Preparation and Characterization of Alginate-Chitosan Nanoparticles as a Drug Delivery System for Lipophilic Compounds.

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by

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DECLARATION

I hereby declare that this dissertation, which I herewith submit for the research qualification

MASTER OF SCIENCE DEGREE IN CHEMISTRY

to the University of Johannesburg, Department of Chemical Technology, is, apart from the recognised assistance of my supervisors, my own work and has not previously been submitted by me to another institution to obtain a research diploma or degree.

_____ on this ____ day of _____

(Candidate)

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(Supervisor)

_____ on this ____ day of _____

(Co-supervisor)

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(Co-supervisor)

DEDICATION

To my parents, thank you for showing me unconditional love.

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- **ABSTRACT**

Despite several decades of extensive research and development in pharmaceutical chemistry, the poor solubility of lipophilic compounds in aqueous media remains a major barrier to their absorption, bioavailability and clinical efficacy. This poor solubility is also a problem in other areas such as the flavour and fragrance industry. In cosmetics, for example, poor aqueous solubility and instability of oily compounds causes problems in formulation and fragrance stability. One approach to overcome these difficulties is to encapsulate oily compounds in biocompatible materials. As a drug delivery system such an approach is attractive if the size of the capsule is reduced to the micrometer or nanometer scale.

Naturally occurring polysaccharides like sodium alginate (NaALG) and chitosan (CS) are generally regarded as safe (GRAS) for use in human use and have therefore gained much attention recently. As a drug delivery system, this polymer matrix can be used to prevent drug degradation in the gastro intestinal tract (GIT) and often provides controlled release of the encapsulant. Cyclodextrins (CDs) on the other hand offer an alternative approach. These cyclic oligosaccharides have the ability to form non-covalent inclusion complexes with a range of organic compounds, and in so doing alter their physiochemical properties such as solubility.

This study was aimed at exploring these concepts by using ALG and CS as an entrapment matrix for an essential oil, tagette oil (used as a model oily drug) that is insoluble in aqueous media. Alginate/chitosan (ALG/CS) nanoparticles were prepared in a 3-step procedure; emulsification of tagette oil in aqueous Na-ALG solution, followed by ionotropic pre-gelation of the ALG core with $CaCl_2$ and further crosslinking with CS. Morphology and particle size measurements were performed by scanning and transmission electron microscopy (SEM and TEM), and Malvern Zetasizer.

Particles obtained at pH 4.7 were spherical, distinct and of the core-shell type. The nanoparticles were negatively charged with an average size of 190 nm, suitable for uptake within the gastrointestinal tract due to their nanosize range and mucoadhesive properties. Polymer interaction and oil incorporation was confirmed by Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). The IR spectra of oil-loaded alginate-chitosan nanoparticles showed ketone absorption bands characteristic of tagette oil and also showed that carboxylic groups of ALG associated with ammonium groups of CS through electrostatic interaction to form the polyelectrolyte complex.

The tagette oil encapsulation efficiency (EE %) within the ALG/CS nanoparticles was 83% and tagette oil was released in a pH-dependent manner under simulated gastrointestinal conditions. The nanoparticles showed a significant controlled release profile extended up to 4 days, with only a total of 55% and 68% being released in pH 1.5 and pH 7.4, respectively. Gas chromatography (GC) analysis on the other, revealed that ALG/CS nanoparticles have the ability to retain tagette oil-volatile compounds (fragrance) and release them in a controlled manner over a period of 24 hours, when used in aqueous cream.

Tagette oil was also incorporated in the ALG/CS nanoparticles as a complex with *beta*-cyclodextrin (*β*CD), in order to improve its solubility and stability, as well as investigate the effect of *β*CD on the release profile. It was demonstrated that tagette oil could be efficiently complexed with *β*CD to from an inclusion complex by the co-precipitation method and formed a 1:1 inclusion. The results of ultraviolet-visible (UV-Vis) spectroscopy as well as comprehensive characterization using proton nuclear magnetic resonance (^{1}HNMR), FTIR spectroscopy, x-ray diffractometry (XRD), DSC and SEM indicated that *β*CD-tagette oil complex has different physicochemical properties from free tagette oil.

*β*CD-tagette oil-loaded ALG/CS nanoparticles were prepared by coating the *β*CD-oil complex with the ALG/CS polyelectrolyte complex. The *β*CD-ALG/CS nanoparticles have their mean size lower than 200 nm (at 109 nm) and can load tagette oil with an EE% up to 93%, which is 10% more than the nanoparticles without *β*CD. It was noted that the cumulative release in acidic medium (pH 1.5)

over a period of 4 days from this nanoparticulate system was significantly (p < 0.05) lower (39%) than that without βCD (55%). Therefore drug loss by degradation in gastric fluid may be reduced by addition of βCD into the ALG/CS nanoparticles. Under pH 7.4, the release was significantly higher (78%) in ALG/CS containing βCD than in ALG/CS nanoparticles without βCD (68%), suggesting that the presence of βCD increases the solubility of lipophilic compounds in a neutral medium, (pH 7.4).

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LIST OF ABBREVIATIONS

αCD	*alpha*-cyclodextrin
βCD	*beta*-cyclodextrin
γCD	*gamma*-cyclodextrin
ALG	Alginate
CANSA	Cancer Association of South Africa
CARISA	Cancer Research Initiative in South Africa
CDs	Cyclodextrins
CS	Chitosan
Da	Daltons
DDS	Dug Delivery System
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
EDX	Electron Diffraction X-ray
EE	Encapsulation Efficiency
FTIR	Fourier Transform Infrared
GC-FID	Gas Chromatography-Flame Ionization Detector
GIT	Gastrointestinal Tract
GRAS	Generally Regarded As Safe
^{1}HNMR	Proton Nuclear Magnetic Resonance
HIV/AIDS	Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome
In vitro	taking place outside a living body
In vivo	taking place in a living body
IR	Fourier Transform Infrared Spectroscopy

Low-G NaALG	Sodium Alginate of low guluronic acid content
M-cells	Microfold cells
mg/L	Milligrams per litre
MRC	Medical Research Council
mV	Millivolts
NaALG	Sodium Alginate
nm	Nanometres
NPs	Nanoparticles
PBS	Phosphate Buffer Saline
PEC	Polyelectrolyte Complex
RNA	Ribonucleic Acid
rpm	Rotations per minute
SEM	Scanning Electron Microscope
TB	Tuberculosis
TEM	Transmission Electron Microscope
US FDA	United States Food and Drug Administration
UV-Vis	Ultraviolet-visible
w/v	Weight per volume
XRD	X-ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Background

In an effort to treat diseases and reduce patient mortality and morbidity, the medical fraternity has tried to improve the clinical experience and knowledge in patient diagnosis and drug prescription. New more efficient drugs being developed and marketed by the pharmaceutical industry are often large hydrophilic molecules. Poor solubility, biodistribution, biokinetics, and stability often sound the death knell for commercialisation of new treatments. The predominant route for delivering these drugs is oral administration and intravenous injections, typically in the form of tablets and emulsions.[1] Nonetheless, even these approved modern and efficient drugs often have certain limitations such as low bioavailability due to immediate degradation of some of the drug in the gastro intestinal tract (GIT), before reaching the target organ in the human body.[2]

Researchers involved in the development of pharmaceuticals have understood that drug delivery is a fundamental part of drug development and a wide range of drug delivery systems (DDS) have been designed. These include liposomes, micelles, dendrimers, microspheres, nanofibers and nanotubes. Ideally, all these systems should improve the stability, absorption and therapeutic concentration of the drug within the target site, while reducing unwanted side-effects.[3] In the last 2-3 decades of medical research, the need for effective drug delivery has emerged as a major concern especially for the treatment of diseases like cardiovascular disorders, cancer, malaria and tuberculosis (TB), which primarily require systematically administered therapies.[2]

A comprehensive body of work has been published over the years in developing “vehicles” that improve the biodistribution of drugs and their accumulation in

specific body sites. Recent technological advances in medicine and nanomaterials, paralleled with knowledge accumulated from the clinical translation of disease-and-drug related data have created a richly fertile ground for nanomedicine to emerge as a new direction in diagnosis and therapy.[4]

Nanomedicine is in a broad sense the application of nano-scale technology to the practice of medicine, namely for diagnosis, prevention and treatment of diseases and to gain an increased understanding of the underlying complex disease mechanisms.[5] The nanometre size of these carrier systems allows efficient crossing of biological barriers, increased tissue tolerance, improved cellular uptake and transport, thus enabling efficient delivery of the therapeutic agents to the target sites like the liver, brain and solid tumours. Nanocarriers can be fabricated to protect drug molecules and preferentially target them to specific anatomical or cellular targets. This "targeting" is the key behind their popularity in drug delivery.[6] It is for these reasons that nanotechnology based drug delivery approaches are the focal point of much of today's therapeutic research.

Common examples of these systems are micron or submicron particles containing drug molecules which may be entrapped, dissolved, encapsulated or attached to the particle surface.[4] For example, microsphere formulations are used to protect agents susceptible to degradation or denaturation while prolonging the duration of action of a drug by increasing systemic exposure or retention of the formulation, whilst nanoparticles on the other hand, have the added advantage of being able to cross membrane barriers, particularly in the absorptive epithelium of the small intestines because of their small size.[7]

The development of nanomaterials has received increased attention generating a large range of applications in several sectors such as industry, agriculture, medicine, pharmacy and biotechnology.[8] Nanoparticles can be designed to have a long shelf life and have been made out of safe materials such as synthetic biodegradable polymers, natural biopolymers, lipids and polysaccharides.[7]

1.2 Problem statement

The principle of pharmacy and medicine is to deliver drugs at the right time in a safe and reproducible manner to a specific target at the required level. These ideal requirements have led to the development of improved drugs as well as of different drug administration routes. Funds to support these developments have been directed into the pharmaceutical and medical industry.[3] For example, the Cancer Association of South Africa (CANSA) in partnership with the Medical Research Council (MRC) and National Health Laboratory Service (NHLS), through the Cancer Research Initiative in South Africa (CARISA), commits more than R5 million to cancer research in South Africa every year.[9] In spite of all these efforts disease still remains a challenge for the 21st century.

Although modern medicine has contributed significantly to improvement of patient/disease management, its current solutions are associated with several shortcomings. These include low bioavailability of drugs, patient non-compliance with treatment, side effects of too little or too much drug, and drug instability in GIT. These shortcomings, in turn lead to the dramatic increase in multi-drug resistance for many human pathogenic micro organisms as well as the appearance of undesirable side effects of certain treatments.[10]

In cancer therapy, currently available cancer therapeutic strategies suffer from severe limitations that frequently result in treatment failure. The underlying basis for such failure is multifactorial including poor oral bioavailability, low therapeutic indices, insufficient targeting of the therapeutic agents, lack of water solubility and consequently emerging side effects and spread of cancer to other organs.[11] The main cause of low oral bioavailability of drugs is often simply poor aqueous solubility. For any drug to be absorbed in the small intestine and be transported to the site of infection, it must possess enough aqueous solubility and be stable enough to pass through the GIT.[12]

In the pharmaceutical industry, a high percentage of drugs with good efficacy/toxicity ratio fail to go through the drug discovery pipeline due to their low intestinal absorption caused by poor aqueous solubility. This is now one of the

major drawbacks in the design of new drug formulations.[13] Paclitaxel, a drug identified by the US National Cancer institute in 1967, is an example of a drug that suffers from formulation issues. It has been found to be a potent anticancer drug against a wide range of cancers, including ovarian, lung and breast cancers. However, the practical chemotherapeutic application of paclitaxel has been limited by its high hydrophobicity and poor solubility in water.[14] Today a number of nano-based formulations of Paclitaxel such as “Albupax”, and “Nanoxel” have partly solved some of these issues. For example, Albupax consist of Paclitaxel in an albumin bound nanoparticle form and is used in the treatment of breast cancer. In the conventional paclitaxel, the solvent polyethylated castor oil is used, which causes severe side effects such as neutropenia, hypersensitivity reactions and neuropathy. Albupax does not contain this solvent and hence can be given in optimum doses for enhanced efficacy.[15]

Lipophilic compounds are also widely used in cosmetic applications, where poor solubility of key drugs (in “cosmeceuticals”), or of fragrance materials in prepared emulsions may lead to similar problems as with lipophilic drugs in pharmaceutical applications. Perfuming cosmetic products is an important part of meeting consumer requirements. The essential functions of fragrance materials are to provide a pleasant odour, to mask the base smell of the product and to give the product an identity.[16] However, since fragrance materials are poorly water soluble or insoluble compounds and usually exist in liquid state, the perfuming process may be difficult. The use of surfactants as solubilizing agents leads to different problems such as causing cloudiness and turbidity on the transparent formulations, skin irritation and sensitization to light. Furthermore the amount of fragrance materials in the product rapidly decreases during storage because of their volatility and poor stability.[17]

Encapsulation of lipophilic compounds into aqueous-soluble nanoparticulate systems may provide a solution to several problems in both the pharmaceutical industry as well as related cosmetic industries by improving the aqueous solubility of key compounds and providing a controlled way to deliver and release drugs with their full potential.

1.3 Justification of study

The development of modern nanoparticulate drug carriers is currently one of the main topics in pharmaceutical research.[3] Nanoencapsulation has the potential to improve the solubility of lipophilic, poorly water-soluble compounds and to protect labile molecules from the biological environment. In addition to these benefits, many drugs are facing the end of their patent protection and new nanoformulations may offer a commercial lifeline for these compounds. Attempts have been made through encapsulation of oily drugs in liposomes, lipophilic and polymeric nanoparticles, biodegradable microspheres, cyclodextrins and hydrogels.[18] In addition, encapsulation offers the possibility of specific drug delivery and transport through mucosal surface after oral administration.[19]

Nanoscale drug delivery systems formulated from biocompatible and biodegradable polymers constitute an evolving approach to drug delivery. What is unique about nanomaterials is that, often known physical and chemical properties are improved or changed, compared to bulk materials.[20] For instance, poorly soluble drugs such as nifedipine, felodipine, ibuprofen and naproxen are examples of common drugs that display low bioavailability after oral administration. Controlled release formulations of these drugs have been designed and shown greater possibility to deliver such drugs with their full potential in humans.[21] The main reason for these differences between nanoparticles and bulk materials is the much greater surface area to volume ratio of the nanoparticles.[20]

Several strategies have been explored so far in the development of these vehicles, among which the design of biodegradable nanoparticles has drawn considerable interest. Biodegradable nanoparticles made of naturally occurring polymers such as polysaccharides are attractive as drug delivery systems since they possess the biocompatibility, biodegradability and non-toxicity required for use in humans.[22]

Biodegradable polymeric nanoparticles, with hydrophilic surfaces, have been designed to have longer circulation periods in the blood. Such systems allow the control of the rate of drug administration which prolongs the duration of the therapeutic effect, as well as the targeting of the drug to specific sites.[23] Alginate

(ALG) and chitosan (CS) are two biopolymers that have received much attention due to their availability and low cost. Entrapment or encapsulation of therapeutic agents including peptides, proteins and polynucleotides within biopolymer systems have been shown to be beneficial on several fronts: the structure and activity of the ingredient is maintained and drugs are protected from enzymatic degradation.[24]

Cyclodextrins (CDs) are also potential candidates for developing carrier materials because of the ease with which they form inclusion complexes both in solution and in the solid state. In the inclusion complex, guest molecules are protected within the hydrophobic environment of the CD cavity. This leads to alteration of physical, chemical and biological properties of guest molecules, leading to enhanced pharmaceutical potential.[25] In addition, CDs may be used in combination with other nanoparticle systems to confer an enhanced effect.

The use of biodegradable polymers as drug carriers has long been of interest in controlled-release technology because these polymers are easily metabolized in the body. As natural biomaterials, ALG and CS are polysaccharides that are highly stable, safe, non-toxic, hydrophilic and biodegradable.[26] The general biocompatibility and biodegradation profiles of polymeric nanoparticles are even more attractive with formulations that require more chronic dosing (such as Cancer, TB and HIV/AIDS therapy) to avoid toxic accumulation of these materials in the human body.[24]

Sustained drug release properties can also be useful for dermal formulations and in cosmetics. For example, for topical antibiotics, it is desirable to maintain high concentrations over a prolonged time on the skin surface and within the skin tissue.[27] Moreover, irritant drugs like benzoyl peroxide or tretinoin, used for acne treatment and typically applied to the affected areas in gel or cream form, turned out to be less irritating if applied in a controlled release device. This also reduces systemic application and uptake of the active agent.[28]

Nanoparticles are also able to enhance the chemical stability of compounds sensitive to light, oxidation and hydrolysis. Enhancement of chemical stability after

incorporation into nanocarriers has been proven for many cosmetic actives e.g. coenzyme Q10, ascorbyl palmitate, tocopherol (vitamin E) and retinol (vitamin A), used in cosmetic anti-ageing products because of their ability to reduce photo-oxidation and wrinkle depth. These cosmetics with a pharmaceutical component are increasingly being referred to as “cosmeceuticals”. The small size of the particles ensures a close contact to the stratum corneum (the outermost layer of the epidermis) and can increase the amount of drug penetrating into the skin, or can deliver the drug to the body in a sustained manner.[29]

In addition, ALG, CS and CDs are generally abundant, naturally available resources whose low cost and ease of processing make them ideal choices as biopolymers. They have hydrophilic groups such as hydroxyl, carboxyl and amino groups which can form non-covalent bonds with biological tissues (mainly epithelia and mucous membranes), and can therefore form good bio-adhesion materials.[26] Nanoparticle carriers made of these bioadhesive, hydrophilic polysaccharides have the potential to increase the solubility of drugs and prolong the residence time and therefore increase the absorption of loaded drugs. All these merits make polysaccharides promising biomaterials in drug delivery.[23]

The need for researchers to investigate the use of these polysaccharides as drug delivery systems for poorly soluble compounds is reaching a critical stage. Ongoing studies are promising but the investigation process is still at its infancy.[3] At present, 95% of all new potential therapeutics have poor pharmacokinetics and biopharmaceutical properties.[30] Therefore there is a need to develop suitable drug delivery systems that allow the distribution of the required therapeutic effect at the site of action.[31]

Many times, the success of a drug is dependent on the delivery method. New drug delivery methods may enable pharmaceutical companies to develop new formulations that can reduce undesirable side effects and increase patient compliance, thus saving money on health care.[31] Furthermore, drug candidates that did not pass through the trial phases because of their low aqueous solubility problems, may be reformulated to be used with new drug delivery systems.[32]

1.4 Objectives of the study

This project was undertaken with the following specific objectives:

i. To synthesize alginate-chitosan (ALG/CS) nanoparticles and characterize them by SEM, EDX, TEM, particle size analyser, FTIR and DSC.

ii. To prepare and characterize ALG/CS nanoparticles loaded with tagette oil (as a model lipophilic drug mixture)

iii. To monitor the *in vitro* release of the encapsulated oil using the dialysis method and UV-Vis spectroscopy.

iv. To prepare *β*CD-tagette oil complex and the complex-loaded ALG/CS nanoparticles and characterize the obtained system by FTIR, ^{1}HNMR, DSC, SEM, EDX, TEM, UV-Vis spectroscopy and XRD.

v. To evaluate the effects of adding cyclodextrins in the delivery system

1.5 Outline of the dissertation

The following outline gives a brief description about the contents of each chapter:

Chapter 1

An introduction to the study with aims and objectives

Chapter 2

Literature reports on the methods of synthesis and characterization techniques. The application of polymeric nanoparticles is also briefly highlighted.

Chapter 3

Deals with the synthesis and characterization of alginate/chitosan nanoparticles containing tagette oil. Results obtained are interpreted and discussed.

Chapter 4

Focuses on the synthesis and characterization of *β*CD-tagette oil inclusion complex. Results obtained are interpreted and discussed.

Chapter 5

Describes the preparation and characterization of ALG/CS nanoparticles containing *β*CD–tagette oil complex. Results obtained are interpreted and discussed.

Chapter 6

Conclusions based on the interpretation of the data obtained are drawn in this chapter. Recommendations for future work are also put forward in this chapter.

Appendix

Selected spectra and other pertinent data are presented in this section.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The development of effective drug delivery systems that can transport and deliver drugs precisely and safely to the site of action is becoming a highly important research area for pharmaceutical researchers. As a result, promising ways of delivering poorly soluble drugs, peptides and proteins are being devised and improved. Although there are many potential improvements to be made in the field of drug delivery and diagnostics, nanotechnology offers advantages that allow a more targeted delivery and controlled release of the therapeutic compounds.[1]

2.2 Nanotechnology in drug delivery

Nanotechnology applies the principles of engineering, electronics, physical and material science and manufacturing at a molecular or submicron level. Thus nanotechnology can be defined as the understanding and control of matter at dimensions between 1 and 100 nm, where unique phenomena enable novel applications.[2] Materials at the nanoscale could be a device, or a system or alternatively, supramolecular structures, complexes or composites. Indeed, the physical and chemical properties of materials can radically change as their size is scaled down to small clusters of atoms.[3] The high surface area to volume ratio and large percentage of surface atoms compared with bulk materials are the results of the small size which have opened new exciting possibilities to solve great challenges in several areas, such as energy, environment, medicine and biology.[2] Practically there is still some debate about the actual definitions and many nano-systems have components larger than 100 nm, or even in the micron range.

2.2.1 Advantages of nanoscale drug delivery systems

The aim of nanotechnology in targeted drug delivery and controlled release is to better manage drug pharmacokinetics, pharmacodynamics, non-specific toxicity, immunogenicity and biorecognition of systems in the quest for improved efficacy. Nanoscale drug delivery systems are considered powerful tools for imaging, diagnosis and therapy due to their intrinsic properties.[2] Some of these properties are outlined below;[4]

- Many nanomaterials can pass through the smallest capillary vessels because of their ultra tiny volume thereby avoiding rapid clearance by phagocytes in the body and prolonging their availability in the blood stream.

- Nanoparticles can more easily penetrate cells and tissue gaps to arrive at target organs such as the liver, spleen, lungs, spinal cord and lymph glands.

- Nanosystems can be constructed to show controlled-release properties using biodegradability, pH, ion and temperature sensibility of materials.

- Nanosystems can improve the utility of drugs and reduce undesired side effects.

2.3 Importance of biodegradable drug delivery systems

Engineering DDS through the combination of biodegradable and biocompatible materials with various functions has been an ever-increasing field in development of novel medical devices and medical care systems. Among the possible strategies to achieve sufficient oral bioavailability of poor aqueous soluble drugs, nanoparticulate carriers made from biocompatible and biodegradable materials represent an exciting approach to increase the uptake and transport of orally administered drugs.[2]

Several nanoparticle vehicles used to deliver drugs and other biomolecules have been developed over the years. These have been used extensively in the pharmaceutical and cosmetic industries because of their ability to break down inside cells once their delivery function has been met. Generally, nanometric carriers include all submicron particles of various morphologies with sizes below 1000 nm.[5] A few examples are discussed below;

2.3.1 **Liposomes**

The development of liposomes as drug delivery vesicles is one of the earliest forms of nanomedicine. Liposomes are defined as vesicles in which an aqueous volume is entirely surrounded by a phospholipid bilayered membrane, with sizes varying from 30 nm to several micrometers. They form spheres that are hollow inside enclosing some of the liquid material in which they are formed. Because of their small size, they can pass through the epidermis and cell membrane and act as carriers for the enclosed substance.[6]

Liposomes may contain hydrophilic or hydrophobic compounds which remain encapsulated in the interior and may escape out of the phospholipid membrane through diffusion.[6] It is postulated that when they reach the outside of a living cell membrane in the body they may become accepted as part of the membrane being of the same composition (**Figure 2.1**). These particles usually break down inside cells once their delivery function has been met. For these reasons they have been extensively used in the pharmaceutical and cosmetic industry.[5]

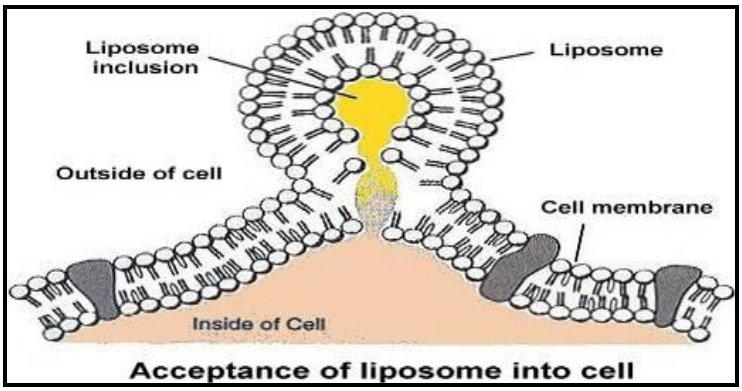

Figure 2.1: Schematic illustration of the uptake of a liposome into a cell.[6]

2.3.2 Micelles

Micelles present similar features as liposomes. Being one of the best ways of delivering hydrophobic drugs, micelles are often smaller than liposomes. They are colloidal dispersions that are constructed from amphiphilic molecules (possessing both hydrophilic and hydrophobic properties) such as lipids, which contain a hydrophobic core allowing for the delivery of drugs, which are often sparingly/poorly soluble in water.[7] In short, most micelles are one layer of lipid and liposomes have two layers (**Figure 2.2**)

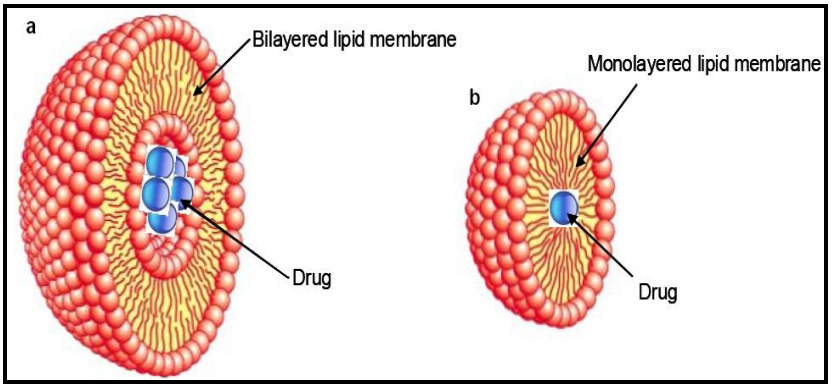

Figure 2.2: An illustration of (a) liposome and (b)micelle[7]

2.3.3 Dendrimers

Dendrimers, a unique class of polymers, are highly branched macromolecules whose size and shape can be precisely controlled. The structure of these polymers is repeated branching around the central core that results in a three dimensional geometrical pattern.[8] The dendritic branching results in a globular shape with a high density of cavities interbranching and presenting a high density of functional groups on the surface. The well defined structure, monodispersity of size, surface functionalization capability and stability are properties of dendrimers that make them attractive drug carrier candidates. Drug molecules can be incorporated into dendrimers via either complexation or encapsulation as shown in **Figure 2.3**.[9]

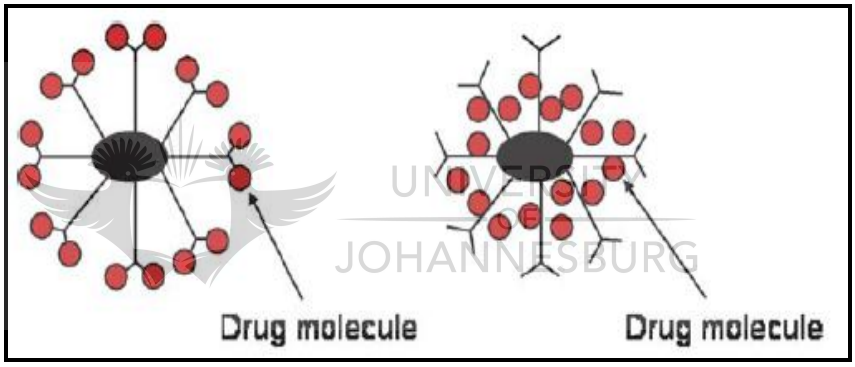

Figure 2.3: Schematic representation of incorporation of drug within a dendrimer structure.[9]

Although all these nanosystems have great potential in the treatment of diseases, their application is still limited by some disadvantages. Liposomes and micelles have limited capability to enhance the stability of the incorporated active compound as well as limited physical stability,[10] whilst dendrimers have multistep chemical synthesis and incorporated compounds may leak out of the branched structure before reaching the target site.[11]

Nanoparticles or polymer-based nanomaterials are superior to liposomes, micelles and dendrimers in that they can be targeted to specific organs/tissue by absorbing and coating their surface with different substances. They also exhibit a long shelf

life and a good stability on storage.[12] Reduced mobility of incorporated drug molecules leads to reduction of drug leakage.[13]

Drug delivery systems are developed to improve the safety and efficacy of drugs, to ensure better patient compliance and to improve the shelf life and stability of the therapeutic products.[14] This research project focuses on the encapsulation of lipophilic (oily) compounds in a polymeric nanoparticle system in order to improve its solubility in aqueous medium and achieve controlled release. The use of controlled release polymeric systems is an approach that holds a promise to improve the duration and effectiveness of the active compound both in pharmaceutical and cosmetic applications.

