045	0.20	1. 0.20 2. 0.20 3. 0.20	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 1:40 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till ± 75 °C.

Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 1) 120 seconds at 8000 rpm.
2) 120 seconds at 8000 rpm.
3) 120 seconds at 8000 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 200 ml
± 100 ml
Three
Vitamin F (24/10/05)

Prepared by: Charlene	Sign.	Date: 2005/10/24
Checked by: Jannie	Sign.	Date: 2005/10/24

Annexure B.2 *Manufacturing sheet of Pheroid vesicles for accelerated stability tests.*

Pheroid Manufacturing

Name of product: Final formula
Date of manufacture: 2006-01-17

Raw Materials	Batch number	Quantity %/100	Weight (g)	Pharm sign	Action sign
Vit F	3046007	2.80	1) 2.82 2) 2.80 3) 2.81 4) 2.80 5) 2.81 6) 2.80	CE	
Cremophor	50369256PO	1.00	1) 1.00 2) 0.98 3) 1.00 4) 1.00 5) 1.01 6) 1.02	CE	
N_2O water	4 Days	96.00	1) 96.00 2) 96.00 3) 96.00 4) 96.01 5) 96.00 6) 96.00	CE	
Vit E	UT03100045	0.20	1) 0.20 2) 0.20 3) 0.19 4) 0.21 5) 0.20 6) 0.20	CE	

Method of manufacture:
Step 1:
In a 50ml glass-container;
Weigh the required amount of Vit E, Vit F and Cremophor.
Heat in Microwave for 1:40 minutes at 900 Watt
or until the mixture is transparent.

Step 2:
Weigh the required amount of water in an Erlenmeyer flask and cover the mouth with parafilm. Heat on stove till 75 °C
Step 3:
Add the heated Oil phase to the Water phase.
Homogenise for 120 seconds at 13500 rpm.

Step 4:
Shake the mixture till roomtemperature (25°C) is reached.

Method of packaging:
Fill jars/bottles
Record fill volume
Record quantity of jars/bottles filled
Lable with appropriate labels

Amber 50 ml
± 50 ml
Ten
Final Formula (17/01/06)

Prepared by: Charlene	Sign.	Date: 2006/01/17
Checked by: Jannie	Sign.	Date: 2006/01/17

ANNEXURE C

EFFECT OF PREPARATION VARIABLES ON THE PHYSICAL PROPERTIES OF PHEROID VESICLES

Table C.1.1 *Characteristics of Pheroid samples prepared at different mixing rates.*

Sample period (days)	Mixing rate (rpm)	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
1	**8000**	1.700	-29.40	6.750	3.97	-198.45	-17.29	20.8	20.8
	13500	1.070	-25.30	7.615	7.12	-192.66	-23.64	39.2	39.2
	24000	0.810	-26.00	9.495	11.72	-246.87	-32.10	46.2	46.2
2	**8000**	1.700	-29.25	7.425	4.37	-217.18	-17.21		
	13500	1.100	-24.40	7.830	7.12	-191.05	-22.18		
	24000	0.840	-27.55	9.990	11.89	-275.22	-32.80		
3	**8000**	1.720	-35.65	7.500	4.36	-267.38	-20.73		
	13500	1.120	-34.40	8.195	7.32	-281.91	-30.71		
	24000	0.840	-35.70	10.555	12.57	-376.81	-42.50		
7	**8000**	1.660	-23.80	6.370	3.84	-151.61	-14.34		
	13500	1.060	-33.05	7.255	6.84	-239.78	-31.18		
	24000	0.850	-27.65	9.570	11.26	-264.61	-32.53		
14	**8000**	1.660	-35.80	6.070	3.66	-217.31	-21.57		
	13500	1.090	-33.00	7.490	6.87	-247.17	-30.28		
	24000	0.850	-34.95	9.970	11.73	-348.45	-41.12		
21	**8000**	1.670	-33.60	6.480	3.88	-217.73	-20.12		
	13500	1.090	-24.55	7.330	6.72	-179.95	-22.52		
	24000	0.840	-32.70	9.965	11.86	-325.86	-38.93		
28	**8000**	1.630	-31.10	5.960	3.66	-185.36	-19.08		
	13500	1.090	-32.05	7.150	6.56	-229.16	-29.40		
	24000	0.840	-30.10	8.605	10.24	-259.01	-35.83		

