ACTIVE ENCAPSULATION OF DICLOFENAC SODIUM INTO LIPOSOMES FOR OPHTHALMIC PREPARATIONS

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the School of Pharmacy, University of the Western Cape.

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KEYWORDS

Active encapsulation

Central composite design

Diclofenac sodium

High pressure liquid chromatography

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Liposomes

Nonsteroidal Anti-inflammatory Drugs

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Responds surface methodology

ABSTRACT

ACTIVE ENCAPSULATION OF DICLOFENAC SODIUM INTO LIPOSOMES FOR OPHTHALMIC PREPARATIONS

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Liposomes as a drug carrier in the pharmaceutical industry has gained currency since its discovery in 1965 by Bangham A. D. Liposomes have been shown to improve bioavailability as they can be delivered to target sites and possess sustained release properties which could be used to mitigate certain weaknesses associated with current diclofenac sodium eye drops. Diclofenac sodium (DNa) eye drop is a sterile Nonsteroidal Anti-inflammatory Drug (NSAID) with diclofenac sodium as its active ingredient. It is indicated for the lessening of ocular pain, prevention of miosis in eye operations, easing of postoperative inflammation and cystoids macular edema. The residence time of eye drops after application has been found to be 1-2 minutes as a result of continuous production of tears diluting the active ingredient, draining the eye drops into the nasolacrimal path, and eliminating it during blinking. As a result of the active ingredient not residing at the target site for the required duration, more frequent administration and medication is required and the risk of non-compliance is increased.

Given the aforementioned potential of liposomes to redress the above weaknesses of current eye drops (dosage form) available for diclofenac sodium ophthalmic application, this study sought to encapsulate diclofenac sodium into liposomes for ophthalmic application.

The main components of liposomes (cholesterol and phosphotidylcholine) and incubation time were set as the independent variables while percentage encapsulation, polydispersity

index (PDI) and drug release profile constituted the dependent variable. Using analysis of variance (ANOVA) and *t*-test statistics, the interaction between the independent variables and their effect on the dependent variables were tested.

Liposomes as a drug carrier where used for sustained release potentials while an *in situ* gel was used for its attachment tendencies due to the formation of gel after the administration. Active encapsulation or acetate gradient was used for encapsulation of DNa in the liposomes and thin film hydration was the method used in the preparation of liposomes. Design of experiment was Response Surface Methodology (RSM) particularly central composite design was used to optimise liposome formulation in order to obtain high percentage of drug encapsulated liposomes and high percentage of drug release from liposomes while obtaining small and homogeneous liposomes. Active encapsulation by acetate gradient method yielded up to 86 % DNa encapsulated into liposome. These liposomes had acceptable range for PDI values (less than 0.5).

Upon optimization there were no significant difference between the predicted and actual values for PDI (41.7 and 40.9), zeta potential (-35.2 and -28.3), percentage encapsulation (79.5 and 78.2), and *in vitro* drug release (41.7 and 40.9).

The optimised preparation was then used to formulate a thermo responsive *in situ* gel. This gel was free flowing at 4 ^{0}C and 25 ^{0}C making it a candidate for ocular administering as it gelled at body temperature (37 ^{0}C) enhancing the residence time with ocular tolerable pH of 7.1±1.

DECLERATION

I declare that *Active Encapsulation of Diclofenac Sodium into Liposomes for Ophthalmic Uses* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Evelyne Nguelweh Alonjang

Thursday, 26 July 2018

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A journey of a thousand miles is said to begin with a step. With the right guidance and support, such a journey eventually comes to a fruitful end. I would like to acknowledge the following people who contributed to the success of this thesis:

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Finally, I thank God for giving me the privilege of living and being able to come this far in life and trusting Him to go further.

DEDICATION

I dedicate every word in this master's thesis to God Almighty, for giving me the strength, patience, resilience, funds, endurance and for strategically placing everyone and everything in my path from the beginning to the end of this work. To you O Lord I raise my hands in thanksgiving.

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ABBREVIATIONS

ANOVA	Analysis of Variance
C	Cholesterol
C:PC	Cholesterol : Phosphotidylcholine
CCD	Central Composite Design
DF	Dry Film Method
DNA	Deoxyribonucleic acid
DOTAP	1,2-Dioleyl-3-trimethyl ammonium propane
DS or DNa	Diclofenac sodium
EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation Efficacy
EI	Ethanol Injection
FM	Film Method
GUV	Giant unilamellar vesicles
LUV	Large unilamellar vesicles
MCV	Microencapsulation Vesicle Method
MGD	Meibomian Gland Dysfunctions (MGD)
MLV	Multilamellar vesicles

MWCO	Molecular weight cut-off
NPs	Nanoparticles
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OLV	Oligolamellar vesicle
PBS	Phosphate Buffer Saline
PC	Phosphotidylcholine
PCL	Poly(epsilon-caprolactone)
PDI	Polydispersity Index
PEG	Poly(ethylene glycol)
P-gp	Glycoprotein P
REV	Reverse Phase Evaporation
rpm	Revolutions per minute
RSM	Response Surface Methodology
SEM	Scanning Electron Microscope
SODI	Soluble Ocular Drug Inserts
SUV	Small unilamellar vesicles
TFH	Thin Film Hydration Method
UHPLC	Ultra High Performance Liquid Chromatography
UHPLC	Ultra High Performance Liquid Chromatography

W/O	water oil
W/O/W	water oil water

CHAPTER 1: INTRODUCTION

1.1. Background and problem definition

In the 21st century nanotechnology has been studied in various disciplines such as biotechnology, computational sciences, physical sciences, social physiology, and health science, to mention a few (Mangematin and Walsh 2012). Nanotechnology originates from the Greek word "nano" which means "dwarf". Liposomes and dendrimers as examples of nanoparticles have found application in drug delivery, gene delivery, as a tool in imaging, molecular diagnostics, cardiac therapy, dental care and orthopaedic application (Sahoo and Labhasetwar 2003).With this vast nature of nanotechnology one can foresee more expansion in the field of pharmaceutical science where new drug delivery systems, dosage forms and treatment devices could emerge.

Liposomes are nanoparticles, first discovered accidentally by Bangham A.D. and Horne R.W. in 1964. In 1972, the first attempt to encapsulate penicillin and other drugs into liposomes was made (Gregoriadis and Ryman, 1972). In 1995 the first liposomal drug, Doxil® was clinically approved. Doxil® is an intravenous cancer preparation containing dorubicin as its active ingredient (Fan and Zhang, 2013). In recent studies liposomes have been extensively employed in cancer treatment and other conditions. In addition to their sustained release potential, liposomes reduce toxicity by mitigating exposure of sensitive tissues to toxic drugs, provide flexibility to actively target specific site by binding to site-specific ligands, facilitate delivery of hydrophobic, hydrophilic and amphipathic compounds and increase pharmacological activities (Anwekar *et al*., 2011, Shashi *et al*., 2012). The down sides of liposomes are its high cost of production and short half-life (Anwekar *et al*., 2011). Liposome encapsulation can either be passive or active. While passive encapsulation method is used for

large protein molecules, active encapsulation or remote loading is a preferred method for small molecules (Xu *et al.*, 2012, Ishida *et al*., 2002).

Diclofenac sodium (DNa) is a non-steroidal anti-inflammatory drug accepted for ophthalmic use. The only ophthalmic preparation of DNa existing in the drug market till date is an eye drop. Nonetheless, it is used extensively for ophthalmic inflammatory conditions with a three to four times-a-day administration. This high frequency of administration can be monotonous and result in non-compliance by some patients. Thus, minimizing the frequency of administration to once or twice a day is crucial to increasing patient compliance (Asasutjarit *et al*., 2011).

As a method for encapsulating drugs into liposomes, acetate gradient has been proven to provide a high encapsulation efficacy of over 90% (Avnir *et al.*,2008). An important issue pertaining to the instability of liposomes has also been dealt with using stability enhancers like polyvinyl alcohol and polyvinyl alcohol derivatives for DNa liposomes. The required ratio (s) of the main components of liposomes (cholesterol (C) and phosphatidylcholine (PC)) varies among researchers ranging from ratios of 8:1 to1:1 (Hironaka *et al*., 2011, Fujisawa *et al*., 2012, Jukanti *et al*., 2011). While there have been attempts to prepare liposome ophthalmic preparations (Hathout *et al*., 2007, Meisner *et al.*, 1995), there is a challenge to optimise the encapsulation process and to get a suitable dosage form to incorporate ophthalmic liposome preparations. Liposome ophthalmic preparations could enhance attachment to the eye surface and prevent undesirable leaching of the active ingredient before desirable penetration into the target site is achieved. *In situ* gels have the potential to prolong pre-corneal attachment time. Therefore, in this study, a combination of liposomes as a carrier and *in situ* gels as a dosage form for DNa could present a suitable novel dosage form to promote corneal penetration and possibly achieve sustained release properties. To obtain a liposome formulation of DNa with a high percentage encapsulation, sustained release

properties, and high stability, there is a need to optimize the encapsulation process. Optimization of the encapsulation process enables the manipulation of various factors that affect the amount of DNa encapsulated into liposomes and DNa release from the liposomal formulation.

Response Surface Methodology (RSM) is a method well explored in optimisation procedures (Chen *et al*., 2010). In this study RSM is used to optimise liposomal formulation of DNa.

To characterize DNa liposomes, its shape, size, polydispersity index, zeta potential, percentage DNa encapsulated and released were studied. The flow properties, pH, gel-sol transition temperature of *in situ* gel of DNa liposomes was also evaluated.

1.2. Aim of the study

The aim of this study was to optimize the process of active encapsulation of diclofenac liposomes and to use the optimised liposomes to prepare a suitable thermo responsive ophthalmic *in situ* gel.

1.3. Objectives

The following were the objectives of the study;

- To develop and validate an UHPLC method for quantification of diclofenac sodium
- To develop a method for active encapsulation of diclofenac into liposomes
- To use design expert® software to determine the numbers of experiments (runs) from input variables (cholesterol: phosphotidylcholine and incubation time)
- To use response surface methodology (RSM) to determine the effect (s) of input variables on output variables (encapsulation efficacy percentage, polydispersity index,

zeta potential and *in vitro* drug release) of liposome formulations obtained from Design Expert® Software

- To determine the combination of input variables required to achieve an optimized formulation of DNa liposomes
- To incorporate the optimised DNa liposomes into *in situ* gel formulation (s)
- To characterize DNa liposome *in situ* gel (s)
- To compare *in vitro* release of *in situ* liposome gel to voltaren® drops

Having established the scope of this study, the next chapter reviews liposome types and other nanoparticles that can be used for ophthalmic drug delivery. Chapter two reviews the common inflammatory conditions which affect the eye and provides a brief overview of RSM. The strengths and weaknesses of the various ophthalmic dosage forms are also discussed and liposomes preparation methods concludes the chapter. Chapter three is a methodology chapter wherein a detailed description of both analytical and experimental methods used is provided. Development and validation of the analytical method -Ultra High Performance Liquid Chromatography (UHPLC) leads to liposome preparation and characterisation through to analysis by RSM. Preparation and characterization of *in situ* gel of DNa liposomes are also discussed in this chapter. Chapter four provides descriptions and illustrations of results obtained from the encapsulation of DNa liposomes to incorporating these liposomes into *in situ* gel. This chapter outlines a comprehensive explanation of the optimization process. Chapter five concludes the thesis by summarizing the study's findings and making recommendations.

CHAPTER 2: LITERATURE REVIEW

With the aim and objectives from Chapter 1 in mind, this chapter will explain DNa with regards to its structure and mechanism of action. The structure of the eye, ophthalmic inflammatory conditions and ophthalmic dosage forms will be discused in this chapter. Also; classification, preparation, and characterization of nanoparticle as well as UHPLC used an a quantitative analytical method is decribed.

2.1. Diclofenac Sodium

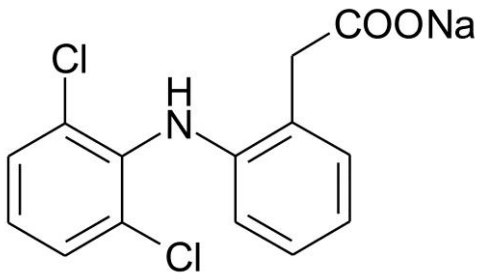

Figure 2.1: Chemical structure of diclofenac sodium (Elzayat *et al*., 2013).

DNa as represented in Figure 2.1 above, is a phenylacetic acid derivation. It is the first drug in the pharmacological class called non-steroidal anti-inflammatory drugs (NSAIDs). DNa was developed to synthesise a NSAID with a high level of activity and good tolerability. DNa was developed after phenyibutazone made an appearance in 1952. Mefenamic acid, ibuprofen and indomethacin NSAIDs were only introduced in the 1960's (Sallmann, 1985). DNa is a salt of a weak acid with a p*K*a of 4, a partition coefficient (*n*-octanol/aqueous buffer, pH 7.4) of 13.4 and has the chemical names 2-[(2, 6-dichlorophenyl) amino] benzene acetic acid sodium salt (Chuasuwan *et al*., 2009, Cardoso *et al*., 2009, Dighe et al., 2006, Žilnik *et al*., 2007). or sodium *o*-(2,6-dichloroanilino) phenyl acetate (Afkhami *et al*., 2016).

2.2. Mechanism of action of DNa (NSAIDs)

Prostaglandins (pain mediators) act on a variety of cells such as vascular smooth muscle cells and spinal neurons. Its actions include muscular constriction and inflammatory mediation. DNa acts by inhibiting the cyclooxygenase, an enzyme which converts arachidonic acids to prostagladins.This leads to reduction of intracellular concentrations of free prostaglandins in leukocytes hence, reducing pain (Kenawi *et al*., 2005). With its (DNa) antipyretic, anti-inflammatory and analgesic properties, DNa is indicated for rhumatoid arthritis, migrain, acute gout, dismenorrhoea and osteoarthritis amongst others (Elzayat *et al*., 2013). DNa is marketed as swallow tablets, dispersible tablets, suppository, topical ointment and ophthalmic drops.

The eye was the organ of choice for this research and inflamation the condition of focus. The target was to get a possible superior formulation for DNa ophthalmic. It is therefore vital to have a look at the anatomy of the eye to provide an understanding of how various inflammatory conditions affect the eye and the different eye parts, for which DNa eye preparation could be the remedy. The different ophthalmic dosage forms as well as the diverse liposome production methods will be discussed in this chapter. Also, a review of the statistiscal method and *in situ* gel (dosage form) used to improve the existing DNa ophthalmic formulation will be explained.

2.3. Basic anatomy of the eye

The eye (figure 2.2) is an important part of the body which aids human appreciation of nature and beauty around**.**

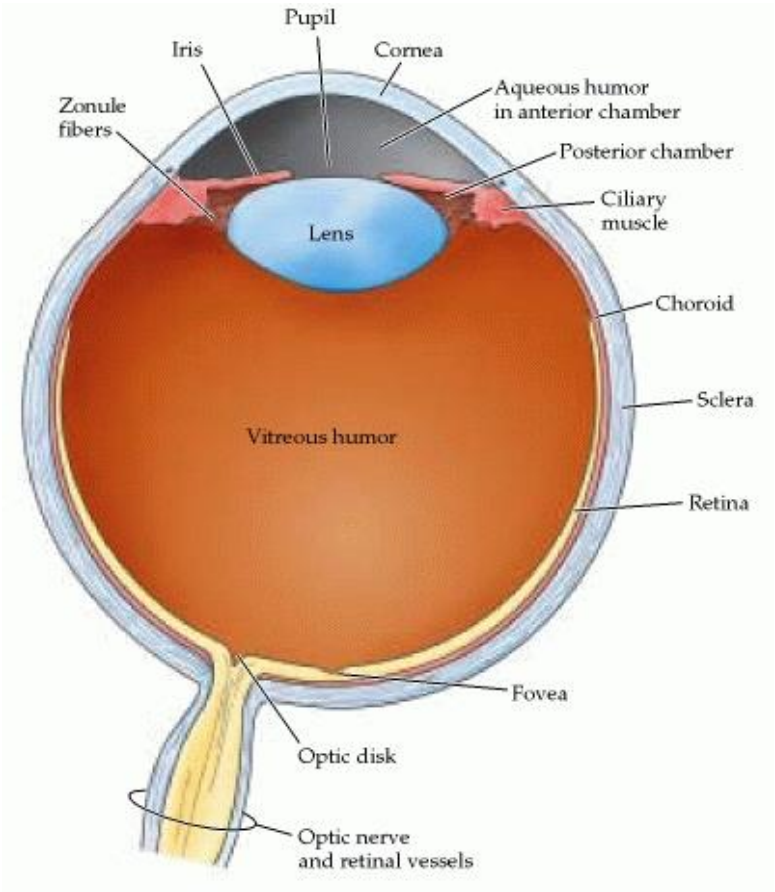

Figure 2.2: Anatomical structure of the eye (Purves *et al*., 2001).

As shown in the figure above the eye comprises of the following parts: (Purves *et al*., 2001).

- Sclera: It consists of most of the outer layer and composes of tough fibrous tissues;
- Cornea: It is a transparent tissue that allows light rays to enter the eye. It is a transformed portion of the sclera located in the front of the eye;
- Iris: It is the coloured part which can be seen through the cornea and functions in adjusting the sizes of the pupil;

- Ciliary muscles: It encircles the lens and is important in adjusting the refractive power of the lens. The ciliary body has ciliary processors that produce fluid which fill the front section of the eye;
- Pupil: It is the opening in the centre of the eye;
- Choroid: It harbours capillaries and serve as a main source of blood supply for the retina;
- Retina: It is the photoreceptor. The inner part of the retina consists of neurons that transmit visual signals and are sensitive to light;
- Aqueous humour: It is produced by the ciliary processes and it is a clear and watery liquid. It supplies blood to the anterior section (the space between the lens and the cornea) of the eye.

The posterior section of the eye is located between the iris and the lens and the space between the lens and the retina is filled with vitreous humour. The vitreous humour constitutes about 80% of the eye volume. It maintains the eye shape and also protects the eye from debris as it has phagocytic cells.

2.4. Inflammatory conditions of the eye

Inflammatory conditions of the eye can result from either localized inflammation in different parts of the eye or systemic condition(s) that causes inflammation in the eye. Common inflammatory eye conditions include: uveitis, scleritis, conjunctivitis, blepharisis, and keratitis.

Uveitis is inflammation of the uvea. The uvea is made of the iris, the ciliary body, and the choroid. Anatomical types of uveitis include: i) anterior uveitis, which relates to inflammation of the iris and anterior chamber, ii) intermediate uveitis, which relates to

inflammation of the vitreous cavity, iii) posterior uveitis, relating to inflammation of the retina and choroid, and iv) pan-uveitis, pertaining to inflammation of all the layers of the uvea (Hollingsworth S. R., 2011).

Scleritis is inflammation of the sclera. It can be caused by local or systemic infections or mediated by other immune diseases. Uveitis and scleritis affects younger people in the most productive stage of their lives (Wakefield *et al*., 2013).

Conjunctivitis commonly called "pink eye" is inflammation of the conjunctiva. This condition could be as a result of viral or bacterial infection, or an allergic condition. **Blepharisis** is a disease of the eyelids. Symptoms will vary depending on which part(s) of the eyelids are affected. Eyelid debris results from inflammation of anterior margin whereas meibomian gland dysfunctions (MGD) results from inflammation of the posterior margin (Malerbi *et al*., 2012). Pathophysiology for this condition is complex involving several factors such as abnormal lid-margin secretions, microbial organisms, abnormality in the tear film. Signs and symptoms vary and are associated with conditions such as dermatitis, rosacea or eczema (Bruce Jackson, 2008).

Keratitis is an inflammation of the cornea with symptoms of intense pain and impaired eyesight. Acanthameoba keratitis (AK) is a serious corneal infection caused by an amoeba. Other types of keratitis are bacterial, fungal and viral keratitis (Panjwani, 2010).

Systemic diseases can also affect the eyes. Diabetic retinopathy and muscular degeneration causes death of retinal cell and this may lead to blindness (Sasaki *et al*., 2013). Rheumatoid arthritis may cause dryness of the eye exposing the eye to infection thus causing inflammation (Sasaki *et al*., 2013).

2.5. Ophthalmic Dosage Forms

The eye is an easily accessible part of the body although it is problematic to administer drugs. Due to pre-corneal loss factors such as tear dynamics, non-productive absorption, and relative impermeability of the corneal epithelium, there is poor bioavailability of ocular drugs. Factors considered in ocular drug delivery are disease process, patient comfort, safety, and compliance (Attama *et al.* 2008*).* Goals for ocular targeting are: enhanced drug permeation, controlled release property, the ability of drugs to reach target sites at a therapeutic level, minimized side effects and timely drug elimination. In addition to *in situ* gel (dosage form of choice for this research) which is well elaborated, other existing ocular dosage forms or ocular drug delivery systems are discussed below:

2.5.1. Eye drops

While a school of thought says 90% of ophthalmic dosage forms are still in the form of eye drops (Asasutjarit *et al*., 2011), another set of researchers hold that 90% of ophthalmic drugs available are eye drops , suspensions and ointments (Le Bourlais *et al*. 1998, Moya-Ortega *et al*., 2013, Lang, 1995). Thus, a huge percentage of commercially available ophthalmic dosage forms are in the form of eye drops. Eye drops are widely accepted by patients because they can be administered relatively easily by most patients and are readily available. There are, however, problems associated with this dosage form: Re-infection can occur if not properly used and elderly patients may struggle to administer the right dose. Also eye drops are not able to get to the posterior section of the eye and cannot reside in the anterior section of the eye. This is because of the protective mechanisms of lacrimation and blinking once eye drops are administered. Nasolacrimal drainage also reduces the required dose and residence time of the drug administered as an eye drop. Only 1-10% of administered eye drops will reach the

target site. Some eye suspensions may even lead to dosage heterogeneity (Asasutjarit *et al*., 2011, Meisner and Mezei 1995).

2.5.2. Ointments and gels

With the thick consistency of this dosage form, residence time of the drug is increased, Blurring is experienced by patients after applying eye gels and ointment (Asasutjarit *et al*., 2011), risking non-compliance to treatment. To apply ophthalmic gels or ointment, hands should be washed before taking the lid off the ointment tube. The head is tipped backward; the lower eyelid is gently pulled down while looking upwards. The tube is held above the eye and 1cm line ointment is gently squeezed along the inside of the lower eyelid. Care is taken for the tip of the tube to not touch the eye or eyelashes. Finally the eye is blinked for the ointment to spread over the surface of the eyeball. Thus, administering eye gels or eye ointments requires the above technique which may be difficult to grasp by some patients especially the elderly. With *in situ* gels, the advantages of increased residence time together with sustained release potential are achieved. Added to this is the fact that no special technique is needed for administration of ophthalmic *in situ* gels, because they are liquid before administration and only convert to gel upon administration to the eye (Marshall, 2014).

2.5.3. Inserts.

There are numerous ophthalmic drug delivery systems that are in the form of inserts some of which are Ocuserts (Ocular inserts), Lacriserts, SODI (Soluble Ocular drug inserts). These are systems made of materials into or onto which active drug substance are loaded. The loaded drugs are delivered over time enabling sustained drug release and reduced frequency of administration. The down side of this drug delivery system is that patients may need training to use inserts or may need to visit trained personnel to administer the inserts, risking

noncompliance (Asasutjarit *et al*., 2011). Non-compliance may also arise with patients' discomfort which comes with instilling a foreign object in the eyes. This discomfort could be physical or psychological (Asasutjarit *et al*., 2011).

2.5.4. Vesicular systems.

Prodrugs, liposomes, niosomes, discomes, and pharmacosomes are some examples of vesicular systems that are used in ophthalmic drug delivery. They present with the advantage of sustained release, and can be delivered as eye drops or inserts. The drawback of these vesicular drug delivery systems is their preparation involves the use of organic chemicals which are difficult to completely remove after the vesicles are formed. Also the vesicular systems have an unstable shelf life and storage of the finished product is a problem (Kavousanakis *et al.,* 2014).

2.5.5. Controlled Delivery systems.

These are implants, iontophoresis, dendrimers, cyclodextrins, contact lenses, collagen shields, microemulsions, nanosuspensions, microneedles, penetration enhancers, mucoadhasive polymers, phase transition systems or *in situ* gel systems. These are relatively new drug delivery systems. They produce sustained release properties but need extensive studies with regards to what active ingredient is suitable for different dosage forms as well as storage conditions and the need for preservatives are yet to be comprehensively explored (Andrés-Guerrero *et al*., 2015).