2.4 Polymeric nanoparticles

These are solid, submicron-sized particles or drug carriers. The term "nanoparticle" here is used as a collective name for both nanospheres and nanocapsules, depending on the method of preparation.[15] Nanospheres have a matrix type of structure with drugs absorbed at the surface or encapsulated within the particle. Nanocapsules are vesicular systems in which the drug is confined to a cavity consisting of an inner liquid core surrounded by a polymeric membrane (**Figure 2.4**).[16]

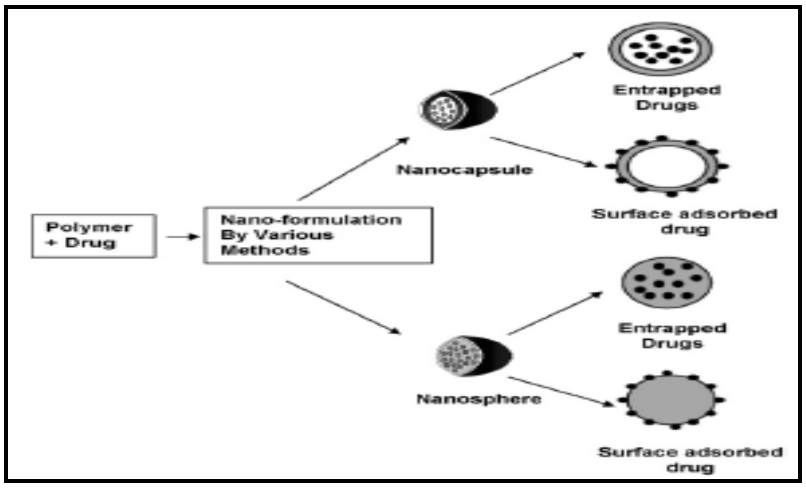

Figure 2.4: Nanocapsules and nanospheres, classified according to structural organization.[12]

Drugs can either be entrapped inside the nanoparticles or adsorbed on their surface. In general, fragile molecules are better preserved from enzymatic degradation occurring in biological medium when they are entrapped in the nanocarriers.[17] In this case, their association with the drug carrier should be done during the preparation of the nanocapsules or the nanospheres.[18] However, if the drug is highly susceptible to degradation, which may occur during the preparation process of the drug carrier or when it does not associate during the preparation of the drug carrier, it can be loaded by adsorption on the surface of already prepared carriers.[19]

Nanocapsules as active substance carriers have added advantages including high drug encapsulation efficiency due to optimized drug solubility in the core, low polymer content compared to nanospheres, drug polymeric shell protection against degradation factors such as pH and light and the reduction of tissue irritation due to the polymeric shell.[18]

Some polymeric nanoparticles are insoluble in water at physiological temperature and pH. They swell considerably in an aqueous medium and are capable of imbibing large amounts of water into the network structure. These nanoparticles are also known as nanogels.[20]

Nanogels are hydrogels that are at the nanoscale. These are materials with water swollen structures composed of mainly hydrophilic polymers.[21] They are insoluble in water due to the presence of chemical or physical cross linkers. The physical cross linkers can be weak associations formed by Van der Waals interactions or hydrogen bonds and these provide the network structure and physical integrity.[22] Being insoluble, polymeric hydrogel nanoparticles can retain a large amount of water that not only contributes to their good compatibility but also maintains a certain degree of structural integrity and elasticity.[23] Hydrophilic functional groups such as -OH, -COOH, $-CONH_2$, and SO_3H present in the hydrogel are capable of absorbing water without causing dissolution.[24]

2.4.1 Stimuli-responsive materials

Hydrogels have been developed as stimuli-responsive materials which can undergo abrupt volume change in response to small changes in environmental parameters, such as temperature, pH, ionic strength etc. (**Figure 2.5**).[24] These unique characteristics of hydrogels are of great interest in drug delivery, cell encapsulation and tissue engineering.[25]

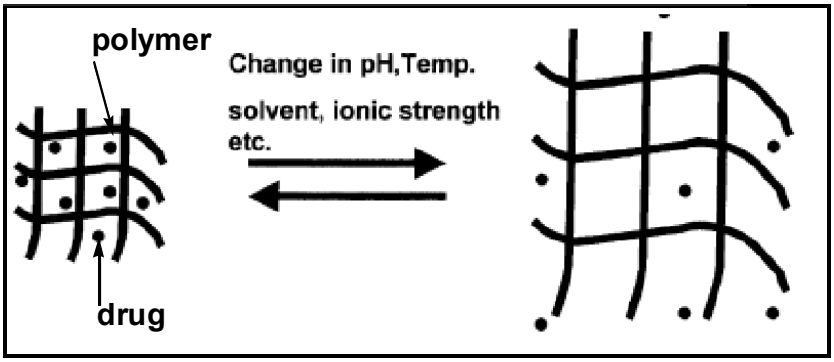

Figure 2.5: Stimuli-responsive hydrogels, modulated release of drug.[24]

The human body exhibits variations of pH along the gastrointestinal tract and also in some specific areas like certain tissues (e.g. tumoral areas) and subcellular compartments. In the case of pH sensitive polymers, the key element of the system is the presence of ionisable weak acidic or basic moieties attached to a hydrophobic backbone. Upon ionization, the coiled chains extend dramatically,

responding to the electrostatic repulsions of the generated charges (anions and cations).[26] Therefore ionic hydrogels (containing carboxylic, sulfonic acidic groups or basic pendant groups such as amines) show either sudden or gradual changes in their dynamic and equilibrium swelling behaviour as a result of changing the external pH.[27] This unique characteristic makes hydrogels especially useful in biomedical applications such as controlled release of drugs.

2.4.2 Controlled release

A very important function of ideal drug carriers is to regulate the release rate of the drug during therapy. Reducing the frequency of drug administration enables the patients to comply with dosing instructions leading to better treatment.[28] Therapeutic drugs are usually delivered in bolus doses, either orally or by injection. These administrations result in initial blood concentrations that are higher than required levels. The rapid increase of the drug concentration in the body is followed by a decrease to sub therapeutic levels due to drug degradation and excretion. Therefore, drugs must be given/taken frequently to maintain therapeutic drug concentrations, **Figure 2.6(a).**[2] The fluctuations of the administered drug might cause an alternating period of ineffectiveness and toxicity.[28]

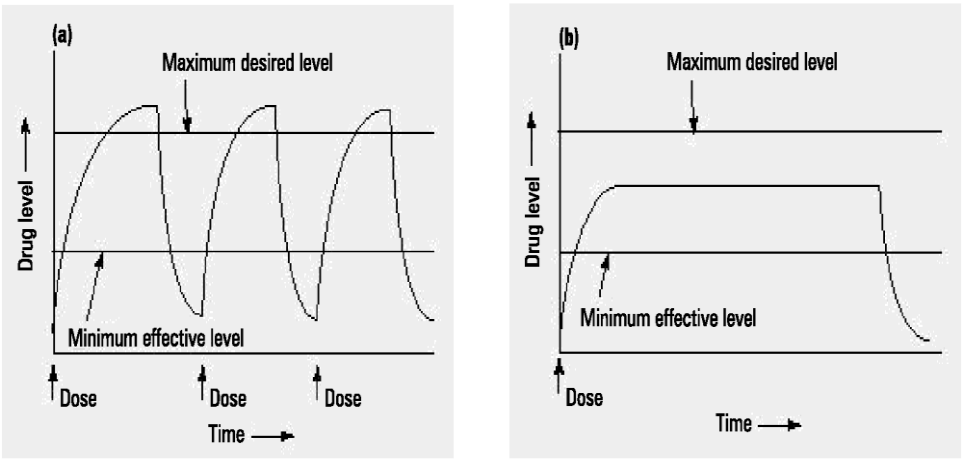

Figure 2.6: Comparison of behaviour of a traditional drug (a) to a nanodrug delivery system (b)[29]

Researchers have since introduced the concept of controlled drug delivery by using carriers to release drugs in a sustained manner. In an ideal case, the therapeutic agents are gradually released from a depot so that the drug concentration is maintained at an effective level over a long period of time (**Figure 2.6(b)**).[30] Controlled drug release also involves the combination of a biocompatible material or device with a drug to be delivered in a way that the drug can be delivered to and released at disease sites in a well designed manner to improve treatment efficiency.[2]

It has been proposed that polymer–based nanocapsules are biphasic systems with a fast initial release phase followed by a slower second phase The initial phase, called burst effect, can be attributed either to desorption of the drug located on the nanocapsule surface or to the degradation of the thin polymeric membrane. The second phase corresponds to the diffusion of the drug molecules from the inner compartment, the reservoir core, to the outer phase.[30]

An example of one such success is "Gliadel", the first Food and Drug Administration (FDA)-approved biopolymeric drug-delivery system for treatment of brain cancer. Gliadel wafers are implanted into the brain after tumour resection. There, the wafers locally release carmustine (a chemotherapeutic drug) for several months.[31]

2.4.3 Nanoparticle uptake by tissues

Compared with other colloidal carries such as submicron emulsions, biodegradable polymeric nanoparticles offer a higher stability when they are in contact with biological fluids.[12] Membrane layers are an obstacle for therapeutic agents attempting to target intracellular structures. During this process, the active compound may be lost due to the hindrance when crossing biological membranes.[32]

Nanoparticles present a solution to avoid this waste by masking the therapeutic agent from its biological environment. This effectively limits the influence of a compound's physical properties on intracellular drug concentrations.[5] Instead the

properties and surface characteristics of the nanoparticles play a greater role in compound delivery and resulting intracellular drug concentrations.[32]

Physicochemical characteristics such as particle size and surface properties play key roles in the cellular uptake of polymer nanoparticles.[33] Nanoparticle uptake could be considered as an adhesion process followed by an internalization process.[34] Polysaccharides coating of polymeric nanoparticles would facilitate the adhesion of particles to the cell membrane due to its biological adhesion properties rendered by the presence of hydroxyl and carboxyl groups in the structure.[35]

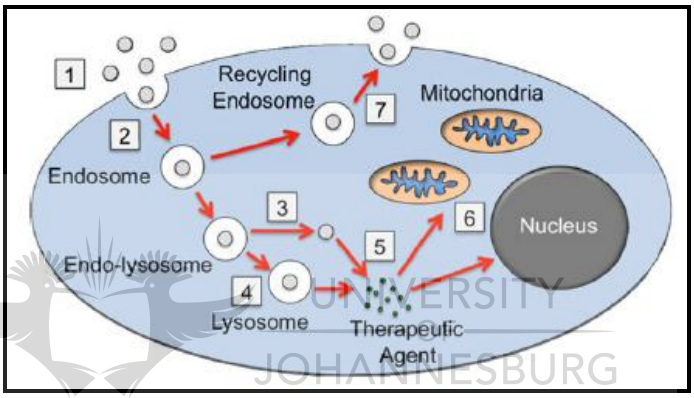

Figure 2.7: Steps detailing the cytosolic delivery of therapeutic agents via nanoparticle carriers.[5]

Larger microparticles are only taken up by phagocytic cells while smaller nanoparticles (50-500 nm) are taken up by virtually all cell types in a process called pinocytosis (the ingestion of sub-micron material and substances in solution).[34] **Figure 2.7** shows a summary of the uptake of nanoparticles by this process; (1) Cellular association of nanoparticles, (2) internalization of nanoparticles via endocytosis, (3) endosomal escape of nanoparticles or (4) lysosomal degradation of nanoparticles, (5) therapeutic agent freely diffuses into cytoplasm, (6) cytoplasmic transport of therapeutic agent to target organelle, (7) exocytosis of nanoparticles.[5]

Moreover nanoparticles based on negatively charged hydrophilic polymers show a strong increase in bioadhesive properties and are absorbed by both microfold (M)-cells (cells that transport organisms and particles from the gut lumen across the epithelial barrier to immune cells) and absorptive enterocytes.[34] A combination of both nanoparticle surface charges and increased hydrophilicity of the matrix material seem to affect the gastrointestinal uptake in a positive sense.[16]

2.4.4 Types of polymers

Several natural polymers (from natural systems such as albumin, gelatin, alginate and chitosan) and synthetic polymers (like polyactide-polyglycolide, polyacrylates and polyaprolactones) have been used in polymeric nanoparticulate systems for drug entrapment. They can protect the labile molecule from degradation in the gastrointestinal tract and promote transport into systemic circulation.[32]

Encapsulation of hydrophobic drugs into an aqueous nanoparticulate system has been attempted so as to deliver such drugs with their full potential. Nanoparticles consisting of synthetic biodegradable polymers, natural biopolymers, lipids and polysaccharides have been developed and tested over the past decades.[32] Water soluble polymers have been used in pharmaceutical formulations for many years and an increase in the drug solubility due to formation of water-soluble drug-polymer complexes has been observed. The polymers can interact with drug molecules via electrostatic bonds (e.g. ion-to-ion, ion-to-dipole or dipole-to-dipole bonds) but other types of forces such as Van der Waal's forces and hydrogen bridges, may frequently participate in the complex formation.[36]

2.4.5 Biodegradable polymers

Polymers used to design nanoparticles for in vivo delivery of drugs need to fulfil several requirements to be used in such an application:[37]

- They need to be biodegradable or at least totally eliminated from the body in a short period of time, allowing repeated administration without any risk of uncontrolled accumulation.

- Must be non-toxic and non immunogenic. Their degradation products, if any, must also be non toxic and non immunogenic.

- Should be formulated under the form of polymer nanoparticles with suitable properties regarding the drug delivery goal for which the nanoparticles are designed.

Polymers containing one or more hydrolysable functional groups are usually biodegradable. Biodegradable polymers can be classified into three categories: biopolymers (e.g. polypeptides, proteins, DNA and RNA); polysaccharides (e.g. cellulose, starch, chitosan and dextran); synthetic polymers (e.g. polyester and polyamide). Biodegradable polymers have been widely used, especially in the fabrication of sutures and implants to support body's recovery systems.[38] Another development is controlled release of drugs with diverse characteristics, such as anticancer drugs; adriamycin, cisplatin, paclitaxel, indomethacin and proteins.[28]

Over the years, a large number of copolymers including polysaccharides were developed. The rationale behind the development of these copolymers came out from the need for nanoparticles with tunable surface properties to modulate their interactions with blood proteins and with mucosa hence controlling their *in vivo* fate. The designed copolymers were also proved efficient to be used as stabilizers to insure nanoparticle stability without the need of other additional surfactants. [39] Due to their biocompatible property, polysaccharides draw a lot of interest in particle formation in order to act as drug carriers.[4]

2.4.6 **Polysaccharides**

Polysaccharides are the polymers of monosaccharides. They are mostly natural biomaterials that are stable, safe, non-toxic, hydrophilic and biodegradable.[4] There are abundant sources of polysaccharides in nature, such as algal based (e.g. alginate), plant based (e.g. pectin), microbial based (e.g. dextran) and animal based (e.g. chitosan) and they are very cheap to process. Polysaccharides have a large number of reactive groups, a wide range of molecular weight (MW) and

varying chemical composition, which contribute to their diversity in structure and in property.[40]

Most of these natural polysaccharides are good bioadhesive materials. They have hydrophilic groups such as hydroxyl, carboxyl and amino groups which could form non-covalent bonds with biological tissues (mainly epithelia and mucous membranes) forming bioadhesion.[40] Nanoparticle carriers made of bioadhesive polysaccharides could prolong the residence time and therefore increase the absorbance of loaded drugs.[41] These properties render polysaccharides a promising future as biomaterials for DDS.

Among the polysaccharides used to this day, chitosan and alginate are known for their biocompatibility, low toxicity, biodegradability and muco-and bioadhesiveness.[4] These two polymers were used in this study in the preparation of mucoadhesive nanoparticle formulations with the aim of improving the dissolution rate of poorly soluble drugs and controlling their release.

2.4.7 Alginate (ALG)

Alginate is a non-toxic, biodegradable, naturally occurring polysaccharide obtained from three species of marine brown algae, namely , *Laminaria hyperborean, Ascophyllum nodosum and Macrocystis pyrifera*. ALG exists as a mixed salt of various cations found in sea water such as Mg^{2+}, Sr^{2+}, Ba^{2+} and Na^{+}.[42]

Sodium alginate is a water soluble sodium salt of alginic acid, a natural polysaccharide and linear polymer composed of 1,4-linked *β*-D-mannuronic acid (M) and *α*-L-guluronic acid (G) residues in varying properties and arrangements (**Figure 2.8**). ALG has a long history of use in numerous biomedical applications including drug delivery systems, as it is a biodegradable, biocompatible and mucoadhesive polymer.[43] This polymer is also hemocompatible and has not been found to accumulate in major organs and show evidence of *in vivo* degradation.[44]

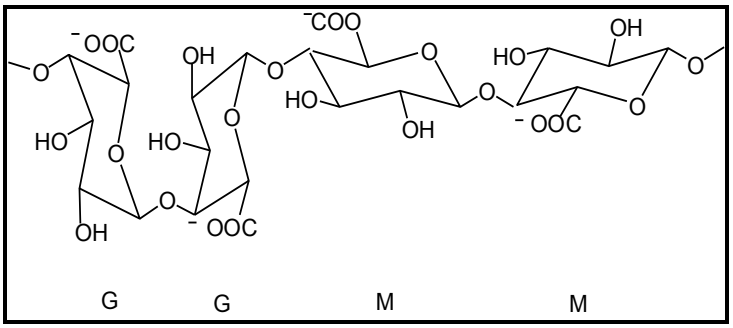

Figure 2.8: Chemical structure of ALG. Shown is a polymer chain of 2 guluronic acid (G) monomers and 2 mannuronic acid (M) monomers, with (1-4) linkages.

2.4.8 Properties of alginate

2.4.8.1 Gel formation

The most important property of ALG, is its ability to form gels by reacting with divalent cations such as Ca^{2+}, Sr^{2+} or Ba^{2+}. Calcium ions have unequal affinity for the guluronic and mannuronic acid units of ALG. As a result, calcium ions initially react with repeating guluronic acid units to form an egg box-shaped structure that stack upon each other. The gelation and crosslinking of the polymer is mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations and the stacking of these guluronic groups to form the characteristic egg-box structure shown in **Figure 2.9.**[45] Each ALG chain dimerizes to form junctions with many other chains and as a result gel networks are formed.

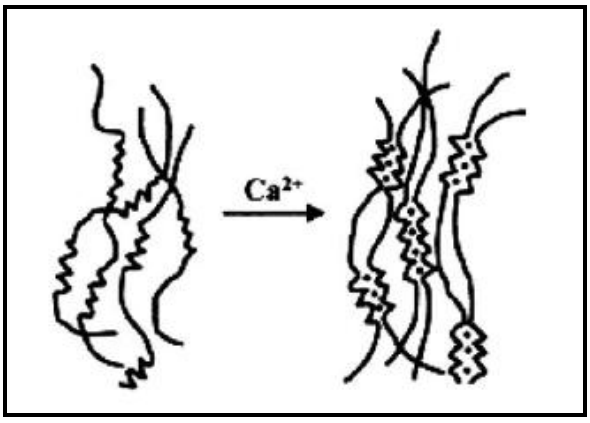

Figure 2.9: Egg-box structure of an ALG gel formed by chelating of Ca^{2+} ions.[48]

2.4.8.2 **Bioadhesiveness**

Mucoadhesive drug delivery systems work by increasing the drug residence time at the site of activity or resorption. Studies have shown that polymers with charge density can serve as good mucoadhesive agents. It has also been reported that polyanion polymers are more effective as bioadhesives than polycation polymers.[46] ALG, being an anionic polymer with carboxyl end groups, is a good mucoadhesive agent. Studies have shown that alginate has the highest mucoadhesive strength as compared to other polymers such as polystyrene, CS and poly(lactic acid). Due to the adherence of alginate particles to the mucosal tissues, drug transit time is delayed and the drug is localized to the absorptive surfaces.[47] This improves drug bioavailability and effectiveness.

2.4.8.3 **Biocompatibility**

Alginates are included in a number of compounds that are generally regarded as safe (GRAS) by the US (FDA) and has been used extensively in food industry as a thickening, emulsifying and stabilizing agent. The oral administration of alginate has not been shown to provoke much immunoresponses and it is reported that ALG is non-toxic and biodegradable.[48] Commercially available ALG when tested after purification by free-flow electrophoresis, did not provoke foreign body

reactions at least up to three weeks after implantation in the peritoneal cavity of rodents.[49]

2.4.8.4 **pH sensitivity**

At low pH medium (e.g. gastric fluid), ALG shrinks and the hydrated sodium alginate is converted into an insoluble alginic skin and the encapsulated compounds are not released. However in higher pH of intestinal tract, the alginic skin is converted to a soluble viscous layer. This pH dependant behavior of ALG can be exploited to customize release profiles for oral delivery.[48]

2.4.9 **Limitations**

ALG has been widely studied for particle formation in the size range of 100 nm – 200 nm for drug delivery. However, low stability (especially in higher pH media) and loss of encapsulated material by leaching through pores of ALG nanoparticles have been observed.[50]

2.4.10 **Modification of alginate nanoparticles**

Researchers have adopted many methods to overcome these limitations and these have been tested for drug delivery purposes. Crosslinking of ALG with aldehydes, thiolation of ALG (e.g. generation of an ALG-cystine conjugate) and hydrophobically modifying ALG (preparing amphiphilic derivatives of sodium ALG by covalent binding long alkyl chains on the polysaccharide backbone via ester functions) to form strong ALG hydrogels have been done successfully.[48]

ALG has also been mixed with other polymers such as pectin, chitosan ethyl cellulose and eudragit, forming polyelectrolyte complexes (PECs) to help solve the problem of drug leaching during preparation. PECs are complexes obtained by mixing aqueous solutions of two polymers carrying opposite charges. Complex coacervation of oppositely charged polyelectrolytes has been extensively used to prepare and strengthen ALG particles.[51]

2.4.11 Alginate-chitosan complexes

ALG has the property of shrinking in low pH and dissolving in higher pH, whereas CS dissolves in low pH and is insoluble in higher pH ranges. In view of these limitations encountered in pure ALG and pure CS (see below) bead systems, the concept of ALG-CS PECs gained acceptance. The PEC between CS and ALG has been widely used in order to obtain microcapsules for cell encapsulation and device for the controlled release of drugs or other substances. The mixing of ALG with CS can have an influence on the pore size and network complexity.[48]

2.4.12 Chitosan

CS is a polysaccharide, similar in structure to cellulose (both made up of *β*-(1-4)-linked D-glucose units) in which there are hydroxyl groups at C2 positions of the glucose rings. Chemically CS is composed of (1→4)-linked 2 amino-2-deoxy-*β*-D-glucose and residual 2-acetamido-2-deoxy-*β*-D-glucose (**Figure 2.10**). The relative amounts of acetylated groups can vary depending on the source and methods of processing. The primary amine groups render special properties that make CS very useful in pharmaceutical applications.[48]

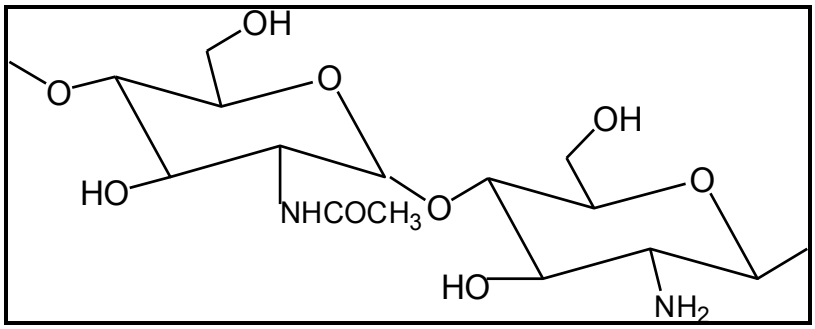

Figure 2.10: The chemical structure of chitosan

2.4.13 Sources of chitosan

CS is obtained from the deacetylation of chitin, a naturally occurring and abundantly available (in marine crustaceans) biocompatible polysaccharide. However, applications of chitin are limited compared to CS because chitin is

structurally similar to cellulose, but chemically inert. The acetamide groups of chitin can be converted into amino groups to give CS. This is usually carried out by treating chitin with a concentrated alkali solution to hydrolyse the amides. The cationic polysaccharide CS contains free amino groups in neutral or basic pH conditions and hence is insoluble in water. In acidic pH, amino groups can undergo protonation thus making it soluble in water, a property used to purify and separate the material.[52]

2.4.14 Properties of chitosan

2.4.14.1 Biocompatibility and biodegradability

CS is biocompatible with living tissues, since it does not cause allergic reactions and rejection. It breaks down slowly to harmless products (amino sugars) which are completely absorbed by the human body. This polymer is known to be biodegraded by several enzymes. Among them, chitinases, which are secreted by intestinal microorganisms, lysozyme, which is highly concentrated in mucosal surfaces and by human chitotriosidase.[48]

2.4.14.2 Mucoadhesiveness

CS is also mucoadhesive, which promotes the absorption of poorly absorbed macromolecules across epithelial barriers by transient widening of cell tight junctions.[49] It has been suggested that chitosan might be valuable for delivery of drugs to specific regions of the gastrointestinal tract like the stomach, small intestine and buccal mucosa. The mechanism of action was suggested to be ionic interactions between positively charged amino groups in CS and the negatively charged mucus gel layer.[52] This property allows CS particles to remain concentrated at the area of drug absorption.

