Stimuli Sensitive Polysaccharide Based Hydrogels as Colon Targeted Drug Delivery Vehicles.

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ABSTRACT

Stimuli Sensitive Polysaccharide Based Hydrogels as Colon Targeted Drug Delivery Vehicles.

By Iman Kavianinia

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Administering drugs orally is by far the most widely used route of administration that will help eliminate the pain caused by injection, psychological barriers associated with multiple daily injections and possible infection from injection sites. However, it is important for oral drug administration to overcome several different obstacles during the delivery through the gastrointestinal tract. The barriers can be morphological barriers and physiological factors such as a wide range of pH and enzymatic activities. The lower water content and fluid mobility of the colon, which leads to longer retention times and also lower proteolytic activity of colon compared to other areas of the gastrointestinal tract, make the colon an ideal site for both systemic and local delivery of drugs. Therefore aggressive research efforts have recently focused on development of new strategies for delivering drugs to the colon.

As a drug delivery systems, hydrogels have received increasing attention due to their outstanding merits. Among the various hydrogels, including natural, synthetic and natural/synthetic hybrid hydrogels, chitosan has attracted significant attention in a broad

range of pharmaceutical and biomedical applications. Chitosan is a hydrophilic polyelectrolyte heteropolysaccharide composed of randomly (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose linked by (1→4)-β-glycosidic bonds. Unlike most known bioadhesive polymers, chitosan displays unique pharmaceutical and biomedical applications due to the large number of hydroxy and amino groups on the backbone of chitosan. These functional groups can be readily modified. This study was commenced with the aim of engineering a carrier with high enough physicochemical stability to reach the colon and to be able to protect a drug from various obstacles throughout the gastrointestinal tract. In this study, a new generation of chitosan derivatives was developed. Furthermore, their viability was investigated for potential applications as drug carriers to the colon. Chitosan based films with improved physical properties from introducing a cyclic imide moiety into the chitosan matrices was developed and characterised. Mechanical, thermal and chemical analyses of these films show that the heterocyclic imide linkage imparts excellent thermal, mechanical and chemical stability to the chitosan film. Additionally, spray dried chitosan microspheres with improved mechanical stability were examined for the controlled drug release of bovine serum albumin as a model protein drug. Additionally, a novel generation of amphoteric crosslinked chitosan derivatives was designed to be pH sensitive and bacterially degradable. Tabletted carriers were designed to protect the drug from the harsh acidic environment of the stomach and the rigorous enzymic activity of the small intestine and deliver the drug to the colon. Tabletted formulation forms of these novel amphoteric derivatives of chitosan showed the excellent potential formulations as colon specific drug delivery vehicles.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AAm	Acrylamide
5-ASA	5-aminosalicylic acid
BSA	Bovine serum albumin
BAPNA	N-α-benzoyl-l-arginine p-nitroanilide
BTEE	N-benzoyl-L-tyrosine ethyl ester solution
BTDA	Benzophenone-3,3′,4,4′-tetracarboxylic dianhydride
CF	Carboxyfluorescein
Cts	Chitosan
CST	Critical solution temperature
CFU	Colony forming unit
CBAA	Amic acid derivative chitosan crosslinked with BTDA
CFAA	Amic acid derivative chitosan crosslinked with FDA
CNAA	Amic acid derivative chitosan crosslinked with NTDA
COAA	Amic acid derivative chitosan crosslinked with ODPA
CPAA	Amic acid derivative chitosan crosslinked with PMDA
CTAA	Amic acid derivative chitosan crosslinked with TMAC
DD	Degree of deacetylation
DMF	Dimethylformamide
DD	Degree of deacetylation
DTG	Differential thermal gravimetric analysis
Da	Dalton
EC	Ethylcellulose
Ea	Electron affinity
EVAC	Ethylenevinyl acetate copolymer
EE	Encapsulation efficiency
ppm	Parts per million
EVAC	Ethylene dimethacrylate
FDA	4,4′-(Hexafluoroisopropylidene) diphthalic anhydride
5-FU	5-Fluorouracil
FTIR	Fourier transform infrared spectroscopy

GIS	Gastrointestinal system
GIT	Gastrointestinal tract
GA	Glutaraldehyde
g	Gram
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HEMA	Hydroxyethylmethacrylate
HPMA	Hydroxypropylmethacrylate
IBD	Inflammatory bowel disease
LCST	Lower critical solution temperature
LC	Loading capacity
LD	Lethal dose
LYZ	Lysozyme
MPEG	Methoxyl poly(ethylene glycol)
Mw	Molecular weight
MAS	Magic angle spinning
MHz	Megahertz
MPa	Megapascal
NTDA	1, 4, 5, 8-Naphthalenetetracarboxylic dianhydride
NaCS	Sodium cellulose sulfate
NMR	Nuclear magnetic resonance spectroscopy
ODPA	4,4′-Oxydiphthalic dianhydride
PBS	Phosphate buffered saline
PAA	Poly (acrylic acid)
PMAAc	Poly (methacrylic acid)
PDMAEMA	Poly (N,N′-dimethylaminoethyl methacrylate)
PL	Poly (lysine)
PHEMA	Poly (2-Hydroxyethyl methacrylate)
PEG	Poly (ethylene glycol)
PNIPAm	Poly (*N*-isopropylacrylamide)
PEO	Polyethylene oxide
PS	Puncture strength
PEGMA	Polyethyleneneglycol methacrylate
PNPAm	Poly (N-n-propylacrylamide)