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size

Table C.1.2 *Characteristics of Pheroid samples prepared at different mixing times.*

Sample period (days)	Mixing time (sec)	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
1	**60**	7.410	-29.30	6.400	0.86	-187.52	-3.95	6.02	55.0
	120	3.180	-21.45	6.030	1.90	-129.34	-6.75	6.50	40.0
	300	1.740	-27.10	5.970	3.43	-161.79	-15.57	6.42	35.4
2	**60**	7.410	-26.15	6.930	0.94	-181.22	-3.55		
	120	3.190	-22.25	6.740	2.11	-149.97	-6.97		
	300	1.850	-18.95	6.550	3.54	-124.12	-10.24		
3	**60**	7.710	-30.75	8.160	1.06	-250.92	-3.99		
	120	3.150	-32.80	7.260	2.30	-238.13	-10.41		
	300	1.820	-35.90	6.030	3.31	-216.48	-19.73		
7	**60**	7.250	-31.60	4.930	0.68	-155.79	-4.36		
	120	3.040	-33.85	5.280	1.74	-178.73	-11.13		
	300	1.800	-26.45	5.900	3.28	-156.06	-14.69		
14	**60**	7.380	-40.70	6.760	0.92	-275.13	-5.51		
	120	3.080	-30.45	5.990	1.94	-182.40	-9.89		
	300	1.780	-23.15	5.980	3.36	-138.44	-13.01		
21	**60**	7.300	-35.50	6.510	0.89	-231.11	-4.86		
	120	3.110	-33.45	5.270	1.69	-176.28	-10.76		
	300	1.780	-30.75	5.380	3.02	-165.44	-17.28		
28	**60**	7.360	-32.40	6.040	0.82	-195.70	-4.40		
	120	3.060	-31.15	5.500	1.80	-171.33	-10.18		
	300	1.830	-34.60	5.750	3.14	-198.95	-18.91		

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size

Table C.1.3 *Characteristics of Pheroid samples prepared at different water phase temperatures.*

Sample period (days)	Temperature Range (°C)	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
1	**55**	1.600	-34.70	7.210	4.51	-250.19	-21.69	6.48	27.55
	65	1.450	-30.55	7.010	4.83	-214.16	-21.07	5.97	56.80
	75	1.470	-32.25	6.840	4.65	-220.43	-21.94	5.95	56.85
	85	1.260	-25.25	7.300	5.79	-184.20	-20.04	6.06	57.15
	95	1.220	-22.40	7.020	5.75	-157.25	-18.36	5.81	65.20
2	**55**	1.260	-28.85	7.080	4.54	-204.11	-18.49		
	65	1.430	-22.35	7.110	4.97	-158.80	-15.63		
	75	1.430	-31.30	7.500	5.24	-234.59	-21.89		
	85	1.300	-31.55	7.900	6.07	-249.09	-24.77		
	95	1.180	-27.10	7.140	6.05	-193.36	-22.97		
3	**55**	1.560	-35.15	7.270	4.66	-255.36	-22.53		
	65	1.520	-36.15	7.150	4.70	-258.29	-23.78		
	75	1.370	-33.80	6.950	5.07	-234.91	-24.67		
	85	1.260	-36.10	7.280	5.78	-262.81	-28.65		
	95	1.170	-34.05	7.650	6.54	-260.48	-29.16		
7	**55**	1.550	-34.70	7.090	4.57	-245.85	-22.39		
	65	1.450	-35.00	6.980	4.81	-244.30	-24.14		
	75	1.410	-23.65	7.420	5.26	-175.36	-16.77		
	85	1.320	-22.50	7.800	5.91	-175.50	-17.05		
	95	1.190	-21.55	7.500	6.30	-161.63	-18.11		
14	**55**	1.560	-34.10	6.970	4.47	-237.68	-21.86		
	65	1.420	-32.70	7.390	5.20	-241.65	-23.03		
	75	1.400	-31.20	7.570	5.40	-236.03	-22.29		
	85	1.240	-30.15	8.050	6.49	-242.71	-24.31		
	95	1.320	-33.30	7.960	6.03	-265.07	-25.23		
21	**55**	1.610	-36.60	7.420	4.61	-271.39	-22.73		
	65	1.410	-35.70	7.670	5.44	-273.64	-25.32		
	75	1.390	-35.30	7.380	5.31	-260.34	-25.40		
	85	1.290	-36.60	8.110	6.28	-296.64	-28.37		
	95	1.170	-37.55	8.180	6.99	-306.97	-32.09		
28	**55**	1.560	-46.05	7.400	4.74	-340.54	-29.52		
	65	1.440	-49.25	7.300	5.07	-359.53	-34.20		
	75	1.410	-40.75	7.650	5.43	-311.74	-28.90		
	85	1.300	-37.65	7.850	6.03	-295.36	-28.96		
	95	1.220	-30.30	8.480	6.95	-256.79	-24.86		