2.5.6. Invasive methods

Invasive methods such as injections will increase the concentration of the active ingredient at the target sites and reduce systemic side effects (Hansen *et al*., 2012). Frequent administration of injections leads to increase intraocular pressure, retinal detachment and

even haemorrhage, cataracts, endophthalmitis and hence an increased possibility of non-compliance (Hansen *et al*., 2012, Bochot & Fattal 2012, and Hironaka *et al*., 2009).

2.6. Nanoparticles / Nanocarriers for ocular delivery

The use of nano carriers for ocular drug delivery started in the 80s but only in the last decade has this field grown substantially, leading to the study of a wide variety of nanostructures and understanding their potential for ocular drug delivery. In this section, the aim is to provide an overview on the main features that characterize each specific type of nanostructure, and analyze the main factors that govern their interactions with the ocular surface after topical administration.

2.6.1. Liposomes and niosomes

Cationic liposomes (figure 2.3.) are composed of stearylamine (SA), 1, 2-dioleyl-3-trimethyl ammonium propane (DOTAP), or coated with certain polymers such as the cationic polysaccharide chitosan. They can interact more efficiently with negatively charged mucins at the ocular surface and be more efficiently internalized by the corneal epithelial cells (Schaeffer & Krohn 1982). This could imply that there is a prolonged residence time with positively charged liposomes leading to an improved therapeutic effect.

Niosomes are particular types of vesicles formed by amphiphilic non-ionic surfactants proposed for topical administration. Depending on their composition, these nanostructures can open tight junctions and modify the permeability properties of the cornea, thereby improving the ocular drug bioavailability (Kaur *et al*., 2004).

Figure 2.3: Structure of a liposome (Reimondez-Troitiño *et al*., 2015).

2.6.2. Nanocapsules

Nanocapsules (figure 2.4.) compose of an oily core surrounded by a PCL (Poly (epsilon-caprolactone) wall. This delivery strategy may combine the advantages of polymer nanoparticles with those of nanoemulsions. Polymer shell of the nanocapsules strongly influences the fate and the interaction of the nanocapsules with the ocular surface. PCL nanocapsules are preferentially internalized by the superficial layer of the corneal epithelial cells. Remarkably, when PCL–PEG (Poly (epsilon-caprolactone)-poly(ethylene glycol) is used, there is a deeper internalization of the nanocapsules into the epithelium (50 µm deep), whereas the coating of PCL nanocapsules with chitosan results in a greater retention at the superficial layers of the epithelia, this could be as a result of the intrinsic behaviour of chitosan (Reimondez-Troitiño *et al*., 2015).

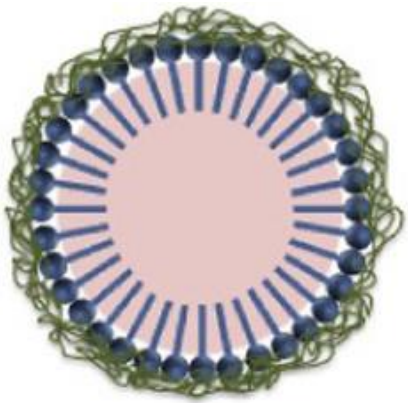

Figure 2.4: Structure of a nanocapsule (Reimondez-Troitiño *et al*., 2015).

2.6.3. Nanoemulsions

Oil-in-water nanoemulsions were proposed for topical ocular delivery in the early 90s (Reimondez-Troitiño *et al*. 2015). The surfactants used to stabilize nanoemulsions play a critical role in their interaction with the ocular surface. For example, the use of polyoxyethylated non-ionic surfactants may lead to an opening of tight junctions and also to an inhibition of the activity of glycoprotein P (P-gp) on the epithelial cells; resulting in an enhancement of the corneal transport of the drug included in the oily droplets (Jiao, 2008). Nanoemulsions (figure 2.5) also interact with the lipid layer of the tear film, remaining in the conjunctival sac for longer times, and consequently acting as a depo drug (Alany *et al*., 2006).

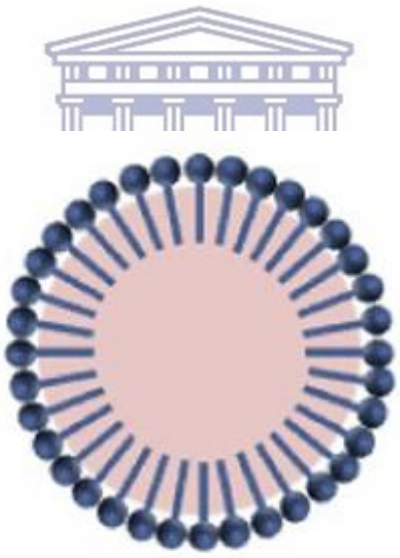

Figure 2.5: Structure of a nanoemulsion (Reimondez-Troitiño *et al*., 2015).

2.6.4. Nanoparticles

Nanoparticles (figure 2.6) made of cationic and bioadhesive materials are those which have exhibited the greatest retention time at the ocular surface after instillation (Klang *et al*. 2000). Plasmid DNA-loaded hyaluronan/chitosan nanoparticles are internalized by receptor-mediated endocytosis without causing any sign of toxicity. This internalization is responsible

for the efficient and long-lasting transfection observed after topical administration to rabbits (Yuan *et al.* 2006).

Figure 2.6: Structure of a nanoparticle (Reimondez-Troitiño *et al*., 2015).

2.6.5. Polymeric micelles

Polymeric micelles (figure 2.7.) are formed by di-block or multi-block amphiphilic copolymers that self-assemble, forming core–shell nanocarriers (Klang *et al.*, 2000). The residence time of polymeric micelles in the ocular surface can be improved by introducing a cationic charge in the hydrophilic shell, or by incorporating reactive groups (Reimondez-Troitiño *et al*., 2015).

Figure 2.7: Structure of a polymeric micelle (Reimondez-Troitiño *et al*., 2015).

2.6.6. Dendrimers

Dendrimers (figure 2.8.) are symmetric structures formed by repetitive branched molecules surrounding a central core and have recently been proposed for their application in topical ocular drug delivery (Kambhampati & Kannan 2013). The performance of these carriers can be enhanced by modifying dendrimer surface using PEGylation or acetylation. Such modifications also result in a reduction of their toxicity (Stasko *et al*., 2007). Dendrimers have led to increased drug residence time in the pre-corneal area, translating into an improved drug bioavailability and thus prolonged therapeutic effect (Vandamme & Brobeck 2005). Anionic high generation PAMAM (Polyamidoamine) dendrimers and neutral and cationic low generation dendrimers promote higher permeability, acting as permeability enhancers (Kitchens *et al*., 2006).

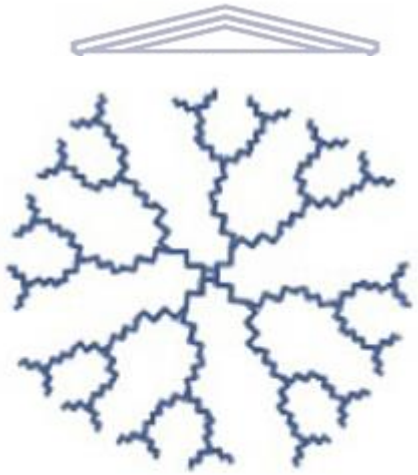

Figure 2.8: Structure of a dendrimer (Reimondez-Troitiño *et al*., 2015).

2.6.7. Nanocrystals

The active compound forms the bulk of the nanostructure of nanocrystals, which are surrounded by excipients that contribute to their stability and may influence their interaction with biological barriers (Junghanns and Muller 2008). Nanocrystals may enhance drug solubility, dissolution rate and may have a high adhesion capacity that can contribute to improving the retention and penetration of drugs into the eye. This adhesion and internalization capacity is determined by the nature of the surfactants. Most commonly used surfactants are poloxamers and polysorbates (Pawar *et al*., 2014).

Overall, the above described (2.6.1-2.6.7) nanocarriers have led to increased retention time of the drug in the ocular mucosa and enhanced penetration of the drug to the eye. Their interaction with the corneal epithelium is favoured when they have a small size and a positive charge. Different mechanisms of interaction with the cornea, which include a disruption of the tight junctions as well as internalization into the superficial layers of the epithelium, have been described. In most cases, the interpretation of the mechanism of interaction has been speculative and based on the drug concentrations attained in different ocular fluids and tissues (Reimondez-Troitiño *et al*., 2015).

The past decades have seen pharmaceutical scientists striving towards more selective drugs or drugs that are incorporated into targeted delivery systems. Much interest has been shown towards liposomes as delivery systems because a multitude of therapeutic agents can be incorporated into their central aqueous core. Liposomes are ideal candidates for the formulation of targeted drug delivery due to their relative ease of preparation (Simões *et al*., 2004). Owing to the above advantage, liposomes were considered for this study. The next section will focus on the liposome structure, types and methods of preparation.

2.7. Liposomes

Liposomes are single or multi-layered spherical vesicles, spontaneously formed when phospholipids are dispersed in an aqueous phase (Gibis *et al., 2014*). Liposomes are formed as a result of an interaction of the lipid head groups of phospholipids with water, when phospholipids are placed in an aqueous medium. In the presence of an aqueous phase the lipophilic tails are shielded from the aqueous medium by the phospholipids spontaneously forming bilayer vesicles (multilamellar or unilamellar). These bilayer vesicles consist of the hydrophilic heads in the enclosed aqueous space and the external aqueous phase while the lipophilic tails face each other away from the aqueous medium (Shashi et al., 2012, Bawarski *et al*., 2008, Vemuri and Rhodes 1995). Active pharmaceutical ingredients (APIs) can be loaded into liposomal vesicles making liposomal drug carrying vesicles or systems. The main components of liposomes are phospholipids and cholesterol (Shashi *et al*., 2012).

2.7.1. Phospholipid

Phosphotidylcholine (PC) and phosphatidylethanolamine are the most common phospholipids in plants and animals and form the main structural part of most biological membranes. The general structure of phospholipids is seen in figure 2.9. The chemical structure consists of a glycerol backbone. The lipid nature of phospholipids is as a result of the esterified long fatty acid chain on position 1 and 2 of the glycerol molecule. At position 3 of the glycerol molecule, a phosphoric acid results from the esterification of the hydroxyl group. Upon further esterification of the one of the remaining oxygen groups of the phosphoric acid, different phospholipids may result like; glycerol in phosphotidylglycerol, choline in phosphotidylcholine, ethanolamine in phosphatidylethanolamine serine in phosphotidylserine and isositol in phosphotidylisositol (Shashi *et al*., 2012, Vemuri and Rhodes 1995). There are also synthetic phospholipids that can be used in liposome preparations such as

dipalmitoyl phosphatidylcholine, distearoylphosphatidylcholine, and Dipalmitoylphosphatidylserine, just to mention a few (Shashi *et al*., 2012).

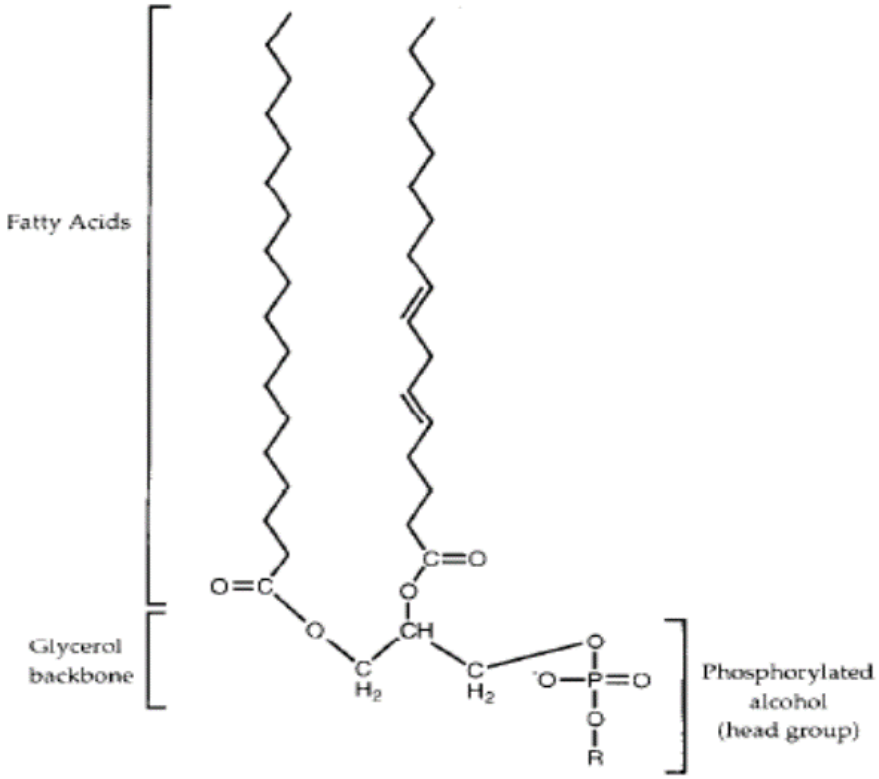

Figure 2.9: Chemical structure of a phospholipid molecule with a fatty acid tail section and a glycerol backbone with a phosphorylated alcohol head (Shashi *et al*., 2012).

2.7.2. Cholesterol

Cholesterol is a steroid molecule (figure 2.10.) found in the mammalian plasma membrane. Cholesterol contributes to the fluidity of the membrane containing it. Cholesterol binds to the inside of the lipid bilayer to enable its polar hydroxyl group to be close to the phospholipids, contributing to the stability and fluidity of the liposome membrane (Kočišová *et al.*, 2013). Liposomes without cholesterol interact with plasma protein as plasma protein extracts a bulk of the outer layer from the liposomes, depleting the outer layer and depressing the physical stability of the liposomes. Cholesterol renders liposomes less fluid, prevents encapsulated

drugs from leaking out of the liposomes by reducing permeability of the liposome membrane, and stabilises liposomes in the presence of plasma in biological systems (Yang *et al*., 2013). Large quantities of cholesterol in liposomal vesicles may protect it against plasma induced drug release (Shashi *et al.*, 2012, Vemuri and Rhodes 1995).

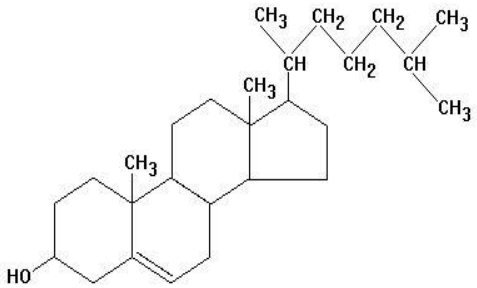

Figure 2.10: Chemical structure of cholesterol (Shashi *et al*., 2012).

2.8. Liposomes Classification

Liposomes are classified either based on composition and mechanism of intracellular delivery or size and lamellarity.

2.8.1. Classification by composition.

In terms of composition and mechanism of intracellular delivery, four major types exist as shown in figure 2.11, namely conventional liposomes, cationic liposomes, immunoliposomes, and long-circulating liposomes (Storm & Crommelin 1998).

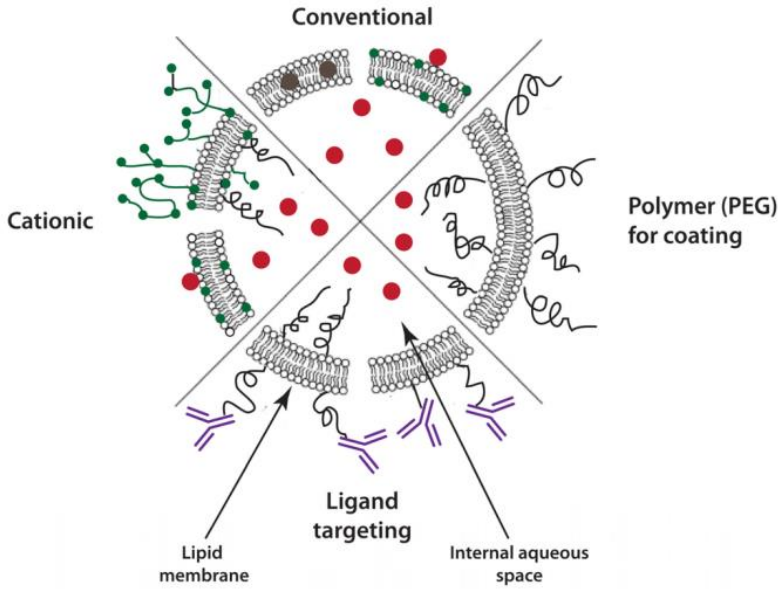

Figure 2.11: Schematic representation of four major liposome types in terms of composition (Storm & Crommelin 1998).

Conventional liposomes are either neutral or negatively charged. They are composed of phospholipids and / or cholesterol.

Cationic liposomes are positively charged liposomes consisting of cationic lipids. They neutralise the negative DNA of the cell, forming lipid-DNA complexes and promoting cellular internalisation.

Immunoliposomes have enhanced binding capability to their target site due to the presence of specific antibodies or antibody fragments on their surface.

Long-circulating liposomes, as implied by the name, have a long blood circulation time. This is due to an attachment of a hydrophilic polymer or polyethylene glycol (PEG) covalently attached to the liposomes' outer surface. They are also referred to as "stealth" or "satirically stabilised" liposomes (Storm & Crommelin 1998).

2.8.2. Classification by size

Vesicle size is a critical parameter in determining the circulation half-life of liposomes, and both size and number of bilayers influence the extent of drug encapsulation within liposomes (Laouini *et al*., 2012). As seen in figure 2.12 below, liposomes can be classified based on its size and number of bilayers as follows:

- Small unilamellar vesicles (SUV): 20–100 nm;
- Large unilamellar vesicles (LUV): > 100 nm;
- Giant unilamellar vesicles (GUV): > 1000 nm;
- Oligolamellar vesicle (OLV): 100–500 nm and
- Multilamellar Vesicle (MLV): > 500nm

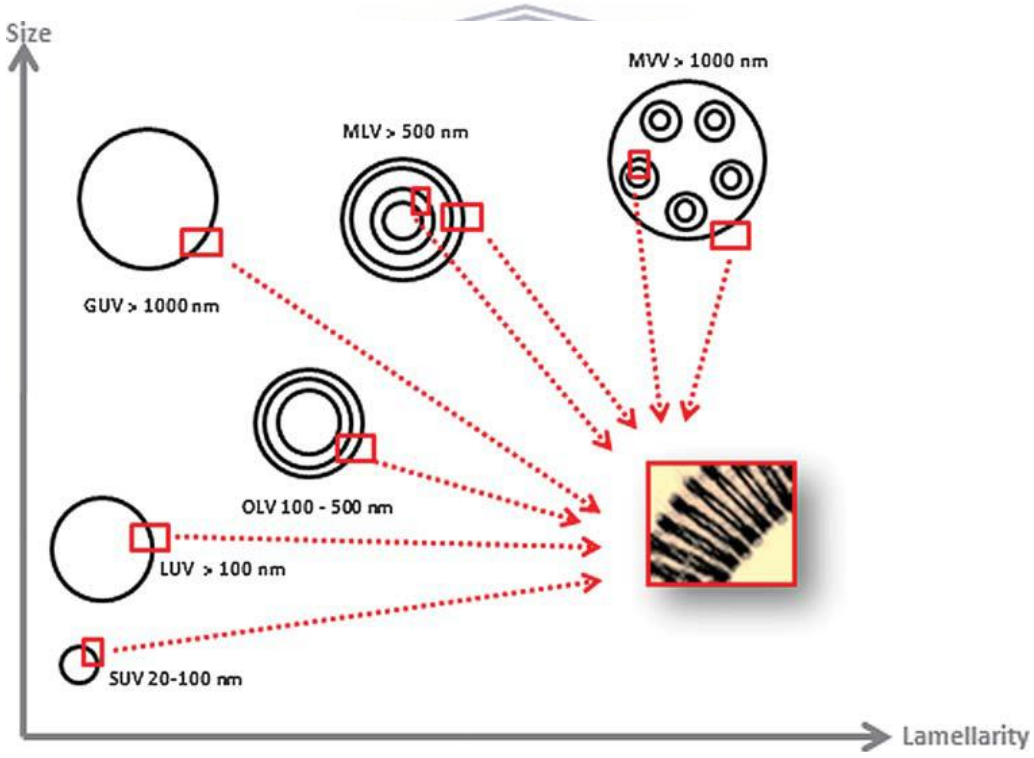

Figure 2.12: Schematic representation of liposomal classification base on size and number of lamellae.(Laouini *et al*. 2012).

2.9. Liposome Preparation Methods

There are two main methods of liposome preparation, namely Thin Film Method (TFM) and Reverse Phase Evaporation method (REV). The method of choice in this work is TFM because it is replicable and can be used with different types of lipid combinations. TFM produces high encapsulation yields and results in homogeneous particle sizes. Although REV similarly produces high encapsulation rates, it is usually accompanied by undesirable heterogeneous particle sizes (Zalba *et al*., 2012). Other methods of liposome preparation include solvent-injection techniques, detergent dialysis, supercritical fluid reverse-phase evaporation, Microencapsulation Vesicle Method (MCV), proliposome method, heating method, freeze drying and spray drying (Laouini *et al*., 2011). Liposomes can also be prepared using a membrane contractor which is reproducible, fast, and can be used in large scale production (Laouini *et al*., 2011). Some methods of liposome preparation are briefly described as follows:

2.9.1. Microencapsulation Vesicle Method (MCV).

There are three main steps in this method; first a w/o emulsion is made by dissolving a phospholipid in an organic solvent and then mixing it with a solution. The second step is the formation of a w/o/w emulsion by dissolving the w/o emulsion in another solution. The last step is evaporation of the organic solvent leading to the formation of liposomes. This method is known for its high rate of encapsulation and formation of homogeneous liposome sizes (Nii & Ishii 2005). Its disadvantage is the inability to completely remove the organic solvent (Nii & Ishii 2005).

2.9.2. Proliposome Method

With this method, ethanol and lipids are mixed, heated to 60 $^{\circ}$C, and cooled, forming proliposomes. A solution of the active pharmaceutical ingredient is added after the heated

solution is cooled. Phosphate Buffered Saline (PBS) is then added drop-wise to form liposomes from the proliposomes (Galović Rengel *et al*., 2002).

2.9.3. Reverse Phase Evaporation Method

With this method, the ingredient to be encapsulated is dissolved in glucose while the lipids are dissolved in a chloroform/ethanol mixture. The lipid mixture is then added to the glucose mixture. After sonication for about 5 minutes, a rotary evaporator is used to remove the organic solvent under reduced pressure. An aqueous suspension is formed by vortexing and extrusion follows (Zalba *et al*., 2012).

2.9.4. Modified Heating Method.

This method is advantageous as it does not make use of solvents which are difficult to completely remove. It makes use of heat. With this method, the liposome component is hydrated in an aqueous medium and then heated at very high temperatures (up to 120 ˚C) in the presence of glycerol (Zalba *et al*., 2012).

2.9.5. Dry Film Method (DF).

This method is similar to the film method described below. By contrast, however, this method uses nitrogen gas to remove excess residue; the dried film is hydrolysed using ionised water; and a vortex is used to suspend the film (Yang *et al*., 2013).

2.9.6. Ethanol Injection Method (EI).

Here the lipids are dissolved in ethanol. The ethanol is then removed leaving behind half of the original solution. Deionized water is then added at a constant volume leading to the formation of liposomes after evaporation (Yang *et al*., 2012).

In general Liposome preparation methods which involve the use of organic solvents such as ethanol and chloroform have a disadvantage of irritation to tissues. It is therefore essential to ensure the complete removal of such solvents (Imura *et al*., 2003).

2.9.7. Film Method (Thin Film Hydration Method, TFH).

Generally, this method (figure 2.13) starts off with the preparation of a mixture of chloroform, methanol and lipids. The solvents are removed using a rotary evaporator at room temperature. For liposomes to be formed, a solution of the active or a suitable solvent is used to hydrate the dry lipid film forming combinations of oligolamellar vesicles, multilamellar vesicles, and giant unilamellar vesicles (Zalba *et al*., 2012). The obtained liposomes are then subjected to a sizing phase to obtain liposomes with similar sizes.