PDEAM	Poly (N,N-diethylacrylamide)
PVA	Polyvinylalcohol
pH	Measure of acidity and basicity in solution
PPS	Sodium polyphosphate
PMDA	Pyromellitic dianhydride
RDZ	Ronidazole
RPM	Revolutions per minute
SCF	Simulated colonic fluid
SEM	Scanning electron microscopy
S %	Swelling percentage
SGF	Simulated gastric fluids
SIF	Simulate gastric fluids
T. foetus	Tritrichomonas foetus
TGA	Thermal gravimetric analysis
TMAC	Trimellitic anhydride chloride
UC	Ulcerative colitis
UCST	Upper critical solution temperature
XRD	X-ray diffraction

LIST OF PUBLICATIONS

1. **Kavianinia, I.;** Plieger, P. G.; Kandile, N. G.; Harding, D. R., In Vitro Evaluation of Spray-Dried Chitosan Microspheres Crosslinked with Pyromellitic Dianhydride for Oral Colon-Specific Delivery of Protein Drugs. *Article first published online:* 13 Feb **2013**.

2. **Kavianinia, I.;** Plieger, P. G.; Kandile, N. G.; Harding, D. R., Preparation and characterization of crosslinked chitosan based films with excellent physiochemical propertie. *International Journal of Biological Macromolecules*. Manuscript under revision.

3. **Kavianinia, I.;** Plieger, P. G.; Kandile, N. G.; Harding, D. R., Fixed-bed column studies on a modified chitosan hydrogel for detoxification of aqueous solutions from copper (II). *Carbohydrate Polymers*. **2012**, 90 (2), 875–886.

4. **Kavianinia, I.;** Plieger, P. G.; Kandile, N. G.; Harding, D. R., New hydrogels based on symmetrical aromatic anhydrides: Synthesis, characterization and metal ion adsorption evaluation, *Carbohydrate Polymers*. **2012**, 87 (1), 881–893.

Papers to be submitted

1. Development of a pH sensitive carrier system based on a novel water soluble chitosan and alginate for colon targeted drug delivery. *Under preparation*

2. Development and evaluation of a novel colon targeting drug delivery system for the treatment of Tritrichomonas foetus intestinal infection in cats. *Under preparation*

3. Synthesis and characterization of a novel generation of amphoteric pH sensitive hydrogels. *Under preparation*

4. Formulation and evaluation of a novel pH and enzyme controlled colon-specific delivery system of 5-ASA using amphoteric chitosan based matrix tablet. *Under preparation.*

5. Preparation and characterization of an amphoteric chitosan based matrix table chitosan based matrix tablet for oral colon-specific drug delivery of protein therapeutics. *Under preparation.*

Chapter One

Introduction

1.0. Hydrogel

Hydrogels have attracted tremendous research interest over many years because of their potential for a wide range of applications, Figure 1-1. Hydrogels are defined as three-dimensional polymeric networks which can absorb from 10% up to thousands of times their dry weight in water or biological fluids without dissolving.[1] The first mention of hydrogels appeared in 1960, when Wichterle and Lim first proposed the use of hydrophilic networks of poly (2-hydroxyethyl methacrylate) (PHEMA) in contact lenses.[2]

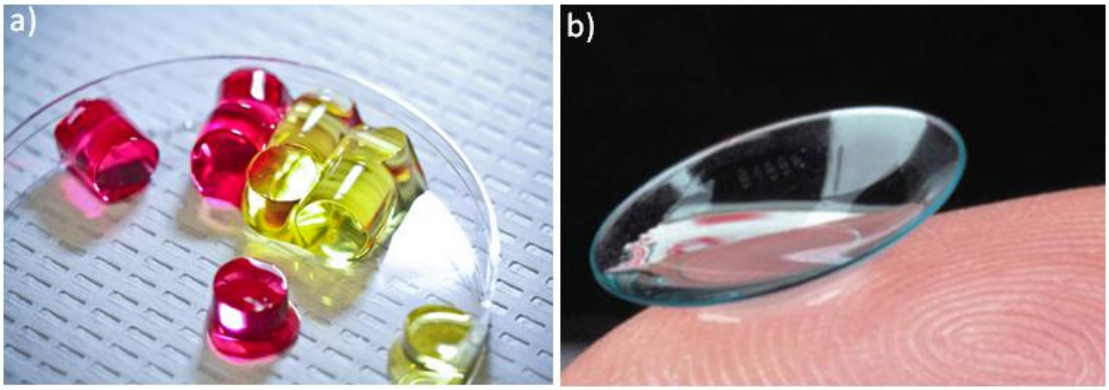


Figure 1-1 Example of hydrogels for biomedical and pharmaceutical applications. a) Hydrogel loaded with a model drug and b) poly (2-hydroxyethylmethacrylate) soft contact lens.