2.4.14.3 Permeation enhancing effect

It has been reported that CS acts as a permeation enhancer by opening epithelial tight junctions. CS is able to enhance the paracellular route of absorption, which is

important for the transport of hydrophilic compounds such as therapeutic peptides and antisense oligonucleotides across the membrane.[49] The mechanism promoting this effect is based on the positive charges of the polymer, which interact with the cell membrane resulting in structural reorganization of tight junction-associated proteins.[52]

2.4.15 Significance of alginate-chitosan complex

As with pure alginate, chitosan by itself forms nanoparticles or beads that are often "leaky" to entrapped drugs because of charge repulsion, but upon mixing, the carbonyl residues (COO^-) of alginate and the amine groups (NH_2^+) of CS ionically interact to form the PEC **(Figure 2.11)**. Complexation of chitosan with alginate reduces the porosity of alginate nanoparticles and decreases the leakage of the encapsulated drugs.[53] The easy solubility of CS in low pH is prevented by the alginate network since alginate is insoluble in low pH conditions. The possible dissolution of alginate at higher pH is prevented by the CS which is stable at higher pH ranges.[49] Swelling of such a system in the stomach should be minimal and thus the drug release will also be minimal. The extent of swelling increases as the hydrogels pass down the intestinal tract due to an increase in pH.[53]

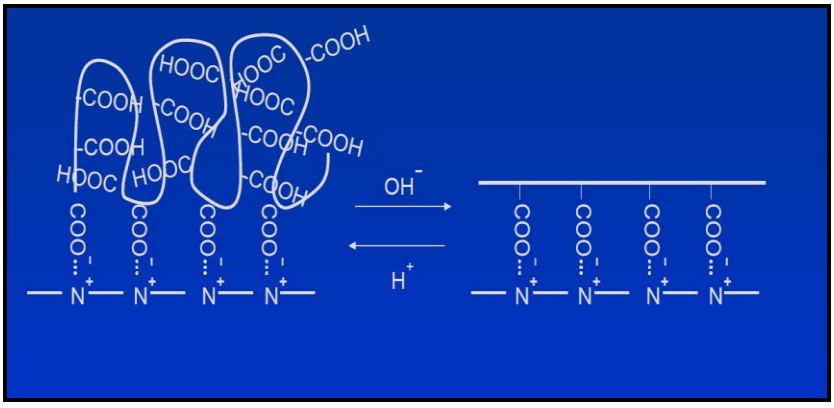

Figure 2.11: Schematic representation of oppositely charged polyions interacting to form a PEC.[51]

2.4.16 CD-containing hydrogels

While significant progress has been made in improving the properties of hydrogels used for drug delivery and in expanding the range of drug kinetics that can be achieved, several challenges remain to improve the clinical applicability of hydrogels for drug delivery.[22] One of major challenges relates to improving the aqueous solubility and extending duration of release of hydrophobic drugs. Cyclodextrins (CDs) are of interest in this context given their hydrophilic exterior, which is useful for maintaining the bulk hydrophilicity and swelling state of the hydrogel and their hydrobophic interior, which can facilitate the entrapment and controlled release of hydrophobic drugs.[54]

Recently, researchers suggested and studied cyclodextrins-based hydrogels because they can combine both the favourable property of CDs to form inclusion complexes and swelling behaviour of hydrogels to combat the problem of low bioavailability of many drugs due to low solubility, low stability at gastric pH and physical barrier of the intestinal epithelium.[55]

2.4.17 Properties of cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of 6, 7 or 8 glucopyranose units and are referred to as alpha (α-), beta (β-) and gamma (γ-) CD, respectively.[56] The glucose units are linked by *α*-1,4-glucosidic bonds, resulting in the formation of toroid or cone shaped molecules with internal hydrophobic cavities and external hydrophilic surface. Primary hydroxyl groups are located on the narrow side of the torus while the secondary hydroxyl groups are located on the wider edge (**Figure 2.12**).[57]

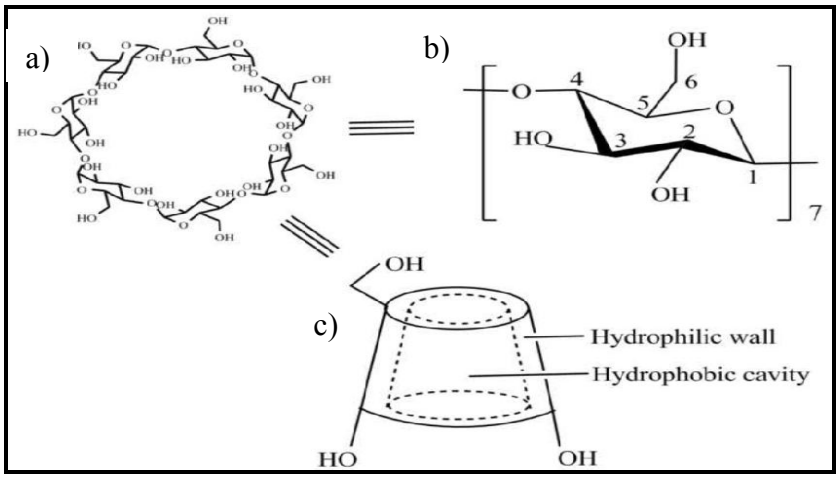

Figure 2.12: An illustration of *β*-Cyclodextrin structure a) a cyclic oligosaccharide b) the 1→4 linked α-D-glucopyranosyl unit and c) a torus shape.[57]

The internal hydrophobic cavities in CDs can facilitate the inclusion of a number of guest molecules stabilized by non covalent interactions.[57] In an aqueous solution, the slightly polar CD cavity is occupied by water molecules which are readily substituted by appropriate "guest molecules" which are less polar than water. Complex formation is a dimension fit between host cavity and guest molecule. The lipophilic cavity of CD molecules provides a microenvironment into which an appropriately sized non-polar compound can enter to form an inclusion complex.[58]

CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs and to increase their bioavailability and stability. In addition, CDs have been used to reduce or prevent gastrointestinal or ocular irritation, and to convert oils and liquid drugs into microcrystalline or amorphous powders.[59]

2.4.17.1 Enhancement of solubility

CDs increase the aqueous solubility of many poorly soluble drugs by forming inclusion complexes with their apolar molecules or functional groups.[60] The resulting complex hides most of the hydrophobic functionality in the interior cavity of the CD while the hydrophilic hydroxyl groups on the external surface remain

exposed to the environment. The net effect is that a water soluble CD-drug complex is formed.[61]

2.4.17.2 Enhancement of bioavailability

CDs enhance the bioavailability of insoluble drugs by increasing the drug solubility, dissolution and/or drug permeability.[62] When poor bioavailability is due to low solubility, CDs are of extreme value. Preconditions for the absorption of an orally administered drug is its release from the formulation in dissolved form. When drug is complexed with CD, dissolution rate and consequently absorption is enhanced.[63] CDs increase the permeability of insoluble, hydrophobic drugs by making the drug available at the surface of the biological barrier, e.g., skin, mucosa or the eye cornea, from where it partitions into the membrane without disrupting the lipid layers of the barrier (**Figure 2.13**).[64]

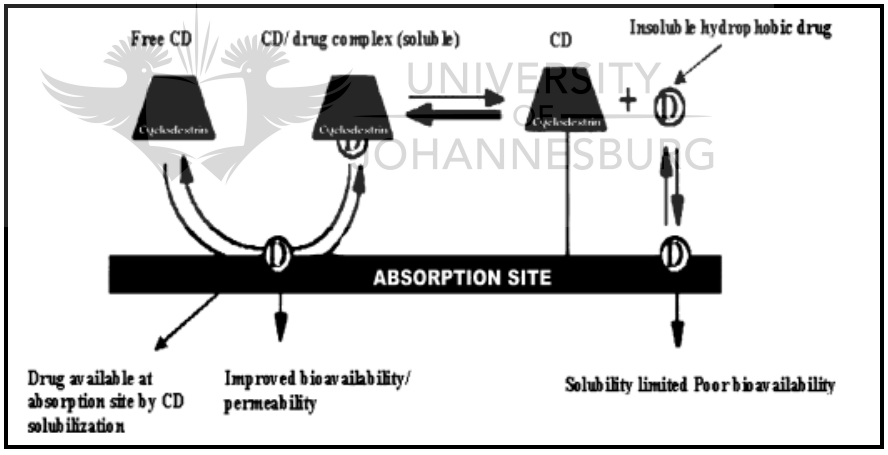

Figure 2.13: Proposed mechanism of mode of penetration enhancement by CDs.[58]

2.4.17.3 Improvement of stability

CD complexation can improve the stability of several labile drugs against dehydration, hydrolysis, oxidation and photodecomposition and thus increase the shelf life of drugs.[65] For an active molecule to degrade upon exposure to oxygen, water, radiation or heat, chemical reactions must take place. When a molecule is

entrapped within the CD cavity, it is difficult for the reactants to diffuse into the cavity and react with the protected guest.[66] By providing a molecular shield, CD complexation encapsulates labile drug molecules at the molecular level and thus insulates them against various degradation processes. Volatile components can be stabilized against loss by reducing the volatility in case of liquids.

The main driving force of complex formation is the release of enthalpy-rich water molecules from the cavity. Water molecules are displaced by more hydrophobic guest molecules present in the solution to attain an apolar-apolar association and decrease of CD ring strain resulting in a more stable lower energy state. The binding of guest molecules within the host CD is not fixed or permanent but rather a dynamic equilibrium.[67]

Due to their unique property to include hydrophobic molecules in their internal cavity, these macromolecules have been used in food, pharmaceutical and cosmetic industries.[67] In cosmetic and pharmaceutical formulations, CDs are mainly used to increase the water solubility of lipophilic materials, to convert liquid or oily materials to powder form and to increase the physical and chemical stability of the guest molecules (by protecting them against decomposition, oxidation, hydrolysis or loss by evaporation) while providing controlled release of the active ingredients.[68]

The remarkable ability to include molecules into their cavity, has promoted the extensive use of CDs in our laboratory to remove organic pollutants in water. In this study, CDs were used to enhance the aqueous solubility of the lipophilic drug and to extend its duration of release. The incorporation of CDs into polymeric drug delivery systems could change the drug-polymer interaction and as a result, the mechanism of drug release may be modified.

CD-containing hydrogels have been prepared using different methods. Most-simply, preformed CD-drug complexes can be loaded into the hydrogel after or during gel cross-linking **(Figure 2.14)**.[22] This strategy improved the loading of estradiol (used as hormone replacement in menopausal women) approximately

500-fold compared to that achieved by simple aqueous loading into hydrogels of similar composition and resulted in release of a therapeutic level of drug for up to a week.[69]

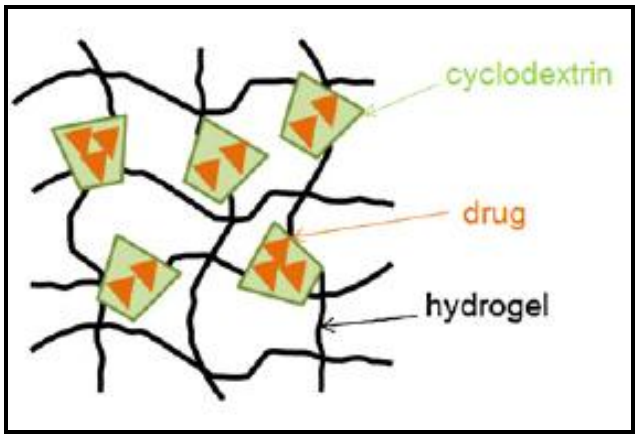

Figure 2.14: Incorporation of cyclodextrin in hydrogels for hydrophobic drug delivery.[22]

2.5 Methods of preparation of nanoparticles

Methods for preparing polymeric nanoparticles generally include two main steps; the preparation of an emulsified system followed by the precipitation or gelation of the polymer.[70]

2.5.1 Preparation of nanoparticles by emulsification-solvent evaporation

In this method, emulsions are formulated with polymer solutions prepared in volatile solvents, such as ethyl acetate, which displays a good toxicological profile. Conversion of the emulsion into a nanoparticle suspension occurs by the evaporation of the polymer solvent, which is allowed to diffuse through the continuous phase of emulsion, **Figure 2.15**. This is a slow process performed under vacuum. The size of the emulsion droplet is reduced during solvent evaporation.[71]

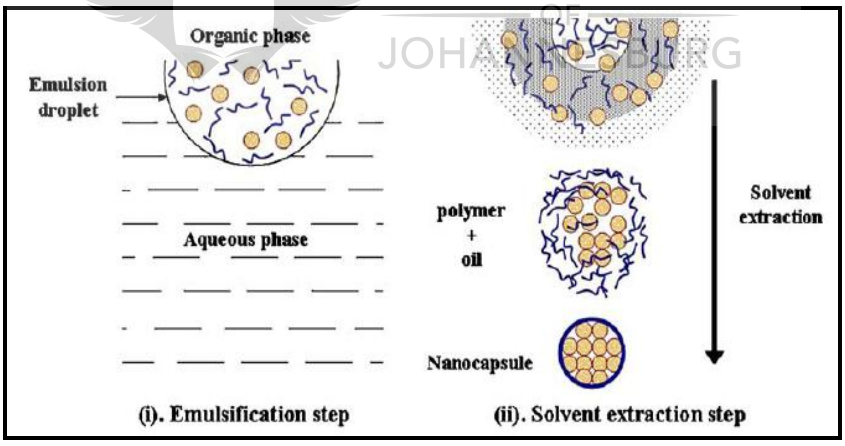

Figure 2.15: Schematic representation of the proposed formation mechanism of nanocapsules by emulsification (i) followed by solvent extraction (ii), where the orange dots and the blue lines are the oil droplets and polymer, respectively.[71]

2.5.2 Preparation of nanoparticles by emulsification-solvent diffusion

This method has been successfully used to prepare biodegradable nanoparticles in an efficient and reproducible manner. For the success of this method, the polymer solvent used to prepare the emulsion needs to be partly soluble in water. The oil-water emulsion obtained using saturated solvents is diluted with an extensive amount of pure water. As a result of this dilution, additional organic solvent from the organic phase contained in the dispersed droplets diffuses out of the droplets leading to the precipitation of the polymer (**Figure 2.16**).[72]

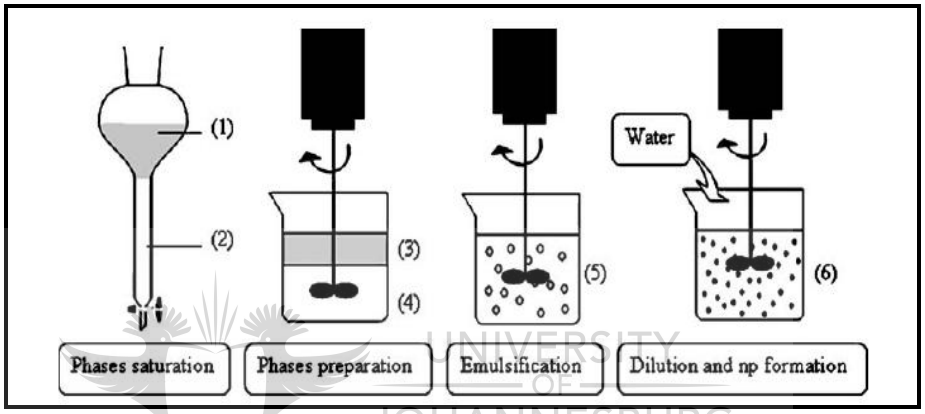

Figure 2.16: Sketch of the manufacturing of nanoparticles by the emulsification-solvent diffusion method.[72]

In contrast with solvent evaporation method, the extraction of the polymer solvent from the emulsion droplets occurs in a time scale of milli-seconds. The mean diameter of the nanoparticles can be reduced by increasing the stirring rate and the concentration of stabilizing agents added in the emulsion.[72]

2.5.3 Formation of polyelectrolyte complexes

Oppositely charged polysaccharides in aqueous solutions interact spontaneously to form PECs when they are mixed. Polyelectrolytes are defined as polyions which contain ionic groups in their repeated units and therefore exhibit electrolyte properties. The driving force for the formation of PECs is the strong electrostatic interaction between oppositely charged polyelectrolytes, which leads to

interpolymer ionic condensation and the formation of aggregates in the nanosized range, called nanospheres (**Figure 2.17**).[58]

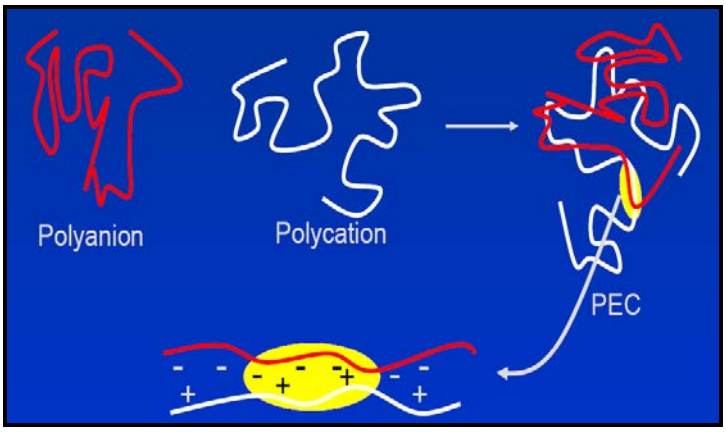

Figure 2.17: Schematic representation of PEC formation.[51]

Chitosan and alginate are polycationic and polyanionic polyelectrolytes, respectively, that have been used to form a PEC to deliver proteins, other drugs and DNA. When the two polysaccharides are mixed, the carboxyl residues of alginate and the amino groups of chitosan ionically interact to form the PEC. Complexation of chitosan with alginate has been found to reduce porosity of alginate beads and decrease the leakage of the encapsulated drug.[48]

Polyelectrolyte complexation of ALG and CS, may be a two-step procedure where the first step is the preparation of a calcium-ALG pre-gel, followed by further crosslinking with CS. The interaction between ALG in dilute solution with calcium ions (Ca^{2+}) occurs at a certain ion concentration. A pre-gel state results with stirring, avoiding the gel point and forming a continuous system.[51]

Subsequent addition of an aqueous polycationic solution (CS), results in a PEC, stabilizing the pre-gel nucleus into individual sponge-like nanoparticles.[73] The nanoparticles are generally characterized by a core/shell structure. The hydrophobic core is composed by the complexed segments whilst the excess of component not incorporated in the PEC is segregated in the outer shell ensuring the colloidal stabilization of the nanoparticles against coagulation and conferring the overall charge of the nanoparticle surface.[58]

Considering ALG nanoparticles, it can be pointed out that the size of the nanoparticles greatly depends on the concentration of ALG and also on the molecular weight of the polycation used to stabilize the nanoparticles.[74] Although ALG complexed with CS alone can form nanoparticles based on the formation of a simple PEC, the formation of a pre-gel phase with calcium before addition of the polycation allow the formation of a more compact structure of the nanogel. This was indicated by the large difference in size between the two nanoparticle systems that formed in each case.[58]

The effect of molecular weight of CS on the nanoparticle size has been investigated using low and medium molecular weight CS (LM and MM-chitosan). The average sizes of the CS-ALG nanoparticles with LM and MM-chitosan were 522 ± 15 and 667 ± 17 nm, respectively, indicating that the nanoparticle size is influenced by the molecular weight of the polycation.[75]

The effect of pH on the size of nanoparticles formed has been investigated and it has been demonstrated that an ALG solution of pH 5 - 5.3 generally produces smaller particles when combined with LM-chitosan (pH 4.5 - 5). Within this pH range, the amine groups of CS are protonated and the carboxyl groups of the ALG are ionized, which is most important for optimum interaction and the polyionic complex formation of more compact and smaller nanoparticles.[76]

2.6 Treatment of nanoparticles after preparation

Several types of treatment can be applied to nanoparticle suspension after synthesis. These include purification, sterilization and drying.

2.6.1 Purification of nanoparticle suspension

Once nanoparticle suspensions are obtained, purification may be further needed to remove impurities and excess of reagents involved during manufacture. Depending on the method of preparation, impurities include solvents, oil, surfactants, large polymer aggregates and residual polymers. Methods of

purification include evaporation under reduced pressure, centrifugation and filtration. [77]

Evaporation under reduced pressure is the most common approach to remove volatile organic solvents and a part of water. This process is usually used after obtaining suspensions by nanoprecipitation, and emulsification-solvent diffusion [77]

Filtration is applied to remove large particles or polymer aggregates which form during preparation. Such purification is mostly applied on nanoparticle suspensions designed for intravenous injections. [78]

Centrifugation at low gravity force can also be applied to remove aggregates and large particles on most of the polymer nanoparticle suspensions. However, it does not warranty the elimination of all particles with a diameter above a very definite size as filtration, since nanoparticles having a high density may sediment with aggregates. [79]

2.6.2 Sterilization

For clinical uses, drug delivery systems have to meet the pharmacopeia requirements of sterility. Sterilization techniques include autoclaving, gamma irradiation, membrane filtration, high hydrostatic pressure sterilization and sterilization by using ethylene oxide or formaldehyde. These methods of sterilization eliminate the risk of microbial contamination.[70]

2.6.3 Drying of nanoparticles

As in the case of many pharmaceutical preparations, storage of nanoparticles as suspensions presents many disadvantages such as risk of microbial contamination, polymer degradation by hydrolysis, physicochemical instability due to particle aggregation and sedimentation and loss of biological activity of the drug. To circumvent such problems, pharmaceutical preparations are stored in dry form.[70] The transformation of a liquid preparation into a dry product can be achieved by freeze-drying or spray drying processes.

2.6.3.1 **Freeze drying**

Freeze drying, also known as lyophilization, is a very common technique of conservation used to ensure long term stability of pharmaceutical and biological products, preserving their original properties.[61] The basic principle of this process depends on removing water content of a frozen sample by sublimation and desorption under vacuum. In general, freeze drying, (carried out by a freeze-drier (**Figure 2.18**)) can be divided into three steps: freezing of the sample (solidification), primary drying, corresponding to the ice sublimation and secondary drying, corresponding to desorption of unfrozen water.[80]

Figure 2.18: A picture of a freeze-drier loaded with drying samples.[80]

2.6.3.2 **Spray drying**

The spray drying technique transforms liquids into dried particles under a continuous process. Nanoparticle formulations submitted to spray drying are generally aqueous suspensions and contain one soluble compound added as drying auxiliary e.g. silicon dioxide, lactose and mannitol. Spray-drying process includes four important steps: 1) atomization of the nanoparticle suspension into a spray, 2) spray-air contact, 3) drying of the spray and 4) separation of product from the drying gas (**Figure 2.19**).[81]

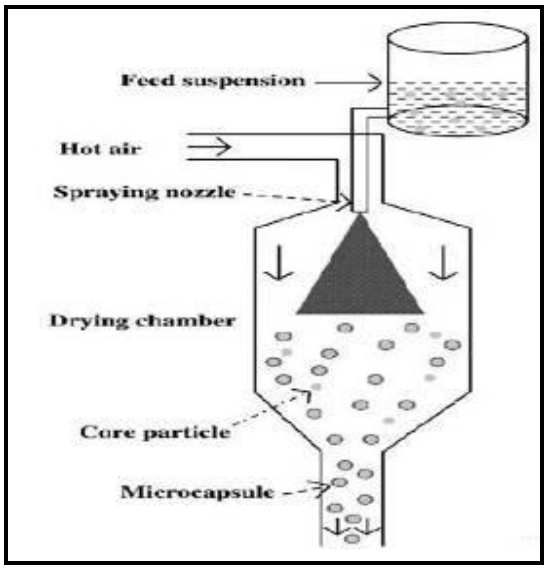

Figure 2.19: Schematic illustrating the process of micro-encapsulation by spray-drying.[81]

2.7 Characterization techniques

Several characterization techniques were used for this study and are briefly outlined in the following sections.