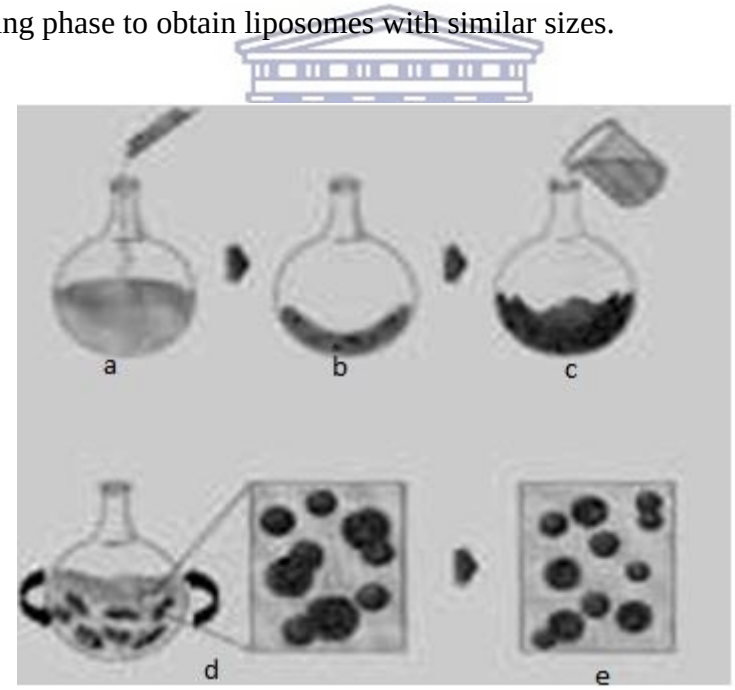

Figure 2.13: Diagrammatic representation of liposome preparation by thin film Hydration (Zalba *et al*., 2012). (From top left and following the arrows: (a) dissolution of lipids in organic solvents, (b) formation of thin film using a rotary evaporator, (c) rehydration, (d) emergence of liposomes and (e) sizing to give homogeneous particle sizes)

2.10. Sizing of liposomes.

Liposomes are sized using sonication, the French pressure cell method or extrusion to produce single unilamellar vesicles. Unilamellar vesicle liposomes are the desired sizes of liposomes for use in drug delivery (Laouini *et al*., 2012).

2.10.1. French Pressure Cell Method

With this method Multilamellar Vesicle, (MLV) are extruded at 20,000 psi at 4 ˚C through a small orifice. This method is simple, rapid and reproducible. The drawbacks of this method are as follows: first, the resulting liposomes are larger than those produced by sonication and extrusion. Second, it is difficult to achieve the desired working temperature. Lastly, getting the required pressure and temperature may require sophisticated and expensive apparatus making this sizing method a costly one (Hamilton and Guo, 1984, Dua *et al*, 2012).

2.10.2 Sonication

Sonication is disruption of Multilamellar Vesicle (MLV) suspensions using sonic energy to produce Small unilamellar vesicles SUV. The most common instrumentation for preparation of sonicated particles is a bath and probe tip sonicators. Probe tip sonicators deliver high energy input to the lipid suspension but suffer from overheating of the lipid suspension, causing degradation. Bath sonicators are the most widely used instrumentation for preparation of SUV – since a large volume can be sonicated at once and the instrumentation is relatively cheap. Sonication of LMV dispersion is accomplished by placing a test tube containing the suspension in a bath sonicator (or placing the tip of the sonicator in the test tube) and sonicating for a desired time. The mean size and distribution of sonicated liposomes is influenced by composition and concentration, temperature, sonication time and power, volume, and sonicator tuning.

The drawbacks of sonication are;

- The lipid suspensions suffer from overheating causing degradation;
- Sonication tips release titanium particles into the lipid suspension which must be removed prior to use;
- Since it is nearly impossible to reproduce the conditions of sonication, size variation between batches produced at different times is not uncommon (Hamilton and Guo, 1984).

2.10.3. Extrusion

Extrusion is performed by forcing a MLV suspension through filters with defined pore sizes. The concentric layers of the multilamellar liposome are forced to pass through the pore, causing a breaking and resealing of the membranes. Repeatedly passing the same liposome suspension through a filter gives rise to a liposome population with a mean diameter that reflects that of the filter pore size. Extrusion is a rapid and convenient way to decrease the size heterogeneity exhibited by most MLV preparations. Extrusion is performed using a hand-held syringe (as will be elaborately discussed in chapter 3). This method is limited by the pressure that can be applied manually. Only low volumes at laboratory scale can be sized using this method, a major setback for extrusion (Hope *et al*., 1993).

2.11. Ultra High Performance Liquid Chromatography as a Quantitative Analysis Method

Ultra High Performance Liquid Chromatography (UHPLC) is a qualitative as well as a quantitative analytical method. For quantitative analysis UHPLC is used to determine the concentration of a known compound in a sample by making use of peak heights or peak areas. In qualitative analysis, UHPLC is used to identify a compound or compounds in a sample by assigning peaks to particular compound(s) (Snyder *et al.,* 2012). For this research,

UHPLC was used for quantitative analysis to determine the concentration of DNa in various samples or preparations. To ensure reliability and reproducibility of results, an UHPLC method was developed and validated.

2.11.1. UHPLC development and validation

Method development and validation provides documented evidence and a high degree of assurance that an analytical method used for a specific test is suitable for its intended use. The parameters that require validation are dependent on the type and applications of the method. These parameters include accuracy, precision, specificity, reproducibility, linearity and robustness.

- Linearity relates to the ability of test results to be directly proportional to the concentration of the compound to be analysed, in this case DNa concentration**.** For the analytical procedures in determining the content of a parent compound, it is recommended that linearity be established at a correlation coefficient (r) level that is not lower than 0.999 (Epshtein, 2004).
- Specificity is the ability to detect a specific compound in a mixture of compounds (Epshtein, 2004).
- Precision and accuracy**:** Accuracy is the ability of a method to correctly determine a concentration close to the actual concentration and precision is the ability of an analytical method to deliver reproducible results (Epshtein, 2004).
- Robustness measures the capacity of an analytical method to remain unaffected by small, but deliberate, variations in method parameters. The same UHPLC instrument was used throughout this work, thus no need for a robustness test was effected (Epshtein, 2004).

2.12. Forced Degradation Studies

To successfully characterise liposomal preparation of DNa, there is a need to determine a suitable analytical method to be used. Subjecting the API under investigation (in this case DNa) to extreme conditions gives an indication of the specificity of the analytical procedure used as well as an identification of likely degradation products (Deshpande and Patel, 2014**).** Extreme stress conditions include boiling, oxidation, acid hydrolysis and base hydrolysis (section 3.2).

2.13. Characterisation of Diclofenac Liposomes

2.13.1. Percentage encapsulation

Percentage encapsulation is the amount API which successfully gets into and/or onto liposomes. Percentage encapsulation is performed by a "direct" or an "indirect" method (Campardelli *et al*. 2016). Calculating directly involves separating the liposomal drug (DNa encapsulated into liposome) from free drug (DNa not encapsulated into liposomes) using the sephadex-50 gel separation method. The indirect method for separating the free drug from the liposomal drug is performed using protamine. While the former method is expensive, cumbersome and time consuming, the latter is relatively cheaper, easy to do and time saving. Both methods yield a similar percentage encapsulation (Kilian, 2011). Another method less frequently used for calculating percentage encapsulation is the centrifugation method. With the centrifugation method, a liposome solution is centrifuged. Then, the concentration of the drug in the supernatant (mg_{drug}) solvent is analysed using UV–Vis spectroscopy or other analytical methods such as UHPLC. The percentage encapsulation or encapsulation efficiency (EE) can then be calculated with respect to the theoretical drug content (mg_{loaded}) using the following equation $EE = 100 – (mg_{drug\ /}\ mg_{loaded}) \times 100$ (Campardelli *et al*., 2016).

2.13.2. Size, polydispersity index and shape determination

Polydispersity index is a measure of the dispersion homogeneity and ranges from 0 to 1. Values closer to 0 indicate homogeneity while values closer to 1 indicate heterogeneity. The particle size, polydispersity index and zeta potential are measured using photon correlation spectroscopy techniques on a Malvern® Zetasizer. This technique measures the time dependent variations in the intensity of scattered light caused by Brownian motion of the particles. Analyses of these fluctuations determine the diffusion coefficient, which then allows for size distribution determination (Pereira-Lachataignerais *et al*., 2006).

Scanning electron microscopy (SEM) is often used to study the surface morphology and shape characteristics of nanoparticles (NPs) (Chaturvedi *et al.,* 2012). An electron beam is generated by an electron gun and passes through the electromagnetic lenses of a column and across the surface of a sample. Electrons interact with atoms on the surface of the sample, producing various signals that can be detected to create an image, providing information about the sample size topography and composition (Chaturvedi *et al.*, 2012). .

2.14. *In vitro* Drug Release study

In vitro drug release is performed to determine the quantity and/or rate of movement from a dosage form of formulation into a receptor. This receptor mimics the physiological conditions of the part of the body where the formulation will be applied. In this study, Franz® diffusion cells were used for release studies. First, free DNa (not encapsulated into liposomes) were separated from liposomes using Sephadex® gel columns. Only components of the formulation containing DNa were used for *in vitro* drug release studies. A detailed separation procedure is outlined in section 3.4.4 (Wissing & Müller, 2002).

2.15. Preparation and Characterisation of *in situ* gels.

In situ gels are dosage forms which go into gel-sol transformation upon administration as solutions or suspensions. This transformation is caused by external stimuli, such as temperature or pH variation (Makwana *et al*., 2015). Despite the fact that the conventional ophthalmic dosage form (eye drop) is relatively easy to administer, it presents major draw backs. When an ophthalmic drug solution (eye drop) is administered, there is a 10-fold reduction in the drug concentration within 4–20 minutes (Maurice *et al*., 1987). Eye drops is the lone ophthalmic preparation for DNa and it is not retained in the eye for a desired length of time to deliver the right dosage for the desired time. The drug is not retained long enough as a function of the following factors: binding of the drug to lacrimal proteins; draining of instilled drops (due to lacrimation or tear turn over); metabolisation of the drug before it reaches the target site; and non-productive absorption of the drug. These factors lead to poor bioavailability of eye drops having motivated research for other dosage forms such as ointments, gels and inserts in recent years. The advantage of ointments and inserts is extended drug residence time in the eye. This advantage is, however, accompanied by blurred vision and risks of patient noncompliance (Nagarwal *et al*., 2009). *In situ* gels on the other hand presents with an advantage of easy administration due to its liquid form (sol form) at the time of administration. The disadvantage of blurred vision associated with the other dosage forms is surmounted with *in situ* gels which is consistent between sols and gels.

Ocular therapy would be considerably enhanced if the pre-corneal residence time of drugs could be increased. Ocular inserts is one of the new preparations that have been developed for ophthalmic use. In addition to prolonging its contact time on the ocular surface, ocular inserts slow down drug elimination. The disadvantages of ocular inserts include the risk of non-compliance (especially by elderly patients) as the inserts cause irritation to the eye and

require technical assistance upon administration. Also, due to their small sizes, the ocular inserts can easily be misplaced (Le Bourlais *et al*., 1998, Ding, 1998, Liu *et al*., 2006).

With the evolution of *in situ* gels, the problem of patient compliance has been mitigated. *In situ* gel ophthalmic drug delivery systems are prepared from polymers that exhibit reversible phase transitions. They have pseudo-plastic behaviour which minimizes interference with blinking. An *in situ* gel is a liquid dosage form suitable for administration by instillation into the eye. Upon exposure to physiological conditions, the liquid changes to the gel phase (Ding 1998) thereby increasing the pre-corneal residence time and enhancing bioavailability (Makwana *et al.,* 2015). Increased residence time and enhanced bioavailability give the *in situ* gel major advantages over other ophthalmic preparations (Makwana *et al., 2015).*

Ciprofloxacin is an antibacterial used in the treatment of corneal ulcers. In a study, ciprofloxacin was used to produce pH triggered *in situ* gel and an ion activated system. This study successfully formulated an *in situ* gel of ciprofloxacin which proved to be a better formulation to conventional ciprofloxacin eye drops and ointments, with respect to ease of administration, sustained release, and improved potential for patient compliance (Makwana *et al., 2015).* In another study of DNa (Asasutjarit *et al*., 2011), *in situ* gel was developed where the formulation showed a potential bioavailability increase in rabbits and a significant reduction in the frequency of administration compared to current ophthalmic eye drops. Thus, the study showed that, the use of *in situ* gel potentially increases patient compliance. Timolol and vitamin A have also been successfully formulated as an *in situ* gel to improve corneal penetration and reduce frequency of administration (Yu *et al*., 2015). With the cited studies in mind, this research used the *in situ* gel as a dosage form and incorporated liposomes as a drug carrier to enhance the ophthalmic preparation for DNa.

Depending on the method employed to transform sol to gel on the ocular surface, the following three types of systems have been recognized:

pH triggered system – pH sensitive polymers contain acidic or alkaline functional groups that respond to changes in pH. Gelling of the solution is triggered by a change in pH at pH 4.4. The formulation is a free-running solution which undergoes coagulation when the pH is raised by the tear fluid to pH 7.4. The pH change after instillation of the formulation (pH 4.4) into the tear film leads to an almost instantaneous transformation of the highly fluid latex into a viscous gel. Swelling of hydrogel increases as the external pH increases in the case of weakly acidic (anionic) groups, but decreases if the polymer contains weakly basic (cationic) groups (Saini *et al*., 2015). The polymers used in this system are pseudolatex - carbomer (Carbopol®) (Srividya *et al.* 2001), and cellulose acetate phthalate latex. (Dol *et al*., 2013; Gurny *et al*., 1985).

Ion-activated induced system – In this method, gelling of the solution instilled is triggered by a change in its ionic strength. The rate of gelation depends on the osmotic gradient across the surface of the gel (that is between the gel and the target site). The aqueous polymer solution forms a clear gel in the presence of the mono- or divalent cations typically found in the tear fluids. The electrolyte of the tear fluid and especially Na^{+}, Ca^{2+} and Mg^{2+} cations are particularly suited to initiate gelation of the polymer when instilled as a liquid solution in the conjunctival cul-de-sac. The polymers which show osmotically induced gelation are Gelrite® or Gellan® gum, hyaluronic acid and alginates (Saini *et al*., 2015, Dol *et al*., 2013, Carlfors *et al*., 1998, Ma *et al*., 2008).

Temperature-dependent system – Temperature is the most widely used stimulus in environmentally responsive polymer systems. The change of temperature is relatively easy to control and also easily applicable both *in vitro* and *in vivo.* Gelling of the solution is triggered

by change in temperature, thus sustaining the drug release. These hydrogels are liquid at room temperature (20–25 °C) and undergo gelation when in contact with body fluids (35– 37 °C). The gelation is caused by an increase in temperature (Saini *et al*., 2015). Due to its unique thermo-reversible gelation properties, Pluronic® F 127 is the poloxamer widely used for this type of *in situ* gel. Plurenic® F 127 is a thermoresponsive gelling agent and also a solubility enhancer for DNa. Cellulose derivatives and xyloglucan are also sometimes used for this kind of *in situ* gel (Liu *et al*, 2006, Dol *et al*., 2014).

Due to the weak buffer capacity of the eye resulting from its tears quantity, pH sensitive *in situ* gels show a rather longer gelation process (Sieval *et al*., 1998). Therefore temperature sensitive (temperature dependent) *in situ* gel which is a preferred type for ophthalmic use (He *et al*., 2013). was chosen for this work.

2.16. Response Surface Methodology (RSM) for Optimization of Formulation

RSM explores the relationships between several control variables and one or more response variables. RSM has been well employed as an optimization procedure (Chen *et al*., 2010). With RSM, an optimum set of variables can be determined for a particular response(s). This allows for reduction of process variability, experimental time, and overall cost with the provision of improved output (Yang *et al*., 2010). RSM is a designed regression analysis intended to forecast the value of a dependent variable based on the precise values of the independent variables (Lee *et al*., 2006). Optimization with factorial designs and analysis of the response surfaces are powerful, efficient, and systematic tools. They shorten the time required for the development of pharmaceutical dosage forms and increase research output (Schwartz *et al*., 2002). Central Composite Design (CCD) suits a quadratic surface which works well for an optimization process (Demirel & Kayan, 2012). The experimental data

obtained from the CCD model experiments can be represented in the form of the following equation:

$$Y = b_o + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j + e_i,$$

Equation 1: Representation of Central Composite model for experimental data (Demirel & Kayan, 2012).

where Y is the predicted response; n is the number of factors; x_i and x_j are the coded variables; b_0 is the offset term; b_i, b_{ii}, and b_{ij} are the first-order, quadratic, and interaction effects, respectively; i and j are the index numbers for the factors; and e_i is the residual error (Demirel & Kayan 2012).

In this study, using the CCD of the RSM, two independent variables were applied viz. cholesterol (C) and phosphotidylcholine (PC) concentration in percentage ratio (C:PC); incubation time. Polydispersity index (PDI) and release profile (percentage of drug release) of the liposomal drug will be used to optimize percentage encapsulation. The total number of experiments (N) needed is determined by $N=2^k +2k + C_0$, where k is the number of factors (in this case k=2, that is C:PC ratio and Incubation time) and C_0 is the centre point's run (in this case $C_0=5$) (Sakkas *et al*., 2010). Using Design Expert® Software version 8.0.7.1, 13 experimental runs will be generated with a quadratic design model for the analysis of an optimized procedure.

CHAPTER 3: METHODOLOGY

This chapter consists of the methodologies applied in this study which includes the development and validation method of the Ultra High Performance Liquid Chromatography (UHPLC) method used to quantify DNa. Forced degradation studies as well as the methods used to prepare and characterise liposomes of DNa are described. The use of RSM and DNa liposome preparation methods will be discussed. In addition, preparation and characterisation methods of *in situ* gels of the DNa liposomes will be explained. Also, the method used comparing the *in vitro* drug releases of *in situ* gels of DNa liposomes with commercial DNa ophthalmic drops is discussed in this chapter.

3.1. Development and Validation of UHPLC method quantifying DNa.

3.1.1. Instrumentation:

The UHPLC apparatus consisted of a Flexar® FX-15 UHPLC pump, Flexar® UHPLC auto sampler programmed at 10 µl per injection, Flexar® solvent manager, Flexar® LC Column oven set at 25 ˚C, and Flexar® FXPDA UHPLC detector. The column used was a 5u C18 100A-column (Phenomenex®) with dimensions 150 mm x 4.6 nm. The mobile phase consisted of an aqueous phase, (A) (0.1 % formic acid in distilled UHPLC grade water) and an organic phase, (B) (0.1 % formic acid in acetonitrile), used in a 15 %: 85 % ratio. To obtain this mobile phase combination, several ratio combinations were used with different mobile phases and the above combination gave a clear and sharp peak in a five minute run time. Injections were performed in duplicate and the average recorded. Each run was five minutes and a wash phase of five minutes between injections applied.

3.1.2. Stock solution:

A stock solution of 1 mg/ml of DNa was prepared in phosphate buffer saline (PBS). From this stock solution, 10 serial dilutions were prepared, with a divisible factor of 0.5 resulting in the following values i.e. 1 mg/ml, 0.5 mg/ml, 2.5x10 mg/ml, 1.25x10 mg/ml, $6.25x10^{-2}$ mg/ml, $3.125x10^{-2}$ mg/ml, $1.56x10^{-2}$ mg/ml, $7.81x10^{-3}$ mg/ml, $3.91x10^{-3}$ mg/ml, and $1.9x10^{-3}$ mg/ml. Using a 0.2 µm syringe filter, each sample was filtered and transferred into 2 ml UHPLC amber vial and analysed with UHPLC to plot a standard curve.

3.1.3. Optimization of UHPLC method:

For optimization, the stock solution of DNa in PBS (1 mg/ml) was used at different ratios of the mobile phase. A ratio of 15:85 (A: B) gave a retention time (RT) of 1.80±1.09 (minutes) for DNa with a run time of 5 minutes. The pump flow rate was set at 1 ml/min, 276 nm was the absorbance wavelength and the oven temperature was maintained at 25 °C. Injections were performed in duplicate and averages recorded.

3.1.4. Validation of the optimized method

To validate the optimized method the following was performed using an adopted method by Dhaneshwar *et al.* 2010.

3.1.4.1. Linearity and standard curve

Each of the following concentrations was injected with a constant injection volume of 10 µl: 1.25x10 mg/ml, $6.25x10^{-2}$ mg/ml, $3.125x10^{-2}$ mg/ml, $1.56x10^{-2}$ mg/ml, $7.81x10^{-3}$ mg/ml, $3.91x10^{-3}$ mg/ml, $1.9x10^{-3}$ mg/ml and blank (distilled water). The area under the curve was plotted against concentration to obtain a calibration curve.

3.1.4.2. Specificity:

To determine specificity, non-DNa (blank) content was injected while keeping all other UHPLC parameters unchanged. Water was the blank while acetate buffer and PBS were also

evaluated by UHPLC. The chromatograms from these solvents were compared to the chromatogram of 1 mg/ml DNa to determine if the solvents used in preparing DNa liposomes interfered with the detection of DNa or quantification of DNa using UHPLC (Dhaneshwar *et al.,* 2010).

3.1.4.3. Precision and accuracy:

This was performed by repeatability and intermediate precision studies. For repeatability studies, three samples were chosen at random and were injected three times on the same day. While for intermediate study, a repeatability study was performed on three different days (Dhaneshwar & Bhusari, 2010).

3.2. Force Degradation Studies

For this study to be carried out, DNa was subjected to the following stress conditions: boiling, oxidation, acid hydrolysis and base hydrolysis (Ngwa, 2010.). This was performed to determine if these conditions brought about any degradation and if any resulting degradation interfered with the UHPLC quantification of DNa.

3.2.1. Boiling:

10 ml of the stock solution of DNa (1mg/ml) was refluxed in a round bottom flask for two hours in a water bath at boiling point. After cooling to room temperature, the mixture was filtered using a 2 µm filter and transferred into an amber UHPLC vial. 10 µl was injected into the UHPLC apparatus and analysed to determine the degree of degradation (Ngwa, 2010).

3.2.2. Acid Hydrolysis:

2 ml of the stock solution was poured into a round bottom flask. Then 2 ml of 1N HCl (Hydrochloric acid) was added and shaken, followed by adding an equal volume (2 ml) of NaOH (sodium hydroxide) to neutralise the acid. 10 µl was injected into the UHPLC

apparatus after filtering using a 0.2 µm syringe filter. The solution was transferred into an amber UHPLC vial for analysis (Ngwa, 2010).

3.2.3. Base Hydrolysis:

2 ml of the stock solution was poured in a round bottom flask. Then 2 ml of NaOH was added and shaken, followed by the addition of an equal volume of 1N HCl. 10 µl was injected into the UHPLC apparatus after filtering with a 0.2 µm filter for analysis (Ngwa, 2010).

3.2.4. Oxidation:

2 ml of the stock solution was poured into a round bottom flask. Then 2 ml of 30 % hydrogen peroxide was added and the reaction was allowed to proceed at room temperature for two hours with intermittent shaking. 10 µl was injected into the UHPLC apparatus after filtering with a 0.2 µm filter once again for analysis (Ngwa, 2010).

3.3. Preparation of Liposomal DNa

DNa liposomes were prepared using a four stepped active encapsulation method. These steps included production of thin lipid film, hydration of the thin film, and sizing of the hydrated film by extrusion and finally active loading by an ion exchange method discussed below.

3.3.1. Production of thin lipid film:

Using a 250 ml round bottom flask, phosphotidylcholine (PC) and cholesterol (C) (ratio combination to total mass of 1 mg) were dissolved in a 10 ml mix of chloroform: methanol (9:1). This homogenous solution was placed in a Rotavapor® (Büchi, Switzerland) at 60 ^{0}C for 3 hours under vacuum for thin film formation to take place in the round bottom flask. Any trace of organic solvent in the thin film formed was gently flushed with nitrogen. The round bottom flask containing the thin film was sealed using Parafilm® and stored below -20 ^{0}C

until needed for hydration. Lipid films were visually inspected for homogeneity to ensure that no aggregates of lipids had formed on the walls of the flask before use.

3.3.2. Hydration:

To hydrate the thin film formed from the preceding step, 5 ml of 150 mM sodium acetate buffer pH 7.9 was added to the thin film and placed in a shaking water bath for 3 hours at 60 ^{0}C. This process led to the formation of large multilamellar vesicles (LMV).

3.3.3. Extrusion:

The method used for extrusion was based on the method used by Hironaka *et al.,* 2011, and Berger *et al.,* 2001. With a 0.2 µm polycarbonate membrane filter, LMV was passed 11 times at 60 ^{0}C using an extruder (Avanti® polar Lipids, U.S.A.), yielding desired small unilamellar vesicles (SUV). The extruder was assembled as shown in figure 3.1 below and fully assembled extruder as seen in figure 3.2. Summarily, one of the two internal membranes was placed (with the O-ring channel facing outward) into the extruder's outer casing and an O-ring was carefully placed into the o-ring channel. A filter support was then placed on the O-ring followed by placing a polycarbonate membrane. A filter support was placed on the second O-ring in the O-ring channel of the second O-ring's internal membrane support. The second internal membrane was then gently placed on the polycarbonate membrane, making sure that the O-rings and filter supports were aligned. A teflon bearing was placed on the opposite ends of the second internal membrane followed by a retainer nut. The retainer nut was screwed to ensure that it was tight and aligned with the extruder outer casing.