1.1. Classifications of hydrogels

Hydrogels can be classified in six categories:

a) According to origin

b) According to the method of preparation

c) According to ionic charge

d) According to the biodegradability

e) According to the method of crosslinking

f) According to physical properties

1.1.1. According to source

Based on the polymer origin, hydrogels can be classified into three major types:[3]

1.1.1.1. Natural hydrogels

Natural hydrogels have been investigated extensively due to their biocompatibility, biodegradability, and good cell adhesion properties. Proteins such as collagen, gelatine and, lysozyme (LYZ) and polysaccharides such as hyaluronic acid (HA), alginate and Chitosan (Cts) are two major types of natural polymers which are used to produce natural hydrogels, Figure 1-2.[4] However, the use of natural hydrogels is often restricted because their mechanical and degradation properties are less controllable.[3]

1.1.1.2. Synthetic hydrogels

Synthetic hydrogels have attracted much attention because they can be engineered to have a much wider range of mechanical and chemical properties than their natural counterparts. As an example, poly (ethylene glycol) (PEG) based hydrogels are one

class of the widely used materials in biomedical applications due to their nontoxicity, high compatibility and low immunogenicity.[5]

1.1.1.3. Hybrid hydrogels

Hybrid hydrogels are usually referred to as hydrogel systems that possess components from at least two distinct classes of molecules. An extreme example might be synthetic polymers and biological macromolecules, although many totally synthetic and natural hybrid gels exist. For instance, to combine the advantages of both synthetic and natural hydrogels, many naturally occurring biopolymers such as Cts, dextran, collagen, and HA have been combined with synthetic polymers such as poly (*N*-isopropylacrylamide) (PNIPAm) and polyvinylalcohol (PVA). Zhang *et al*.[6] prepared a biodegradable hybrid hydrogel with a combination of a hydrophilic dextran derivative of allyl isocyanate and hydrophobic poly (D,L) lactide diacrylate macromer. Temperature sensitive hybrid hydrogels can be obtained by combining natural polymers with PNIPAm.[3, 7] Combinations of polyacrylic acid with natural polymers led to pH sensitive hydrogels.[8]

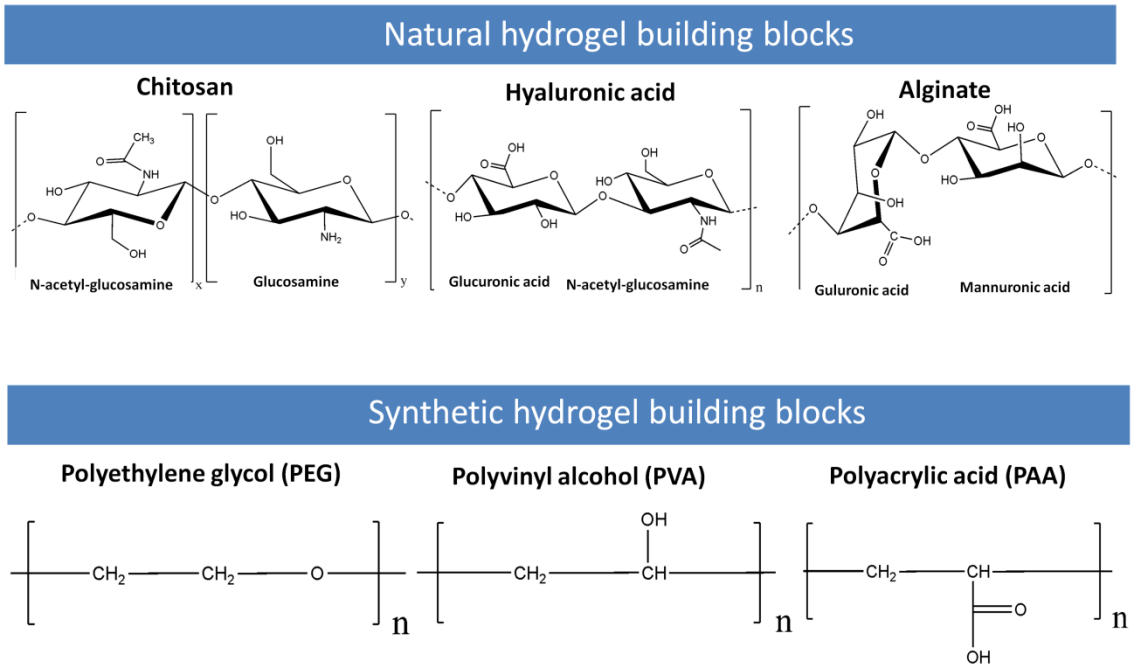


Figure 1-2 Molecular structures of typical polymers used for natural and synthetic hydrogel preparation.

1.1.2. According to the method of preparation

1.1.2.1. Homopolymers

Homopolymer hydrogels are crosslinked networks of a hydrophilic monomer unit to form three dimensional network structures.

1.1.2.2. Copolymers

Copolymer hydrogels are produced by crosslinking two or more monomeric units where at least one of the monomers is hydrophilic. The properties of copolymer hydrogels depend upon the varied combination of polymeric units and respective arrangements of these polymeric networks.

1.1.2.3. Interpenetrating polymer network hydrogels

Interpenetrating polymeric hydrogels are produced when one polymeric network swells in the network of another polymer and cannot separate due to physical entanglements.[9]

1.1.3. According to ionic charge

Hydrogels may be categorized into four groups on the basis of the presence or absence of electrical charge located on the crosslinked chains:

1.1.3.1. Non-ionic hydrogels

Non-ionic hydrogels such as agarose and dextran which have neutral monomeric units which crosslink to form three dimensional networks.[10]

1.1.3.2. Ionic hydrogels

Ionic hydrogel swelling is dependent on the pH of the aqueous medium, which determines the degree of dissociation of the ionic chains.[11] Cationic hydrogels, such as

Cts, display superior swelling in acidic media and anionic hydrogels such as pectin and alginic acid swell more at higher pH.[12]

1.1.3.3. Ampholytic Hydrogels

Ampholytic hydrogels such as collagen, gelatin and carboxymethyl chitin possess both positive and negative charges, Thus they allow a considerable spectrum for manipulating the swelling of their hydrogels.[13]