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size

Table C.1.4 *Characteristics of Pheroid samples gassed for different days.*

Sample period (days)	Number of days gassed	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
1	**0**	1.150	-41.10	8.190	7.12	-336.61	-35.74	7.39	-22.95
	1	1.180	-37.60	8.090	6.86	-304.18	-31.86	5.56	82.10
	3	1.190	-34.95	8.740	7.34	-305.46	-29.37	6.11	51.90
	4	1.390	-36.15	7.160	5.15	-258.83	-26.01	8.60	90.60
2	**0**	1.160	-35.15	8.690	7.49	-305.45	-30.30		
	1	1.160	-34.45	8.090	6.97	-278.70	-29.70		
	3	1.190	-35.15	8.620	7.24	-302.99	-29.54		
	4	1.360	-33.00	6.690	4.92	-220.77	-24.26		
3	**0**	1.230	-36.85	8.620	7.01	-317.65	-29.96		
	1	1.150	-35.05	8.370	7.28	-293.37	-30.48		
	3	1.180	-31.90	8.710	7.38	-277.85	-27.03		
	4	1.320	-34.60	7.290	5.52	-252.23	-26.21		
7	**0**	1.150	-36.70	8.600	7.48	-315.62	-31.19		
	1	1.150	-34.65	8.120	7.06	-281.36	-30.13		
	3	1.240	-35.25	9.090	7.33	-320.42	-28.43		
	4	1.410	-32.40	7.200	5.11	-233.28	-22.98		
14	**0**	1.190	-31.35	8.980	7.55	-281.52	-26.34		
	1	1.150	-32.85	8.470	7.37	-278.24	-28.57		
	3	1.200	-37.35	9.020	7.52	-336.90	-31.13		
	4	1.370	-24.15	7.100	5.18	-171.47	-17.63		
21	**0**	1.260	-54.10	8.410	6.67	-454.98	-42.94		
	1	1.200	-51.00	8.570	7.14	-437.07	-42.50		
	3	1.200	-39.30	9.050	7.54	-355.67	-32.75		
	4	1.400	-33.15	7.110	5.08	-235.70	-23.68		
28	**0**	1.130	-33.55	8.780	7.77	-294.57	-29.69		
	1	1.170	-33.95	8.470	7.24	-287.56	-29.02		
	3	1.220	-38.30	9.210	7.55	-352.74	-31.39		
	4	1.390	-39.15	7.390	5.32	-289.32	-28.17		

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size

Table C.1.5 *Characteristics of Pheroid samples prepared with different concentrations of Cremophor® RH 40.*