To facilitate extrusion, the extruder stand/heating block was placed onto a hot plate. A thermometer was inserted in the well provided on the block and the hot plate was allowed to reach a desired 40 ^{0}C temperature. Extrusion was performed in the following steps:

Firstly, the hydrated sample from section 3.3.2 was placed in one of the gas-tight syringes and carefully placed in one end of the mini-extruder. An empty 1 ml air-tight Hamilton® syringe was placed into the other end of the extruder while making sure the syringe plunger was set to zero. The empty 1 ml Hamilton® syringe automatically filled as the hydrated sample was extruded through the membrane.

Secondly, the fully assembled extruder apparatus was inserted into the extruder stand.

Thirdly, the temperature of the hydrated liposome suspension was allowed to equilibrate to the temperature of the heating block (approximately 5-10 minutes).

Fourthly, the plunger of the filled syringe was gently pushed until the liposome suspension was completely transferred into the alternate syringe.

Fifthly, the plunger of the alternate syringe was gently pushed to transfer the solution back into the original syringe.

The fourth and fifth steps were repeated to a total of 11 passes through the membrane and the final extrusion filled the alternate syringe.

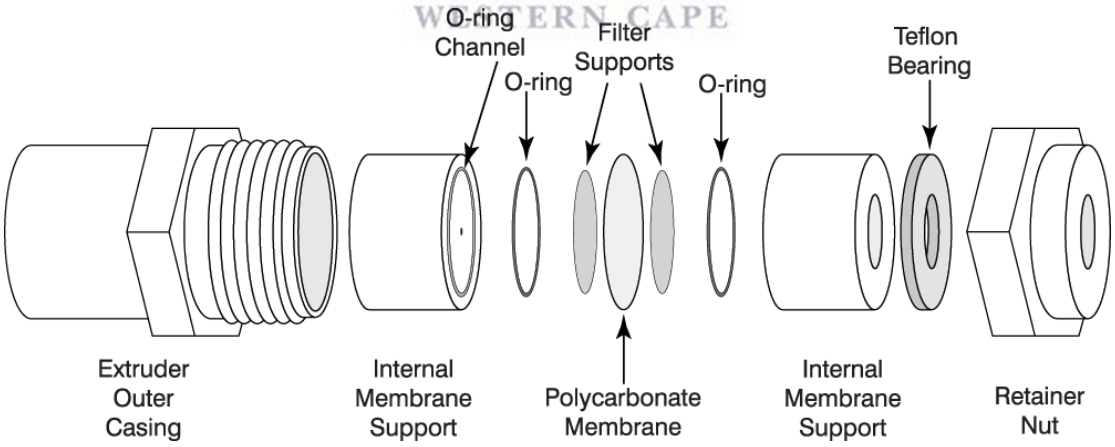

Figure 3.1: A diagram to identify the parts of a mini extruder. It shows the order in which to assemble the various parts of the mini extruder with a polycarbonate membrane supported on both sides by a filter support

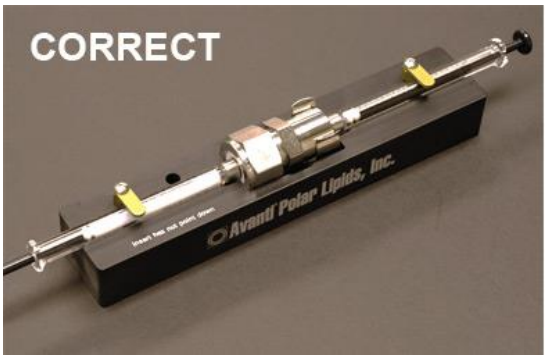

Figure 3.2: An assembled extruder just before it is placed on a hot plate for the extrusion process

3.3.4. Remote/ active loading by ion exchange:

A transmembrane pH of liposomes was created in two dialysis steps. This was to create an external pH 4.0 of liposomes while keeping their internal pH at 7.9.

For the dialysis steps, one litre of 1/10 (that is 100 ml of PBS and 900 ml of distilled water) PBS (pH 7.4) was adjusted to pH of 4 using hydrochloric acid (HCl). Small unilamellar vesicles (SUV) liposomes were placed in synthetic Snakeskin® (U.S.A.) dialysis tubing (3500 MWCO) and using regular sewing thread both ends of the membrane were tied to prevent the content from leaking. The tied sample was completely immersed into the one litre (1:10 PBS) solution for one hour, and repeated using a fresh sample of external medium (1:10 PBS Solution).

A DNa solution of 1 mg /ml was prepared in PBS (1 in 10 parts) solution of pH 4. The DNa solution was added to extruded liposomes from section 3.3.3 in a 1:1 ratio and placed in an oven at 40 ^{0}C for active encapsulation to take place.

3.3.5. Mechanism of active encapsulation

The calcium acetate gradient method (Hironaka *et al*., 2011) was used to prepare DNa liposomes. While cations are membrane impermeable, protonated acetic acid are membrane

permeable. This is due to the permeability coefficient difference between cations and anions. In active encapsulation (figure 3.3 below), the following occur (Hironaka *et al*., 2011): The first step is rehydration of thin film with acetate gradient as described in chapter 3 section 3.3.2. This leads to equilibrium in acetic acid in and out of liposomes (part A of figure 3.3). The second step is a creation of membrane gradient by dialysis (part B of figure 3.3). In this stage cations remain inside the liposomes while protonated acetic acid leak out of the liposomes creating an increased internal liposomal pH. This pH difference is the driving force for active encapsulation of DNa into liposomes. The last step is the incubation step (part C of figure 3.3). Here, DNa is loaded into empty liposomes. Uncharged DNa molecules are pumped into liposomes. While inside the liposomes, DNa assumes a positive charge and become impermeable and cannot leave liposomes once entrapped. This happens until the liposomes are saturated with DNa.

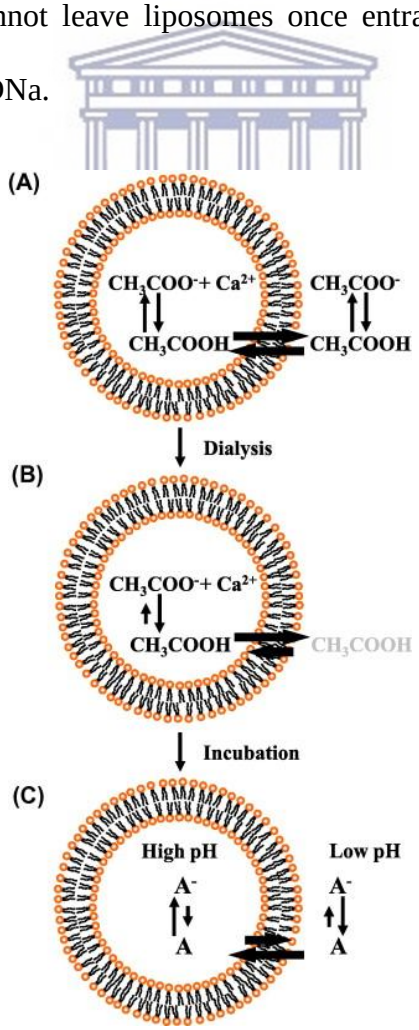

Figure 3.3: Schematic representation of active encapsulation (Hironaka *et al*. 2011)

3.4. Characterisation of DNa Liposomes.

3.4.1. Percentage encapsulation

Percentage encapsulation was performed to determine the amount of DNa entrapped into liposomes. Protamine was used as an aggregating agent. Protamine aggregated with liposomes to form a dense plug at the bottom of the vial leaving only un-encapsulated or free DNa in the supernatant. To facilitate sedimentation of liposomes by centrifugation, the sizes of liposomes were increased using protamine (Torchilin and Weissig, 2003). In using protamine, equal volumes of liposomal diclofenac solution and protamine sulphate solution (10 mg/ml) were mixed in a 2 ml centrifuge vial and left to react for 10 mins. After10 mins., this mixture was centrifuged for 15 mins. at 13000 rpm. The supernatant liquid (unencapsulated or free DNa) was analysed using UHPLC. The amount of DNa encapsulated was calculated by deducting the amount not encapsulated from the original diclofenac concentration (Chan *et al*., 2004). That is, Percentage Encapsulation = (T-C)/T ×100 (Where T is total concentration or start concentration and C is the concentration detected in the supernatant).

3.4.2. Size, polydispersity index and zeta potential determination

The zeta potential, PDI and size determination of liposomes were investigated based on a method by Odeh *et al*., 2012. A correlation spectroscopy technique on a Malvern® Zetasizer Nano ZS (Malvern Instruments, UK) was conducted. This technique measures the sizes of particles in a nanometer range and gives the size distribution (PDI) of vesicular structures (Goll *et al.,* 1982). To perform these measurements, 1 ml of hydrated sample (liposomes) was placed in a cuvette in a zetasizer. The zetasizer recorded the size, zeta potential and PDI of

the liposomes. Measurements were conducted at an angle of 173° with the following experimental parameters: medium refractive index of 1.330; medium viscosity of 1.0 cps; dielectric constant of 79 and at a temperature of 25 ^{0}C. Measurements were performed in triplicate and the mean diameter and mean polydispersity index recorded.

3.4.3. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was performed to determine the morphology of liposomes. To prepare samples for SEM, a drop of liposomal samples was dispersed on carbon adhesive tape applied on an aluminium stub, then dried completely under a fume hood. The dried liposome was coated with gold palladium for 30 seconds using Emitech K550X (England) sputter coater and viewed with the Auriga HR-SEM F50 (Zeiss, Germany) with a voltage of 5 kV.

3.4.4. *In vitro* drug release study.

3.4.4.1. Separation of free DNa from loaded Liposomes

For *in vitro* drug release of the DNa liposome, free DNa (that is DNa not encapsulated into liposomes) was separated from DNa loaded liposomes in the liposome suspension loaded with DNa (loaded liposomes). Failing to perform this separation might have led to a higher than normal percentage drug release as a result of free DNa permeating together with DNa loaded liposomes across the Franz® diffusion cell (used for *in vitro* drug release studies). A mini column centrifugation (gel chromatography) method was used to separate free DNa from the loaded DNa (Kumar *et al*., 2010). To prepare the centrifugation column, 10 % w/v Sephadex® G-50 was prepared in PBS (pH 7.4) and left for 48 hours to completely swell. A barrel of a 1 ml disposable syringe was plugged with moist filter paper to prevent Sephadex® from leaking out of the syringe. To form a gel filtration column, swollen Sephadex® was carefully packed into the plugged syringe avoiding air bubbles. This prepared column was

attached to a 2 ml centrifuge vial and spun in a centrifuge. In the first step of separation of free DNa from liposomes containing DNa (loaded liposome), excess water was removed from the prepared Sephadex® column into the centrifuge vials by centrifuging at 2000 rpm for three minutes. Secondly, 100 µl of liposome sample (containing both free drug and loaded drug) was slowly added to the column and centrifuged at 5000 rpm for three mins. Finally, 100 µl of PBS at pH 7.4 was passed through the column and centrifuged for three mins. at 5000 rpm; this final step was a wash phase to remove any trace of DNa liposomes left in the column. Free DNa bound to the Sephadex® in the column while DNa loaded liposomes passed through. The eluted liposomes were collected and used for drug release.

To confirm that free DNa was trapped in the Sephadex® column while loaded liposomes passed through, 100 µl of 1 mg/ml DNa solution was passed through the column following the same procedure as above. This resulted in less than 1 % of DNa passing through the Sephadex® column while the rest of the DNa was retained in the Sephadex® column, indicating that the Sephadex® column retained free DNa while loaded liposomes passed through the column to be used for the *in vitro* drug release study.

3.4.4.2. Drug release study using Franz® Diffusion cells.

For the drug release study, a bi-chambered donor / receiver compartment model (Franz® diffusion cell apparatus) was used as shown below (figure 3.4). Snakeskin® (synthetic) dialysis membrane (3500 MWCO with 22 mm internal diameter) was used as a dialysis membrane. Before use, the dialysis membrane was soaked in PBS (pH 7.4) for three hours to remove any preservatives present. To ensure that DNa passed freely through the dialysis membrane, a preliminary study was performed as follows:

Pure 1 mg/ml DNa solution was used in the donor compartment and 2 ml of PBS at physiological pH of 7.4 in the receptor compartment. A 12 hour study was performed at 3

hour intervals, each time removing all the content of the receptor compartment and replacing with fresh PBS to maintain sink conditions. After 12 hours the cumulative reading (area under the curve) of samples from the receptor compartment was recorded. A cumulative of 78.65 % DNa passed through the membrane. The dialysis membrane was then used for drug release studies as there was relatively free movement of DNa across the membrane.

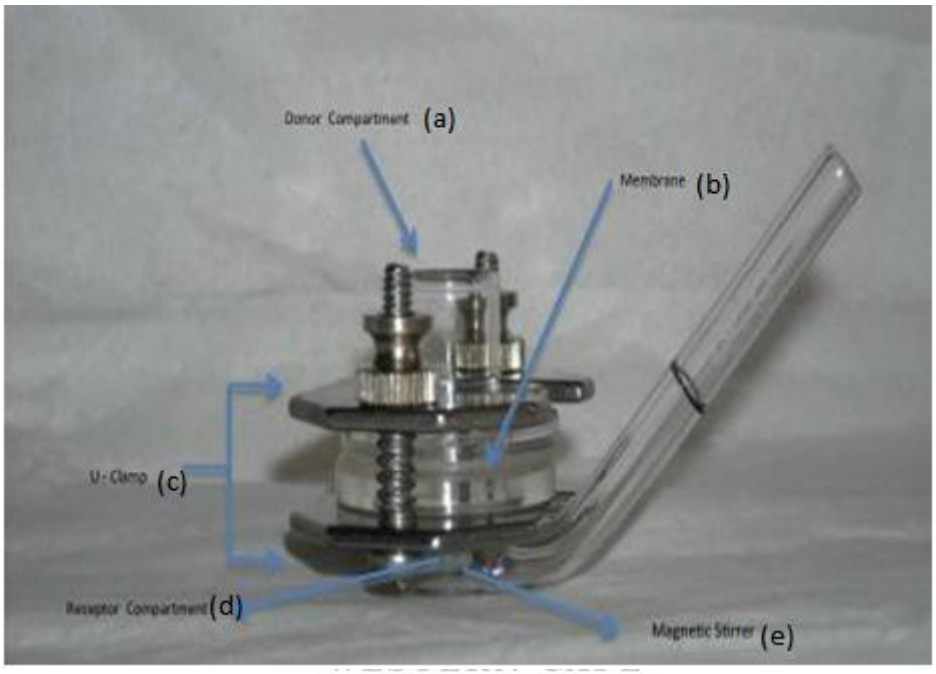

Figure 3.4: *In vitro* drug release set up with bi- chambered Franz® diffusion cell
((a) donor compartment, (b) membrane,(c) u-clamp, (d) receptor compartment and (e) magnetic Stirrer).

For *in vitro* drug release (figure 3.4) the above set up was used; 200 µl of "pure" (only DNa loaded liposomes, that is after free DNa removed using Sephadex® column) DNa liposomes was placed in the donor compartment of the Franz® cell and 2 ml PBS at pH 7.4 in the receptor compartment. Parafilm® was used to seal the top of the donor compartment to prevent evaporation. Temperature was maintained at 37 ^{0}C with the use of a circulating water bath apparatus. The Franz® cell placed on a multi-magnetic stirrer stand, continuously stirred using a magnetic stirrer bar at 10 rpm. 2 ml was carefully withdrawn from the receptor and placed into an UHPLC vial at 3 hours, 6 hours, 9 hours and 12 hours' time intervals. Each 2

ml was replaced with fresh PBS after each withdrawal to maintain sink conditions. Experiments were performed in duplicate and averages recorded.

3.5. Design of statistical method – Response Surface Methodology (RSM)

RSM has been well employed as an optimization procedure (Chen *et al*. 2010). With RSM, an optimum set of variables is determined for a particular response(s). In this study the responses are % encapsulation, % *in vitro drug* release, PDI and zeta potential. Using the central composite design (CCD) of the RSM, two independent variables cholesterol (C) and phosphotidylcholine (PC) concentration in percentage (C:PC ratios), and incubation time are used to optimize percentage encapsulation, polydispersity index (PDI) and release profile (percentage of drug release) of the liposomal drug. Using Design Expert® Software version 8.0.7.1, 13 experimental runs where generated with a quadratic design model and performed. To determine what values where used for the dependent variable, first arbitrary values were chosen to obtain preliminary result. It is from these preliminary results that a final set of values were chosen in order to get valuable results.

3.5.1. Optimization of liposomal diclofenac sodium (DNa).

Optimization was performed with Design Expert® software by adjusting or controlling variables to determine the suitable factors that retained the best possible and desired outcomes or responses. Optimization done to obtain as much as possible of DNa encapsulated into liposomes that are stable and can readily leach the encapsulated DNa during *in vitro* release studies. An optimum can be determined by RSM (Sakkas *et al*., 2010). For optimization to be effectively performed, two experimental protocols were established. First, a study of the design space was undertaken to determine how variables were interrelated. Second, a study was executed to ascertain response behaviour(s) and get a desired optimum

response by manipulating the variables studied in the first step. All variables could be set at one of the following six categories goals: in range, maximum, minimum, equal to value, less than or more than a value. The overall impact was then measured using desirability. Desirability is an objective function ranging from 0 to 1 which concurrently determines the settings of all input variables that will give optimum levels of the responses. Several attempts were performed to get the best desirability possible. With every desirability value obtained were predicted results for a corresponding combination of independent variables (C : PC ratio and incubation time). Experiments were then performed (for the chosen desirability) and the results obtained were compared to the predicted result. Finally, comparing the predicted to the actual (experimental) values gave a ascertain the possibility of using these procedure (RSM and design expert®) to predict experimental outcomes.

3.6. Stability of liposomes at storage temperature (4^{o}C)

To evaluate the stability of liposomes prepared, runs from the 13 experimental runs generated by Design Expert® software for RSM, that is low, medium and high cholesterol content, were used. The size and PDI of these samples were measured weekly for four weeks.

3.7. Preparation of DNa liposome *in situ* gel

DNa liposome *in situ* gel was prepared and characterised using a method for preparing ophthalmic *in situ* gels by Asasutjarit *et al*., 2011). Briefly, Pluronic® F127 was completely dissolved in cold phosphate buffer pH 7.4. Then, Carbopol® 980 and DNa liposomes were respectively added into the solutions of gelling agents and stirred continuously until homogeneous solutions were obtained. Several mass ratio combinations of Pluronic® F 127 (BASF, Germany) and Carbopol® 980 NF (Lubrizol, USA) were used to prepare *in situ* gels to determine what ratio of the excipients will be liquid (sol) at room temperature (25 °C) and gels at close to body temperature 35±2 °C (table 3.1).

Table 3.1: Formulation combinations of *in situ* gel using different ratios of PBS and Carbopol®

Formulations	Pluronic® (g)	Carbopol® (mg)	DNa liposomes(ml)	PBS 7.4(ml)
1	4.0	70.0	12.5	12.5
2	2.0	7.5	7.5	7.5
3	2.0	10.0	5.0	5.0
4	4.0	70.0	25.0	25.5
5	2.0	35.0	5.0	5.0

The preferred *in situ* gels formulations were characterized as follows:

3.7.1. Determination of flowability of diclofenac sodium liposomes *in situ* gels

A test tube inverting method was carried out to determine the phase behaviour of DNa liposomes *in situ* gel at room temperature (25 ^{o}C), storage temperature (4 ^{o}C), and at precorneal temperature (35 ^{o}C). This method was based on Jeong *et al*.,2002). 2 ml sample of DNa liposomes *in situ* gel was placed in a test tube and set upside down. Samples flowing at 4± 1 °C (storage temperature) and 25 ± 1 °C (room temperature) but not at 35 ± 1 °C (precorneal temperature) within 30 seconds were accepted as optimum thermo responsive *in situ* gels (Asasutjarit *et al*., 2011). Flowability test was performed in triplicate and the mean was recorded.

3.7.2. pH determination of DNa liposome *in situ* gels

Formulations to be instilled into eye should be non-irritating to the eye. Eye preparations should have the same pH (7.4) as that of lacrimal fluid (Dol *et al*., 2013, Nayak *et al.*, 2012). The pH of the prepared *in-situ* gelling system after addition of all the excipients was measured using a digital pH meter. Readings were performed in triplicate and the mean standard deviation was recorded.

3.7.3. Gel capacity test DNa liposomes *in situ* gel

Gelling capacity was determined by placing a drop of the sample (about 20 µl) into a test tube containing 2 ml of PBS (pH 7.4) equilibrated at 35 ± 1 °C. The visual assessment of gel formation was performed in triplicate (Asasutjarit *et al*., 2011).

3.7.4. Determination of visual clarity of DNa liposomes.

Clarity is an important characteristic feature of ophthalmic preparations. The formulation was inspected for visual appearance and clearness by visual observation against a white and black background to check the presence of any particulate matter (Asasutjarit *et al*., 2011).

3.7.5. Comparing *in vitro* drug release profile, commercial Voltaren® eye drops and prepared *in situ* gel of DNa liposomes.

To conclude characterisation of the preferable *in situ* gel formulation, a comparative *in vitro* drug release study was carried out for the formulated DNa liposomes *in situ* gels and commercial DNa ophthalmic eye drops (Voltaren®). The same method as in subsection 3.4.4.2 was used. In summary, 1 ml of sample to be studied (Voltaren® eye drops or DNa liposome *in situ* gel or DNa liposome) was placed in the donor compartment and 2 ml of PBS at physiological pH of 7.4 in the receptor compartment. A 12 hour study was performed at 3 hour intervals, each time removing all the content of the receptor compartment and replacing with fresh PBS to maintain sink conditions. After 12 hours, the reading (area under the curve) of DNa from the receptor compartment was recorded.

To recap, Chapter Three described all the experimental procedures performed in this study. That is from instrumentation and validation of UHPLC apparatus to preparation characterization of DNa liposomes and *in situ* gel of DNa liposomes. The next chapter will elaborate on the results obtained.

CHAPTER 4: RESULTS AND DISCUSSION

This work was performed in the following order; UHPLC method development and validation to ascertain UHPLC as a suitable quantification method for DNa. DNa was subjected to stress condition to ensure that reagents did not interfere with the quantification of DNa. Response surface methodology (RSM) and design expert® software was used to generate experimental runs and to determine interrelations of factors involved in the preparation and characterisation of diclofenac sodium liposomes. Diclofenac sodium liposomes *in situ* gel preparation and characterisation. Finally, comparison study of *in vitro* drug release profile of commercial DNa eye drops with *in situ* gel of DNa was performed. This chapter will be discussed in the same order as above.

4.1. UHPLC Validation

To ensure that the UHPLC used in quantifying DNa was suitable, the UHPLC method was validated by checking for linearity, specificity, accuracy and precision.

4.1.1. Linearity

Linearity was studied to illustrate a proportional relationship between peak areas of chromatograms at different concentration of DNa. A serial dilution of DNa in HPLC grade water (table 4.1) was used to plot a standard curve.

Table 4.1: Serial dilutions of diclofenac sodium versus mean peak Area (n=3) at 276 nm

Concentration of DNa (mg/ml)	Mean peak Area (n=3)
0.125	1764206.0
0.0625	929487.7
0.03125	478028.2
0.015626	319361.9
0.007813	130710.7
0.003906	94035.86

A standard curve (figure 4.1) shows a linear relationship between diclofenac sodium concentration and the absorbance reading (area under the curve) with an acceptable correlation (R^2) value of 0.997 (Shabir, 2004). This standard was used to determine the concentration of DNa in all other solutions throughout this study.

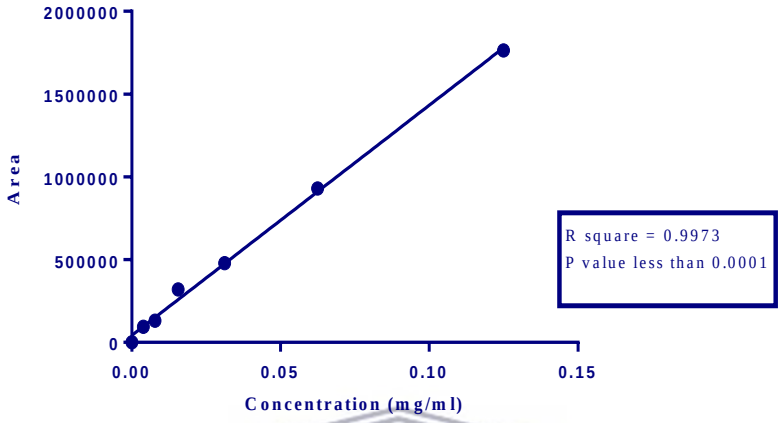

Figure 4.1: Standard curve for UHPLC of diclofenac sodium (n = 2), R^2=0.9973

4.1.2. Specificity

Specificity sought to ensure that solvents used in the preparation of DNa liposomes did not interfere with the quantification of DNa by UHPLC. There was no UHPLC chromatogram peak when, PBS of pH 7.4 (figures 4.2), acetate buffer of pH 7.4 (figures 4.3), PBS of pH 4.0 (figures 4.4) or distilled water (figures 4.5) were analysed. The chromatograms (figure 4.2, 4.3, 4.4, and 4.5) indicated a baseline with no peak. This was an indication that the UHPLC method was selective to DNa. It was imperative that reagents used in this study did not interfere with UHPLC analysis of DNa. Thus, the UHPLC method used was specific.