1.1.4. According to the biodegradability

1.1.4.1. Biodegradable hydrogels

Hydrogels which can be degraded under physiological conditions, either enzymatically or chemically, have attracted tremendous attention in various areas such as drug delivery systems, cell carriers, and scaffolds for tissue engineering. Many polymers created in nature are biodegradable, such as Cts, HA, fibrin and agar.[14] Polyanhydrides, poly (aldehyde guluronate) and poly (*N*-isopropylacrylamide) are examples of synthetic biodegradable polymers.[15]

1.1.4.2. Non-biodegradable hydrogels

Non-biodegradable hydrogels have been extensively used for engineering bone and cartilage.[16] Various vinylated monomers or macromers such as methoxyl poly (ethylene glycol) (MPEG), 2-hydroxyethylmethacrylate (HEMA), 2-hydroxypropylmethacrylate (HPMA) and acrylamide (AAm) are widely applied in the preparation of non-biodegradable hydrogels.[17]

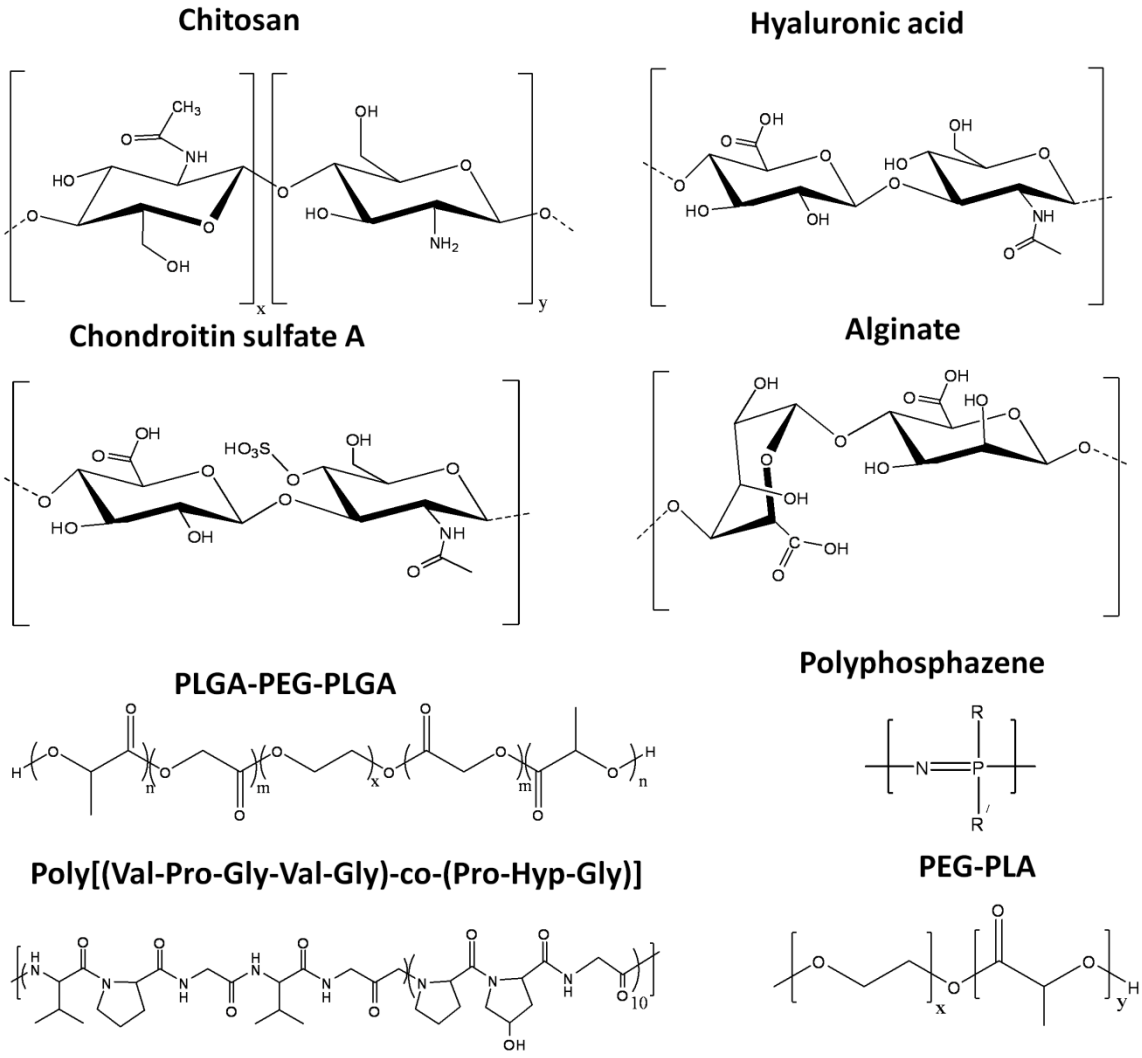


Figure 1-3 Formula of some typical natural and synthetic biodegradable polymers.

1.1.5. According to method of crosslinking

Hydrogels can be divided into two categories based on the chemical or physical nature of the crosslink junctions.

1.1.5.1. Physically crosslinked hydrogels

Physically crosslinked hydrogels are held together by molecular entanglements, and/or secondary forces including ionic interactions, hydrogen bonding or hydrophobic interactions.[18] Many of the reconstituted biological proteins and polysaccharide hydrogels are assembled in this way.[10a, 19]

1.1.5.2. Chemically crosslinked hydrogels

The mechanical strength of physically crosslinked hydrogels is generally low. Chemically crosslinked hydrogels which generally have higher strengths have been extensively studied. In chemically crosslinked hydrogels, polymer chains are held together by covalent bonds. Traditional methods for the synthesis of covalently crosslinked hydrogels include crosslinking copolymerization, crosslinking of reactive polymer precursors, and crosslinking via polymer-polymer reactions.[20]

1.1.6. According to physical properties

1.1.6.1. Conventional hydrogels

Conventional hydrogels are defined as hydrogels that do not show significant sensitivity to environmental changes – examples of conventional hydrogels include HEMA and polyethyleneneglycol methacrylate (PEGMA) based gels.