2.7.1 Fourier transform infrared (FTIR) spectroscopy

FTIR is a powerful tool for identifying types of chemical bonds (functional groups) in a molecule by producing an infrared absorption spectrum that is like a molecular finger print. When IR radiation is passed through a sample, some of the IR radiation is absorbed by the sample and some of it is passed through (transmitted). The wavelength of light absorbed is characteristic of a specific chemical bond. The resulting spectrum represents the molecular absorption and transmission creating a molecular fingerprint of the sample. Like a fingerprint, no two unique molecular structures produce the same IR spectrum.[82] This makes it possible to identify the various functional groups in a sample using the different wavelengths at which each functional group absorbs IR radiation. Therefore IR analysis has been used in structure determination and to examine interactions between polyelectrolytes in PEC formation.[83]

2.7.2 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy has become the pre-eminent technique for determining the structure and purity of organic compounds, and measures the resonance frequency of NMR-active nuclei when placed in a strong magnetic field. Since the NMR peaks obtained in this way are very sensitive to changes in the local environment, NMR offers the most direct evidence for the inclusion of a guest into a cyclodextrin cavity in solution. ^{1}H-NMR may also be used to determine the direction of penetration of guest molecules into the cyclodextrin cavity. The resonance peaks for the H-3 and H-5 atoms of cyclodextrin, which are directed towards the interior of the cyclodextrin will often show a significant shift if inclusion does indeed occur. Similarly, the peaks for H-1, H-2, and H-4, located on the exterior of the cavity will show only marginal shifts during inclusion formation, but large shifts if the inclusion does not occur.[81]

The chemical shift change ($\Delta\partial$) is defined as the difference in chemical shift in the presence and absence of the other molecules. In cases of chemical shift change, positive sign means a downfield shift and negative sign means an upfield shift. The spectrum of the guest molecule may also be changed upon inclusion complex formation.[84]

2.7.3 Ultraviolet-visible (UV-Vis) spectroscopy

Ultraviolet and visible radiation interacts with matter which causes electronic transitions (promotion of electrons from the ground state to a high energy state). The ultraviolet region falls in the range between 190-380 nm, the visible region fall between 380-750 nm. UV-Vis absorption spectroscopy measures the amount of radiation that is absorbed by a compound at each wavelength. The wavelength and amount of light that a compound absorbs depends on its molecular structure and the concentration of the compound used.[85]

UV-Vis spectroscopy is widely used in organic chemistry to investigate the extend of multiple bond or aromatic conjugation within molecules. This technique is also ideal for quantitative analysis of solutions where the concentration of an analyte in

solution can be determined by measuring the absorbance at a selected wavelength and applying the Beer-Lambert law.[82]

2.7.4 Differential scanning calorimetry (DSC)

By observing the difference in heat flow between a sample and a reference, calorimeters are able to measure the amount of energy absorbed or released during any transitions or transformations in a material. The result is a plot of the differential heat flow as a function of temperature: a DSC curve. Generally, the differential heat flow is calculated by subtracting the sample heat flow from the reference heat flow. Thus in this case, exothermic processes will show up as positive peaks, while peaks resulting from endothermic processes are negative.[86]

DSC can be used to characterize the thermal behavior of PECs and biomolecules which is correlated to their structure, hydrophilic properties and association state. This is made possible by obtaining the complete temperature profile of the Gibbs energy change associated with the loss of water in polymers, with depolymerization at high temperatures. Shifts of exothermic and endothermic peaks are usually associated with interaction between the polymers and interaction between the drug and polymers.[81]

2.7.5 X-ray diffractometry (XRD)

X-ray powder diffraction is a rapid analytical technique primarily used for phase identification of a crystalline material. The analysed material is finely ground, homogenized and average bulk composition is determined. X-rays are generated by a cathode ray tube and the interaction of these rays with the sample produces diffracted rays. These diffracted x-rays are then detected, processed and counted. Conversion of the diffraction peaks to d-spacing allows identification of the material.[87]

Powder x-ray diffractometry may also be used to detect inclusion complexation in the solid state. When the guest molecules are liquid, since liquids have no diffraction pattern of their own, then the diffraction pattern of a newly formed

substance clearly differs from that of uncomplexed material (such as cyclodextrin). The difference of the diffraction patterns indicates the complex formation.[88]

2.7.6 Scanning electron microscopy (SEM)

The scanning electron microscope is an electron microscope that images the sample surface by scanning it with a high energy beam of electrons (instead of light waves used in conventional light microscopes). When the beam of electrons strikes the surface of the specimen and interacts with the atoms of the sample, signals in the form of secondary electrons (back scattered electrons) are generated that contain information about the samples surface topography.[89]

The beam may also cause x-rays to be emitted from the material, which are used to determine the elemental composition of the sample by a technique known as energy dispersive x-ray (EDX) spectroscopy, where the energy of the x-rays emitted depends on the material under examination.[86]

SEM requires that the specimen should be conductive for the electron beam to scan the surface for conventional imaging. Non-conductive solid specimens are generally coated with a layer of conductive material by low vacuum sputter coating. Images are used in analytical SEM along with the spectra obtained from the characteristic x-rays as clues to the elemental composition and morphology of nanoparticles formed.[89]

2.7.7 Transmission electron microscopy (TEM)

TEM is a microscopy technique similar to SEM, whereby a beam of electrons is directed at a specimen under vacuum. Unlike SEM the electron beam is transmitted through an ultra thin specimen and interacts as it passes through the sample. An image is formed from the transmitted electrons on a phosphorescent screen or CCD camera. The image can be magnified and focused by an objective lens and appears on an imaging screen. In TEM the sample interacts with the electron beam mostly by diffraction rather than by absorption.[87] The morphology and dispersion of nanoparticles can be investigated using TEM. The size of

nanoparticles can also be estimated using this technique. Other advanced analyses such as EDX can also be performed in some cases.

2.7.8 Particle size

One of the most common methods for determining size is by using a zetasizer instrument, which provides the ability to measure the size, zeta potential and molecular weight of particles or molecules in a liquid medium. This system determines the size of particles by measuring the Brownian motion of the particles in a sample using dynamic light scattering.[90] It is known that small particles move quickly in a liquid whilst large particles move slowly. Therefore after each measurement if there has been minimal movement and the particle positions are very similar, then the particles in the sample will be large. Similarly if there has been a large amount of movement and the particle positions are quite different, then the particles in the sample are small. Using this knowledge and the relationship between diffusion speed and size, the size can be determined.

Particle size is the most important parameter when using nanoparticles as drug carriers since it is determinant in mucosal and epithelial tissue uptake of particles and in the intracellular traffic of the particles. If the particle size is too large, the surface area decreases and the absorptivity rapidly decreases. If the particle size is too small, it begins to form compounds with vaccine particles and the particles are easily decomposed making it impossible to provide continuous drug delivery. The appropriate size suggested in theory is 100-400 nm.[88]

2.7.9 Zeta potential

The zeta potential is the electrostatic potential that exists as the shear plane of a particle, which is related to both surface charge and the local environment of the particle. It is measured using a technique called laser Doppler electrophoresis. This method measures how fast a particle moves in a liquid when an electrical field is applied i.e. its velocity. Once the velocity of the particle and the electrical field applied are known and using constants of the sample; viscosity and dielectric constant, the zeta potential can be obtained.[87]

The zeta potential of a sample determines whether the particles within a liquid will tend to flocculate (stick together) or not. For small particles a high zeta potential will confer stability i.e. the solution or dispersion will resist aggregation. Particles with zeta potential more positive than +30 mV or more negative than -30 mV are normally considered stable. Zeta potential of nanoparticles also has an effect or influence on the cellular/tissue uptake of the nanoparticles.[91]

2.7.10 Gas chromatography (GC)

Gas chromatography is one of the most widely used techniques for qualitative and quantitative analysis. In gas chromatography, the components of a vaporized sample are separated as a consequence of being partitioned between a mobile gaseous phase and a liquid or solid stationary phase held in a column. In performing a gas chromatography separation, the sample is vaporized and injected onto the head of a chromatographic column. Elution is brought about by the flow of an inert gaseous mobile phase called the carrier gas. Helium is the most common mobile-phase gas, although argon, nitrogen and hydrogen are also used.[92]

Gas chromatograms are used to establish the purity of organic compounds. Contaminants, if present, are revealed by the appearance of additional peaks. The areas under these peaks provide rough estimates of the extent of contamination. In theory, GC retention times should be useful for identifying components in mixtures. Quantitative GC is based on comparison of either the height or the area of an analyte peak with that of one or more standards.[93]

2.8 Applications of polymeric nanoparticles

Over the years, nanoparticle drug delivery systems have shown huge potential in biological, medical and pharmaceutical applications. The novel properties of these carriers suggest that they will have an important role in the biomedical field. In anticancer therapy, one of the worst problems is the low tumor answer to treatment, because of the non-specific bioavailability of administered anticancer agents. Use of nanoparticles as vehicles for anticancer agents achieves

bioaccumulation of drug in the target tissue. Paclitaxel and doxorubicin are examples of anticancer drugs that have been successfully encapsulated in polymeric nanoparticles (polyalkylcyanoacrylate nanoparticles) and used in the treatment of cancer.[6]

One major concern with protein delivery is the loss of therapeutic efficacy of the protein due to the degradation/denaturing of the protein. Insulin-loaded ALG/CS nanoparticles produced by polyelectrolyte complexation method have been shown to provide enhanced intestinal absorption of insulin following oral administration. The physiological effect was observed for more than 18 hours per dose. Thus ALG/CS nanoparticles are promising as an oral delivery system for proteins and other drugs.[94]

Nanoparticles have also been used in the food and cosmetic industries. In cosmetic formulations, nanoparticles have been used to increase the stability and water solubility of fragrance materials, to provide controlled release and to convert substance from liquid to powder form by preparing their inclusion complexes with CDs.[95] The use of CDs has also been reported to reduce or prevent skin irritation. They achieve this by increasing or decreasing the absorption of various compounds into the skin and by stabilizing emulsions/suspensions avoiding the breaking down of labile molecules into products with undesired effects. In food industry, nanoparticles are used as stabilizers for flavoring agents and to reduce unpleasant odors and taste.[96]

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CHAPTER 3

PREPARATION AND CHARACTERIZATION OF TAGETTE OIL-LOADED ALGINATE/CHITOSAN NANOPARTICLES

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3.1 Introduction

Polymeric nanoparticles have been used widely to deliver drugs, proteins, genes and for the sustained release of fragrances and other active compounds in cosmetic industry.[1] This is on the basis that nanoparticles can entrap drugs or biomolecules into their structures and/or absorb drugs or biomolecules onto their exterior surfaces. In this way they improve the properties of the incorporated molecules, such as increasing their aqueous solubility and promote their transport into the systemic circulation consequently improving efficacy.[2]

Essential oils are a complex mixture of several substances, normally liquids at room temperature. They are used as fragrances and for their antibacterial, antifungal and insect repellent activities in pharmaceutical and cosmetic industries.[3] However, like many lipophilic drugs, these concentrates are not water soluble and are sensitive to light and high temperatures. Also, the use of an oil mixture allows us to evaluate the stability of a complex mixture of lipophilic compounds for application in cosmetics and other areas. Due to the poor solubility and stability it is necessary to transform these compounds, for their utilization and full potential application in industries.[4] Encapsulation helps improve aqueous solubility of oily compounds and protects the molecules from the biological environment. Such an approach is even more attractive if the size of the capsule is reduced to a nanometer scale.[5]

This chapter focuses on the preparation of nanoparticles of ALG and CS origin, loading these with an essential oil, tagette oil (as a model drug mixture) and monitoring the release of the oil from the particles. The production of biopolymeric

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nanoparticles may be useful in a variety of applications including cosmetic preparations and as a drug delivery system.

3.2 Experimental procedure

3.2.1 Materials

All chemicals and solvents were of analytical grade and they were used as supplied without further purification. Materials used include low-G NaALG and low molecular weight (LMW) CS, calcium chloride (anhydrous), and Tween 80®, all purchased from Sigma Aldrich, South Africa. Commercially available tagette oil, was kindly provided by Clive Teubes cc, South Africa. Aqueous cream (fragrance and colourant free) was purchased from Clicks, South Africa (PTY) LTD. Distilled water was used throughout the project, and any other solvents were analytical-grade unless otherwise indicated.

3.2.2 Preparation of tagette oil-loaded alginate/chitosan nanoparticles.

3.2.2.1 Preparation of solutions

Solutions of NaALG and $CaCl_2$ were prepared by dissolving the relevant amounts of polymer and $CaCl_2$ in distilled water. The pH of the NaALG solution was adjusted to 4.9 using 1% HCl. CS solution was prepared by dissolving the required amount of CS in 1% acetic acid solution and the pH adjusted to 4.6 using 1M NaOH. The NaALG and CS solutions were filtered through 0.47 and 0.22µm membranes under vacuum before use in nanoparticle preparation.

3.2.2.2 Preparation of tagette oil loaded ALG-CS nanoparticles

ALG/CS nanoparticles were prepared in a two-step procedure based on the ionotropic pre-gelation of polyanion (ALG) with $CaCl_2$ followed by polycationic (CS) crosslinking, which results in the formation of a PEC membrane on the surface of the nanoparticle. The method used was adapted from Lertsutthiwong method with some modifications as follows.[5]

Tagette oil (1.22 g) dissolved in ethanol (6 ml) was added dropwise into a beaker containing 200 ml ALG solution (0.063% w/v) containing 1 ml Tween 80®. After sonicating for 15 minutes, 40 ml of 0.198% (w/v) $CaCl_2$ solution was added dropwise for 15 minutes under gentle stirring (800 rpm) into the ALG-oil emulsion, to provide an ALG pre-gel. Then 40 ml CS solution (0.05% w/v) was added dropwise into the pre-gel over 30 minutes whilst stirring. The nanoparticle suspension was left standing overnight to equilibrate.

Blank nanoparticles were prepared using the same procedure, excluding the addition of tagette oil, for comparison.

3.2.3 Purification and drying of nanoparticles

The ethanol used to dissolve the oil was removed from the nanoparticle suspension by rotary evaporation at 40°C for 20 minutes, under reduced pressure. The nanoparticles were collected by centrifugation (Hettich EBA III, Tuttingen Germany) at 5000 x g for 60 minutes. The pellet obtained was frozen in liquid nitrogen and freeze dried overnight using a freeze dryer (VirTis Bench Top K Freeze dryer, SP Industries).

3.2.4 Variation of parameters in the formulation

The optimal ALG:CS mass ratio for nanoparticle preparation was determined using ratios of 1.3:1, 1.6:1, 2:1, 10:1 and 20:1. The effects of ALG/CS mass ratio, pH, and storage time on the particle size, zeta potential, encapsulation efficiency (EE%) and percentage yield (%) were examined.

3.2.5 Nanoparticle characterization

3.2.5.1 Particle size and zeta potential

Nanoparticle size and zeta potential were determined by using a Malvern Zetasizer and particle analyser (Zetasizer Nano Series, Malvern Instruments). Measurements were made on aqueous dilute samples of 2 ml which were

sonicated for 10 minutes in an ultrasonic bath, placed in the analyser chamber and measured. Collective 12 readings were performed 3 times on a sample of particles at 25^{o}C with a detection angle of 90^{o}

3.2.5.2 **Scanning electron microscopy (SEM)**

To study the morphology of the nanoparticles, such as shape and occurrence of aggregation, the Scanning Electron Microscope (SEM) was used. Samples of nanoparticles were mounted on glass slides with carbon tape, carbon coated under vacuum and then examined on a JEOL JSM 6700F SEM. The instrument was also equipped with an Energy-Dispersive X-ray spectroscopy (EDX) analyser, used for the determination of elements present in the samples. The analysis was performed on two marked spots on the surface of the nanoparticles.

3.2.5.3 **Transmission electron microscopy (TEM)**

The nanoparticles were also observed using Transmission Electron Microscope (TEM, model JEM-2100, Japan). After freeze drying the nanoparticles were redispersed in methanol and sonicated for 20 minutes. The nanoparticle suspension was deposited on a Formvar-coated copper grid and stained with 1% uranyl acetate solution and examined using TEM.

3.2.5.4 **Fourier transform infrared (FTIR) Spectroscopy**

The Fourier transform infrared spectrometer was employed to characterize the potential interactions of the polymers in the nanoparticles. The FTIR spectra of tagette oil, ALG/CS nanoparticles and their individual components, were collected between 4000 and 600 cm^{-1} using a Perkin Elmer spectrum 100, FTIR spectrometer.

3.2.5.5 **Differential scanning calorimetry (DSC)**

Thermograms were obtained using a Mettler Toledo DSC 822e differential scanning calorimeter. Lyophilized samples were dried in a vacuum desiccator;

approximately 5 mg of the dried powder was crimped in a standard aluminium pan and heated from 25 - 350°C at a heating rate of 10°C/min under constant purging of nitrogen at 20 ml/min. All samples were run with a reference.

3.2.6 Quantitative analysis of loaded tagette oil

3.2.6.1 Preparation of standard solutions

Stock solution of tagette oil was prepared by dissolving 2000 mg tagette oil in 1 L chloroform (2000 mg/L). The tagette oil stock solution was further diluted with chloroform to obtain the different standard solutions. The standard solutions were prepared at concentrations of 20.48, 51.20, 128.00, 320.00 mg/L. The standards were then run on the Shimadzu 2450 UV-Visible spectrometer at a wavelength range of 300-260 nm, in triplicates. A standard calibration curve was obtained.

3.2.6.2 Assay of drug content

Accurately weighed (1000-1500 mg) nanoparticles were transferred into a test tube. Then tagette oil was extracted from the ALG/CS nanoparticles by 10 ml phosphate buffer saline (PBS) solution (pH 1.5) containing tween 80®. This solution was then vortexed vigorously for 15 minutes. Chloroform (5 ml) was added into the solution and shaken for 1 hour at 171 rpm.[6] The solution was then centrifuged at 5000 x g for 60 minutes, and the yellow supernatant was collected and quantified spectrophotometrically at 300-260 nm range.

3.2.6.3 Encapsulation efficiency (EE%) and percentage yield (%) of nanoparticles

To determine the EE% of nanoparticles, the nanoparticle suspension (obtained after the preparation of oil-loaded nanoparticles) was centrifuged at 5000 x g for 60 minutes, to separate nanoparticles from the aqueous medium containing nonassociated tagette oil. The free tagette oil in the supernatant was extracted using chloroform (5 ml) and quantified spectrophotometrically. The tagette oil EE% of the nanoparticles was calculated using the following equation[7];

$$EE\% = \frac{Total\ amount\ of\ T.oil - Free\ T.oil}{Total\ amount\ of\ T.oil} \times 100 \quad \text{.....3.1}$$

Direct GC analysis of the encapsulated nanoparticles that remained allowed for a quick verification of the EE%

The percentage yield (w/w) was calculated from the weight of dried nanoparticles recovered (w_1) and the sum of the initial dry weight of starting materials (w_2).[8]

$$Yield\ (\%) = \frac{W1}{W2} \times 100 \quad \text{.....3.2}$$

3.2.7 *In Vitro*-release studies

3.2.7.1 **PART A For oral administration**

The oil release study was performed using the dialysis method.[9] In short, lyophilized tagette oil-loaded nanoparticles obtained from one set of preparations were re-dispersed in 20 ml PBS solution (pH 1.5 and 7.4). This suspension was filled in a dialysis bag (dialysis tubing cellulose membrane D9777-100 FT, cut-off point 1000 Da) and the bag placed in 100 ml PBS solution (pH 1.5 and 7.4). For time-dependent release study at time intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 48, 60, 72, 84, 96 and 108 hours. The set up was kept at 37 °C under gentle stirring (100 rpm). At the periodic intervals, samples of the release medium were taken out and spectrometrically assayed for the amount of tagette oil released at 250-300 nm, and the volume was replaced with fresh dissolution medium. These studies were carried out in triplicate. The data represent the average from the three independent experiments.

The % release was calculated as follows;

$$Release\ \% = \frac{Released\ T.oil}{Total\ T.oil} \times 100 \quad \text{.....3.3}$$

3.2.7.2 **Determination of total tagette oil released**

The total tagette oil released from the nanoparticles was determined using the following equation:

$$\sum[\%\ Analyte\ 2 - 0.8\ (\%\ Analyte\ 1)] = Total\ \%\ Released \quad \text{.....3.4}$$

Where analyte 1 is the amount of tagette oil found in the first sample of the release medium and analyte 2 is the amount of tagette oil found in the second sample.

3.2.7.3 **PART B For topical administration**

ALG/CS nanoparticles may also be used for controlled-release of fragrances in the cosmetics industry. To study the release of tagette oil volatiles (fragrance) in a topical administration, equal amounts of aqueous cream (3 g) were placed in two vials, A and B. Free tagette oil was added in vial A and tagette oil-loaded ALG/CS nanoparticles (with the same concentration of tagette oil as that of the free tagette oil) were added in vial B. The vials were then placed in a water bath at 30°C. At specific time intervals (6, 12, 18, 24 hours), tagette oil volatiles were trapped in a GC (gas chromatography) syringe and injected into the GC.

GC analysis of the oil volatiles was conducted using GC (SRI 8610 GC) with a flame ionization detector. A split inlet was used to inject volatiles into a Restek MXT-5 column (0.25 mm id x 15 m and 25 μm film thickness). The oven temperature was raised from 60°C to 200°C at 10°C/min with nitrogen as the carrier gas at a constant flow of 1 ml/min. The split ratio of 1:50 was used for all analyses.

The quantity of essential oil components and their principal components in GC were determine by the external standard method. The standard calibration curve was generated by analysis of known concentrations of tagette oil dissolved in chloroform. Quantitative data was obtained from FID peak areas. The components of the oil were identified by comparison with data published in literature.[10]

3.2.7.4 **Statistical analysis**

All experiments were performed in triplicates and data are expressed as mean values ± standard deviation (SD). Statistical analysis was performed using Graph Pad software with *p* value less than 0.05 considered as an indication of statistical significance.[11]

3.3 Results and discussion

Preparation of ALG/CS nanoparticles containing tagette oil was successfully carried out using a multistep process of o/w emulsion, gelification and solvent removal. Dispersion of diluted to oil in an aqueous ALG solution containing Tween 80® caused immediate formation of micelles with an oil core. The ALG shell was then solidified by crosslinking with $CaCl_2$ and CS, which occurs spontaneously via electrostatic interaction between the negatively charged carboxylate groups on ALG and the positively charged calcium ions and/or protonated amine groups on CS.[5] Finally, the solvent, ethanol used to dilute the oil was removed from the system by evaporation under reduced pressure. The following sections discuss the results obtained under varying conditions.

3.3.1 Effect of the ALG:CS mass ratio on the average size and zeta potential of nanoparticles.

The size and zeta potential of nanoparticles play a key role in their mechanism of cellular uptake and the stability of nanoparticle suspension. Particles with a diameter of 50-500 nm have been proven to have the best properties for cellular uptake, with a range of 100-400 nm indicated as ideal.[12] In this study it was observed that the nanoparticle size and zeta potential were strongly dependent on the amount of ALG added (**Figure 3.1 (a), Appendix 1 and 2**), with a higher amount of ALG in the formulation producing larger nanoparticles. The minimum average size of 190.5 nm, corresponds to the lowest ALG:CS mass ratio (1.3:1) and the maximum average size, 589.7 nm, corresponds to the highest ALG:CS mass ratio (20:1). These results confirm that smaller nanoparticles result when the

availability of the functional groups on the two polymers for interaction are in stoichiometric proportion.[13]

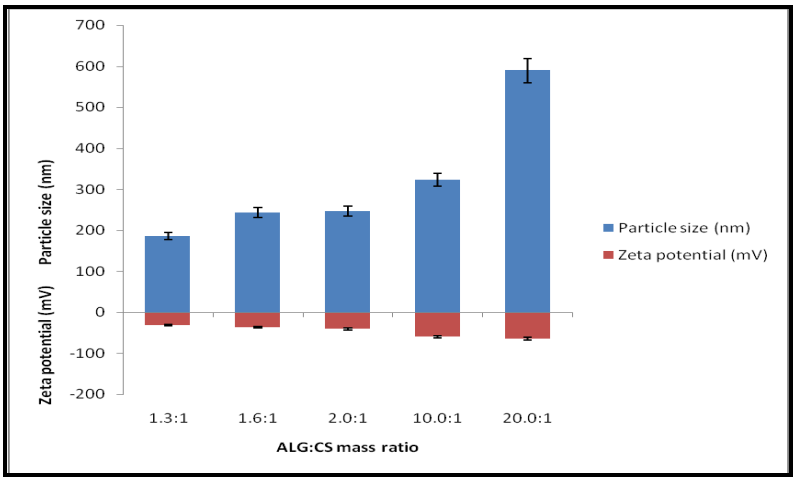

Figure 3.1 (a): Effect of ALG:CS mass ratio on size and zeta potential of blank ALG/CS nanoparticles

On the other hand, the zeta potential of the nanoparticles was also less strongly affected by the amount of ALG added, but followed a similar trend. As the polymer concentration increased from 0.06% to 1% there was a corresponding increase in zeta potential from -30.6 to -63.9 mV (**Figure 3.1 (a)**). This can be ascribed to the higher availability of negatively charged carboxylate groups with increasing ALG concentration. This higher electric charge on the surface of the nanoparticles can produce strong repellent forces among the particles to prevent their aggregation in solution, so the particles can all be considered as stable.[14] This higher charge may also partly explain the increase in size, with adjacent polymer chains remaining further apart, producing larger (and possibly less dense) particles.