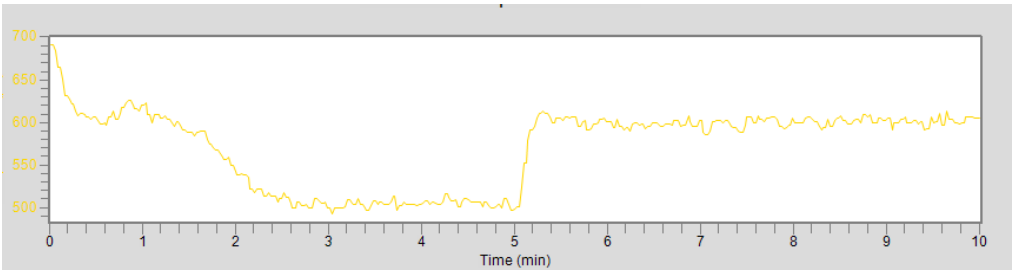

Figure 4.2: UHPLC chromatogram of PBS pH 7.4 with no peak height detected

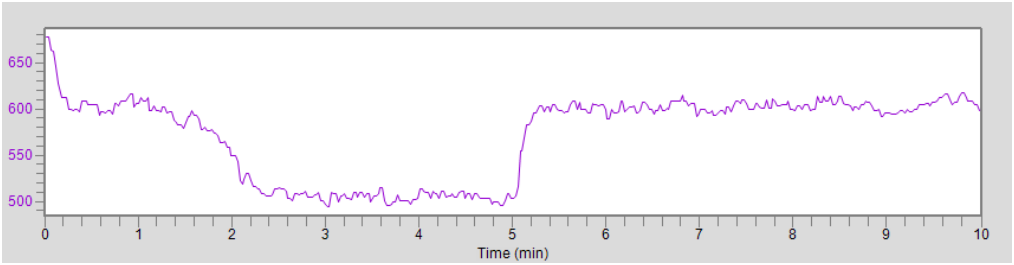

Figure 4.3: UHPLC chromatogram of acetate buffer pH 7.4 with no peak height

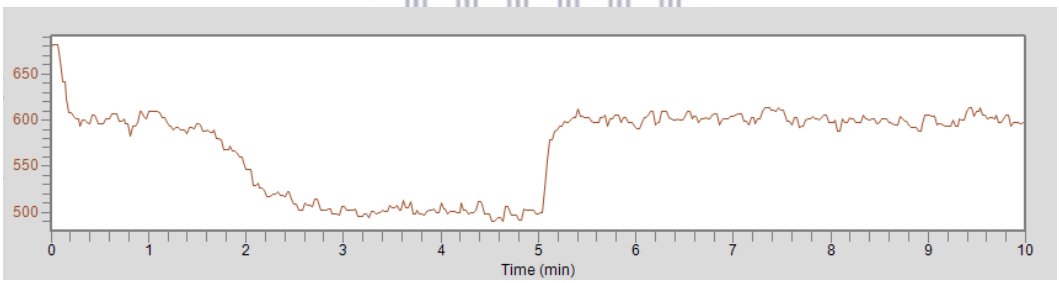

Figure 4.4: UHPLC chromatogram of PBS pH 4 with no peak height detected

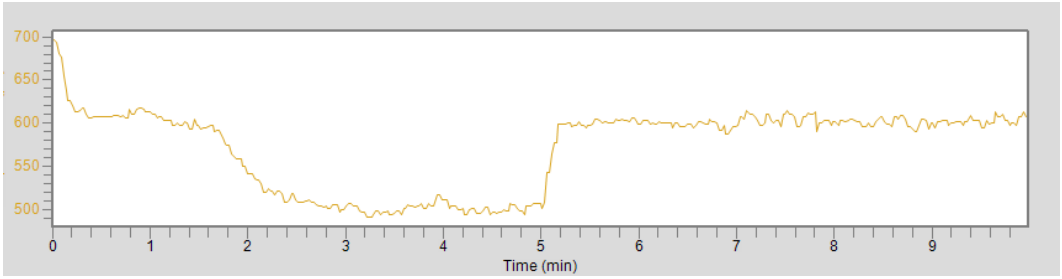

Figure 4.5: UHPLC chromatogram of distilled water with no peak height detected

4.1.3. Accuracy

An accuracy criterion for an assay method requires the mean recovery to be 100 ± 2% at each concentration over the range of 80 to 120% of the target concentration (Shabir, 2004). Accuracy was determined by single injection of three concentrations within the linearity range. The three concentrations of DNa were analysed on UHPLC. The actual concentrations were compared with the concentration measured by UHPLC. None of the responses showed more than 2% deviation in terms of recovery, indicating that the UHPLC method had a high degree of accuracy (table 4.2). The mean recovery percentage was within the accepted range, implying that this UHPLC protocol was accurate.

Table 4.2. Accuracy table for diclofenac sodium showing an acceptable Percentage deviation of less than 2%

Actual concentration(mg/ml)	Measured concentration(mg/ml)	Mean percentage recovery	Percentage deviation
0.125	0.127	101.6	0.16%
0.0312	0.0314	100.64	0.35%
0.00391	0.00387	99.97	1.75%

4.1.4. Precision

Precision was studied by an intra-day and inter-day studies:

For the intra-day study, three concentrations of DNa (1mg/ml, 0.5 mg/ml and 0.25 mg/ml) of DNa were randomly chosen. UHPLC analysis was performed on these samples, three times a day, for three days. Samples were analysed in triplicate and the average area under the peak height recorded (table 4.3). The results obtained showed a RSD (Relative Standard Deviation) value of less than 10 which is an acceptable RSD value (Shabir, 2004).

Table 4.3: Intra-day variance of 1mg/ml of DNa

	Day 1(n=3)	Day 2	Day 3
First injection	7899834.0	7444306.0	6769516.0
Second injection	8048325.0	7546924.0	7607577.0
Third injection	7830563.0	7499271.0	7592729.0
Mean	7926241.0	7496834.0	7323274.0
SD	111257.1	51352.7	479626.0
RSD	**1.403**	**0.0685**	**6.317**

Regarding the inter-day study, three concentrations of DNa within the linearity range (from the standard curve) were analysed. A UHPLC analysis was performed on three different days, in duplicate each day and average reading recorded. The results as shown in the inter-day table above (table 4.4) revealed a RSD value of less than 10 which is an acceptable RSD value (Shabir, 2004). The inter-day and intra-day results indicated that the UHPLC method was precise.

Table 4.4: Inter day readings for three concentrations

	1.0mg/ml	0.5mg/ml	0.25mg/ml
Day 1	7899833.8	5409985.5	3071944.7
Day 2	7444305.7	5342029.5	3070401.1
Day 3	7607577.0	5346026.9	3129772.1
Mean	7650572.0	5366014.0	3090706.0
SD	230787.6	38132.9	33841.0
RSD	**3.017**	**0.711**	**1.094**

4.2. Force degradation studies

Force degradation or stress testing of DNa was performed to identify the occurrence of interference of degradation product(s) of DNa (if any) with quantification of DNa by UHPLC. Furthermore, it was performed to determine the stability of DNa under stress conditions and to validate the stability and specificity of the UHPLC analytical procedures (Sistla *et al*., 2005). First DNa was dissolved in UHPLC grade water and analysed, then DNa subjected to different stress conditions and analysed. The stress conditions were; treating DNa with HCl , NaOH, and hydrogen peroxide. The UHPLC chromatograms of the DNa subjected to stress conditions were then compared with DNa dissolved in water to see the effect of stress conditions on DNa

4.2.1. DNa dissolved in water

DNa was dissolved in UHPLC grade water and analysed with UHPLC to determine the nature and height of its chromatogram when no stress condition is applied on it. This chromatogram was then used as a reference to compare chromatograms after subjecting DNa to stress conditions.

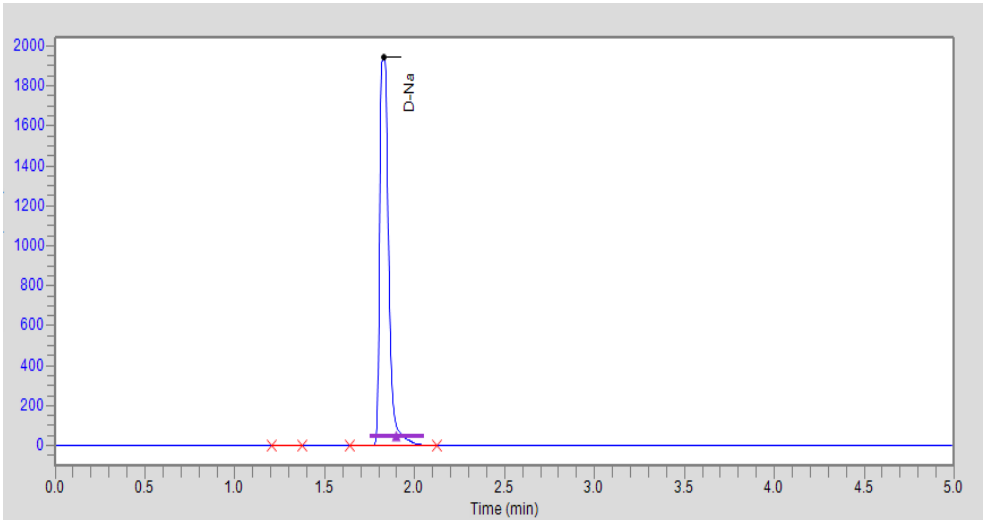

Figure 4.6: UHPLC chromatogram of diclofenac sodium (1mg/ml) dissolved in distilled water with peak detected at 1.9 minutes

The UHPLC chromatogram for DNa (1 mg/ml) dissolved in UHPLC grade water showed a peak with retention time at 1.90 minutes. The peak showed a good degree of symmetry, that is, the peak was clear and sharp (figure 4.6). Also 84.04% of the dissolved DNa was recovered by UHPLC (table 4.5) implying very little of DNa was lost during dissolution and chromatographic processes. This chromatogram (figure 4.6) obtained was used as a baseline to check for degradation occurrence when DNa was subjected to stress conditions.

Table 4.5: DNa solution (1mg/ml) treated with water, acid, base, hydrogen peroxide and analysed (in UHPLC) to determine degradation of DNa occurs.

Force Degradation Condition	Start Concentration Before Degradation Studies (1 mg/ml)	Concentration(mg/ml) Retained After Degradation Study	Percentage DNa Retained
UHPLC grade water	1	0.8404387	84.04
In acid	1	0.009650827	0.965
In base	1	0.01836051	1.836
Hydrogen peroxide	1	0.005718959	0.571
Heating (refluxing in distilled water)	1	0.8826619	88.266

4.2.2. DNa dissolved in water and refluxed for 60 minutes

The chromatogram for DNa dissolved in distilled water and refluxed for one hour showed a peak with retention at 1.90 minutes. The neat and sharp peak (denoted as DNa) showed a good degree of symmetry (figure 4.7). The chromatogram and retention time was similar to that of DNa dissolved in water (figure 4.6). In the degradation data for DNa (table 4.5), 84% of DNa was recovered after dissolving DNa in distilled water and similarly 88% was recovered after refluxing in distilled water. This suggested that DNa underwent minimal (2 to 6 %) degradation under reflux conditions.

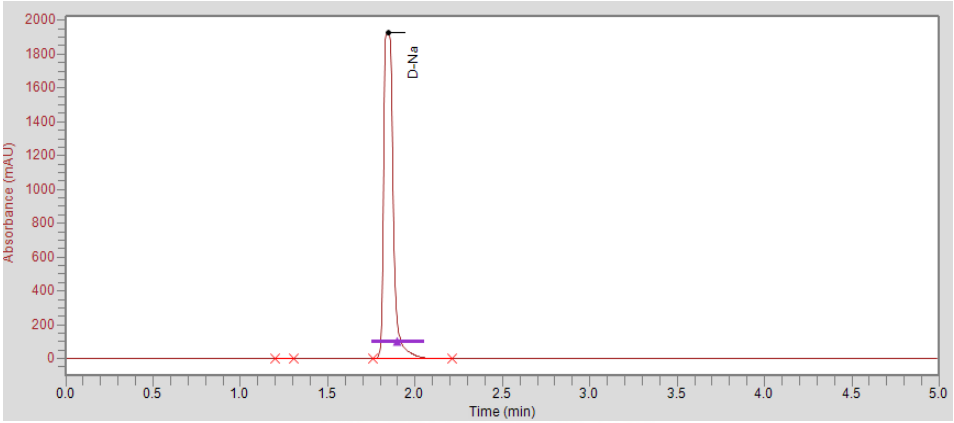

Figure 4.7: UHPLC chromatogram of DNa reflux in water for 60 minutes with DNa peak at 1.9 minutes

4.2.3. DNa dissolved in water and treated with 1M hydrochloric acid for 15 minutes

The chromatogram for DNa treated with 1M hydrochloric acid showed two peaks. A degradation peak (peak B, figure 4.8) was detected at 1.2 minutes and DNa peak (peak A, figure 4.8) detected at 1.7 minutes since the spectra of peak A (figure 4.8) was the same as the spectra of the DNa chromatogram in figure 4.7. From table 4.5, 0.965 % of DNa was retained confirming with the chromatograms (figure 4.8) that degradation occurred but did not interfere with the UHPLC detection and quantification of DNa. Degradation of DNa occurred due to acid-catalysed hydrolysis.

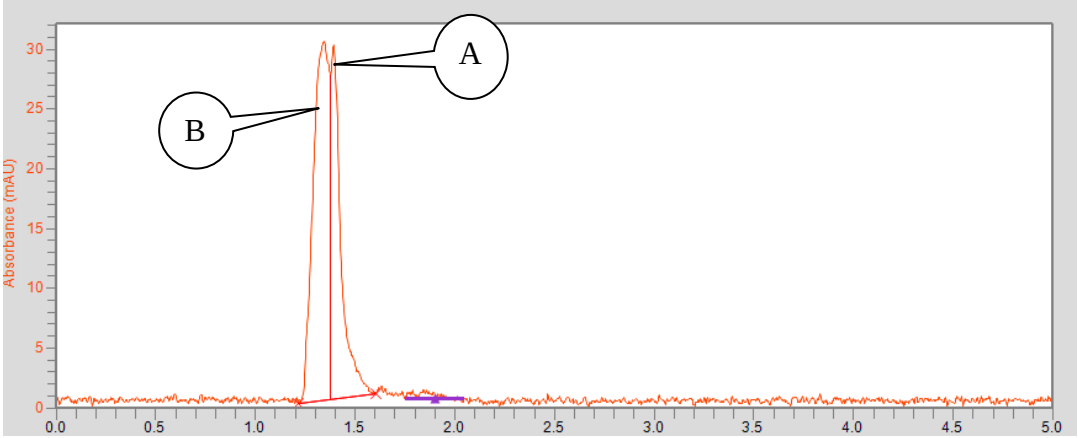

Figure 4.8: UHPLC chromatogram of diclofenac sodium after undergoing acid reflux for 15 minutes with 1 M HCl. Peak A (Diclofenac sodium) and peak B (degradation product).

4.2.4. DNa dissolved in water and treated with 1N sodium hydroxide for 15 minutes

The chromatogram for DNa treated with 1N NaOH showed two peaks. A degradation peak (peak B, figure 4.9) was detected at 1.3 minutes and DNa peak (peak A, figure 4.9) detected at 1.5 minutes. From table 4.5, 1.8 % of DNa was recovered confirming with the chromatograms (figure 4.9) that degradation occurred but did not interfere with the HPLC

detection and quantification of DNa. Degradation of DNa occurred due to base-catalysed hydrolysis.

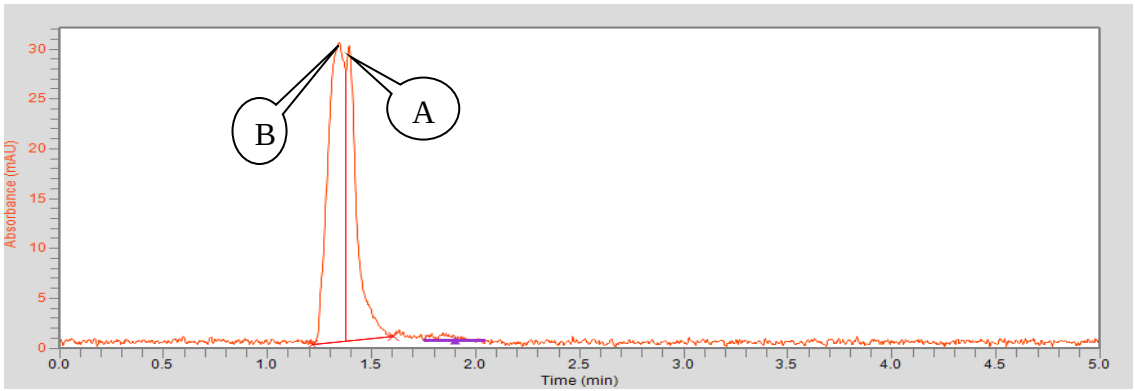

Figure 4.9: UHPLC chromatogram of diclofenac sodium after base reflux for 15 minutes with 1N NaOH. Peak A (Diclofenac sodium) and peak B (degradation product).

4.2.5. DNa dissolved in water and treated with 1M Hydrogen peroxide for 15 minutes

The chromatogram for DNa treated with 1M hydrogen peroxide showed two peaks. A degradation peak (peak A, figure 4.10) was detected at 1.6 minutes and DNa peak (D-Na figure 4.9) detected at 1.8 minutes. From table 4.5, 0.6 % of DNa was retained confirming with the chromatograms (figure 4.10) that degradation occurred but did not interfere with the HPLC detection and quantification of DNa.

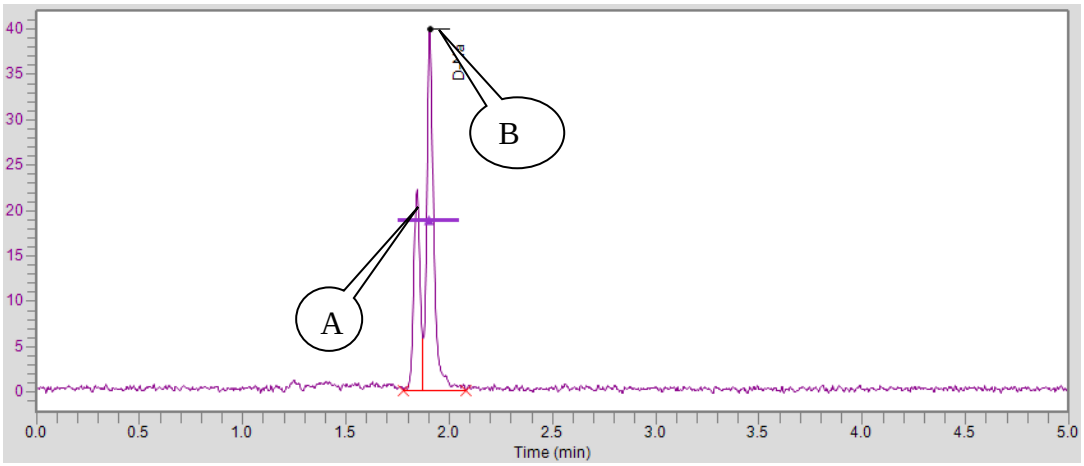

Figure 4.10: UHPLC chromatogram of DNa after refluxing with peroxide form 15minute in 1M hydrogen peroxide. (Peak A is the degradation product while peak B is diclofenac sodium)

In conclusion, the results obtained from force degradation studies showed that when diclofenac sodium was subjected to stress conditions, its detection was not affected even in the presence of degradation product(s). Therefore, UHPLC was used throughout this work for the detection of DNa.

4.3. Evaluation of the effects of incubation times and cholesterol to phosphotidylcholine ratios on percentage encapsulation, *in vitro* drug release, size and polydispersity index of diclofenac sodium liposomes

Analysis of variance (ANOVA) provides statistical results and diagnostic checking to evaluate the adequacy of a model. Analysis of variance was used for graphical analyses of the data obtained. Central composite design (CCD) of response surface methodology (RSM) was used to design the experiments, build models and determine the conditions for optimization. The statistical design was based on two independent factors (input factors); cholesterol to phosphotidylcholine (C: PC) ratio in percentage and incubation time (in minutes) and four responses (output factors), that is polydispersity index (PDI), percentage encapsulation

(%EE), *in vitro* drug release in percentage and zeta potential (mV). Design Expert® Software (version 8.0.7.1) was used for the statistical design of experiments and data analysis.

Using Design Expert® Software, the quality fit of the polynomial model was expressed by the coefficient of determination R^2, and its statistical significance was checked by the Fisher's *F*-test. This software determined the best model to evaluate the data input. Model terms were evaluated by the *P*-value (probability) with 95% confidence level. Three-dimensional plots and their respective contour plots were obtained based on effects of C : PC ratio and incubation time on PDI, percentage encapsulation, *in vitro* drug release and zeta potential. Significant model terms were desired to obtain a good fit in a selected model. It is vital to highlight other statistical terms and their meaning as used in Design Expert® Software for statistical analysis.

The lack of fit (LOF) *F*-test described the variation of the data around the fitted model. If the model did not fit the data well, the LOF *F*-test value was significant. If there was a large *P* value for lack of fit (>0.05), then *F*-statistic was insignificant, implying significant model correlation between the variables and process responses. *P*-value was a probability of error. The R^2 coefficient gave the proportion of the total variation in the response predicted by the model. A high R^2 value close to 1 was desired as it implied the empirical models closely fitted the actual data (Lee *et al*., 2006).

Preliminary experiments (Runs) were generated by design expert software® using arbitrary limits as shown in table 4.6 below. This gave a trend of what combination of C: PC ratios and incubation time were to be used for different runs. For preliminary runs (table 4.7) the limits set for C: PC (20 to 40) ratio and Incubation time (15 minutes to 60 minutes). Results of these preliminary runs are seen in table 4.7, it was noticed that the responses of runs 1, 2, 3, 5, 6, 7, 8, 11 and 12 were similar to each other possibly because the range of values used for C : PC

(%) was narrow (20 to 40). Due to the closeness of the results obtain from this preliminary runs, the effects of C : PC and incubation time on percentage encapsulation, polydispersity index and *in vitro* drug release was not clear.

Table 4.6: Summary of dependent variables, their upper (max.) and lower (min.) limits as generated by design expert® Software to determine the number of preliminary experiments (runs) and combination of variables for each run

Factor	Name	Units	Types	Min.	Max.	Coded values	
A	Incubation time	Minutes	Numeric	5.68	69.32	FALSE	1=60
B	C : PC ratio	%	Numeric	15.86	44.14	FALSE	1=40

Table 4.7: Preliminary experiments (runs) generated by design expert® Software using the variable in table 4.6. It shows ratios and incubation time used for each run.

Runs	Incubation time (minutes)	C:PC (%)	Percentage encapsulation (%)	Polydispersity Index	In vitro drug release (%)
1	37.50	15.86	86.00	0.233	18.6
2	37.50	30.00	85.00	0.142	14.5
3	37.50	30.00	87.70	0.120	12.0
4	69.32	30.00	75.70	0.300	10.0
5	15.00	20.00	80.00	0.238	1.7
6	37.50	30.00	80.00	0.116	12.0
7	15.00	40.00	84.86	0.300	6.7
8	37.50	30.00	82.00	0.120	11.0
9	37.50	30.00	80.00	0.140	12.2
10	5.68	30.00	94.00	0.305	11.5
11	60.00	40.00	85.44	0.392	11.7
12	37.50	44.14	85.44	0.229	42.8
13	60.00	20.00	93.40	0.235	1.2

To generate a second set of experiments (runs) the lowest possible lower limits (table 4.8) were used for the independent variable, that is; 10 % for C : PC and 15 minutes for incubation time. Any values lower than these (10 % and 15 minutes) resulted in negative values which were not suitable for use. The maximum values were set at 60 minutes for

incubation time and 50 % for C : PC (table 4.8). Again 13 runs were generated (table 4.9). The data from this second set of parameters gave statistically significant results as shall be individually discussed in the next sections.