1.1.6.2. Stimuli responsive hydrogels "Smart hydrogels"

Stimuli responsive hydrogels which are also called intelligent, smart or environmentally sensitive hydrogels are defined as polymer networks able to respond to small environmental changes resulting in abrupt changes in their swelling behavior, network structure, permeability and/or mechanical strength, Figure 1-4.[21] The utility of smart hydrogels have become increasingly important not only because of their unique properties, but also because of their potential for significant technological and biomedical applications.[8, 22]

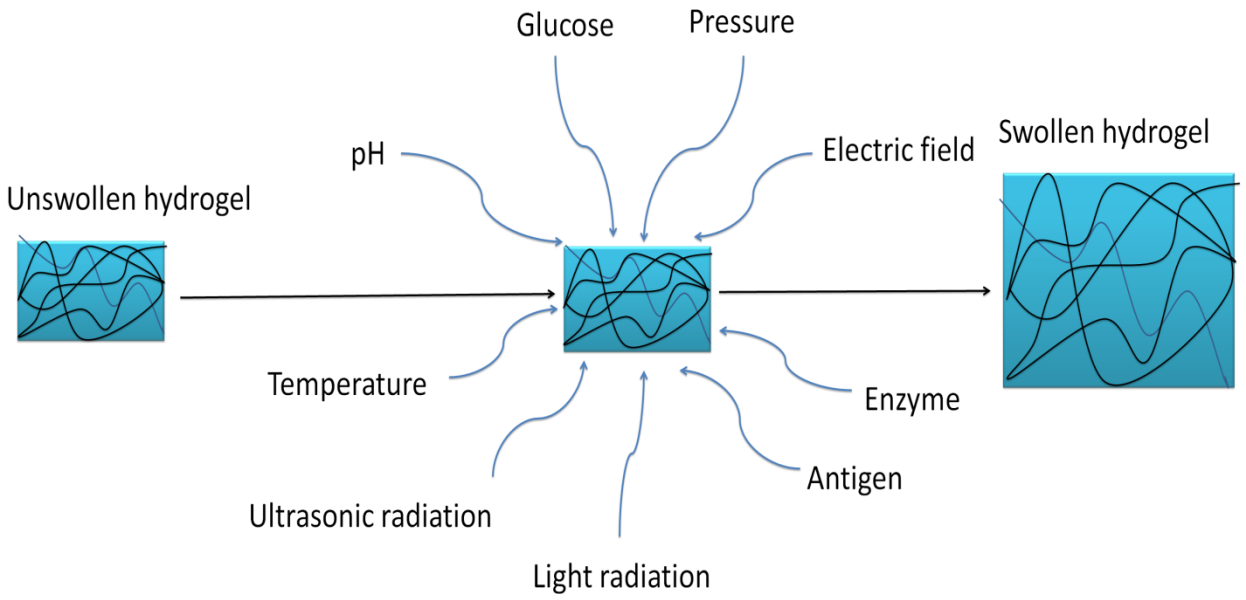


Figure 1-4 Stimuli responsive swelling of hydrogels.

1.2. Stimuli responsive hydrogel classification

1.2.1. Chemical responsive hydrogels

Chemical stimuli will change the interactions between polymer chains or between polymer chains and solvent at the molecular level.[23]

1.2.1.1. pH responsive hydrogels

pH sensitive hydrogels are made of a polymeric backbone with ionic pendant groups that can either accept or release protons in response to changes in environmental pH.[24] The ionized pendant groups generate fixed charges on the polymer network that result in the development of electrostatic forces. pH sensitive hydrogels can be anionic such as poly (acrylic acid) (PAA) and poly (methacrylic acid) (PMAAc)[25] or cationic such as Cts, poly (lysine) (PL), poly (N,N′-dimethylaminoethyl methacrylate) (PDMAEMA) depending on the nature of the ionizable moieties on their backbones, Figure 1-5.[26]

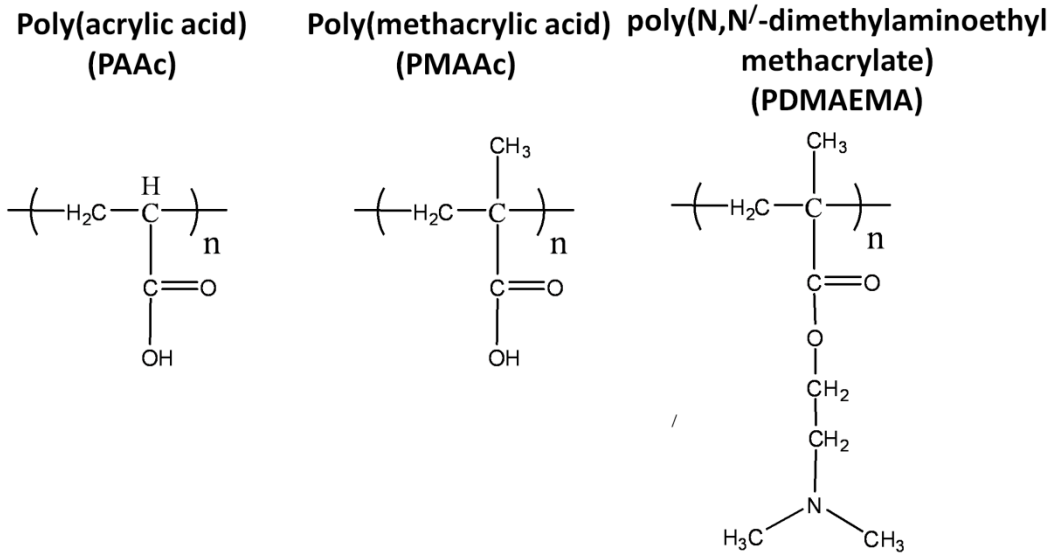


Figure 1-5 Structures of some pH sensitive polymers.