The loading of tagette oil into the nanoparticles did not have a significant influence (p>0.05) on the average size of the nanoparticles. However the presence of the oil slightly decreased the zeta potential of the nanoparticles, e.g. for 0.06% ALG formulation, the zeta potential decreased from -30.6 to -26.1 mV for blank nanoparticles and oil loaded nanoparticles, respectively (**Figure 3.1 (b)**). This reduced negative potential of oil loaded nanoparticles confers a reduced repulsive force between the negative biological cells and the lipophilic drug-loaded

nanoparticles thus minimizing the undesirable rapid elimination of nanoparticles from the blood circulation and facilitate their accumulation in infected cells (such as tumour sites).[15]

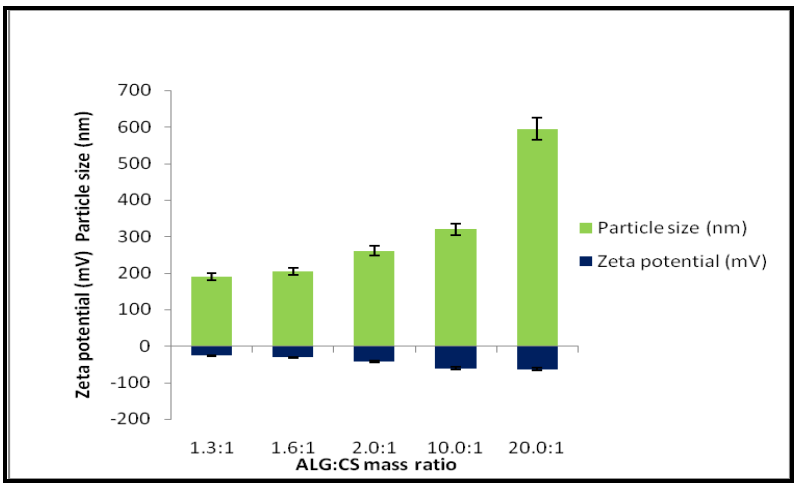

Figure 3.1 (b): Effect of ALG:CS mass ratio on size and zeta potential of tagette oil-loaded ALG/CS nanoparticles

3.3.2 Effect of pH on nanoparticles

From **Table 3.1**, it can be observed that pH 4.7 was the most ideal for nanocomplex formation of the polyelectrolytes, since the smallest size, high EE% and yield (%) were observed at this pH. At pH 4.7 the carboxyl groups of the ALG are ionized and the amine groups of the CS are protonated, which is most important for optimum interaction and the polyionic complex formation.[16] When the pH was reduced to 4.2, much larger particles were obtained. At this pH ALG approaches its pKa value (~3.5) and starts to precipitate as alginic acid. This precipitation and aggregation of ALG leads to the increased particle size and decrease in EE % of nanoparticles. Similar observations were made when the pH was increased to 5.2. At this pH, CS approaches its pKa value (~6) and precipitation may occur, resulting in less CS available for nanoparticle formation.[17]

Table 3.1: Effect of pH on size, EE% and yield (%) of nanoparticles obtained with ALG:CS mass ratio 1.3:1 (n=3, ± SD).

pH of nanoparticle formulation	Size (nm)	EE(%)	Yield (%)
4.2	1245 ± 23.1	46 ± 5.6	49 ± 2.7
4.7	190 ± 20.5	83 ± 5.1	72 ± 4.2
5.2	798 ± 11.9	52 ± 4.8	58 ± 5.5

3.3.3 Physical stability of the optimized tagette oil-loaded nanoparticle suspension

Change in average size and zeta potential were also used to determine the physical stability of the tagette oil-loaded ALG/CS nanoparticles after storage at 4^{o}C and 25^{o}C for a period of four months. The physical stability data in **Table 3.2** show that the size and zeta potential of the tagette oil loaded nanoparticles did not differ significantly (p> 0.05) at either temperature during storage. These results suggest that the use of CS under the optimized conditions gives nanoparticles with good physical stability. This may be due to the stability effect of the polyelectrolyte complex of ALG and CS, which reduces the porosity of ALG particles.[5]

Table 3.2: Physical stability of ALG/CS nanoparticles containing tagette oil (at 1.3:1, ALG:CS mass ratio) after storage at 4°C and 25°C for a period of 4 months.

Time (days)	Average size (nm) ± S.D.		Zeta potential (mV) ± S.D.	
	4°C	25°C	4°C	25°C
0	190 ± 23.6	190 ± 23.6	-26 ± 3.3	-26 ± 3.3
60	187 ± 20.3	185 ± 43.6	-26 ± 0.5	-24 ± 0.4
120	205 ± 39.8	200 ± 21.5	-28 ± 2	-27 ± 8.3

3.3.4 Morphology studies

3.3.4.1 TEM analysis

The microscopy analysis confirmed the presence of nanoparticles and provided morphological information of the typical tagette oil loaded ALG/CS nanoparticles. With the TEM, particles were seen to be spherical, distinct and regular (**Figure 3.2**). The nanoparticle coating, plays a predominant role in protecting the active substance incorporated and in the release profile.[18] The nanoparticles loaded with tagette oil, did not show a smooth surface but a rough appearance (**Appendix 3**).

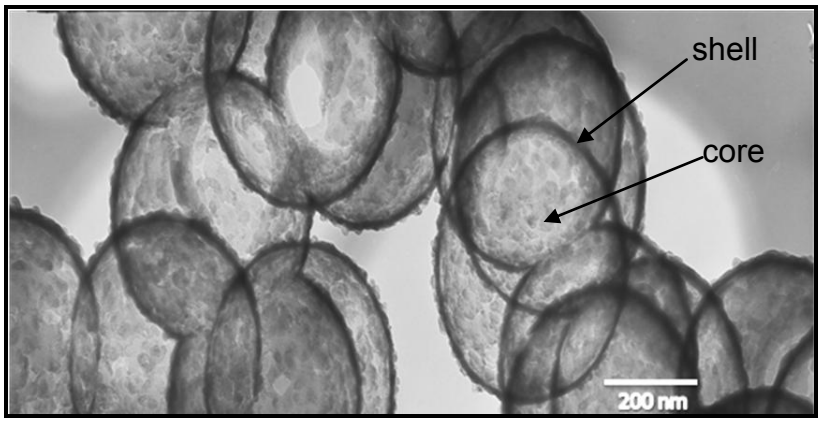

Figure 3.2: TEM image of oil loaded ALG/CS nanoparticles: magnification x50 000

3.3.4.2 **SEM analysis**

Figure 3.3 (a) below shows the SEM images of the ALG/CS particles. Nanoparticles' surface showed a regular shape and morphology related to a generic spherical shape. The electron microscope was coupled to an EDX spectrometer and the spectra obtained (**Figure 3.3(b)**) was used to confirm elements present in the nanoparticle matrix. The EDX spectra of the nanoparticles revealed that the main elements C, O, Ca, Cl and Na were present in the samples, indicating that the expected composites from ALG and CS and $CaCl_2$ used during nanoparticle preparation exist in the matrix. The presence of C could be determined by coating the particles with a thin film of gold rather than the usual sputtered carbon.

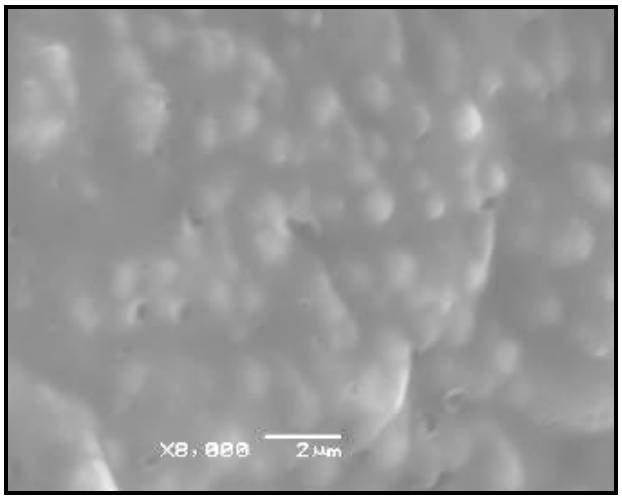

Figure 3.3 (a): SEM micrograph of ALG/CS nanoparticles

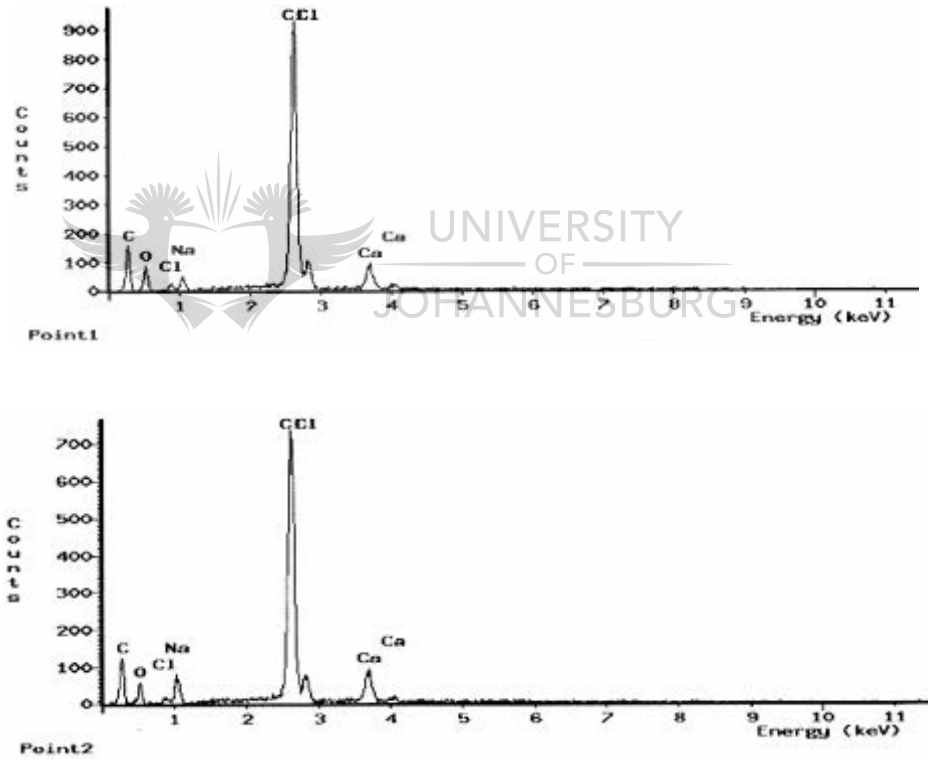

Figure 3.3 (b): EDX spectra of two batches of ALG/CS nanoparticles.

3.3.5 Structure analysis

3.3.5.1 FTIR evaluation

In order to examine the relationship between components of nanoparticulate systems, more attention was paid on polyelectrolytes interactions and tagette oil entrapment, since it has been well established that the carboxyl group (COO^-) of the anionic polymer may interact with the amino group ($-NH_3^+$) of chitosan and form an ionic complex between the two compounds.[19] Changes in the FTIR spectra in the absorption bands of the amino groups, carboxyl groups and amide bonds were monitored.

Figure 3.4 (a), represent FTIR spectra of NaALG, CS and blank ALG/CS nanoparticles. In the spectrum of CS, the broad band at 3349 cm^{-1} corresponds to the amine (N-H) and hydroxyl (OH) groups. The bending vibrations of the N-H (N-acetylated residues, Amide II band) were observed at 1587 cm^{-1}. The bands at 1594 cm^{-1} and 1406 cm^{-1} present in the spectrum of NaALG were assigned as asymmetric and symmetric stretching peaks of carboxylate salt (COO^-) groups, respectively.

Consequently in the IR spectrum of blank ALG/CS, it was observed that the asymmetric stretching of COO^- groups shifted to 1572 cm^{-1} and the symmetric stretching of COO^- groups shifted to 1408 cm^{-1}. The absorption band at 1587 cm^{-1} for the amide band of CS shifts to 1572 cm^{-1} after the reaction with ALG. The stretching vibration of OH and NH_2 at 3349 cm^{-1} shifts to 3264 cm^{-1} and broadens and the peak absorbance of amino groups of CS at 1150 cm^{-1} was present after complexation at 1100 cm^{-1}

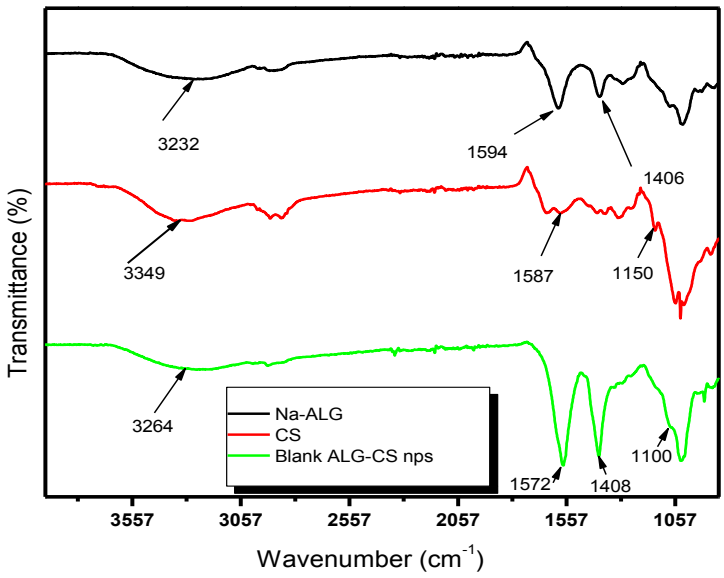

Figure 3.4 (a): IR spectra of NaALG, CS and blank ALG-CS nanoparticles

These results, also summarized in **Table 3.3 (a)** below, suggest an effective interaction between the two polymers i.e. the carboxylic groups of ALG associated with ammonium groups of CS through electrostatic interactions to form the polyelectrolyte complex.

Table 3.3 (a): Diagnostic IR bands for ALG-CS PEC formation

Functional groups in Na-ALG and CS	Band position in components (cm^{-1})	Shift in blank nanoparticles (cm^{-1})
Asymmetric stretching COO-	1594	1572
Symmetric stretching COO-	1406	1408
Amine (N-H) and Hydroxyl (OH)	3349 (Broad)	3264
Secondary amine (N-H)	1587	1572
Amino (C-N)	1150	1100

The introduction of tagette oil into nanoparticles was also investigated by FTIR analysis of oil loaded nanoparticles and tagette oil (**Figure 3.4 (b)).** Pure tagette oil displayed peaks at 2965 and 2913 cm^{-1} assigned to alkyl groups, 1700 cm^{-1} (C=O Ketones), 1674 cm^{-1} (COO^- Carboxylic acids), 1620 cm^{-1} (C=C alkenes) and 1441 cm^{-1} (C-C aromatic rings). All these characteristic absorption bands of tagette oil appeared in the oil loaded ALG/CS nanoparticles, with slight shifts due to the interaction between the polymers and the oil. This also indicates that tagette oil molecules were incorporated in the polymeric network. **Table 3.3 (b)** summarizes the most important bands of tagette oil and tagette oil loaded nanoparticles after IR characterization.

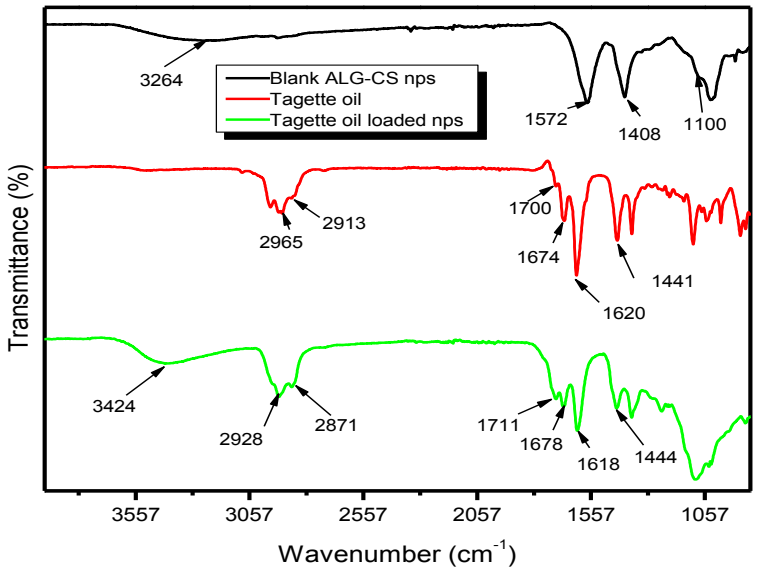

Figure 3.4 (b): IR spectra of tagette oil, oil loaded and blank nanoparticles

Table 3.3 (b): Diagnostic IR bands for incorporation of tagette oil in nanoparticles

Functional groups in tagette oil	Band position in tagette oil cm^{-1}	Shift in oil loaded nanoparticles cm^{-1}
Alkyl groups (C-H)	2965	2928
Aromatic ketones	2913	2871
Ketones (C=O)	1700	1711
Carboxylic acids (COO-)	1674	1678
Alkenes (C=C)	1620	1618
Aromatic rings	1441	1444

3.3.5.2 **DSC analysis**

The thermogram of ALG/CS physical mixture (made by mixing 1.3 g of ALG with 1 g of CS prior to analysis) showed a broad endothermic peak at 98°C which probably represents the combination of the two endothermic polymer peaks for the expulsion of water. Exothermic peaks at 240°C and 306°C resulted from individual contributions of ALG and CS, respectively, which are identical to their respective endothermic peaks as pure compounds. It was observed, however, that the endothermic and exothermic peaks of the complexes shifted from those of physical mixture to form one broad exothermic peak at around 260°C. Moreover, although peaks of physical mixture were a combination of each polymer, they were different from those of the nanoparticles, because complexation of polyelectrolytes results in new chemical bonds formed (**Figure 3.5**).

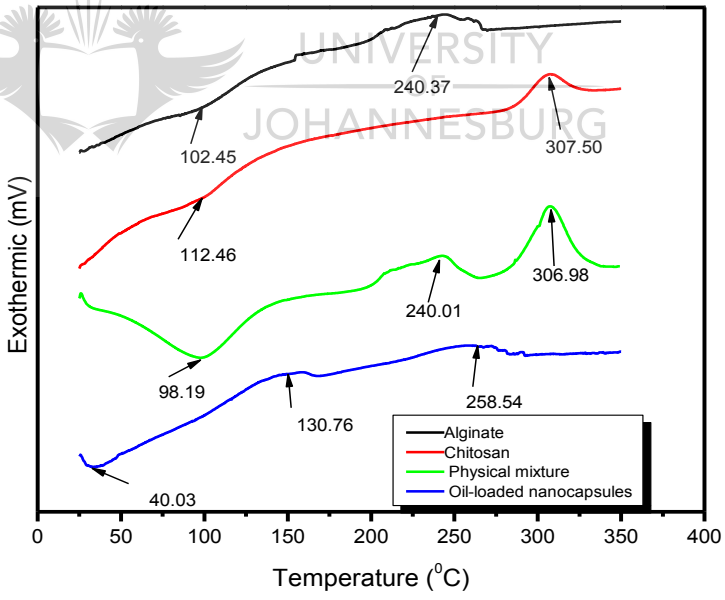

Figure 3.5: DSC thermograms of ALG, CS and physical mixture of ALG and CS and tagette oil loaded nanoparticles

Thermograms of tagette oil loaded nanoparticles displayed an endothermic peak at a very low temperature (40°C) compared to the physical mixture. The tagette oil

is a mixture of components, so its boiling range is around 180°C, but in the nanoparticles this decreases significantly to give an endothermic peak from 130°C to about 170°C. Thus an interaction between the oil and the polyelectrolytes is postulated to have occurred and the oil was incorporated in the complex.

The results for the characteristic endothermic and exothermic peaks for ALG and CS used to make nanoparticles are summarized in **Table 3.4.**

Table 3.4: Diagnostic endothermic and exothermic peaks for complexation of ALG and CS

Components	Endothermic peak (°C)	Exothermic peak (°C)
ALG	103	240
CS	113	307
Physical mixture	98	240, 307
Oil-loaded ALG-CS nanoparticles	40, 150	259

3.3.6 Assay of drug content in nanoparticles

The amounts of free and encapsulated tagette oil (representing the free and encapsulated drug, respectively) were determined using UV-Vis spectroscopy and GC/FID analysis. No interferences were observed from blank nanoparticles directly or in solution, neither was there any interference from the other components used during preparation (such as Tween 80, ethanol, and trichloromethane). The content of tagette oil was determined from calibration curves ($y = 0.009x + 0.086$) and $r^2 = 0.9765$ for the UV-Vis analysis and ($y = 216.9x$

- 2.756, r^2 = 0.995) for GC (**Appendix 4**). Samples were analysed in triplicate, and the average (EE%) of the optimized nanoparticles was found to be 83.6%.

The EE% of the nanoparticles decreased when the concentration of ALG used was increased. Also, there existed an inverse relationship between the particles size and tagette oil encapsulation (**Figure 3.6**). This unusual result can be explained on the basis of the fact that larger nanoparticles are likely to have more water making up the bulk of the nanoparticle matrix, and therefore presenting both a less favourable environment for the oily drug, and a more "leaky" capsule. An attempt to verify this by integrating the exothermic water peak for various nanoparticles was not successful due in part to the broad nature of the peak as explained above.

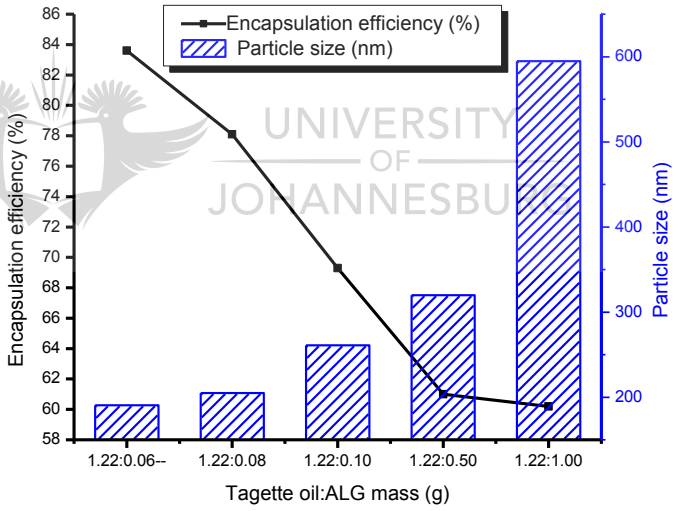

Figure 3.6: Effect of drug : polymer ratio on particle size and EE%

3.3.7 *In vitro* release study

3.3.7.1 Part A: For oral administration

Figure 3.7 shows the release profiles of tagette oil from ALG/CS nanoparticles at different pH (at 37 ± 0.5 °C) as a function of time. It was observed that the release of the nanoparticles occurred in a controlled manner. In the first 12 hours only 21%

of tagette oil was released from the nanoparticles at pH 1.5 (and a significantly higher 30% at pH 7.4). The release profile was characterised by an initial “burst” effect in the two media within the first 10 hours, followed by a gradual increase up to 36 hours and finally by a continuos and sustained release phase up to 108 hours.

The initial burst release indicate that a significant amount of tagette oil associated with nanoparticles remained on their surfaces held by weak interactions forces between polyelectrolytes and oil and was displaced rapidly by the solvent. The tagette oil encapsulated within the matrix would face an additional physical barrier and take longer to be washed free of the particles, which has been observed in the literature but cannot be confirmed in this study.[20]

The release of tagette oil from ALG/CS nanoparticles in the medium at pH 7.4 was much faster than that for nanoparticles in the medium at pH 1.5, in the same period. This can not only prevent potential drug loss in an acid environment (e.g. the gastric fluid) but also controls drug release in the GI tract. In total over the 108 hours, only 56% was released at pH 1.5, while 69% was released at pH 7.4. This suggests that the drug release of ALG/CS nanoparticles is pH sensitive and more of the drug will be released in the blood system (pH 7.4) than in the acidic stomach (pH 1.5).[6] Therefore more of the drug will be absorbed into the blood system and in turn transported in the blood circulatory system to the site of infection more efficiently than when the drug is not encapsulated in the nanoparticles. By extrapolation of the graph at pH = 7.4, and considering the same steady-state release, we could estimate that it would take around 250 hours to completely release all the drug.

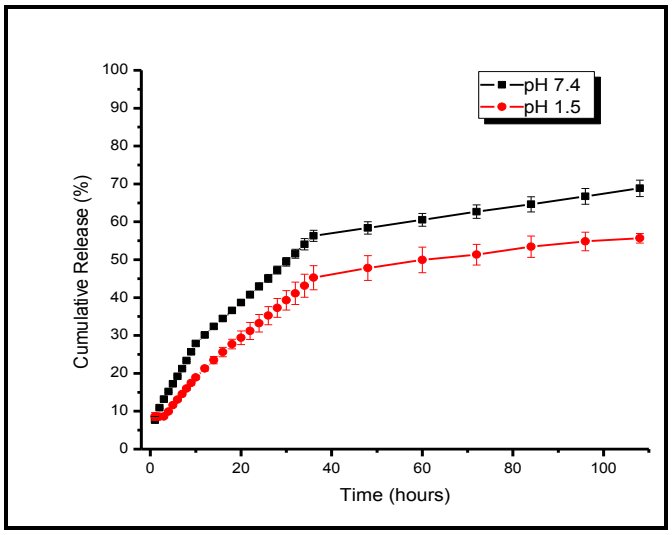

Figure 3.7: The cumulative release curves of tagette oil from ALG-CS nanoparticles in various pH (7.4 and 1.5) at 37 °C

3.3.7.2 **PART B: For topical administration**

The major component of tagette oil is a terpene called ocimene, which is responsible for the sharp floral smell of tagette oil. This component was used to monitor the amount volatiles released from a formulation of the nanoparticles in aqueous cream. This was determined from a calibration curve of ocimene (y = 216.9x - 2.756, r^2 = 0.995), see **appendix 4.**

Figure 3.8 and Appendix 5 show that a higher amount of the volatile component, ocimene was released from the aqueous cream with free tagette oil when compared to that with tagette oil encapsulated in ALG/CS nanoparticles in the first 6 hours. The concentration dropped drastically after 12 hours. Whilst the tagette oil volatiles encapsulated in ALG/CS nanoparticles were released in a slow and controlled manner. This implies that the fragrance/perfume released could be slowed down by oil incorporation in nanoparticles instead of being delivered in oil droplets. The nanoparticles prevent the rapid evaporation of the fragrance components to create a once-a-day application with a prolonged effect over several hours. Anecdotal evidence for this effect was collected by asking colleagues to smell the creams with and without nanoparticles. Those formulations

with no nanoparticles lost their smell within a day, while the other formulation was still distinctly floral after three days.

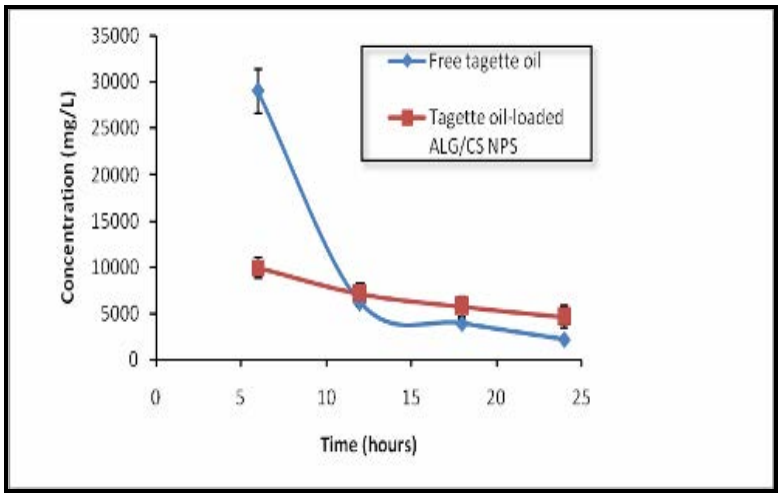

Figure 3.8: The release profile of tagette oil volaties in aqueous cream.