Table 4.8: Summary of dependent variables, their upper (max.) and lower (min.) limits as generated by Design Expert® Software to determine the number of experiments (runs) and combination of variables for each run

	Name of factor	Units	Types	Min.	Max.	Coded values	
A	Incubation time	Minutes	Numeric	5.68	69.32	FALSE	1=60
B	C : PC ratio	%	Numeric	1.72	58.28	FALSE	1=50

Table 4.9: Experiments (runs) generated by Design Expert® Software using the variable in table 4.8. It shows ratios and incubation time used for each run.

Runs	Factor1A:incubation (minutes)	Factor 2B: C : PC (%)
1	69.32	30
2	37.5	30
3	15	10
4	37.5	30
5	37.5	30
6	15	50
7	37.5	30
8	5.68	30
9	37.5	58.28
10	60	10
11	60	50
12	37.5	1.72
13	37.5	30

4.3.1. Percentage Encapsulation

The effect(s) of C: PC ratio and incubation time on the amount of DNa encapsulated into liposomes was analysed. Expressed in percentage and termed percentage encapsulation (EE %). Percentage encapsulation was calculated using the formula: EE% = (T-C)/T ×100 (where T is total concentration or start concentration of DNa and C is concentration detected in the supernatant.

Table 4.10: Percentage encapsulation (EE %) for different Cholesterol to phosphatidylcholine (C: PC) ratio and different Incubation Times (IT)

Runs	EE (%)	IT (Minutes)	C: PC (%)
1	**71.6**	69.32	30
2	**72.8**	37.5	30
3	**86.4**	15	10
4	**69.2**	37.5	30
5	**65.2**	37.5	30
6	**68.4**	15	50
7	**55.6**	37.5	30
8	**64.8**	5.68	30
9	**54.8**	37.5	58.28
10	**82**	60	10
11	**56.8**	60	50
12	**79.2**	37.5	1.72
13	**68.8**	37.5	30

As seen in table 4.10, a decrease in the cholesterol content generally led to an increase in percentage encapsulation. Similarly, Kaiser *et al*., (2003) reported a decrease in the quantity of cholesterol resulted in a dramatic increase in %EE. The possible reason for this trend is that the increased content of PC with a corresponding reduction in cholesterol content leads to an increase in the quantity of larger liposomes and/or decrease in number of lamellae of the liposomes (Kaiser *et al.,* 2003). This possibly resulted in larger encapsulation space of the liposomes and hence the tendency for more DNa to be encapsulated, as such an increase in percentage encapsulation.

It was also observed that initially increasing cholesterol concentration from 1.72 % (run 12) to 10 % (run 3 and run 10) led to an increase in percentage encapsulation. Increasing cholesterol content to a level above 10% led to a drop in percentage encapsulation. At higher concentration, cholesterol interferes with the packing of the liposome structure resulting in

increased fluidity, leakage of entrapped DNa out of the liposome, and a reduction in percentage encapsulation (Ramana *et al*., 2010).

Using Design Expert® Software, ANOVA analysis of the model for response showed that the (linear) model chosen for this analysis had a significant fit, with an F-value of 9.61(P-value 0.0047). Values of "Prob> F" less than 0.0500 indicated model terms were significant while values greater than 0.1000 indicated the model terms were insignificant. Accordingly, C: PC ratio was a significant model term with Prob> F value of 0.0014. For incubation time, Prob> F-value of 0.6891 was not significant. This was an indication that C: PC ratio affected the percentage encapsulation while incubation time did not.

A "Lack of Fit F-value" of 0.86 implied the Lack of Fit was not significant. Non-significant lack of fit was good as the model was required to fit. The "Pred R-Squared" value of 0.4276 was in reasonable agreement with the "Adj R-Squared" value of 0.5894. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 was desirable. In this case a ratio of 9.090 indicated an adequate signal. This meant the model could be used to navigate the design space and make valuable predictions.

The normal plot of residuals (figure 4.11), showed a straight line pattern, indicating that the data was normally distributed (good). The predicted versus actual plot (figure 4.12) also showed a straight line, a confirmatory indication that this model could be used to make reasonable predictions about the system.

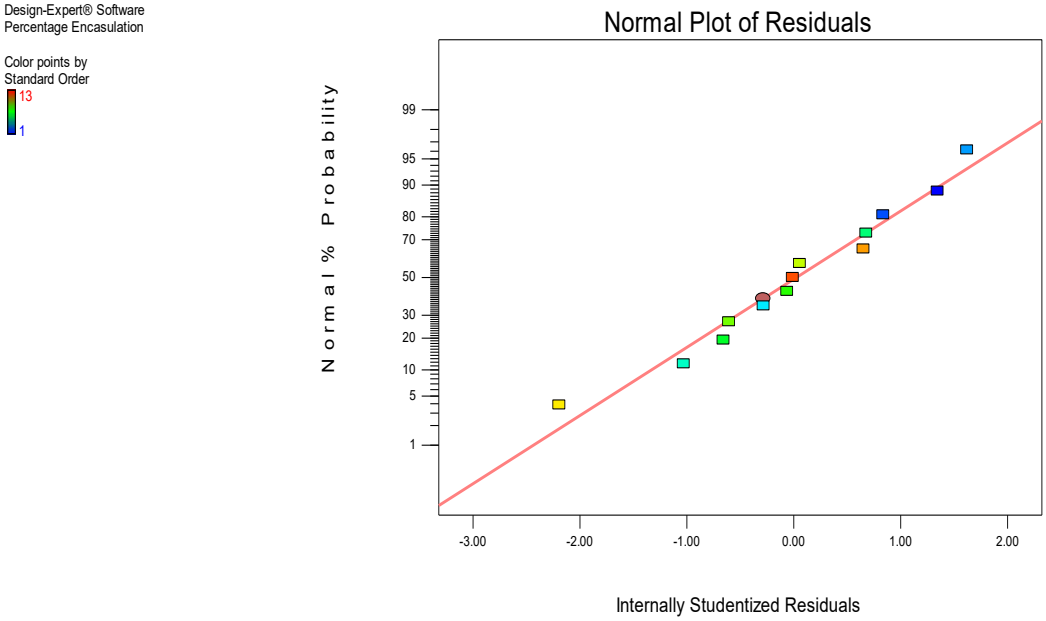

Figure 4.11: Normal plot of residuals for percentage encapsulation.

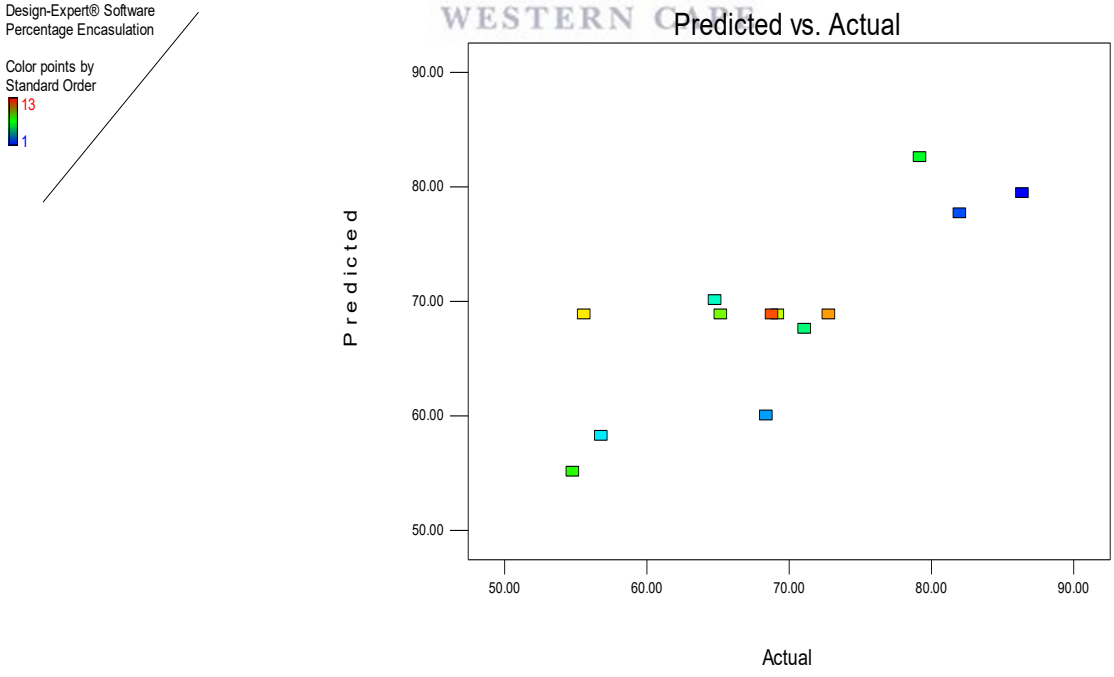

Figure 4.12: Plot of predicted versus actual values for percentage encapsulation

4.3.2. Polydispersity Index PDI:

The polydispersity index is used to assess the homogeneity of liposomes (Pereira-Lachataignerais *et al*., 2006). It was necessary to assess how each factor (C: PC ratio or incubation time) could be varied to decrease the polydispersity index of a liposomes for a more homogenised particle size distribution. A polydispersity index below 0.5 is considered good. As seen on table 4.11 below, there was one outlier value of 0.71 for run 5. The levels of the variable were not suitable to give desirable reponses. All other PDI values were in an acceptable range, undermining the relevance of the outlier PDI value with respect to its relationship with incubation time or C: PC ratio. PDI is related to size and sizing technique. The same sizing technique (extrusion method) was used for all 13 runs (DNa liposome preparations) and explains the similarity in PDI values. PDI was therefore left in range during the optimization phase as the PDI values obtained were in an acceptable range (less than 0.5) regardless of variations in incubation times and C:PC ratios.

Table 4.11: Polydispersity index (PI) for different C: PC (Cholesterol: phosphatidylcholine) ratios at different incubation times (IT)

Runs	PI	IT (minutes)	C: PC (%)
1	**0.481**	69.32	30
2	**0.35**	37.5	30
3	**0.494**	15	10
4	**0.433**	37.5	30
5	**0.718**	37.5	30
6	**0.215**	15	50
7	**0.154**	37.5	30
8	**0.393**	5.68	30
9	**0.508**	37.5	58.28
10	**0.458**	60	10
11	**0.227**	60	50
12	**0.347**	37.5	1.72
13	**0.341**	37.5	30

4.3.3. *In Vitro* Drug Release study

To evaluate the effects of C: PC and incubation time on *in vitro* release of DNa from DNa liposome, ANOVA was used. ANOVA analysis of the model for response showed that the (linear) model chosen by Design Expert® Software for this analysis had a significant fit with an F-value of 5.10 (P-value = 0.0298). Values of "Prob> F" less than 0.0500 indicated model terms were significant, while values greater than 0.1000 indicated the model terms were not significant. Cholesterol to phosphotidylcholine ratio had a significant model terms with "Prob> F" value of 0.0298. The "Lack of Fit F-value" of 0.15 implied the Lack of Fit was not significant which was acceptable as the model was required to fit. The "Pred R-Squared" of 0.3267 was in reasonable agreement with the "Adj R-Squared" of 0.4057. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 was desirable. For this analysis the ratio was 6.580, an indication of an adequate signal. This model could therefore be used to navigate the design space and make predictable outcomes.

Table 4.12: Drug release (DR) for different C: PC (Cholesterol : phosphotidylcholine) ratio and Different Incubation times (IT)

Runs	DR (%)	IT (minutes)	C: PC(%)
1	**19.55**	69.32	30
2	**26.1**	37.5	30
3	**32.25**	15	10
4	**28.9**	37.5	30
5	**10.77**	37.5	30
6	**20.4**	15	50
7	**5.4**	37.5	30
8	**23.1**	5.68	30
9	**1.82**	37.5	58.28
10	**35.37**	60	10
11	**5.35**	60	50
12	**56.81**	37.5	1.72
13	**54.4**	37.5	30

From table 4.12 above, high cholesterol content (run 6, 9 and 11) showed a lower drug release while low cholesterol content had a higher drug release. High cholesterol content was associated with a low drug release most likely because liposomes with a high content of cholesterol had greater fluidity, allowing DNa to easily leach out of the liposomes before initiation of drug release. Also lower cholesterol content gave higher percentage encapsulation and vice versa. A possible implication of this was that more active compound was loaded promoting a higher potential to be released hence higher percentage of drug release.

The normal plot of residuals (figure 4.13) shows a fairly straight line indicating that the data is normally distributed (good). The predicted versus actual plot (figure 4.14) also shows a straight line, indicating that the model could be used to make reasonable predictions about the system.

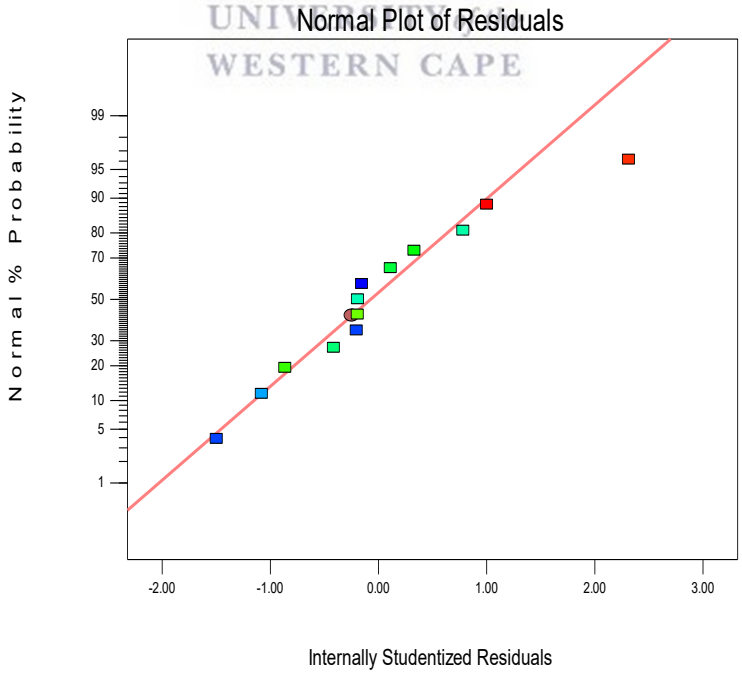

Figure 4.13: Normal plot of residuals for *in vitro* drug release

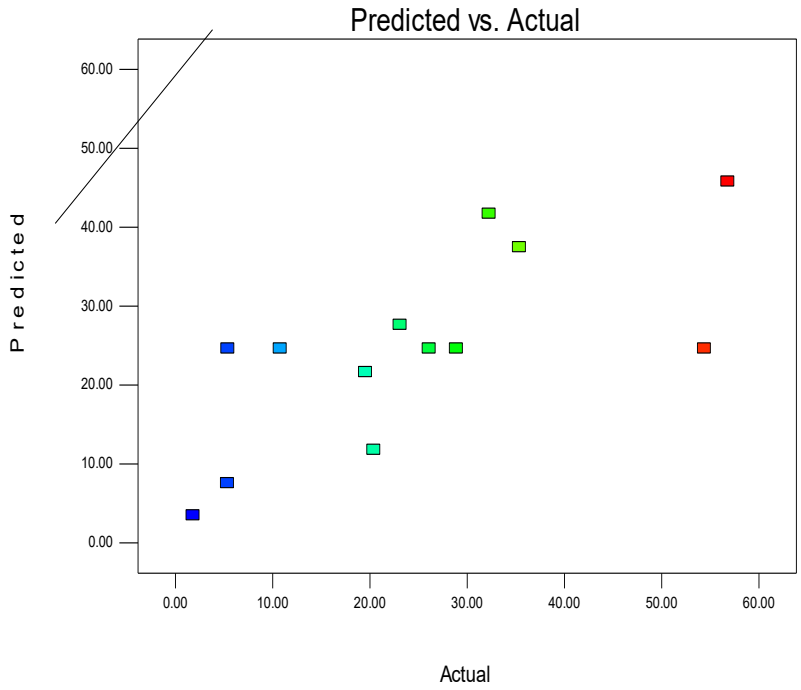

Figure 4.14: Plot of predicted versus actual values for drug release.

Interaction plots (figure 4.15) for the model showed that there was significant interaction between the factors, meaning one factor cannot be changed without the other being affected. So a change in either factors (for instance C: PC) will bring about a change in the other factor (drug release). In figure 4.15, red lines indicate concentration of C: PC greater than 50% while black lines indicate C : PC below 10%. These black and red lines crossing imply interaction occurs below 10% and above 50%. This is portrayed in figure 4.15 by the two lines intersecting. A three dimensional plot of these interactions is seen in figure 4.16.

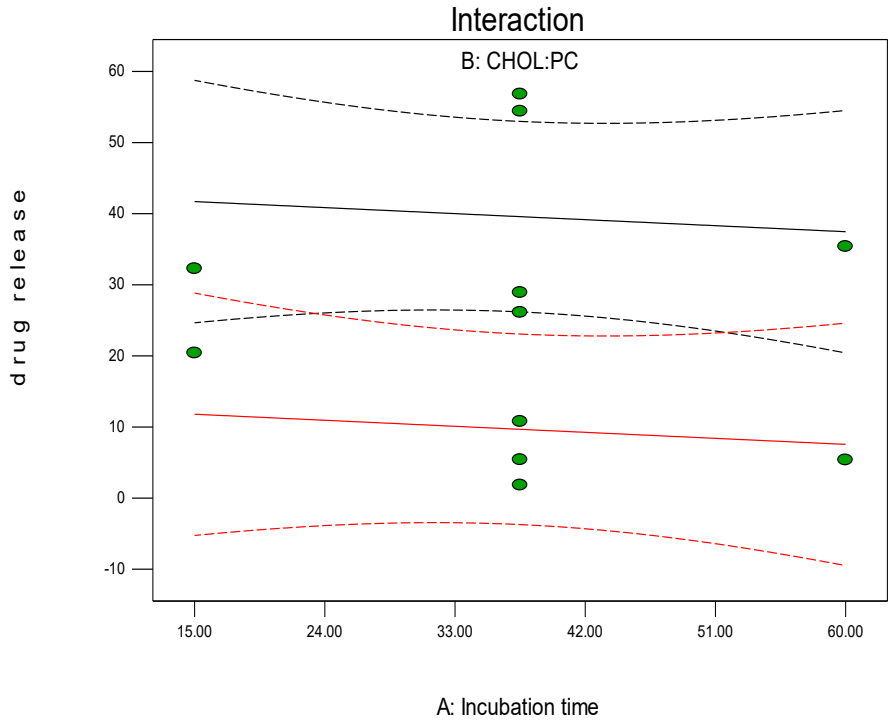

Figure 4.15: Interaction plot for drug release for DNa liposomes, red lines indicate C: PC greater than 50 % while black lines indicate CHOL: PC below 10 %. These black and red lines crossing imply interaction occurs below 10% and above 50%.

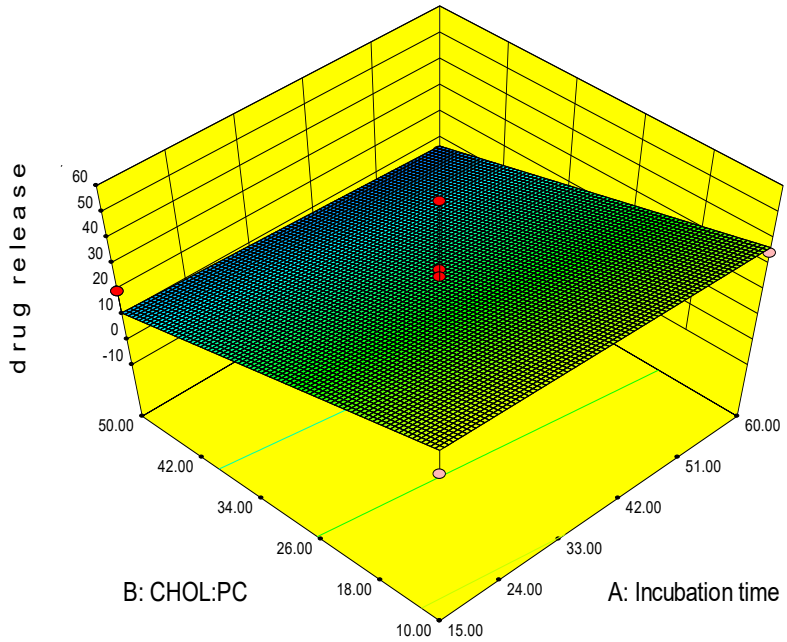

Figure 4.16: Three dimensional response surface plot of drug release for DNa liposomes

4.3.4. Zeta Potential

ANOVA analysis of the model for response showed that the (quadratic) model chosen by Design Expert® Software for this analysis had a significant fit with an F-value of 5.92 (P-value of 0.0186). Values of "Prob> F" less than 0.0500 indicated that model terms were significant, while values greater than 0.1000 indicated model terms were not significant. Cholesterol to PC ratio) were significant model terms with "Prob> F" value of 0.0186. The "Lack of Fit F-value" of 4.66 implies the Lack of Fit was not significant. Non-significant lack of fit was desired because the model is statistically required fit. A negative "Pred R-Squared" (-0.1233) implied that the overall mean was a better predictor of this response than the current model. "Adeq Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable. In this case, 6.816 indicated an adequate signal and this model could therefore be used to navigate the design space.

There was an increase in zeta potential with increase in cholesterol content when time was kept constant. As shown in table 4.13, increasing cholesterol percentage content from 1.72% (Run 12) to 30% (Runs 2,4,5,7 and 13) resulted in an increase in zeta potential. But a further increase in cholesterol led to a decrease in zeta potential. These observations were similar to a previous work (Liu *et al*., 2000). In their study, the experimental data also indicated that the absolute zeta potential became more negative as the molar ratio of cholesterol changed from 0% to 33% in relation to its component contents within the lipid vesicle. However, the decrease of zeta potential did not significantly change as the molar ratio of cholesterol became 50%. This is due to the cholesterol structural arrangements within the PC bilayer. Cholesterol lessens the surface binding affinity among the cations in the buffer solution and its bilayer surface by fusing in the bilayer (Liu *et al*., 2000). The structural arrangements of the PC bilayer became irregular as a result of the incorporation of the cholesterol, thus affecting the binding ability between the PC vesicular surface and the cation as the molar ratio of cholesterol reaches 50% in the PC vesicle. The weak electrostatic repulsive force between the PC vesicles and incorporation of cholesterol into the system can elevate the negative zeta potential.

Table 4.13: Zeta potential (zet. Pot.) for different (C: PC) (Cholesterol : phosphotidylcholine) ratios and Incubation times (IT)

Runs	Zet. Pot.	IT (minutes)	C: PC (%)
1	**-34.033**	69.32	30
2	**-36.9**	37.5	30
3	**-29.43**	15	10
4	**-36.2**	37.5	30
5	**-35.3**	37.5	30
6	**-23.5**	15	50
7	**-40.6**	37.5	30
8	**-38.3**	5.68	30
9	**-21.9**	37.5	58.28
10	**-31.5**	60	10
11	**-15.9**	60	50
12	**-34.9**	37.5	1.72
13	**-41.4**	37.5	30

The normal plot of residuals (figure 4.17) showed a reasonably straight line indicating that the data is normally distributed (good). The predicted versus actual plot (figure 4.18) also showed a reasonable straight line, indicating that the model could be used to make reasonable predictions about the system.

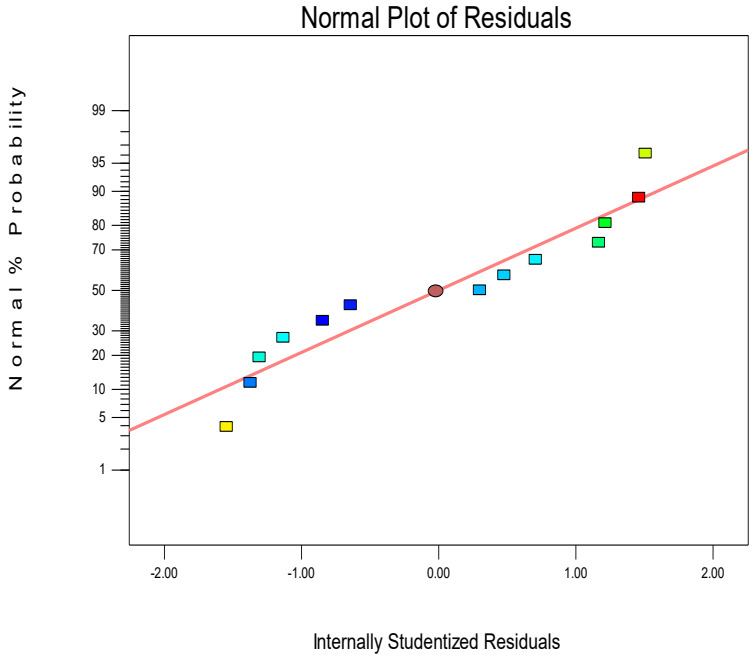

Figure 4.17: Plot of residuals for zeta potential showing a normal distribution.