1.2.1.2. Glucose responsive hydrogels

Glucose sensitive hydrogels have attracted considerable attention in the biochemical and biomedical field, since they can be responsive to the environmental glucose concentration.[27] Glucose sensitive hydrogels are useful for development of self-regulated delivery systems which can deliver the necessary amount of insulin in response to blood glucose concentration. Several regulating systems such as those containing phenylboronic acid groups have been prepared and investigated for insulin release.[28]

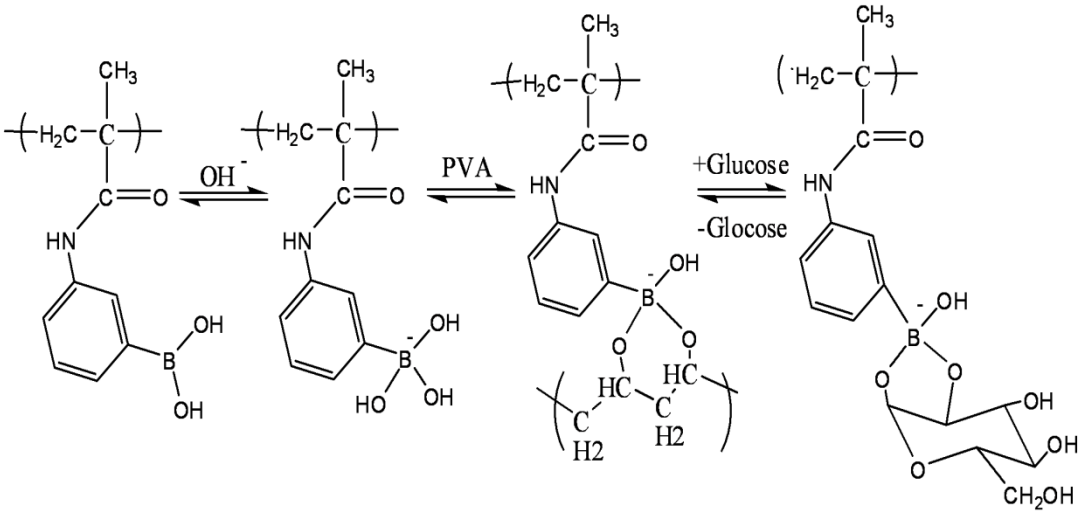


Figure 1-6 Synthesis of the complex between a phenylboronic acid complex and glucose in aqueous solution.

1.2.2. Physically responsive hydrogels

Physical stimuli will depend on the level of various energy sources and will alter molecular interactions at critical onset points.[23]

1.2.2.1. Pressure responsive hydrogels

Pressure responsive hydrogels have been investigated as a form of hydrogels that undergo volume phase transitions in response to the changes of external pressure.[29] The concept that hydrogels may undergo pressure induced volume phase transitions was firstly proposed by Marchetti (1990) through theoretical calculation.[30] According to his theory, hydrogels which are collapsed at low pressure would expand at higher pressure. Poly (n-propylacrylamide) (PNPAm**)**, poly (N,N-diethylacrylamide) (PDEAM) and PNIPAm are samples of hydrogel that show pressure sensitivity.

1.2.2.2. Temperature responsive hydrogels

Temperature sensitive hydrogels are a well accepted form of hydrogels that are able to swell or shrink in response to changes in the environmental temperature, Figure 1-7.[31] Thermosensitive hydrogels undergo phase transitions above or below a certain temperature called the critical solution temperature (CST). Temperature sensitive hydrogels are categorized into positive or negative temperature sensitive systems. Positive temperature sensitive hydrogels swell in water with an increase at temperatures above the critical point called upper critical solution temperature (UCST) and contract upon cooling below the UCST.[32] Polymer networks of PAA and poly (acrylamide-co-butyl methacrylate) have a positive temperature dependence of swelling. Negative temperature sensitive hydrogels have a lower critical solution temperature (LCST) and contract upon heating above the LCST. Copolymers of N-isopropylacrylamide are usually used for negative temperature release.[33]