3.4 Conclusion

ALG/CS nanoparticles, a prospective carrier for sustained release of bioactives were prepared by a modified ionic pre-gelation method and characterized. The small size and negative zeta potential for tagette oil-loaded ALG/CS nanoparticles and blank ALG/CS nanoparticles indicate their potential to provide bioadhesion and easy cellular uptake in the GIT if used as carriers for hydrophobic drugs. FTIR analysis of the nanoparticles demonstrated the evidence of crosslinking between positively charged amino groups and negatively charged carbonyl groups. It also showed evidence for the incorporation of tagette oil components into the nanoparticle matrix. The nanoparticles also showed good efficiency for entrapment of tagette oil and clear evidence that the mass ratio of the two polymers and pH of their solutions are crucial for particle size, zeta potential, encapsulation efficiency and yield.

Two potential applications of these nanoparticles were demonstrated through the release of a mixture of oily compounds under pH sensitive conditions, and through the slow release of a fragrance (ocimene) in a cosmetic application.

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CHAPTER 4

PREPARATION AND CHARACTERIZATION OF

β-CYCLODEXTRIN-TAGETTE OIL INCLUSION COMPLEX

*

4.1 Introduction

In the previous chapter, tagette oil loaded ALG/CS nanoparticles were found to have an ideal size and zeta potential for delivery of lipophilic drugs. However, while the encapsulation efficiency (EE%) of the nanoparticles was quite high, 83%, a lot of tagette oil was lost during preparation. More importantly, we felt that we might be able to improve the release profile of the tagette oil. In acidic medium the release was greater than 50% of the total loaded oil within 48h, and showed a "burst release" profile within the first few hours, possibly due to some oil on the surface of the nanoparticles. As a result the amount of oily drug released in neutral or alkaline media (and say absorbed into blood circulatory system) may not be ideal under therapeutic conditions.

The use of cyclodextrins and other guest molecules to encapsulate lipophilic water-insoluble drugs has gained in popularity recently. Much of our interest in CDs arose precisely from the ease of forming encapsulated inclusion complexes with hydrophobic molecules of suitable size, where such complexes alter physical, chemical and biological properties of the encapsulated guest molecules (**Figure 4.1**).[1] This method is also simpler and cheaper than most other methods of encapsulation.[2]

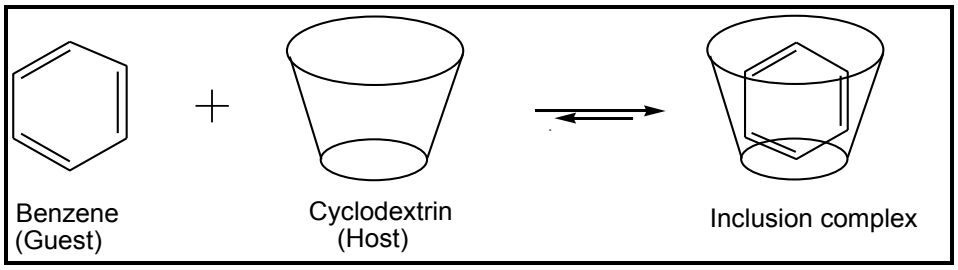

Figure 4.1: Schematic representation of an inclusion complex formation

Of the commonly available CDs, the intermediate sized *β*CD and its derivatives has been used in pharmaceutical formulations to enhance solubility, dissolution rate, membrane permeability and bioavailability of water insoluble drugs.[2] It has been proposed that the incorporation of CDs into polymeric drug delivery systems could change the drug-polymer interaction and as a result the mechanism of drug release may be modified.[3]

By taking advantage of CD complexation with organic compounds, an attempt was made to increase the encapsulation efficiency (EE%) and achieve a more controlled release of tagette oil. The aim of this chapter and of **Chapter 5** is to investigate the effects of adding *β*CD to the structure and properties of the ALG-CS nanoparticle system. This chapter deals specifically with the preparation of *β*CD-tagette oil inclusion complex, while the next chapter examines the incorporation of the complex in the ALG-CS system. Changes in the physicochemical properties provide methods to confirm whether guest molecules are really included in the CD cavity.

4.2 Experimental procedure

4.2.1 Materials

*β*CD was purchased from Sigma Aldrich, South Africa and commercially available tagette oil, was kindly provided by Clive Teubes cc, South Africa. All other chemicals were of analytical grade and purchased from Sigma Aldrich, South Africa.

4.2.2 Preparation of *β*CD-tagette oil inclusion complex

An inclusion complex of tagette oil and *β*CD was prepared by the co-precipitation method. Tagette oil (0.67 g in ethanol 1 ml), was slowly added drop wise to a solution of *β*CD in water (0.67 g in 200 ml) at 55°C. The solution was stirred continuously at 330 rpm for 4 hours as the solution was slowly cooled to room temperature and then to 4°C. The mixture was then kept at 4°C overnight. The cold precipitated material was recovered by centrifugation, frozen in liquid nitrogen and freeze dried for 24 hours. The powder was stored in airtight sample vials and kept in a desiccator at room temperature prior to analysis.[1]

4.2.3 Characterization of *β*CD-tagette oil inclusion complex

4.2.3.1 Fourier transform infrared (FTIR) spectroscopy

FTIR was used to determine the potential interactions between *β*CD and tagette oil. The FTIR spectra of tagette oil, *β*CD, their physical mixture and the inclusion complex were collected between 4000 and 600 cm^{-1} using a Perkin Elmer spectrum 100, FTIR spectrometer.

4.2.3.2 UV-Vis spectroscopy

The UV-Vis absorption spectra were recorded for tagette oil, *β*CD, their physical mixture and the inclusion complex. Each sample was dissolved in methanol (to give 0.100 mg/L concentration of tagette oil) at room temperature. The solutions were scanned from 400 to 200 nm to obtain UV-Vis absorption spectra.

4.2.3.3 ^{1}HNMR spectroscopy

Proton NMR analysis was performed on a Bruker DRX-400 spectrometer at 25 °C. The residual signal of DMSO-d_6. (∂ = 2.50 ppm for ^{1}H) was used as the internal reference. The samples were prepared by dissolving a small amount of the samples in approximately 0.5 ml of DMSO-d_6 solvent. Proton chemical shifts are reported in parts per million (ppm).

4.2.3.4 **Powder x-ray diffraction (XRD)**

XRD patterns were obtained using a X'Pert PAN analytical diffractometer with Cu Kα radiation (40 kV). All powder samples were mounted on sample holders and scanned between 2θ = 4° and 60°.

4.2.3.5 **Differential scanning calorimetry (DSC)**

DSC analysis was carried out for *β*CD and the inclusion complex. Each dry powder (5-6 mg) was heated in a crimped aluminium pan at a scanning rate of 10°C/min from 25 to 350°C under a nitrogen flow of 20 ml/min. An empty pan sealed in the same way was used as a reference.

4.2.3.6 **Scanning electron microscopy (SEM)**

The surface morphology of the CD-oil complex was examined by SEM, JEOL JSM-7500F. Prior to examination, samples were prepared by mounting about 0.5 mg of powder onto a 5 x 5 mm double sided carbon tape, on an aluminium stub. The powder was then sputter-coated for 40 seconds with carbon and then the samples were examined with SEM.

4.3 Results and discussion

An analysis of the supernatant from the preparation of the CD complex showed that there was no tagette oil nor ocimene (the major constituent of tagette oil) remaining in solution. This implies that there was a 1:1 (w/w) complex formed. Since tagette oil is a mixture it is difficult to determine what the molar ratio of the complex is, but given that the molecular weight of ocimene ($C_{10}H_{16}$) is 136.24 g/mol, and that of *β*CD ($C_{42}H_{70}O_{35}$) is 1135 g/mol, it is safe to estimate that we should have a 1:8 molar ratio of oil to CD.

4.3.1 FTIR analysis

The FT-IR spectra of the *β*CD, tagette oil, their physical mixture and inclusion complex are presented in **Figure 4.2**. The spectrum of tagette oil shows strong absorption bands at 2965 cm^{-1} (alkyl C-H stretch), 1700 cm^{-1} (ketone C=O stretch), 1681 cm^{-1} (carboxylic acid C=O stretch) and 1620 cm^{-1} (aromatic C=C bending). The IR spectrum of the physical mixture shows a superimposition of the peak of *β*CD and tagette oil.

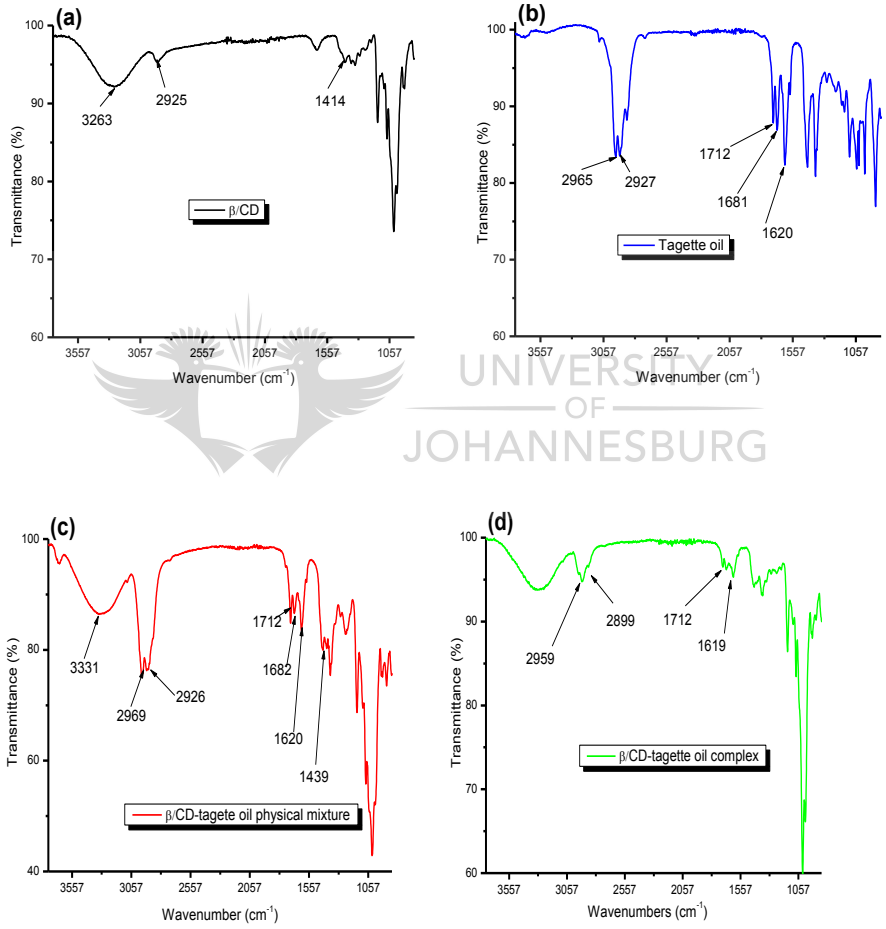

Figure 4.2: FTIR spectra of (a) *β*-CD, (b) tagette oil, (c) their physical mixture and (d) inclusion complex

However, the FTIR spectrum of the inclusion complex predominantly showed the absorption bands of *β*CD. The C-H and OH absorption peaks of tagette oil

originally at 2965 and 2927 cm^{-1} slightly shifted to 2958 and 2899 cm^{-1} and greatly reduced in intensity. So did the stretching vibration bands of C=O and -C=C- which shifted from 1700 to 1712 cm^{-1} and 1620 to 1619 cm^{-1} respectively. The reduction in intensity and shift of tagette oil absorption peaks suggest the possible formation of an inclusion complex where compounds of tagette oil are incorporated inside the *β*CD cavity.[4]

4.3.2 **UV-Vis analysis**

The obtained absorption spectra of tagette oil, *β*CD and tagette oil physical mixture and *β*CD/oil complex are presented in **Figure 4.3** below. *β*CD had no UV absorption, since it does not contain any chromophore in the 200 to 400 nm region. The maximum absorbance wavelength of tagette oil was 294 nm, representing the UV absorption band for a conjugated diene system (**Figure 4.3(a)**). The spectrum of tagette oil and *β*-CD physical mixture (**Figure 4.3(b**)) showed a peak at (293 nm) which was almost identical with that of tagette oil alone. However, the absorption maxima of *β*CD/oil complex is significantly hypsochromically shifted to 274 nm compared to that of tagette oil alone. This large blue shift of ca. 20 nm for tagette oil, strongly suggests that an interaction between tagette oil and *β*CD existed.[5] This shift has been linked to an increase in hydrogen bonding as the guest enters the restricted cavity of the cyclodextrin. It is known that H-bonding lowers the energy levels of some orbitals resulting in a shorter wavelength transition. This was observed, for example in the complexation of hydrocortisone butyrate[6]

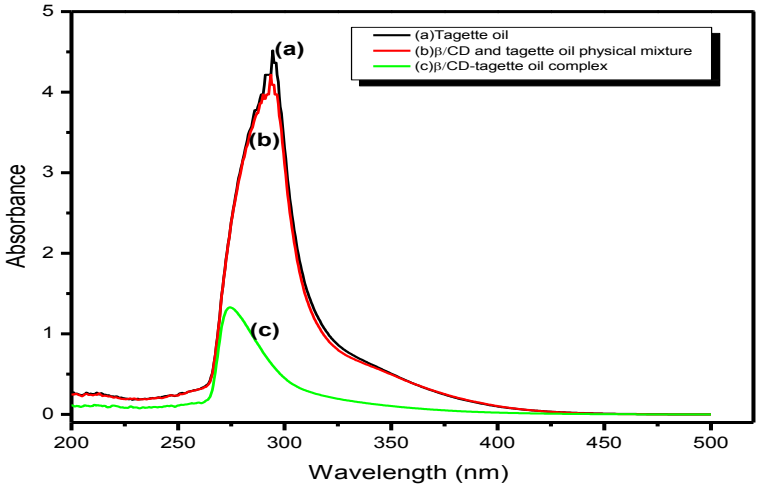

Figure 4.3: UV-Visible spectra of (a) tagette oil, (b) *β*CD-tagette oil mixture and (c) *β*CD-tagette oil complex

4.3.3 ^{1}H NMR analysis

The ^{1}H NMR spectra of *β*-CD, and βCD-tagette oil inclusion complex and the NMR chemical shifts are shown in **Figure 4.4** and **Table 4.1**, respectively. See **appendix 6** for ^{1}HNMR spectra of tagette oil and βCD-tagette oil physical mixture. From **Table 4.1** and **Figure 4.4 a) and b)**, it can be observed that the H-3 and H-5 protons of *β*CD, located inside the hydrophobic cavity, shifted downfield during the formation of the inclusion complex. The other *β*CD protons (H-1, H-2, H-4, H-6) presented relatively small and insignificant changes in the chemical shifts. The downfield shifts of the protons located within the *β*CD cavity (H-3 and H-5) as well as the presence of minor peaks of tagette oil compounds in the *β*CD oil complex spectrum, confirmed the inclusion of tagette oil compounds into the *β*CD cavity. The changes in the position of the two protons signals located inside the cavity are often taken as indications of successful incorporation of guest compounds into the CD cavity.[7]

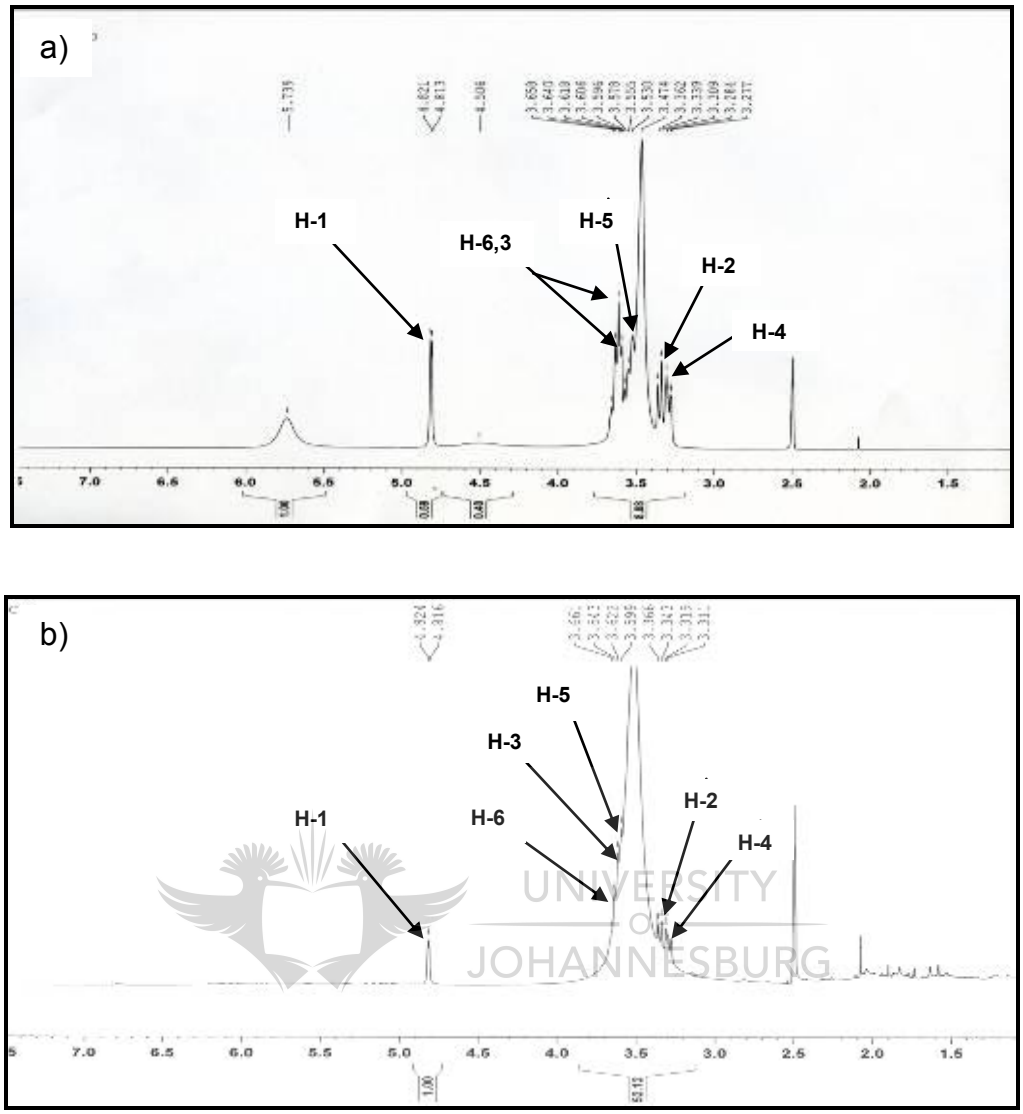

Figure 4.4: ^{1}H NMR spectra of (a) *β*CD and (b) *β*CD-tagette oil complex

Table 4.1: Chemical shifts (∂) of βCD and βCD-tagette oil complex.

Proton of β-CD	β-CD ∂ (ppm)	Complex ∂ (ppm)	Δ∂ (ppm)
H-1	4.821	4.824	0.003
H-2	3.362	3.366	0.004
H-3	3.555	3.622	0.067
H-4	3.339	3.343	0.004
H-5	3.530	3.599	0.069
H-6	3.640	3.643	0.003

Δ∂ = ∂(complex)-∂(β-CD)[8]

4.3.4 XRD analysis

The powder x-ray diffraction (XRD) patterns of βCD and the inclusion complex of βCD and tagette oil are illustrated in **Figure 4.5.** The XRD pattern of βCD (**Figure 4.5 (a)**) showed intense, sharp peaks that indicate the crystalline nature of the compound. On the other hand, the XRD pattern of βCD-oil complex (**b**) revealed a greatly reduced crystallinity in the complex system as evidenced by fewer and broader peaks of lower intensity. This indicates the formation of the inclusion complex between βCD and tagette oil, which has probably disrupted the crystal lattice of βCD.[9]

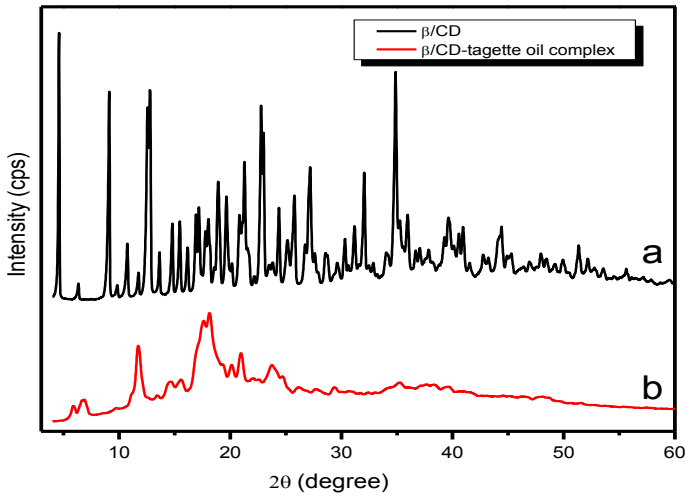

Figure 4.5: The X-ray diffraction of (a)βCD and (b)βCD/oil complex

4.3.5 DSC analysis

The thermal curves of βCD and βCD/oil complex are shown in (**Figure 4.5**). Owing to its crystalline nature, βCD showed a broad endothermic peak at 125°C. CDs do not really have a melting point but rather decompose at around 300°C. A different pattern was observed in the thermogram of the βCD/oil complex. A new small peak at 219°C together with the shifting and broadening of β-CD characteristic band (from 125 to 116°C) is indicative of a change in the substance structure and an interaction between tagette oil and βCD.[10] In many cases the band from the guest has a much lower intensity or is lost altogether, which is again taken as an indication of guest-host complexation.

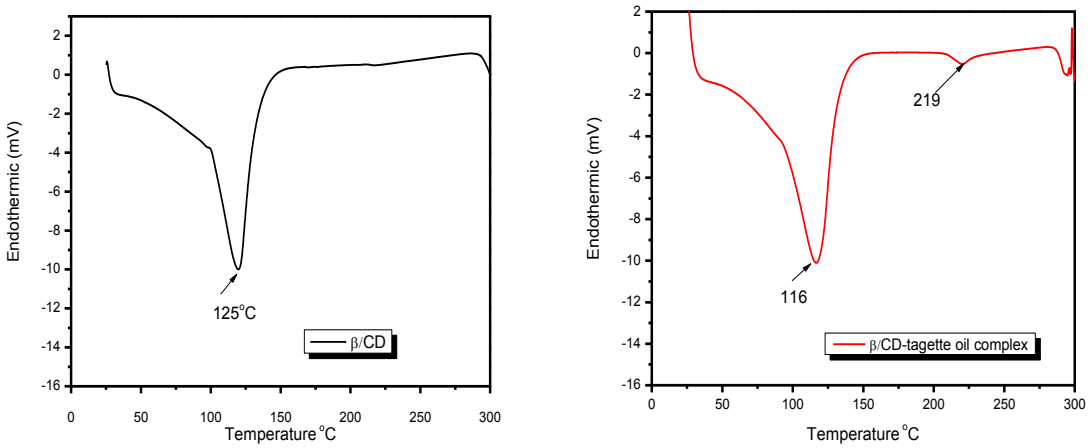

Figure 4.6: DSC thermograms of (a) *β*-CD and (b) *β*-CD/tagette oil complex

4.3.6 SEM analysis

The surface morphology of the powders derived from *β*-CD, *β*-CD/oil complex, were assessed by SEM. As illustrated in **Figure 4.7**, the conformation of the complex (b) is different from that of the *β*CD alone (a). *β*CD has a rough surface, with pits and crests, whilst the *β*CD/oil complex has a regular brick-like or rectangular shape. This shows that the inclusion complex is structurally distinct from the isolated components, *β*CD and tagette oil.

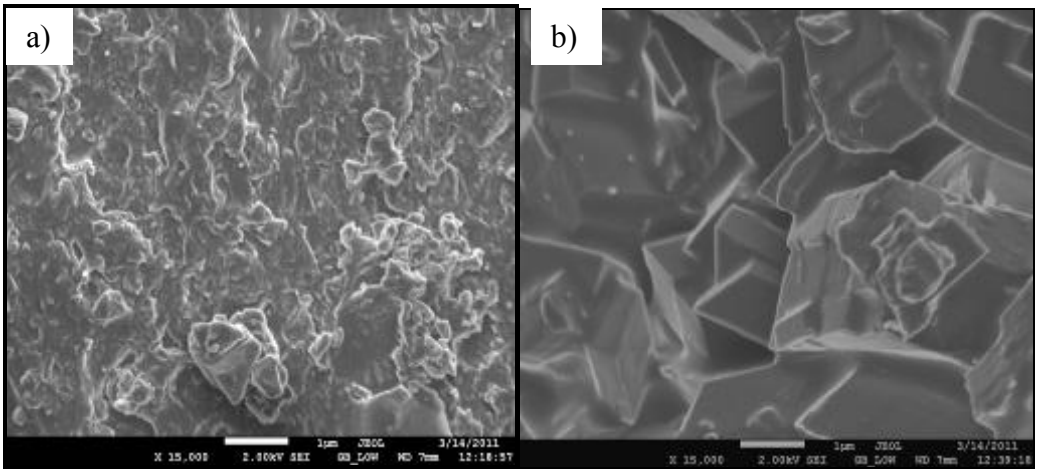

Figure 4.7: SEM images of (a) *β*-CD and (b) *β*-CD/tagette oil complex

4.4 Conclusion

The inclusion complex of tagette oil and *β*CD was successfully prepared by the co-precipitation method. The different analytical methods considered (FTIR, UV-Vis, DSC XRD, SEM and ^{1}HNMR) in combination provided evidence of the complex formation, particularly with NMR making clear the complexation effect on the H-3 and H-5. Whilst the XRD showed that the process of inclusion results in new patterns on the molecular structure as the *β*CD interacts with tagette oil. All the characterization techniques demonstrated that the *β*CD/tagette oil complex has different physicochemical characteristics from free tagette oil, suggesting that the tagette oil is incorporated into the *β*CD cavity and thus behaves differently.

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CHAPTER 5

PREPARATION AND CHARACTERIZATION OF βCD-TAGETTE OIL LOADED ALGINATE/CHITOSAN NANOPARTICLES.