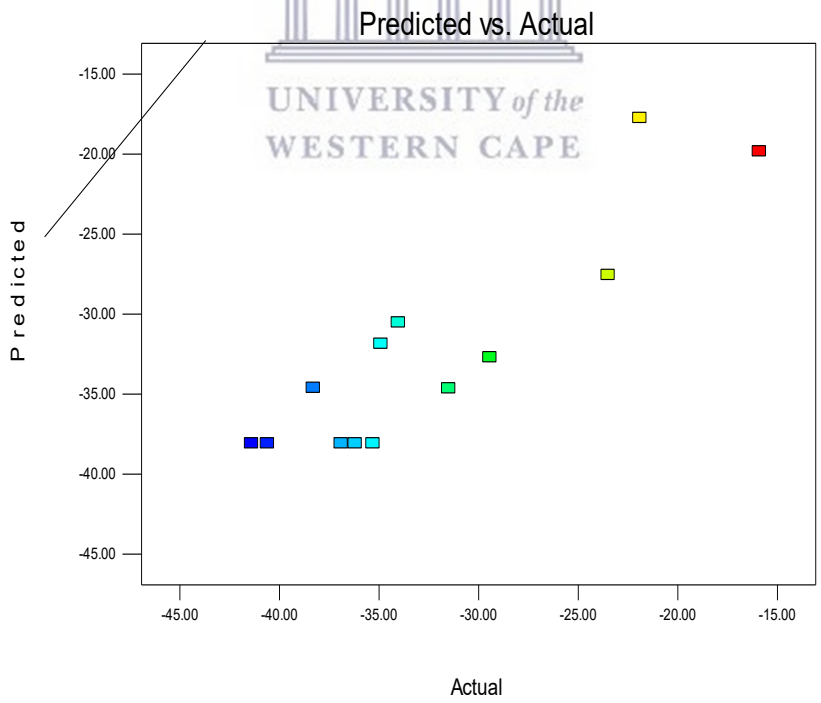

Figure 4.18: Plot of predicted versus actual plot for zeta potentials of DNa liposomes

Interaction plots for the model showed that there was significant interaction between the two factors: one factor cannot be changed without the other being affected. Put differently, a change in either factor will bring about a change in the other factor. This was confirmed in the ANOVA analysis and is shown in figure 4.19 by the two lines intersecting. The red lines indicate C: PC greater than 50% while the black lines indicate C : PC below 10%. These black and red lines crossing suggest interaction occurs below 10% and above 50% and a three dimensional plot of these interaction is seen in figure 4.20. Three dimensional (3D) plots were made for the estimated responses, which were the bases of the model polynomial function for analysis to investigate the interactive effect of the two factors (incubation time and PC : C %) on Zeta potential.

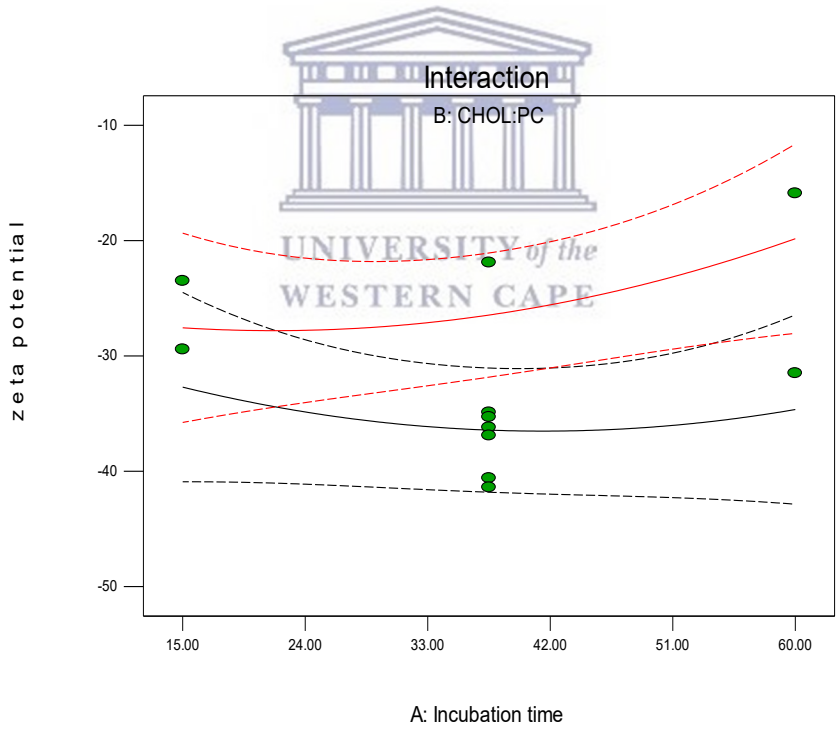

Figure 4.19: Interaction plot for Zeta potentials for DNa liposomes, red lines indicate C: PC greater than 50% while black lines indicate CHOL:PC below 10%. These black and red lines crossing suggest interaction occurs below 10% and above 50%

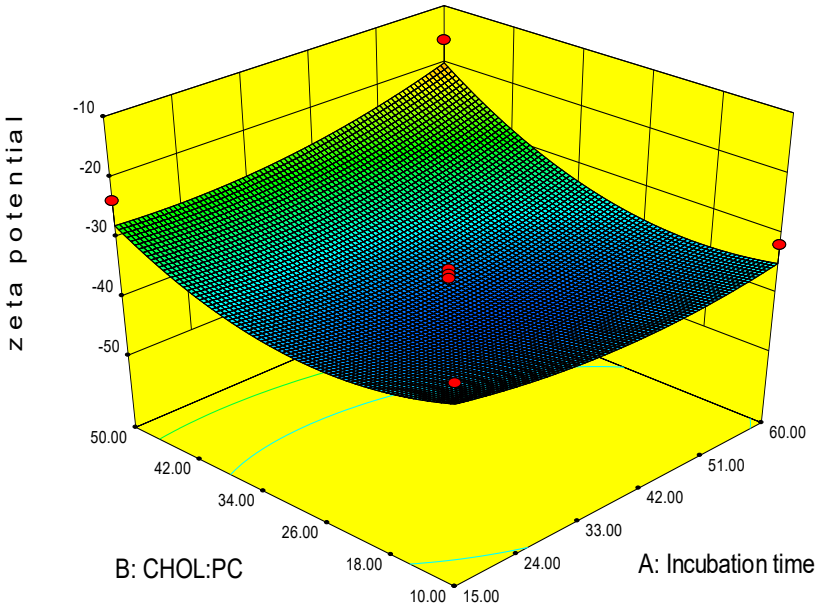

Figure 4.20: Three-dimensional response surface plot for DNa liposomes with respect to Zeta Potential

4.3.5. Scanning Electron Microscopy (SEM)

SEM was used to study the morphology of empty liposomes (liposomes without DNa) and liposomes with DNa (loaded liposomes) (figure 4.21). Samples for microscopy were prepared using the procedure described in section 3.4.2. Liposomes are approximately spherical, with a wrinkled surface (Campardelli *et al*., 2016). The loaded and empty liposomes were spherical in shape. The loaded liposomes were individually separated from each other while the empty liposomes occurred in clusters. This was possibly due to a repulsive force that existed between loaded liposomes and a lesser repulsive force between the empty liposomes. This was confirmed by the large negative zeta potential of loaded liposomes (-28.9mV) compared to the zeta potential of its equivalent empty liposomes (-0.02mV). The greater the zeta

potential value from 0, the greater the repulsive force between liposomes, and thus the more dispersed the liposomes as is the case with loaded liposomes on the left of figure 4.21.

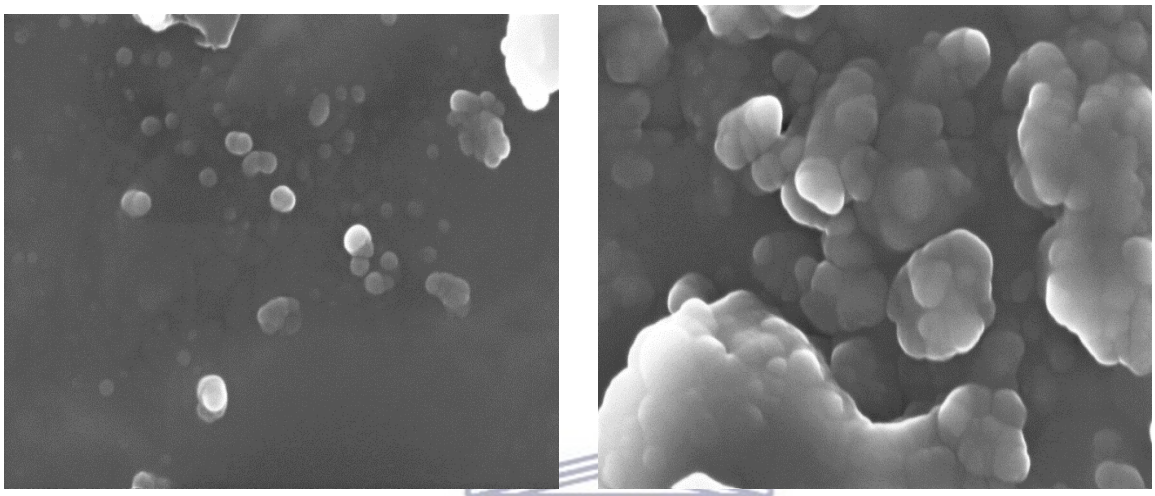

Figure 4.21: Morphology of loaded DNa liposomes (left) and empty liposomes (right) as seen using Scanning Electron Microscopy.

4.3.6. Optimization

Optimization was performed by varying the independent variables [incubation time and Cholesterol (C) to phosphotidylcholine (PC) ratio] in order to determine a suitable factor combination that would retain the best possible desired outcomes or responses (the outcomes being percentage encapsulation, zeta potential and percentage *in vitro* drug release). Usually, the optimization process can be performed either by simplex optimization or Response Surface Methodology (RSM). An exact optimum is determined by RSM (Sakkas *et al*., 2010). For this study, for optimization to be effectively performed, two experimental protocols were established. First, a study of the design space was performed to determine how the variables interrelated (section 4.3.1 to section 4.3.5). Then, to get desired optimum responses (high percentage encapsulation and high *in vitro* drug release) the variables studied

in the first step were varied and described below. Using Design Experts® software, all variables could be set at one of six goals: in range, maximum, minimum, equal to a number, less than or more than a certain value. For this work the variables and goals used are indicated in table 4.14 below. The level of importance of all the variables was set at 3 as changing the level of importance did not change the desirability at all. Level of importance ranged from 0 to 5 (0 being 'not important' and 5 being 'most important'). The incubation time was left in range because there was generally a high percentage encapsulation (above 50 %) for all the 13 runs of experiments (table 4.10). Also, Design Expert® Software did not report any influence of incubation time on other factors (section 4.3.1 to section 4.3.5). Cholesterol to Phosphotidylcholine (C: PC) ratio was set as minimize (with the range 10 % to 50 %). This was because there was an increase in percentage encapsulation and increase drug release (table 4.12) with a decreasing ratio of C: PC towards 10 %. The PDI and Zeta potential for all 13 runs of experiments was in an acceptable range and so PDI and zeta potential were kept in range.

The overall impact of goals set for the various variables was then measured using "desirability". Desirability is an objective function which concurrently determines the settings of all input variables that will give optimum levels of the response(s). Desirability ranges from 0 to 1. The closer to 1 the desirability is, the better the outcomes and variable combination would be. Several trials were made (Appendix 1 to 8) to get desirability as close to 1 as possible.

One of the aims was to optimize percentage encapsulation and percentage drug *in vitro* release, thus, these two responses were set to maximum. After several trial and error attempts (see Appendix 1 to 8) to get desirability as close to 1 as possible, the highest desirability of 0.83 was obtained and used (table 4.15)

Table 4.14: Summary of Criteria for optimization process in Response Surface Methodology using Design Expert® software

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
A:Incubation time	In range	15	60	1	1	3
B:Chol:PC %	Minimize	10	50	1	1	3
% Encapsulation	Maximize	54.8	86.4	1	1	3
PDI	In range	0.154	0.718	1	1	3
Drug release	Maximize	1.82	56.81	1	1	3
Zeta potential	In range	-41.4	-15.91	1	1	3

Obtaining a 41.7 % *in vitro* drug release over a 12 hour period with a single application as stipulated by Design Expert® software (table 4.14) will be a potential improvement to the existing dosage form. As ophthalmic DNa drops available in the market is indicated for six hourly applications with a 0 to 10 % bioavailability (Asasutjarit *et al*., 2011, Meisner and Mezei. 1995)., implying 20 % after a 12 hour period.

The best possible experimental run (number one from table 4.15) was "selected" by Design Expert® Software. A comparison was performed for the responses predicted by the software and actual experimental responses. The closer the actual values were to the predicted values, the stronger the potential of design space to predict acceptable outcomes, that is Response Surface methodology can then be used to predict experimental outcomes.

Table 4.15: Predicted solutions generated by Design Expert® Software using the criteria on table 4.14 above. Number 1 was selected by the software to be evaluated

Number	IT	C:PC%	% E	PDI	DR	Zeta pot.	Desirability	
1	15.00	10.00	79.45	0.39	41.70	-32.70	0.83	Selected
2	15.21	10.00	79.45	0.39	41.69	-32.76	0.83	
3	20.48	10.00	79.24	0.39	41.19	-34.12	0.82	
4	15.00	10.37	79.27	0.39	41.43	-32.90	0.82	
5	45.37	10.00	78.26	0.39	38.85	-36.43	0.79	

IT=incubation time, C: PC=cholesterol to phosphotidylcholine ratio in percentage, PDI=polydispersity index, DR = *in vitro* drug release and Zeta pot.= Zeta potential.

To compare the predicted value with the actual experimental values the statistical t- test was used. If there was no significant difference between the predicted and the actual values, this meant that RSM and Design Expert® software can be used to make acceptable experimental outcomes. Figures 4.22, 4.23, 4.24 and 4.25 represents the difference between the actual and the experimental values from different output factors.

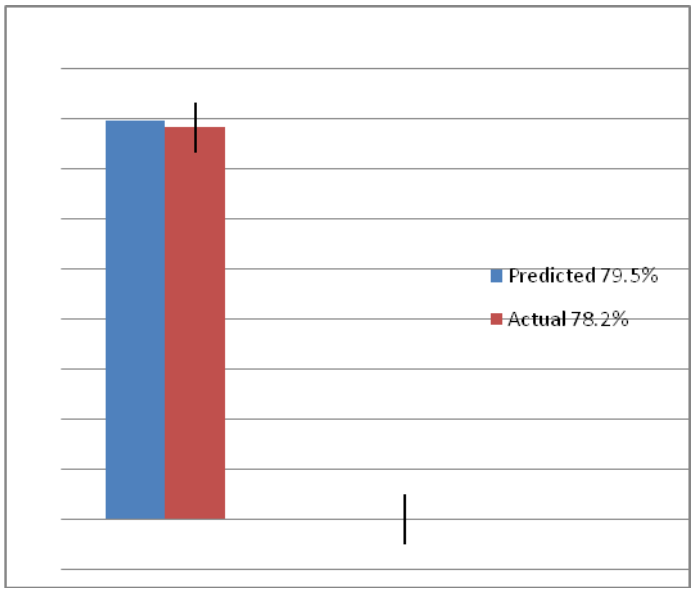

Figure 4.22: Predicted versus actual values for percentage encapsulation.
(n=2, predicted value= 79.5%, Actual experimental value =78.2%, σ= 0.92.)

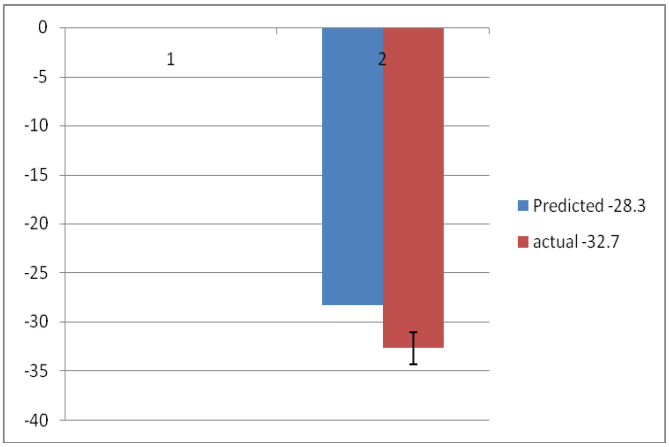

Figure 4.23: Predicted versus actual values for zeta potential of DNa liposomes.
(n=2, Predicted value = -28.3, Actual experimental value = -32.7, σ=3.11.)

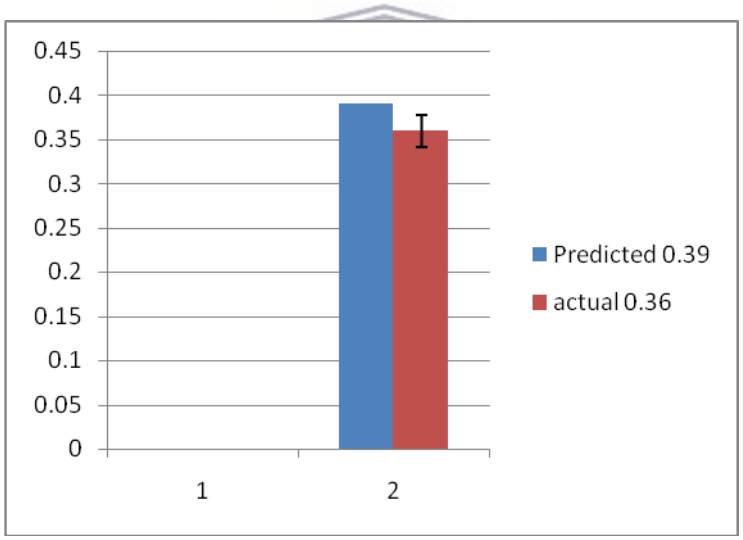

Figure 4.24: Predicted versus actual values for polydispersity index of DNa liposomes
(n=2 , Predicted value = 0.36, Actual experimental value = 0.36, σ= 0.02.)

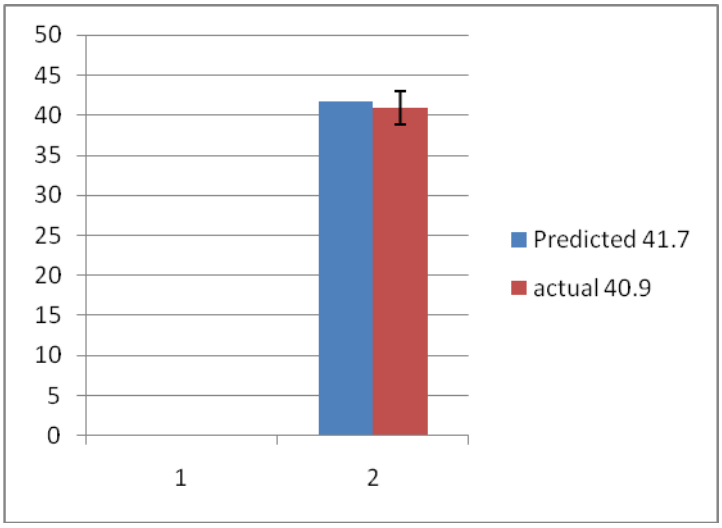

Figure 4.25: Predicted versus actual values for *in vitro* drug release
(n=2, Predicted value = 41.7%, actual experimental value = 40.9%, σ = 0.57.)

For percentage encapsulation, polydispersity index, zeta potential and *in vitro* drug release, there was insignificant difference found between the actual and the experimental values (figures 4.22, 4.23, 4.24 and 4.25). Therefore, Design expert® can be used to make acceptable predictable outcomes.

4.4. Stability study of diclofenac sodium liposomes at 4 °C

Prepared DNa liposomes were examined to determine how PDI would behave over time with respect to storage temperatures. These changes in PDI indicate a degree of physiological stability. For this study, low (1.72 %), medium (30.0) and high cholesterol (58.28) content were used. This is because the function of cholesterol is to add stability (Pietzyk & Henschke, 2000). The PDI was measured once a week for four weeks. During the four-week study, the liposomes were kept at 4 ^{0}C (a recommended storage temperature for liposomes).

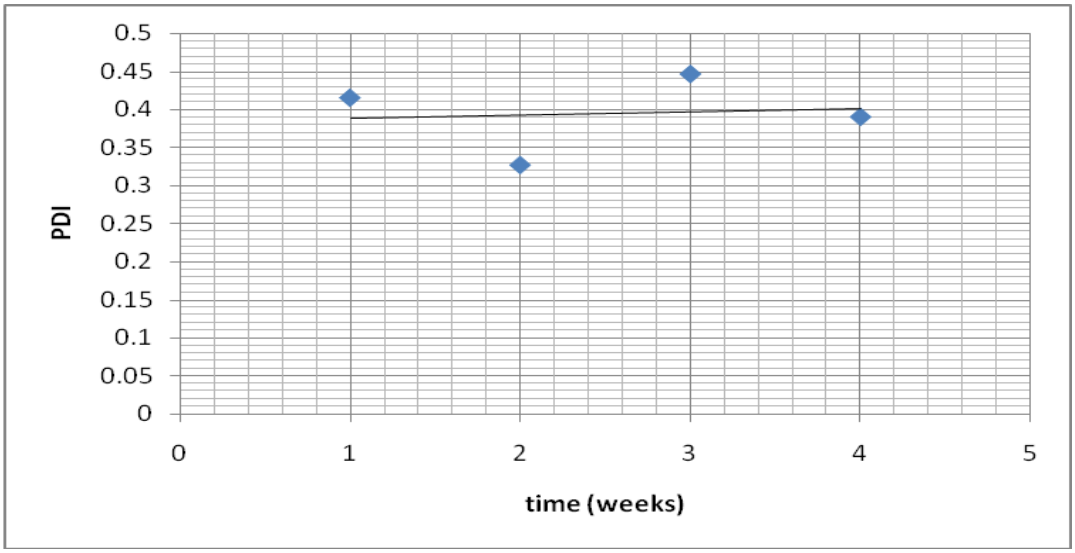

Figure 4.26a: Changes in PDI of liposomes stored at 4 °C for four weeks for C:PC = 1.72 (n=3, average=0.393, standard deviation = 0.049).

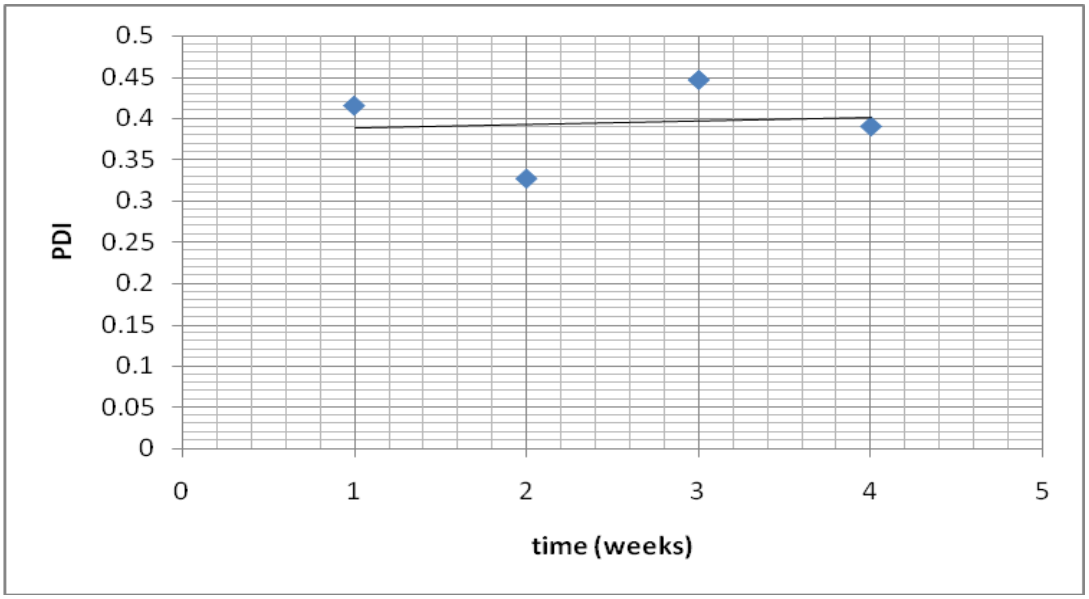

Figure 4.26b: Changes in PDI of liposomes stores at 4 °C 4 for four weeks for C : PC = 30 (n=3, average=0.364, standard deviation = 0.035).