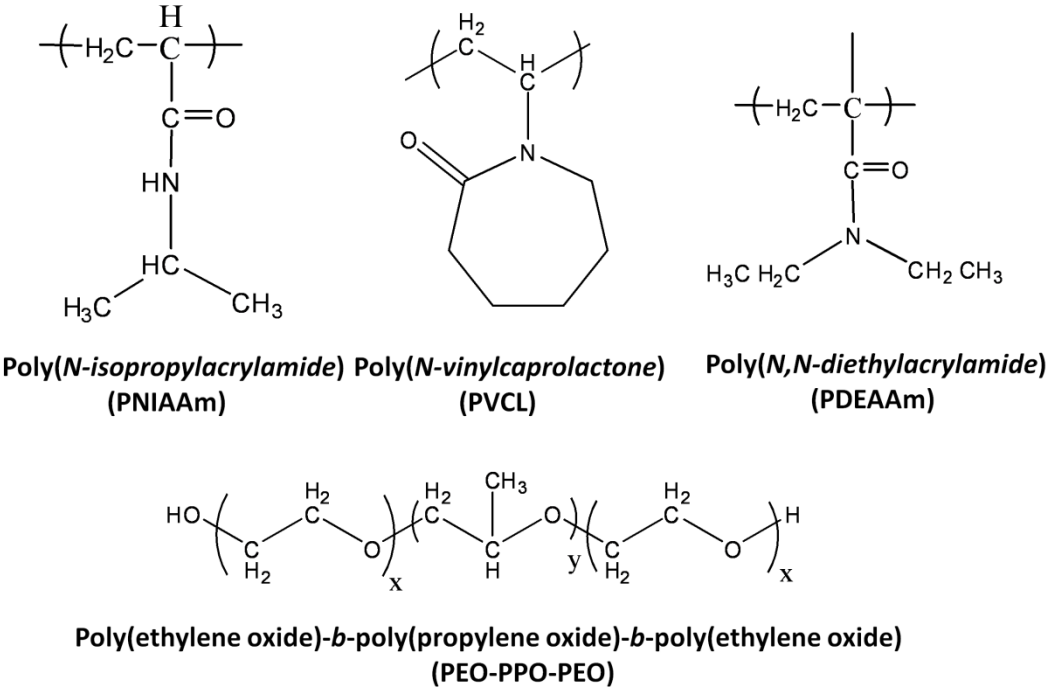


Figure 1-7 Chemical formulas of polymers that form or are part of thermoresponsive hydrogels.

1.2.2.3. Ultrasound responsive hydrogels

Ultrasonically responsive hydrogels are widely employed as drug carriers because ultrasound is able to penetrate into the interior of the body and yet is non-invasive.[34] Ethylenevinyl acetate copolymer (EVAC) is an example of an ultrasound responsive hydrogel that has been used as a 5-fluorouracil (5-FU) carrier.[35] 5-FU is an established drug for treating stomach cancer.[36]

1.2.2.4. Field responsive hydrogels

Field responsive hydrogels generally undergo a reversible change in their rheological properties in the presence of an external field such as an electric or magnetic field.[37] These hydrogels attracted great interest in various applications such as drug delivery systems, artificial muscles or biomimetic actuators. For example, incorporating colloidal magnetic particles into functional polymer hydrogel networks, field responsive hydrogels can be prepared.[38]

1.2.2.5. Light responsive hydrogels

Light responsive hydrogels are an attractive class of hydrogels that can be used in the development of photo-responsive artificial muscle tissue, drug delivery systems or *in situ*, forming gels for cartilage tissue engineering.[39] In general, light responsive hydrogels have a light sensitive moiety such as azobenzene and 2-nitrobenzyl groups as side groups or chain ends in the polymer backbone.[40]

1.2.3. Biochemically responsive hydrogels

Biochemical responsive hydrogels are appealing biomaterials for pharmaceutical, biotechnological and biomedical applications because of their sensitivity to antigens, enzymes, ligands, and other biological agents.[41]

1.2.3.1. Antigen responsive hydrogels

Hydrogels that are responsive to antigens can be used to fabricate antigen sensing devices for biomolecules, protein or drug delivery to desired sites.[42] In these hydrogels, antigens are grafted on to hydrophilic polymeric backbones. In the absence of a free antigen, the hydrogel structure shrinks due to the intra-chain antigen-antibody binding in the polymer network.[43]

1.2.3.2. Enzyme responsive hydrogels

Enzyme responsive hydrogels are increasingly investigated in the context of applications in drug delivery, as they undergo changes triggered by particular enzymes or combinations of enzymes. Biodegradable polymers such as Cts are promising candidates for preparing enzyme responsive hydrogels since they are degraded by specific enzymes.[44]

1.3. Biomedical applications of stimuli-responsive hydrogels

Hydrogels are three dimensional, hydrophilic, polymeric networks capable of imbibing large amounts of water or biological fluids**.** Since the pioneering work of Wichterle and Lim in the 1960s on the crosslinked three-dimensional polymers obtained by the copolymerization of HEMA with ethylene dimethacrylate (EDMA), polymeric hydrogels have attracted tremendous research interest.[45] High water absorption capacity and in most cases biocompatibility of hydrogels make them an attractive candidate for biomedical applications.[22]

1.3.1. Applications of hydrogels in tissue engineering

Almost 40 years have passed since the term 'tissue engineering' was created. In general, tissue engineering seeks to fabricate a living replacement part for the body. The necessity of tissue engineering is expanding as the other therapeutic sources like organ and tissue donation and conventional treatment methods are not meeting demands.[46] A variety of synthetic and naturally derived materials have been used to form hydrogels for tissue engineering, Figure 1-8. Among the materials used for tissue engineering, hydrogels are receiving increasing attention due to their ability to imbibe a large volume of water, offer structures similar to the macromolecular components in the body and hence provide biocompatibility. Hydrogels to be used for tissue engineering must meet a number of design criteria to function appropriately and promote new tissue formation. These criteria include both physical parameters as well as biological performance parameters.[4, 47]