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5.1 Introduction

An ideal oral drug delivery system should provide a stable environment to ensure that the main fraction of the therapeutic compound will be biologically active during both particle processing and drug release.[1] Nanoparticles are stable systems suitable for providing targeted drug delivery and to enhance the efficacy and bioavailability of poorly soluble drugs. However the efficacy of nanoparticles are sometimes limited by their low entrapment efficiency that may lead to excessive administration of polymeric material or their poor release profiles (such as sudden or "burst" release) that could lead to undesired effects.[2]

In drug delivery, the concept of entrapping a drug as a guest-host complex and in turn encapsulating these complexes in nanoparticles combines the advantages of both CDs (such as increasing the stability of drugs) and nanoparticles (such as targeting of drugs) into a single system.[3] It has been proposed that the incorporation of CDs into polymeric drug delivery systems could change the drug-polymer interaction and as a result the mechanism of drug release may be modified.[4]

This chapter reports on a formulation of βCD/tagette oil-loaded alginate/chitosan nanoparticles as shown in **Figure 5.1**. The βCD forms a guest complex with the oil, and retains it within the core of ALG/CS nanoparticles, which may decrease degradation of the encapsulated lipophilic compounds in gastric environment and

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allow controlled release.[5] The encapsulation efficiency and *in vitro* release profile of this system were also investigated.

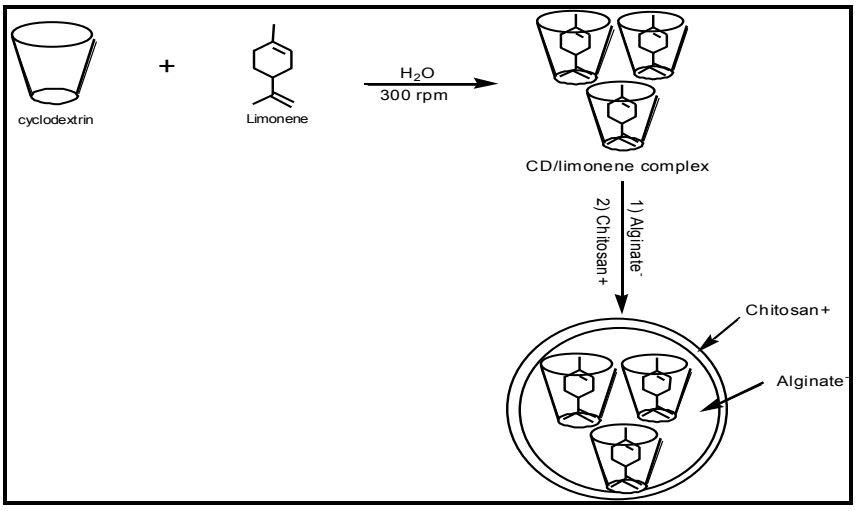

Figure 5.1: Schematic representation of the complexation of tagette oil compound by *β*CD in alginate/chitosan nanoparticles

5.2 Experimental

5.2.1 Preparation of *β*CD/tagette oil loaded ALG/CS nanoparticles

The *β*CD/tagette oil inclusion complex was prepared as described in **section 4.2.2** To the inclusion complex solution, 10 ml of ALG solution (0.063%) was added dropwise, while stirring (at 500 rpm) for 15 minutes. Then 4 ml of $CaCl_2$ (1%) solution was added dropwise into the solution for 15 minutes while stirring. Finally, 4 ml CS solution (0.05%) was dropped into the pre-gel. The nanoparticle suspension was maintained under stirring for 30 minutes to improve curing. This solution was then kept at 4°C overnight to equilibrate. After which the nanoparticles were collected by centrifugation at 10 000 x g for 60 minutes and freeze dried for 24 hours.[5]

5.2.2 Characterization

5.2.2.1 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to determine the potential interactions between the *β*CD/oil complex and the ALG and CS. The infra-red spectra for the complex and the nanoparticles were obtained using a Perkin Elmer spectrum 100 FTIR spectrometer.

5.2.2.2 Nanoparticle size and zeta potential

To measure the size the nanoparticles, 5 ml of sample was sonicated after redispersing in water and placed into the analyzer chamber. Readings were collected at 25°C with a detector angle of 90° using a Malvern Zetasizer and Particle Analyzer 5000 (Malvern Instruments, UK).

5.2.3 Morphology study

5.2.3.1 TEM

The morphology of the nanoparticles was studied by a Transmission Electron Microscope (TEM) using a JEOL Electron Microscope JEM 2100 (200.00 kV). The samples were dispersed in ethanol, sonicated for 10 minutes and mounted on carbon coated copper grids before examination.

5.2.3.2 SEM

The nanoparticles were also observed using Scanning Electron Microscope (SEM). Samples of nanoparticles were mounted on glass slides with carbon tape, carbon coated under vacuum and then examined on a JEOL JSM 6700F SEM.

5.2.4 Quantitative analysis of loaded tagette oil

Experimental procedure to determine the amount of tagette oil loaded into nanoparticles, the encapsulation efficiency (EE%) and experimental yield (%) of nanoparticles was carried out as described in **Section 3.2.6.2** and **3.2.6.3**, respectively.

5.2.4.1 *In vitro* release studies

To study the release profile for the nanoparticles, the method described in **Section 3.2.6.4** was followed.

5.2.4.2 Statistical analysis

All experiments were performed in triplicates and data are expressed as mean values ± standard deviation (SD). Statistical analysis was performed using Graph Pad software with *p* value less than 0.05 considered as indicative of statistical significance.[6]

5.3 Results and discussion

As with the previous ALG-CS nanoparticles (**Chapter 3**), the synthesis, yield and isolation of the nanoparticles was dependent on a number of factors especially the ratio of ALG to CS, but also temperature and pH. In order to compare the effects of adding CD to the system, the ideal conditions from Chapter 3 (e.g. ALG/CS = 1.3:1) were followed in these investigations, and the effects are discussed in the following sections.

5.3.1 FTIR analysis

FTIR analysis was used as an indication of the incorporation of the three components. The FTIR spectra of *β*CD/oil complex and *β*CD/oil-loaded ALG/CS nanoparticles are shown in **Figure 5.2**. In the spectrum of nanoparticles the broad band at 3349 cm^{-1} for the stretching vibration of OH (hydroxyl groups) broadens

and has increased intensity owing to the increased number of OH groups, due to the presence of the three polysaccharides. The bending vibration of N-H, amide II band at 1587 cm^{-1} and the asymmetric stretching of COO^- groups at 1572 cm^{-1} are characteristic of the presence of ALG and CS, respectively.

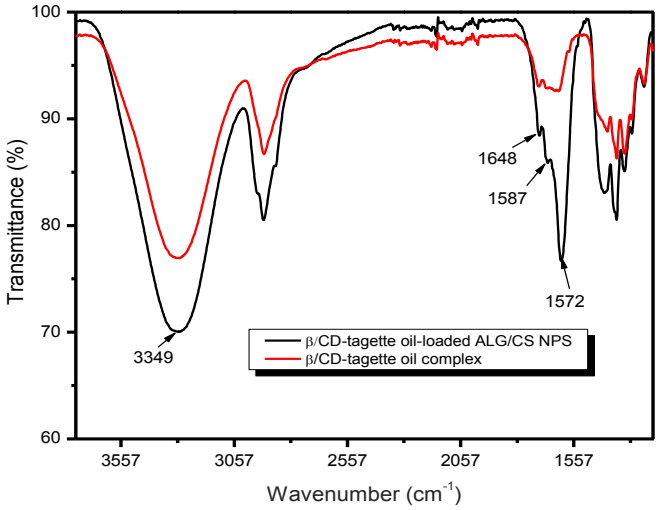

Figure 5.2: FTIR spectra of *β*CD/oil loaded ALG/CS nanoparticles and *β*CD/oil complex

5.3.2 TEM analysis

The TEM images (**Figure 5.3**) show that most particles are of irregular but generally spherical shape. This effect has been observed in our laboratory on the addition of cyclodextrin to nanoparticle systems, but has not yet been reported. The particles have a size that ranges from 50-100 nm. It was also observed that the particles have a tendency to agglomerate which may affect their stability.

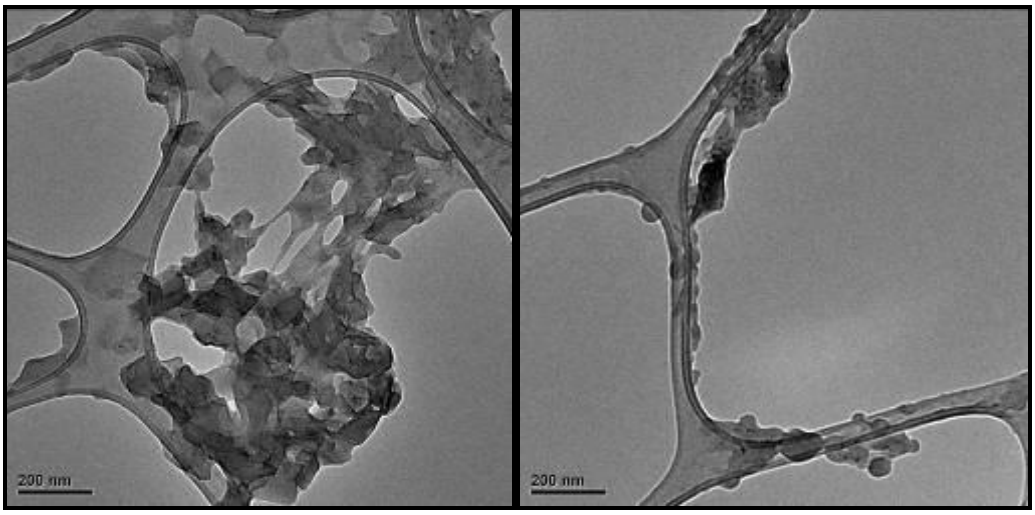

Figure 5.3: TEM images of *β*-CD/oil loaded ALG/CS nanoparticles

5.3.3 SEM analysis

From the SEM images (**Figure 5.4**), it was observed that the *β*CD/oil complex (a) has a regular brick-like or rectangular morphology. On the other hand, the particles (b) produced after coating the inclusion complex with ALG and CS were spherical in shape with a smooth surface. The difference in morphology of the complex from the nanoparticles show that the addition of ALG and CS brings some significant change to the system.

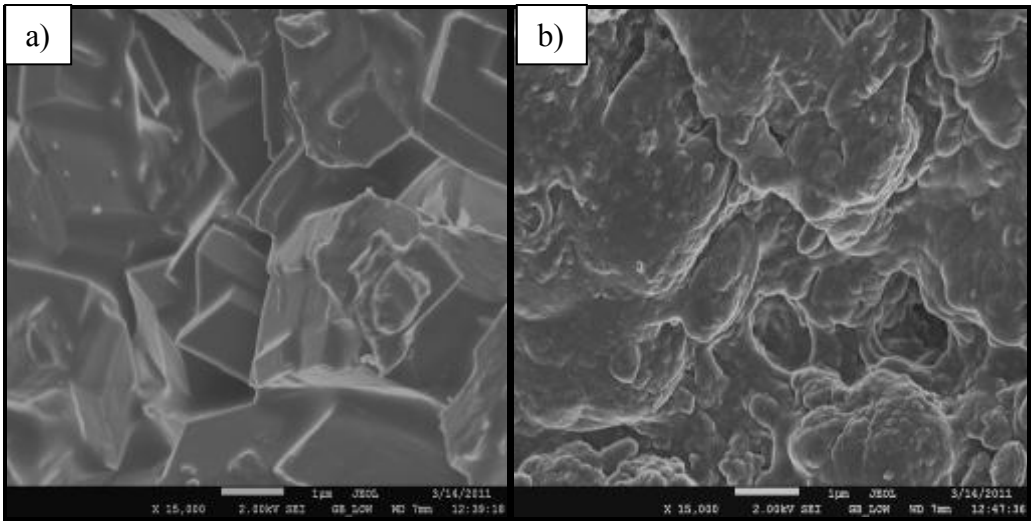

Figure 5.4: SEM images of a) *β*-CD/oil complex, b) *β*-CD/oil-loaded ALG/CS nanoparticles

5.3.4 Nanoparticle size and zeta potential

The mean size of tagette oil loaded nanoparticles was affected by the ALG/CS mass ratio and the *β*CD content as shown in **Table 5.1**. It seems that *β*CD exerts a significant influence on the particle size, since all the systems loaded with *β*CD/tagette oil complex are much smaller than those loaded with tagette oil alone. This may be because the strong electrostatic attraction between *β*CD and the lipophilic compounds of the tagette oil can condense the nanoparticle into a more compact system.[5] In addition, the results from Chapter 3 indicate that the tagette oil has a slightly positive zeta potential and causes an increase in the particle size when added to the ALG-CS nanoparticles. Therefore, the complexation of the oil within the cyclodextrin should "neutralise" this positive charge effect and hence cause the nanoparticles to contract.

Table 5 1: Comparing the particle size and zeta potential of *β*CD/oil loaded ALG/CS nanoparticles at pH 4.7

	(*β*-CD:ALG:CS) mass ratio	**Particle size (nm)**	**Zeta potential (mV)**
1	**2:1.3:1**	109 ± 12.1	-32 ± 2.5
2	**6:1.3:1**	160 ± 13.0	-37 ± 5.1
3	**6:0:0**	100 ± 15.8	-3 ± 2.9
4	**0:1.3:1**	190 ± 20.5	-31 ± 3.6

The negative zeta potential values were obtained due to the presence of polymer terminal carboxylic groups (COO^-) of ALG. All the zeta potential values are more negative than -30 mV (-32, -37 and -31 mV) except *β*CD-tagette oil complex (-3 mV), which means that the *β*CD-ALG/CS nanoparticles can be predicted to be stable and not likely to agglomerate due to the high-energy barrier between

particles.[7] The addition of CD to the system (e.g. **Table 5.1** row 1 vs row 4) caused a decrease in particle size and an increase in the value of the zeta potential.

5.3.5 Assay of drug content in nanoparticles

Preparation of nanoparticles from preformed inclusion complexes of tagette oil and *β*CD proved to be an effective method to enhance drug loading to nanoparticles. The data in **Table 5.2** expressed in terms of encapsulation efficiency suggest that nanoparticles containing *β*CD have a higher encapsulation efficiency (93%) than ALG/CS alone.[3] This may be attributed to the highly hydrophobic cavity of the *β*CD which increases the affinity of tagette oil compounds for the *β*CD thus resulting in more tagette oil compounds being incorporated in the ALG/CS nanoparticles containing *β*CD. The experimental yield (%) of nanoparticles obtained when *β*CD were added into the system, also increased by 15% when compared to ALG/CS alone.

Table 5.2: The difference in the yields (%), EE% and amount (%) tagette oil released of the three formulations.

			Drug release (%) in 108 hours	
Formulations	**Yield (%)**	**EE (%)**	**pH 7.4**	**pH 1.5**
ALG/CS NPs	72 ± 4.2	83 ± 2.6	69 ± 5.6	56 ± 3.0
***β*-CD/oil complex**	82 ± 4.7	95 ± 3.8	95 ± 0.3	40 ± 0.1
***β*-CD/oil-ALG/CS NPs**	87 ± 5.6	93 ± 0.4	78 ± 0.4	39 ± 0.1

5.3.6 *In vitro* release studies

The release patterns from the different nanoparticle formulations were biphasic, comprising of an initial quick release followed by a sustained, continuous phase. The first phase was attributed to the immediate dissolution and release of the portion of the drug located on or near the surface of the nanoparticles, whilst the second continuos phase is due to the slow drug release from the CD cavity and ALG/CS polymer matrix.[3] Future work (see **Chapter 6**) could include verifying and minimising this effect.

In vitro release profiles of tagette oil from the two types of nanoparticles were assessed using the dialysis method. It was demonstrated that the encapsulated oil was released from the nanoparticles in its stable form for more than 4 days. All the nanoparticle systems exhibited an initial release of approximately 8.5% and the remaining oil was liberated with a controlled release profile as shown in **Figure 5.5.** The *β*-CD/oil complex released tagette oil at a higher rate than the *β*CD/oil-ALG/CS nanoparticles.

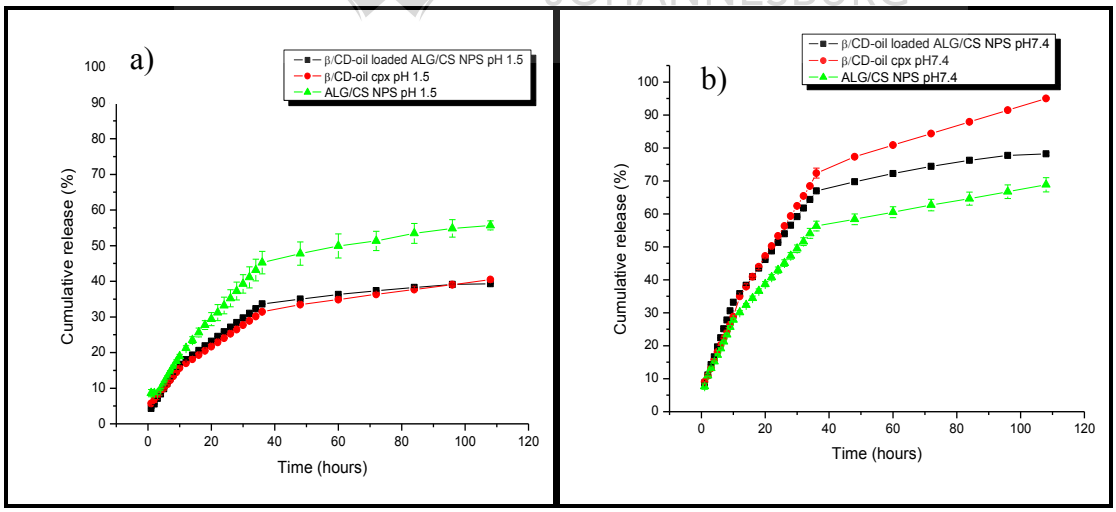

Figure 5.5: *In vitro* release profile of tagette oil from nanoparticles into PBS solution, pH 1.5 (a) and 7.4 (b), (n=3 ± S.D.).

In vitro release of tagette oil was clearly delayed from nanoparticles containing *β*CD. This is most obvious at lower pH when compared with the previous study

performed with ALG/CS nanoparticles in acidic medium. In the case of the CD-containing system, only 26% of the oil was released after 24 hours, whilst 33% was released in ALG/CS nanoparticles without CDs. This suggests that *β*-CD nanoparticles can be more effective in protecting the loaded drug in the gastric fluid, allowing a greater percentage of the drug to reach the small intestines which is the main absorption site into the blood system.[8]

However, when *β*CD-ALG/CS nanoparticles were placed in higher pH media (pH 7.4) it was observed that they released the oil at a faster rate than ALG/CS nanoparticles alone. In 24 hours 52% oil and 42% was released from *β*CD-ALG/CS nanoparticles and ALG/CS nanoparticles, respectively. This may be attributed to the fact that drug solubility affects the release rate. For a drug with low solubility, CDs can enhance its release and for a drug with higher solubility, CDs can retard its release. In the case of a drug with low solubility (such as tagette oil), the formation of inclusion complex can increase its solubility, leading to drug concentration and the release rate increasing in aqueous media.[9] This allows easy absorption and transportation of the lipophilic drug by the blood into infected body cells. Again future work could include determining if the CD-oil complex is released from the nanoparticles before the oil is liberated, or whether the oil is released within the nanoparticle itself.

5.3.7 **Statistical analysis**

The paired t-test was employed to compare the amount (%) of drug released from ALG/CS nanoparticles and *β*CD-ALG/CS nanoparticles.[10] This allowed us to determine whether the addition of *β*CD in ALG/CS nanoparticles had an effect on the behaviour of the nanoparticles in terms of their release profile in acidic and neutral media.

From the data in **Table 5.3** it was observed that the mean difference of the amount of tagette oil released at pH 1.5 and pH 7.4 was -7.16% and 6.99% with a confidence interval (CI) of -9.03 to -5.29 % and 5.60 to 8.37 %, respectively. The *p*-value for these relationships was found to be less than 0.05 which indicates that

the difference between these two groups was unlikely to be due to chance. The data therefore suggests that *β*CD-ALG/CS nanoparticles release less of the lipophilic drug in acidic media when compared to ALG/CS nanoparticles alone. Whilst in higher pH conditions the *β*CD-ALG/CS nanoparticles release more of the drug as compared to ALG/CS nanoparticles.

Table 5.3: The statistical difference between ALG/CS nanoparticles and *β*CD-ALG/CS nanoparticles

	ALG/CS NPS			***β*CD-ALG/CS NPS**				
Variable	**M%**	**SD**	**SEM**	**M%**	**SD**	**SEM**	**95% C I**	**P value**
pH 1.5	30.12	±15.99	2.93	22.96	±11.03	2.05	-7.16	<0.0001
pH 7.4	38.72	±18.43	3.42	45.71	±21.97	4.08	6.99	<0.0001

5.4 Conclusion

*β*CD-tagette oil loaded ALG/CS nanoparticles were synthesized by encapsulating the *β*CD-tagette oil complex with the ALG/CS PEC. The *β*CD formed a complex with the tagette oil and retained it within the core of ALG/CS PEC which greatly reduced the size of the nanoparticles. The inclusion complex formation led to a higher yield, and better EE (%) of *β*CD-ALG/CS nanoparticles (10% higher than the EE (%) of ALG/CS nanoparticles alone). Tagette oil release rate was lower in the acidic medium when compared to neutral pH. This suggests that the addition of *β*CD to ALG/CS nanoparticles is more effective in protecting the loaded drug in the gastric fluid (acidic medium) and increases the solubility of the hydrophobic drug in the intestinal and blood fluid, and could allow improved absorption and bioavailability of the drug *in vivo.*

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CHAPTER 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS.

*

6.1 General conclusions

The following conclusions were drawn based on the aims and objectives of the study:

- ALG/CS nanoparticles were prepared successfully by ionotropic pre-gelation of an ALG core with $CaCl_2$ followed by crosslinking with CS for polyelectrolyte complexation. This was confirmed by TEM, SEM, IR and DSC analysis. The characterization techniques revealed that the nanoparticles were spherical in shape with a mean diameter of 190 nm and a zeta potential more negative than -30 mV, which ensures their physical stability and render them suitable for uptake by cells in the human body system. The optimum conditions for the formation of these particles was determined and found to rely strongly on pH, ratio of ALG:CS and temperature.

- PEC formation between ALG and CS and tagette oil loading was confirmed by FTIR, DSC, XRD, and UV-Vis spectroscopy. The IR spectra of oil-loaded ALG/CS nanoparticles showed ketones absorption bands characteristic of tagette oil and also showed that carboxylic groups of ALG associated with ammonium groups of CS through electrostatic interactions to form the PEC. Exothermic peaks of physical mixture were different from those of the nanoparticles, which also indicate that complexation of the PECs (ALG and CS) results in new chemical bonds formed.

- The EE (%) of the ALG/CS nanoparticles was 83.6% and it was observed that these nanoparticles released the tagette oil in a pH-dependent manner.

This implies that the ALG/CS nanoparticles are stimuli-responsive hydrogels which show sudden changes in their swelling behaviour as a result of changing the external pH. This property allows for modulated release of the incorporated drug. The release pattern was biphasic comprising of an initial burst effect followed by a sustained, continuous phase.

- GC analysis confirmed that tagette oil evaporation was significantly reduced by being incorporated into ALG/CS nanoparticles, when compared with a conventional emulsion formulation of free tagette oil and aqueous cream. The nanoparticles prevent the rapid evaporation of the fragrance components to create a once-a-day application with a prolonged effect over several hours.

- An inclusion complex between *β*CD and tagette oil was formed using the co-precipitation method in a mass ratio of 1:1. The results of the UV-visible spectroscopy, FTIR, DSC and XRD demonstrated that *β*CD/tagette oil complex has different physicochemical characteristics from free tagette oil. ^{1}HNMR spectroscopy showed downfield shifts of *β*CD cavity protons (H-3 and H-5), indicative of successful incorporation of tagette oil compounds into the CD cavity. This complex formation was confirmed using several spectroscopic techniques.

- *β*CD/tagette oil-loaded ALG/CS nanoparticles were successfully prepared by coating the *β*CD/oil complex with the ALG/CS PEC. The particle analyser showed that the addition of *β*-CD to ALG/CS nanoparticles reduced their size and made the zeta potential more negative from (-31 to -37 mV). This make *β*CD-ALG/CS nanoparticles more stable against agglomeration and more effective in promoting the uptake of lipophilic drugs by the human body cells.

- The incorporation of *β*CD into ALG/CS nanoparticles also enhanced the experimental yield (%) and EE (%) (increased by 15% and 10%,

respectively) of the nanoparticles. It also showed improvement in the *in vitro* release profiles of the ALG/CS, by releasing the oil in a more controlled and sustained manner. The *β*-CD ALG/CS nanoparticles released less oil in acidic medium, which means less of the lipophilic drug would be released in gastric environment, thus drug is protected from degradation in the stomach and is delivered in the small intestines, where more absorption of molecules into the blood occurs.

- These results indicate that ALG/CS nanoparticles are a promising carrier system for the controlled delivery of hydrophobic drugs and the incorporation of *β*CD can enhance the overall performance of the system by reducing the particle size whilst increasing the encapsulation efficiency and reducing the amount of drug released in an acidic medium, allowing a larger percentage of the drug to be absorbed into the blood and carried to site of infection resulting in more efficient disease treatment.

6.2 Recommendations

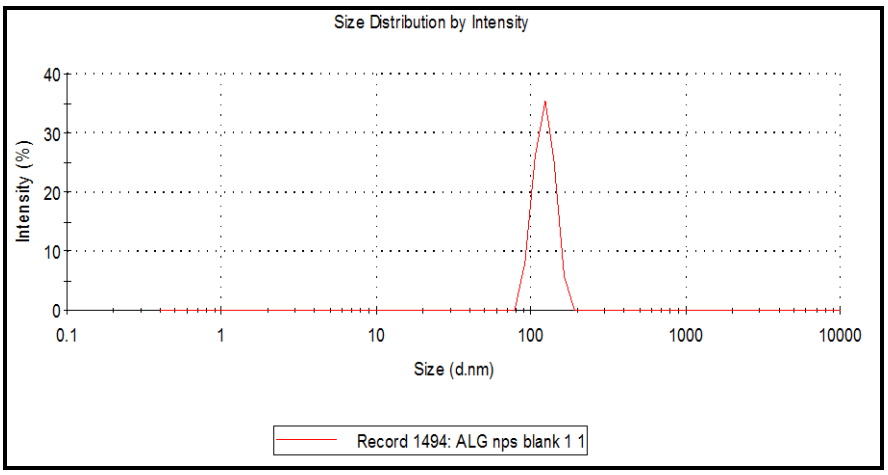
- A clinically significant drug that is poorly soluble in water could be used in this study to evaluate the pharmacological activity of the drug before and after loading in nanoparticles. To determine if the efficacy of the associated bioactive compound is not affected by the preparation processes of the nanoparticles. Since a lot of work has been done on anti-cancer drugs like paclitaxel, this is one option for further investigation.