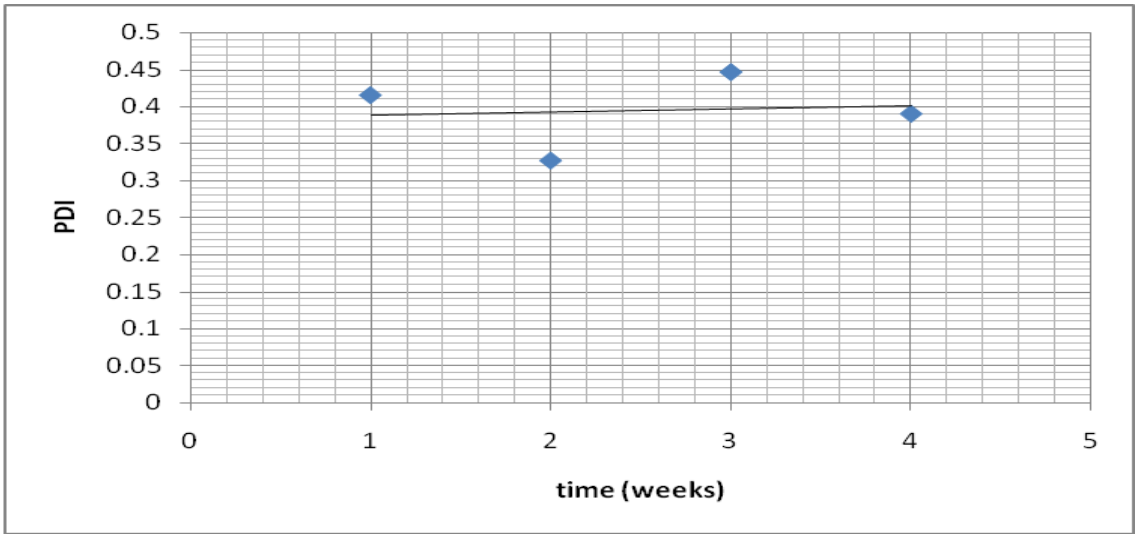

Figure 4.26c: Changes in PDI of liposomes stores at 4 at 4 °C for four weeks for C:PC = 58.28 (n=3, average=0.163, standard deviation = 0.032).

The trend lines from the graphs above (figure 4.26a, 4.26b and 4.26c) indicate that over a four week period very little physical changes (a function of the PDI) occurred. This is an indication of stability of liposomes at 4 °C. This result is similar to Pietzyk & Henschke (2000) who reported that cholesterol containing liposomes have a high stability when stored at 4 °C. Therefore, liposomes are stable at 4 °C, regardless of their concentrations of cholesterol. Therefore 4 °C is a suitable storage temperature for liposomes.

4.5. Physical Characterisation of *in situ* gel with and without diclofenac sodium liposome

The goal was to get an *in situ* gel preparation of DNa liposome with the following quality; pH values the eye can tolerate (6 to 8.5), sol-gel transition at body temperature, very good flowability, uniform, transparent and free of particulate matter. Thus, *in situ* gel preparations with and without DNa liposome were observed for these characteristics. To prepare DNa liposomes *in situ* gels, different excipients were used as indicated in table 4.16 below. For

example, to prepare formulation 1, 4g of Pluronic®, 70.0mg of Carbopol®, 12.5ml of DNa liposomes and 12.5ml of PBS were used.

Table 4.16: Formulation table for *in situ* gel of DNa liposomes.

	Pluronic® (g)	**Carbopol® (mg)**	**DNa liposomes(ml)**	**PBS 7.4(ml)**	**$T_{sol-gel}$ (^oC)**
Formulation 1	4.0	70.0	12.5	12.5	34.0 ± 2
Formulation 2	2.0	7.5	7.5	7.5	30.0 ± 2
Formulation 3	2.0	10.0	5.0	5.0	28.5± 2
Formulation 4	4.0	70.0	25.0	25.5	35.0 ± 2
Formulation 5	2.0	35.0	5.0	5.0	26.0 ± 2

$T_{sol-gel}$ (^oC) is the transition temperature from solution to gel

Formulation 1 and formulation 4 had a sol – gel transition temperature of 34.0 ^oC ± 2 ^oC and 35 ^oC ± 2 ^oC respectively and for this reason were chosen for evaluation. Formulation 1 and formulation 4 were replicated without DNa liposomes and both sets of preparations (with and with DNa) used for further evaluation as discussed below

Table 4.17: pH and gel-sol transition temperature of thermo-responsive *In situ* gel without liposomal DNa

	pH	**$T_{sol-gel}$(^oC)**
Formulation 1	7.1 ± 0.0	34 ± 2
Formulation 4	7.1 ± 0.1	35 ± 2

$T_{sol-gel}$ (^oC) is the transition temperature from solution to gel

Table 4.18: pH and gel-sol transition temperature of thermo-responsive liposomal DNa ophthalmic *in situ* gel

	pH	$T_{sol-gel}$ (^oC)
Formulation 1	7.1 ± 0.0	34 ± 2
Formulation 4	7.1 ± 0.1	35 ± 2

$T_{sol-gel}$ (^oC) is the transition temperature from solution to gel

From table 4.17 and table 4.18 above, inclusion of DNa liposomes affected neither the sol-gel transition temperature nor their pH. Both formulations (without and with DNA liposomes) had an acceptable pH range (pH 6±2). The eye can tolerate preparations over a range of pH values from about 6.5 to about 8.5 (Gonnering *et al*. 1979). Depending on the degree of pH change, corneal cellular damage or structural and functional injury can occur at pH values outside the acceptance range (Gonnering *et al*. 1979).

Table 4.19: Transparency and flowability evaluation of thermo responsive *in situ* gel without liposomal DNa

	Transparency at three temperatures (^0C)			Flowability at three temperature (^0C)		
	4±1	27± 1	35±1	4±1	27± 1	35±1
Formulation 1	+++	+++	+++	+++	+	_
Formulation 4	+++	+++	+++	+++	++	+

+++ = transparent, ++ = slightly translucent, + = translucent, and - = turbid.
For flowability: +++ = very good, ++ = good, + = average, and - = not flow

Table 4.20: Transparency and flowability evaluation of thermo responsive Liposomal DNa ophthalmic *in situ* gel

	Transparency at three temperatures (^{0}C)			Flowability at three temperatures (^{0}C)		
	4 ± 1	**27 ± 1**	**35 ± 1**	**4 ± 1**	**27 ± 1**	**35 ± 1**
Formulation 1	_	_	_	+++	+	_
Formulation 4	_	_	_	+++	++	+

+++ = transparent, ++ = slightly translucent, + = translucent, and - = turbid.
For flowability: +++ = very good, ++ = good, + = average, and - = not flow

To test for clarity of the formulations, "+++" meant he preparation was transparent, "++" meant it was slightly translucent, "+" meant it was translucent, and "-" meant the formulation was turbid. From table 4.19 both formulations without DNa liposomes were transparent while formulations with DNa liposomes (table 4.20) were turbid however neither formulations contained particles. Ophthalmic preparations should be particle free (Uddin M. S. et al. 2017) to avoid irritation to the eye that could lead non-compliance.

To evaluate flowability, "+++" meant "very good" flowability, "++" meant "good" flowability, "+" meant "average" flowability, and "–"meant "no flowability". Generally, a decrease in flowability with an increasing temperature was observed for *in situ* gels with and without DNa liposomes (table 4.19 and 4.19). Formulation 4 had better flowability than formulation 1 at 4^0 C and 25^0 C, making it easy to store (4^0 C) and administer (25^0 C). Formulation 1 had no flowability at 35^0 C, this thus was a suitable formation of choice as it could present with slow release potential. It is imperative that an ophthalmic drops / gel readily flows at storage temperature (4^0 C), room temperature (25^0 C) for easy storage and administration and gel only once administered (35^0 C). This was the case with formulation 4. Decrease in flowability due to gelling results in an increase residence time and possibly controlled release of DNa. Gelation with increased temperature is caused by the interaction

between molecules as temperature increases (Prajapati N. B. & Goyal A. 2013). At a lower temperature, molecules are hydrated and there is little polymer–polymer interaction other than simple entanglement hence more flowability tendencies. Decrease flowability occurs as the temperature is increased because the molecules lose their water of hydration (Prajapati, & Goyal, 2013).

4.6. Comparing *in vitro* drug release profile, commercial Voltaren® eye drops and prepared *in situ* gel of DNa liposomes.

To check for a potential improvement of *in situ* gel of ophthalmic DNa liposomes over commercially available DNa ophthalmic eye drop, an *in vitro* drug release study was performed. The *in vitro* drug release study samples were Voltaren® eye drops, thin and thick *in situ* gel consisting of DNa liposomes

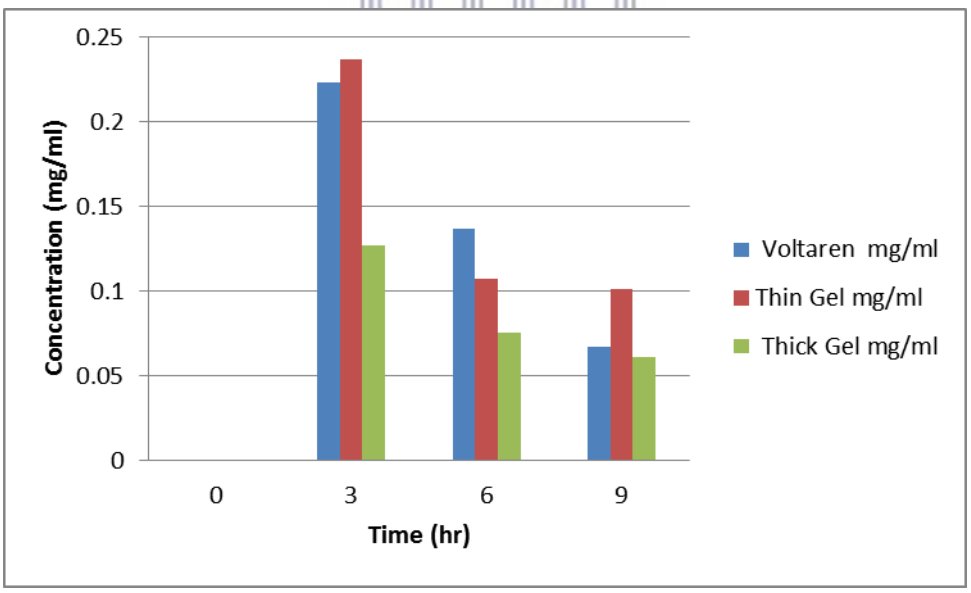

Figure 4.27: *In vitro* drug release comparison of Voltaren® eye drops, thin and thick *in situ* diclofenac sodium liposome.

As observed from figure 4.27, all three preparations had DNa present at 3, 6, and 9 hours in the receptor compartment of the Franz cell upon permeating the synthetic snake skin dialysis tubing. The release for all preparations showed an initial burst (higher concentration) at 3

hours, and then reduced release concentrations at 6 and 9 hours. The concentration difference between the donor and receptor compartment was highest between 0 and 3 hours, i.e. a maximum concentration gradient, resulting in more DNa moving from the donor to receptor compartment compared to the 6 and 9 hour intervals. This could have contributed to the decrease over time.

When observing the Voltaren® formulation concentration in the receptor compartment of the Franz cell over the 9 hour period, there is more or less a 50% drop in concentration across the 3 to 9 hour time interval. However, when observing the two gel formulations (thin and thick), after an initial higher release at 3 hours, the 6 hours and 9 hours concentration values were relatively constant for both gels. This difference compared to the Voltaren® drops could be attributed to the formulation matrix of the liposome-gel formulation, which lends itself to having possible desirable sustained release properties (Khatera *et al.*, 2016). The thin gel will be preferred as it had a higher initial concentration a 3 hours and its relative constant release at 6 hours and 9 hours when compared to the thick gel formulation.

It is also noted that the thin gel had a higher 3 hour and 9 hour permeation than the Voltaren® drops. This in an interesting observation and could be attributed to the liposomes having a better potential to permeate the synthetic membrane (Duangjit *et al.*, 2010).

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

Objectives:

- To develop and validate an UHPLC method for quantification of diclofenac sodium
- To develop a method for active encapsulation of diclofenac into liposomes
- To use design expert® software to determine the numbers of experiments (runs) from input variables (cholesterol: phosphotidylcholine and incubation time)
- To use response surface methodology (RSM) to determine the effect(s) of input variables on output variables (encapsulation efficacy percentage, polydispersity index, zeta potential and *in vitro* drug release) of liposome formulations obtained from Design Expert® Software
- To determine the combination of input variables required to achieve an optimized formulation of DNa liposomes
- To incorporate the optimised DNa liposomes into *in situ* gel formulation (s)
- To characterize DNa liposome *in situ* gel (s)
- To compare *in vitro* release of *in situ* liposome gel to voltaren® drops.

The following conclusions and recommendations are summarized below.

A linear, specific, accurate, and precise UHPLC method was successfully developed and validated for quantification of DNa.

A successful method of active encapsulation by calcium acetate gradient was developed. Cholesterol to phosphotidylcholine ratio was set at 1.72 to 58.28 % while incubation time

was set at 5.68 to 69.32 minutes. This resulted to encapsulation of DNa into liposomes of up to 86 %. These liposomes had acceptable PDI values of less than 0.5

Design Expert® software was effectively used to study the interrelation between encapsulation times, percentage of C: PC, percentage encapsulation, PDI, Size, Zeta potential and percentage drug release. C: PC ratio had an impact on percentage of DNa encapsulated into liposomes and *in vitro* dug release. Summarily, a decrease in C : PC (from 58.28 to 1.72) ratio led to an increase in percentage encapsulation (54.8% to79.2% respectively) and a corresponding increase in *in vitro* drug release (1.82% to 56.81% respectively).

Optimization of encapsulation of DNa liposomes was accomplished using Design Expert® Software as there were insignificant differences between predicted values (generated by Design Expert® software) and actual experimental values registered in the laboratory. The predicted versus actual (experimental) values were; percentage encapsulation (79.5 versus 78.2), zeta potential (-23.3 versus -32.7), polydispersity index (0.39 versus 0.36) and *in vitro* drug release (41.7 versus 40.9) respectively.

The *In situ* gel of DNa liposomes produced was free flowing at 4^0 C and 25^0 C and gelled at 35 ^{0}C, making storage (at 4^0 C) and administration (at 25^0 C) feasible and presenting potential long residence time in the eye respectively.

A three hourly *in vitro* release study for diclofenac *in situ* gel gave a broad sense of its release profile. What happens at shorter time intervals is not known but could be important. This will assist to better understand the *in vitro* release profile. Thus, shorter time intervals (hourly or lesser) is recommended for further studies.

Having used Response Surface Methodology (RSM) with Design Expert® software to successfully predict experimental outcomes, optimising *in vitro* release studies using the

same procedure is recommended. This studies will contribute towards successfully formulating a better ophthalmic preparation for diclofenac sodium.

APPENDICES

APPENDIX A: DESIGN EXPERT GENERATED CONDITIONS AND RESPONDS OBTAINED

Design Summary

File Version 8.0.7.1

Study Type	Response Surface	**Runs**	13
Design Type	Central Composite	**Blocks**	No Blocks
Design Mode	Quadratic		

Factor	Name	Units	Type	Subtype	Minimum	Maximum	Coded	Values	Mean	Std. Dev.
A	Incubation time	minutes	Numeric	Continuous	5.68	69.32	-1.000=15.00	1.000=60.00	37.50	17.65
B	CHOL:PC	%	Numeric	Continuous	15.86	44.14	-1.000=20.00	1.000=40.00	30.00	7.84

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Percentage En	%	13	Polynomial	75.7	94	84.58	5.23174	1.24174	None	Mean
Y2	PDI		13	Polynomial	0.116	0.392	0.226154	0.0910017	3.37931	None	Quadratic
Y3	Drug Release	% released in	13	Polynomial	1.178	42.85	12.766	10.2073	36.3752	None	Quadratic

Select	Std	Run	Factor 1 A:Incubation ... minutes	Factor 2 B:CHOL:PC %	Response 1 Percentage ... %	Response 2 PDI	Response 3 Drug Release % released i...
	7	1	37.50	15.86	86	0.233	18.6
	10	2	37.50	30.00	85	0.142	14.5
	11	3	37.50	30.00	87.7	0.12	12
	6	4	69.32	30.00	75.7	0.3	10
	1	5	15.00	20.00	80	0.238	1.7
	9	6	37.50	30.00	80	0.116	12
	3	7	15.00	40.00	84.86	0.3	6.73
	13	8	37.50	30.00	82	0.12	11
	12	9	37.50	30.00	80	0.14	12.2
	5	10	5.68	30.00	94	0.305	11.5
	4	11	60.00	40.00	85.44	0.392	11.7
	8	12	37.50	44.14	85.44	0.299	42.85
	2	13	60.00	20.00	93.4	0.235	1.178

APPENDIX B: LIST OF REAGENTS AND SOLUTIONS

Acetate buffer (pH 7.7)
Sodium Acetate (Saarchem, Krugersdorp, RSA) 4.10 g

Sodium chloride (Saarchem, Krugersdorp, RSA) 8.77 g

HCl 1 M qs

RO water to 1000 ml

The sodium acetate and sodium chloride were dissolved in 800 ml water and adjusted to pH 7.7 with HCl. The solution was then made to volume with water.

Phosphate-buffered saline (PBS) (pH 7.4)
NaCl (Saarchem, Krugersdorp, RSA) 8 g

KH_2PO_4 (Protea Laboratory Services, Jhb, RSA) 0.2 g

$Na_2HPO_4.12H_2O$ (Saarchem, Krugersdorp, RSA) 1.44 g

KCl (Saarchem, Krugersdorp, RSA) 0.2 g

EDTA (Sigma-Aldrich, St Louis, USA) 0.2 g

HCl 1 M qs

RO water to 1000 ml

The components were dissolved in 800 ml water and the pH adjusted to 7.4 with 1 M HCl and made to volume with water.

Phosphate-buffered saline (PBS) (pH 4.0)
NaCl (Saarchem, Krugersdorp, RSA) 8 g

KH_2PO_4 (Protea Laboratory Services, Jhb, RSA) 0.2 g

$Na_2HPO_4.12H_2O$ (Saarchem, Krugersdorp, RSA) 1.44 g

KCl (Saarchem, Krugersdorp, RSA) 0.2 g

EDTA (Sigma-Aldrich, St Louis, USA) 0.2 g

HCl 1 M qs

RO water to 1000 ml

The components were dissolved in 800 ml water and the pH adjusted to 4.0 with 1 M HCl and made to volume with water.

Protamine solution

Protamine (from salmon) (Sigma-Aldrich, St Louis, USA) 10 mg

PBS (pH 7.4) to 10 ml

Sephadex G50

1 g Sepahadex G50 (Sigma-Aldrich, St Louis, USA) was hydrated for 24 hours at 25 ^{0}C

PBS to 100 ml

APPENDIX C: OPTIONS FOR OPTIMIZATION PROCESS USING DESIGN EXPERT 8.0.7.1

OPTION 1

Name	Goal	Lower Limit	Upper Limit	Importance
A:IT	in range	15	60	3
B:CHOL:PC	equal to 10	20	60	3
% EE	maximize	54.8	86.4	3
PDI	is in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	is in range -	41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	% EE	PDI	DR	Zeta Pot	Desirability	
1	15.00	10.00	79.4535	0.393769	41.7058	-32.7003	0.752	Selected
2	17.73	10.00	79.346	0.393769	41.4489	-33.4462	0.748	

OPTION 2

Name	Goal	Lower Limit	Upper limit	Importance
A:IT	in range	15	60	3
B:CHOL:PC	equal to15.	20	60	3
% EE	maximize	54.8	86.4	3
PDI	is in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	is in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	15.00	77.0252	0.393769	37.9671	-34.9624	0.680	Selected
2	16.13	15.00	76.9807	0.393769	37.8609	-35.2504	0.678	

OPTION 3

Name	Goal	Lower Limit	Upper Limit	Importance
A:IT	in range	15	60	3
B:CHOL:PC	equal to 20	20	60	3
% EE	maximize	54.8	86.4	3
PDI	in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	20.00	74.5968	0.393769	34.2285	-36.3945	0.608	Selected

OPTION 4

Name	Goal	Lower Limit	Upper Limit	Importance
A:IT	in range	15	60	3
B:CHOL:PC	minimize	20	60	3
% EE	maximize	54.8	86.4	3
PDI	in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	20.00	74.5968	0.393769	34.2284	-36.3946	0.717	Selected
2	16.65	20.00	74.5318	0.393769	34.0729	-36.7669	0.715	
3	32.12	20.00	73.9225	0.393769	32.6163	-38.8136	0.697	
4	53.33	20.00	73.0869	0.393769	30.6187	-37.3829	0.672	

OPTION 5

Name	Goal	Lower Limit	Upper Limit	Importance
A:IT	in range	15	60	3
B:CHOL:PC	equal to 7.50	20	60	3
%EE	maximize	54.8	86.4	3
PDI	in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	7.50	80.6677	0.393769	43.5752	-31.258	0.788	Selected
2	17.49	7.50	80.5696	0.393769	43.3407	-31.9753	0.785	
3	18.14	7.50	80.5442	0.393769	43.2799	-32.1503	0.784	
4	20.63	7.50	80.4459	0.393769	43.0451	-32.7836	0.780	
5	22.99	7.50	80.3528	0.393769	42.8224	-33.3216	0.776	
6	23.60	7.50	80.3289	0.393769	42.7652	-33.45	0.776	
7	24.39	7.50	80.2978	0.393769	42.6909	-33.6107	0.774	
8	25.25	7.50	80.2638	0.393769	42.6096	-33.7787	0.773	
9	27.48	7.50	80.176	0.393769	42.3997	-34.1753	0.770	
10	27.97	7.50	80.157	0.393769	42.3542	-34.254	0.769	
11	30.14	7.50	80.0713	0.393769	42.1494	-34.5773	0.766	

OPTION 6

Name	Goal	Lower Limit	Upper Limit	Importance
A:IT	in range	15	60	3
B:CHOL:PC	equal to 5	20	60	3
%EE	maximize	54.8	86.4	3
PDI	in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	is in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	5.00	81.8818	0.393769	45.4445	-29.6081	0.825	Selected
2	15.26	5.00	81.8714	0.393769	45.4196	-29.691	0.824	
3	18.20	5.00	81.7556	0.393769	45.1427	-30.5621	0.820	
4	19.28	5.00	81.7134	0.393769	45.0417	-30.8563	0.818	
5	19.73	5.00	81.6954	0.393769	44.9988	-30.9777	0.818	
6	19.94	5.00	81.6873	0.393769	44.9793	-31.0318	0.817	
7	20.26	5.00	81.6746	0.393769	44.949	-31.1153	0.817	
8	23.11	5.00	81.5623	0.393769	44.6805	-31.8062	0.812	

OPTION 7

Name	Goal	Lower Limit	Upper Limit	Importance
A:Incubation time	in range	15	60	3
B:CHOL:PC	minimize	10	50	3
%EE	maximize	54.8	86.4	3
PDI	in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	10.00	79.4535	0.393769	41.7058	-32.7003	0.827	Selected
2	15.21	10.00	79.4452	0.393769	41.686	-32.7607	0.827	
3	20.48	10.00	79.2375	0.393769	41.1895	-34.1171	0.821	
4	15.00	10.37	79.2749	0.393769	41.4308	-32.8949	0.821	
5	45.37	10.00	78.257	0.393769	38.8454	-36.4316	0.794	

5 Solutions found

OPTION 8

Name	Goal	Lower Limit	Upper Limit	Importance
A:Incubation time	in range	15	60	3
B:CHOL:PC	minimize	10	50	3
% EE	maximize	54.8	86.4	3
PDI	in range	0.154	0.718	3
drug release	maximize	1.82	56.81	3
zeta potential	in range	-41.4	-15.9	3

Solutions

Number	IT	CHOL:PC	%EE	PDI	DR	Zeta pot.	Desirability	
1	15.00	10.00	79.4535	0.393769	41.7058	-32.7003	0.827	Selected
2	15.00	10.34	79.2908	0.393769	41.4553	-32.8778	0.821	
3	21.29	10.00	79.2056	0.393769	41.1133	-34.2984	0.820	
4	34.60	10.00	78.6814	0.393769	39.86	-36.2582	0.806	
5	45.37	10.00	78.257	0.393769	38.8454	-36.4316	0.794	

5 Solutions found

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