Sample period (days)	Chremophor® RH 40 (g)	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
1	**0.25**	3.130	-36.25	2.930	0.93	-106.03	-11.58	5.30	94.40
	0.50	3.620	-31.30	3.690	1.02	-115.50	-8.65	5.48	84.70
	1.00	1.370	-37.90	5.150	3.76	-195.19	-27.66	6.81	7.90
	2.00	1.670	-33.30	1.930	1.16	-64.27	-19.94	6.47	27.80
	4.00	2.460	-26.00	0.580	0.23	-14.95	-10.57	6.37	33.30
2	**0.25**	3.090	-41.00	3.030	0.98	-124.23	-13.27		
	0.50	3.730	-33.70	3.650	0.98	-122.84	-9.03		
	1.00	1.190	-36.15	5.080	4.26	-183.46	-30.38		
	2.00	1.830	-38.10	2.010	1.10	-76.58	-20.82		
	4.00	1.780	-30.40	0.720	0.40	-21.74	-17.08		
3	**0.25**	3.060	-34.45	2.910	0.95	-100.25	-11.26		
	0.50	3.850	-32.25	3.640	0.95	-117.39	-8.38		
	1.00	1.220	-29.85	5.050	4.14	-150.59	-24.17		
	2.00	1.890	-28.80	2.010	1.06	-57.74	-15.24		
	4.00	1.770	-20.90	0.690	0.39	-14.42	-11.81		
7	**0.25**	3.040	-43.05	2.780	0.91	-119.68	-14.16		
	0.50	3.650	-32.60	3.700	1.01	-120.46	-8.93		
	1.00	1.190	-32.20	5.140	4.32	-165.35	-27.06		
	2.00	1.450	-31.20	2.130	1.47	-66.46	-21.52		
	4.00	1.290	-27.05	0.720	0.55	-19.34	-20.97		
14	**0.25**	3.200	-37.60	2.920	0.91	-109.79	-11.75		
	0.50	3.940	-38.30	3.840	0.97	-146.88	-9.72		
	1.00	1.190	-33.35	5.000	4.20	-166.58	-28.03		
	2.00	1.610	-33.10	2.040	1.27	-67.52	-20.56		
	4.00	1.540	-27.75	0.760	0.49	-21.09	-18.02		
21	**0.25**	3.090	-37.20	2.870	0.93	-106.58	-12.04		
	0.50	3.770	-33.45	3.740	0.99	-125.10	-8.87		
	1.00	1.270	-33.10	5.270	4.15	-174.44	-26.06		
	2.00	1.570	-31.45	2.310	1.47	-72.49	-20.03		
	4.00	2.190	-27.60	0.940	0.43	-25.94	-12.60		
28	**0.25**	3.180	-39.15	2.790	0.88	-109.23	-12.31		
	0.50	3.750	-37.05	3.610	0.96	-133.75	-9.88		
	1.00	1.190	-36.05	5.250	4.41	-189.26	-30.29		
	2.00	1.660	-35.45	2.210	1.33	-78.17	-21.36		
	4.00	45.77	-31.10	0.920	0.02	-28.61	-0.68		

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size

Table C.1.6 *Characteristics of Pheroid samples prepared with different concentrations of Vitamin F Ethyl Ester CLR.*