- The mechanism of incorporation of the drug in the CD/ALG/CS system needs to be investigated so the release profiles can be better understood and tailored as needed.

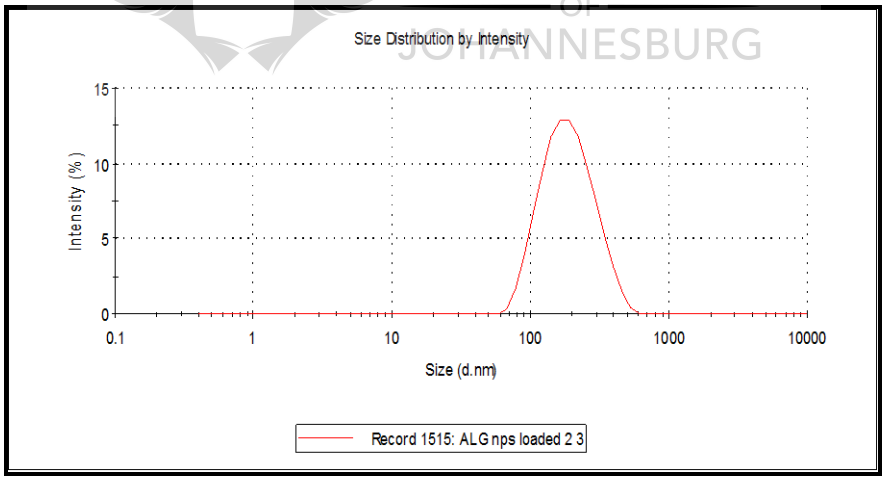
- Release profiles for the system were only carried out *in vitro*. There is still a need to test the feasibility of the application of *β*CD-ALG/CS nanoparticles *in vivo*. This would also allow for the testing of toxicity and compatibility of the systems.

APPENDIX 1

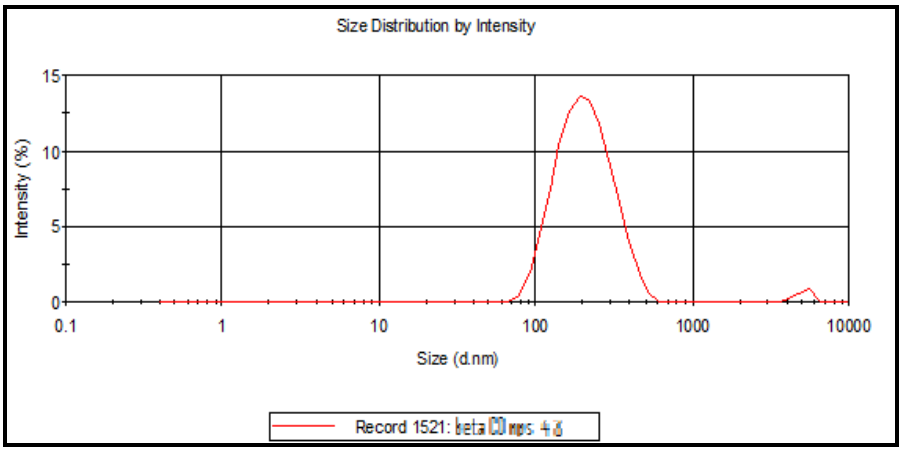
SIZE DISTRIBUTION CURVES



Size distribution curve for blank ALG/CS nanoparticles



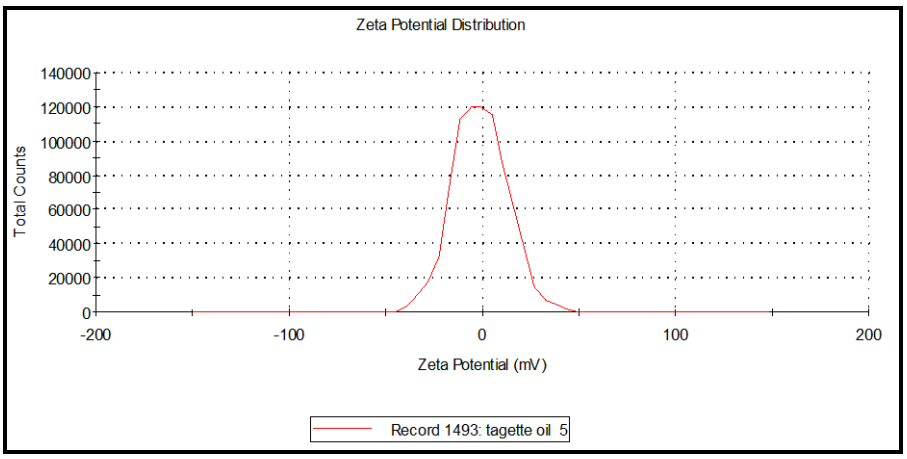
Size distribution curve for tagette oil-loaded ALG/CS nanoparticles



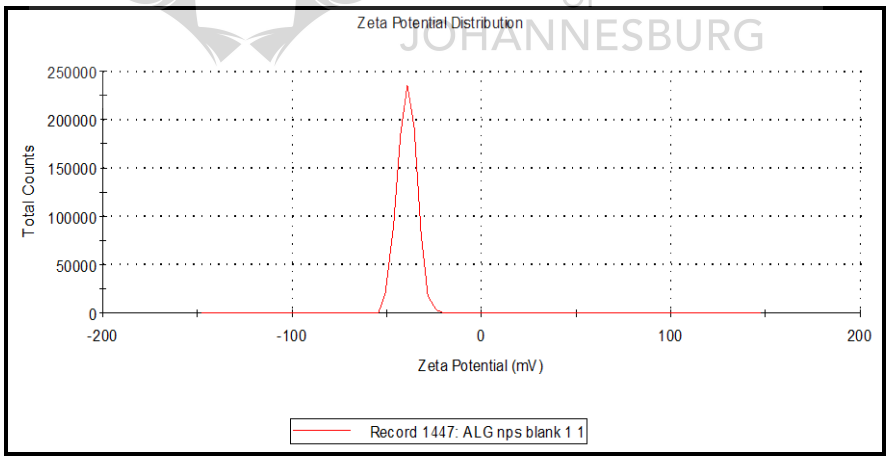
Size distribution curve for βCD/tagette oil-loaded ALG/CS nanoparticles

APPENDIX 2

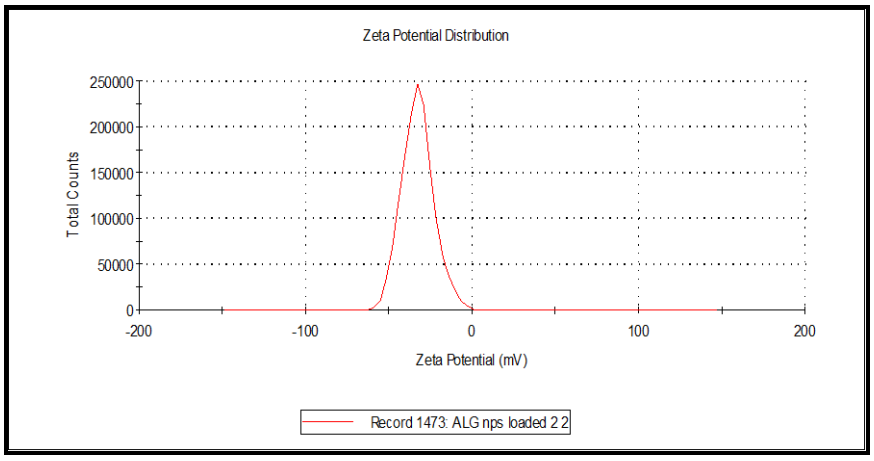
ZETA POTENTIAL DISTRIBUTION CURVES



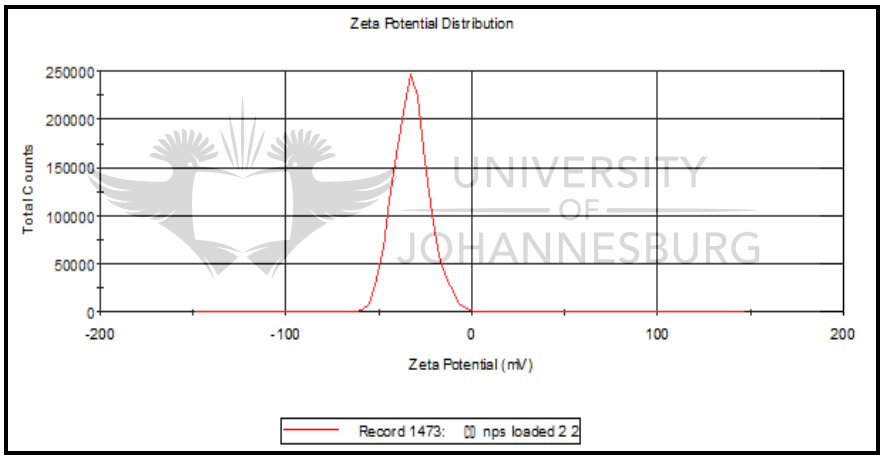
Zeta potential distribution curve for tagette oil



Zeta potential distribution curve for blank ALG/CS nanoparticles



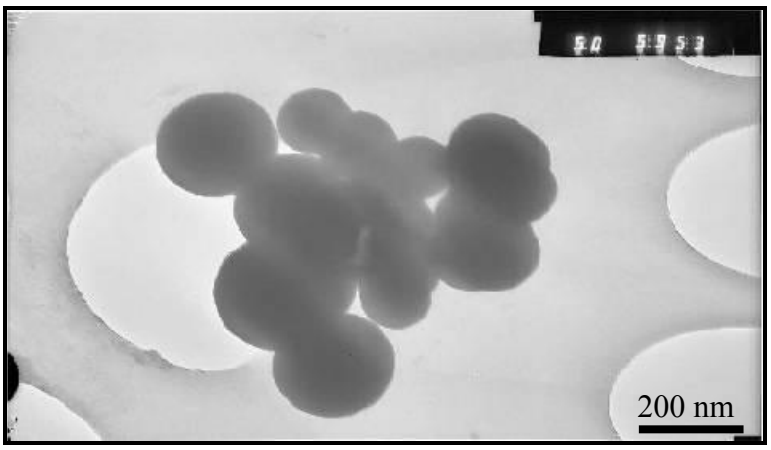
Zeta potential distribution curve for tagette oil-loaded ALG/CS nanoparticles



Zeta potential distribution curve for βCD/tagette oil-loaded ALG/CS nanoparticles

APPENDIX 3

TEM IMAGES OF BLANK ALG/CS NANOPARTICLES

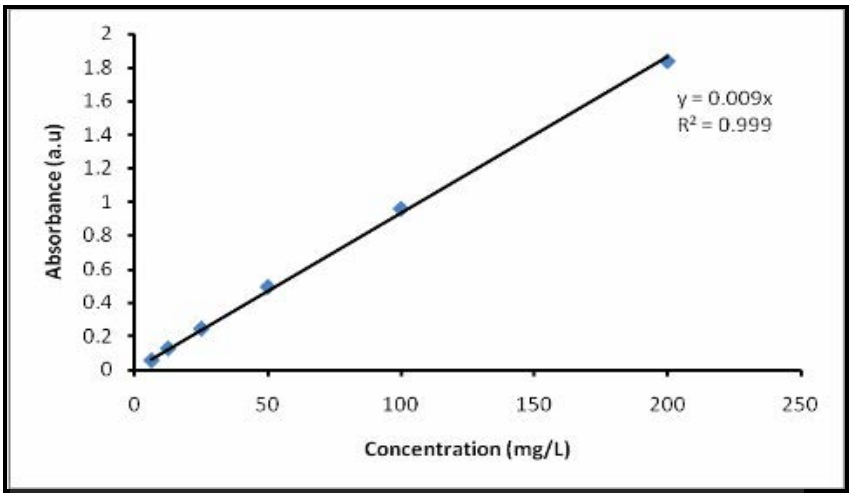


TEM image of blank ALG/CS nanoparticles at 50 000 x magnification

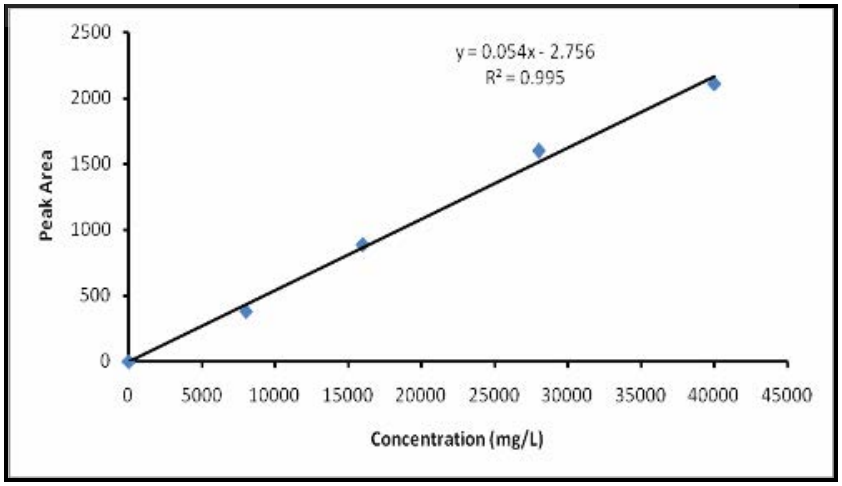
TEM image of blank ALG/CS nanoparticles at 55 000 x magnification.

APPENDIX 4

CALIBRATION CURVES



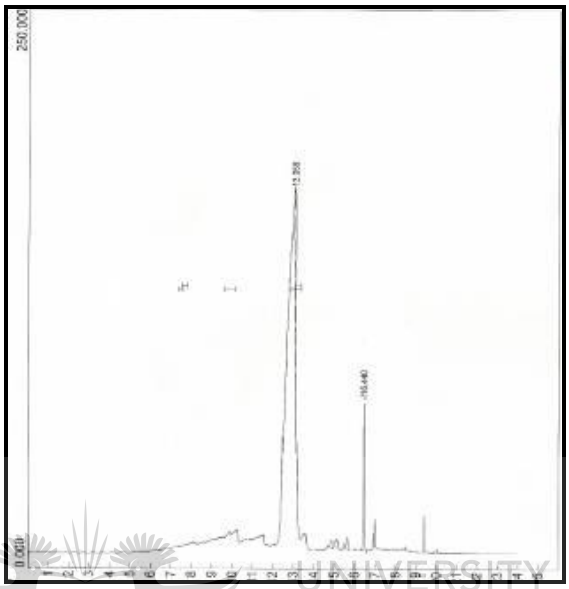
UV-Visible calibration curve for tagette oil standards



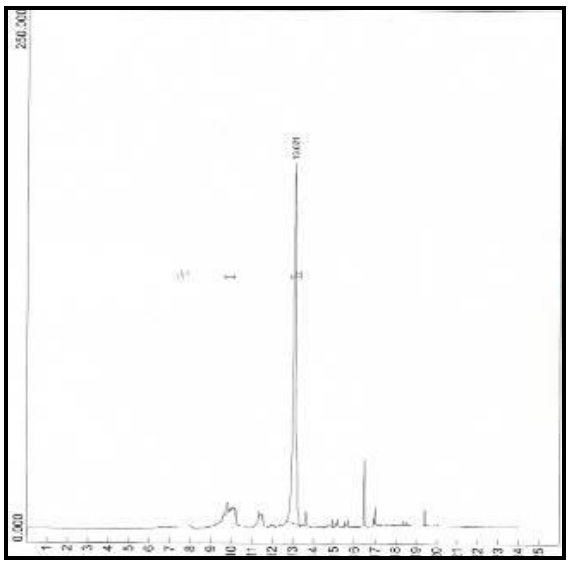
GC calibration curve for tagette oil volatiles

APPENDIX 5

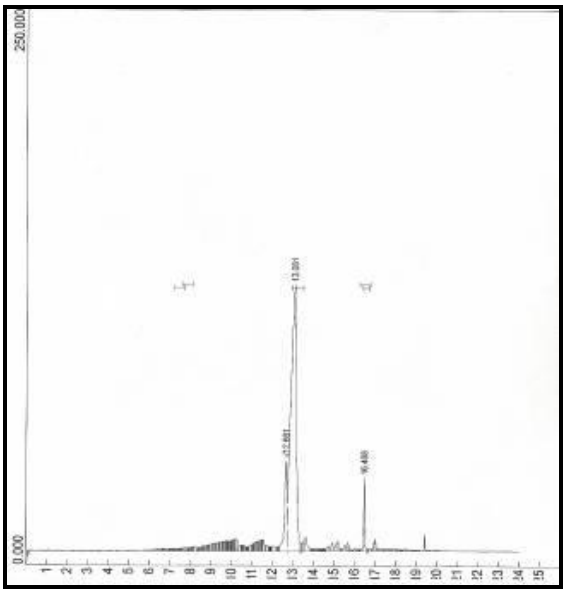
GC CHROMATOGRAMS FOR *IN VITRO* RELEASE STUDIES



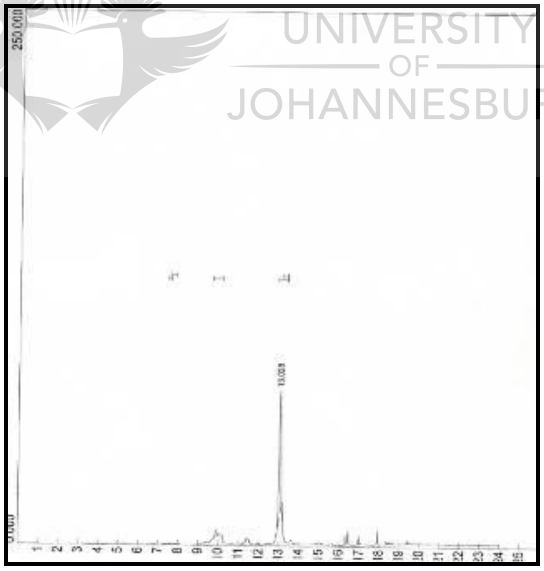
GC chromatogram for tagette oil volatiles after 6 hours



GC chromatogram for tagette oil volatiles after 12 hours

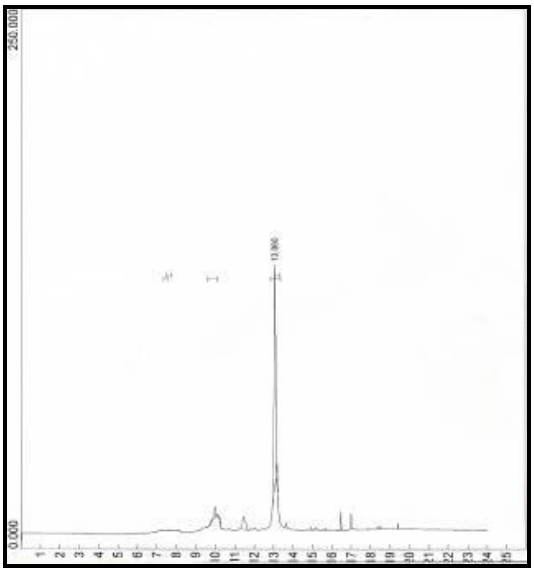


GC chromatogram of tagette oil volatiles after 18 hours

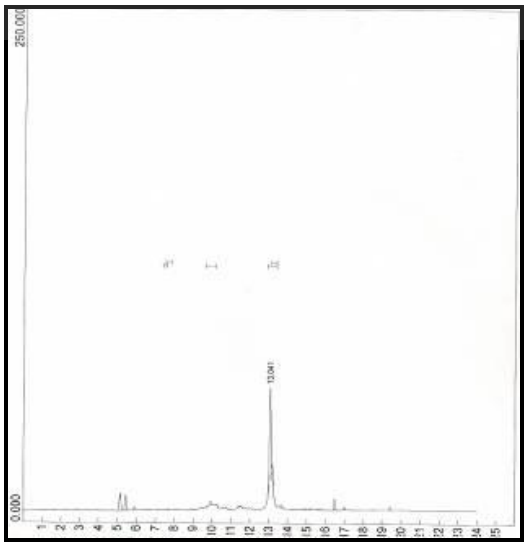


GC chromatograms of tagette oil volatiles after 24 hours

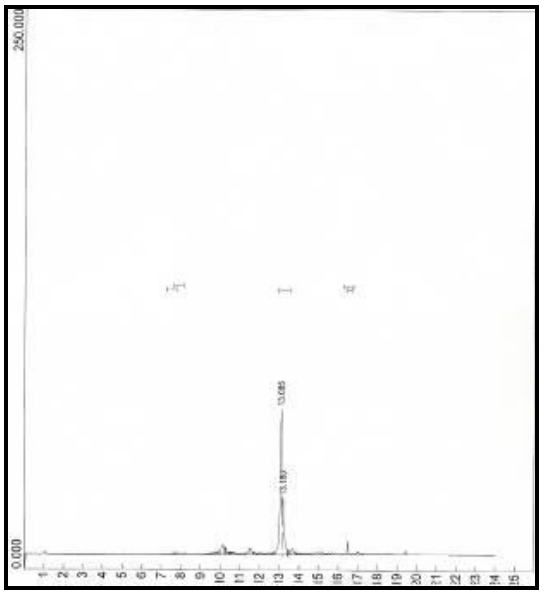
GC chromatograms of tagette oil volatiles released from tagette oil-loaded ALG/CS nanoparticles



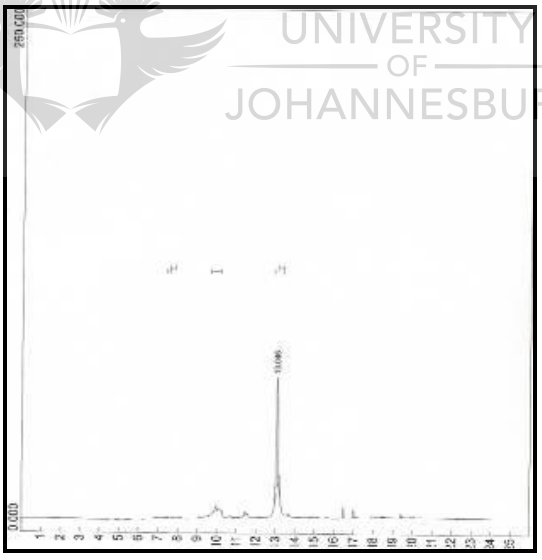
GC chromatogram for tagette oil volatiles after 6 hours



GC chromatogram for tagette oil volatiles after 12 hours



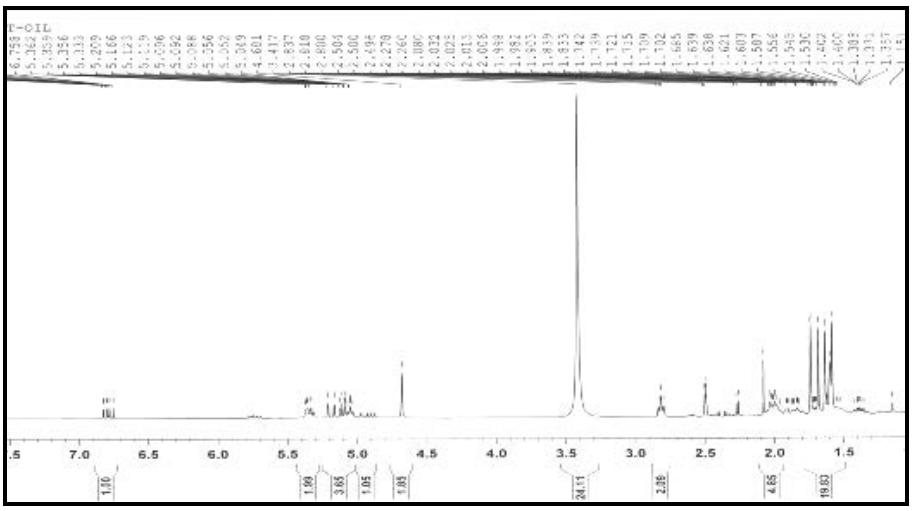
GC chromatogram of tagette oil volatiles after 18 hours



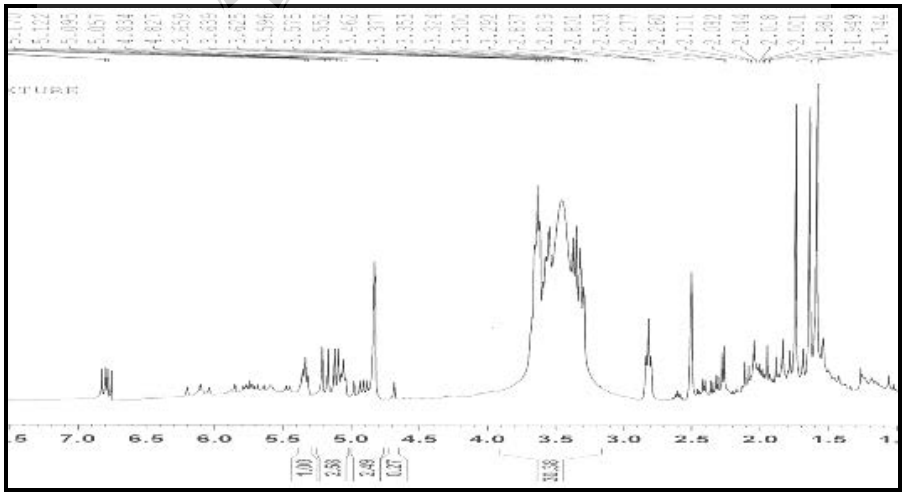
GC chromatograms of tagette oil volatiles after 24 hours

APPENDIX 6

^{1}H NMR spectra



^{1}H NMR spectrum of tagette oil



^{1}H NMR spectrum of β-CD and tagette oil physical mixture