Sample period (days)	Vitamin F Ethyl Ester CLR (g)	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
1	**1.00**	1.980	-37.55	0.710	0.36	-26.66	-18.96	8.33	-76.15
	2.00	1.210	-33.95	5.620	4.64	-190.63	-17.15	7.04	-2.35
	2.50	1.020	-36.40	7.930	7.77	-288.65	-30.08	6.78	12.45
	2.80	1.460	-33.90	8.780	6.01	-297.47	-33.24	6.44	31.75
	3.25	1.700	-30.85	8.240	4.85	-254.20	-21.13	6.53	26.45
	3.50	2.020	-32.90	8.490	4.20	-279.32	-19.35	6.23	43.95
2	**1.00**	2.270	-21.70	0.610	0.27	-13.13	-10.74		
	2.00	1.070	-25.70	5.460	5.10	-140.32	-11.32		
	2.50	1.080	-23.60	7.510	6.95	-177.12	-22.06		
	2.80	1.140	-36.50	9.020	7.91	-329.23	-33.80		
	3.25	1.670	-36.65	8.200	4.91	-300.35	-32.15		
	3.50	2.060	-36.55	8.120	3.94	-296.60	-21.89		
3	**1.00**	2.190	-36.40	0.580	0.26	-21.11	-17.67		
	2.00	1.050	-35.00	5.260	5.01	-184.10	-15.98		
	2.50	1.040	-36.50	7.490	7.20	-273.20	-34.76		
	2.80	1.080	-38.65	8.630	7.99	-333.55	-37.16		
	3.25	1.600	-37.75	7.470	4.67	-281.99	-34.95		
	3.50	2.060	-37.30	6.900	3.35	-257.18	-23.31		
7	**1.00**	29.350	-22.65	0.780	0.03	-17.67	-11.60		
	2.00	1.310	-40.20	5.500	4.20	-221.10	-1.37		
	2.50	1.050	-35.00	7.480	7.12	-261.80	-26.72		
	2.80	1.170	-32.45	9.040	7.72	-293.19	-30.90		
	3.25	1.590	-37.60	7.690	4.83	-288.96	-32.14		
	3.50	2.010	-35.90	7.690	3.82	-275.89	-22.58		
14	**1.00**	1.040	-29.40	0.770	0.74	-22.49	-14.63		
	2.00	1.100	-31.90	6.250	5.68	-199.22	-30.67		
	2.50	1.940	-33.95	7.960	4.10	-270.07	-30.86		
	2.80	1.160	-36.60	9.190	7.92	-336.35	-18.87		
	3.25	1.540	-35.25	8.290	5.38	-292.22	-30.39		
	3.50	1.960	-37.95	7.250	3.70	-274.95	-24.64		
21	**1.00**	17.710	-39.50	0.570	0.03	-22.52	-20.15		
	2.00	1.140	-36.20	5.640	4.94	-203.99	-2.04		
	2.50	1.020	-37.95	9.330	9.15	-354.07	-33.29		
	2.80	1.130	-38.55	9.210	8.15	-354.85	-37.79		
	3.25	1.610	-39.15	9.210	5.72	-360.38	-34.65		
	3.50	1.980	-37.10	7.730	3.90	-286.60	-23.04		
28	**1.00**	1.960	-45.55	0.650	0.33	-29.61	-23.01		
	2.00	1.270	-39.95	5.450	4.29	-217.82	-20.38		
	2.50	1.090	-37.15	8.950	8.22	-332.66	-29.25		
	2.80	1.120	-43.20	8.840	7.89	-381.87	-39.63		
	3.25	1.550	-46.55	8.840	5.70	-411.49	-41.56		
	3.50	1.990	-39.45	7.440	3.74	-293.39	-25.45		

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size

Table C.2 *Accelerated stability test results.*

Sample period (months)	Stability point	Mean particle size (μm)	Zeta potential (mV)	Turbitidy (NTU)	T/P	TZ	Z/P	pH 24.0 °C	Current (mV)
Release		0.880	-33.31	5.440	6.18	-181.21	-37.85	6.645	8.00
1	**5°C**	0.890	-35.40	5.900	6.63	-208.86	-39.78	6.175	43.35
	25°C / 60%RH	0.860	-36.25	6.135	7.13	-222.39	-42.15	6.385	31.60
	40°C / 75%RH	0.860	-36.05	5.900	6.86	-212.70	-41.92	6.620	17.75
2	**5°C**	0.880	-34.05	6.120	6.95	-208.39	-38.69	6.180	42.65
	25°C / 60 % RH	0.860	-33.05	6.220	7.23	-205.57	-38.43	6.060	49.50
	40°C / 75%RH	0.860	-39.60	4.140	4.81	-163.94	-46.05	2.860	231.65
3	**5°C**	0.880	-35.80	5.980	6.80	-214.08	-40.68	7.300	-19.35
	25°C / 60%RH	0.850	-34.65	5.690	6.69	-197.16	-40.76	4.520	140.3
	40°C / 75%RH	0.830	-34.40	6.000	7.23	-206.40	-41.45	6.480	27.95

T/P = Turbidity/Mean particle size; TZ = Turbidity x Zeta potential; Z/P = Zeta potential/Mean particle size