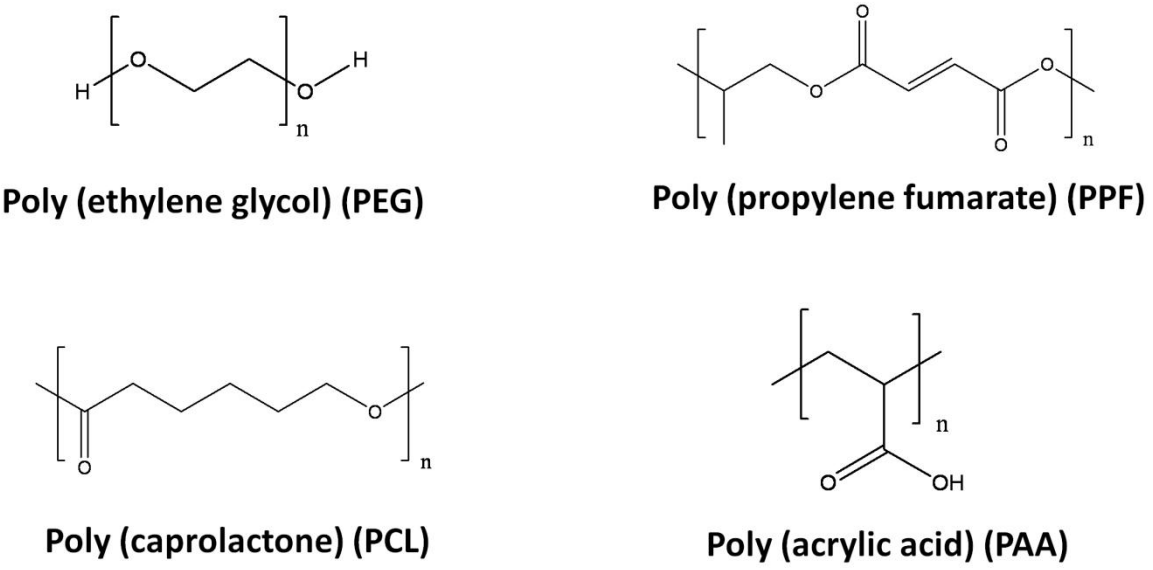


Figure 1-8 Structure of some polymers commonly used in tissue engineering.

1.3.2. Applications of hydrogels in wound healing

Before the 1960s wound dressings were considered to be only passive products having a minimal role in the healing process.[48] The study by Winter (1962) initiated the concept of an active involvement of a wound dressing in establishing and maintaining an optimal environment for wound repair.[49] A desirable wound dressing should create and keep a moist environment, protect the wound from bacterial infection, absorb the wound fluids and exudates and also be biocompatible. Among the many different materials that are in use as a wound dressing, hydrogel based wound dressings have attracted special attention because of their properties such as biocompatibility and ability to keep the wound area moist, Figure 1-9.[50]

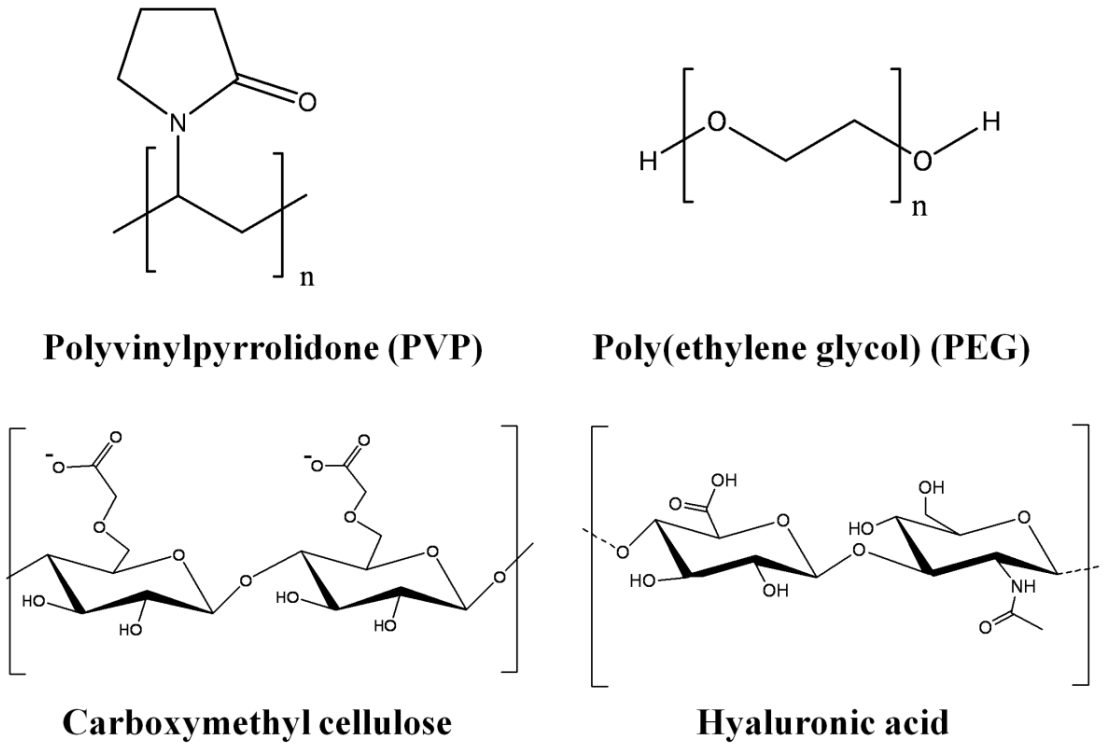


Figure 1-9 Structure of some polymers commonly used in wound healing.

1.3.3. Applications of hydrogels in drug delivery

Controlled release systems were first used in medical research in the 1960s. The earliest drug delivery systems were first introduced in 1970s and were based on polymers formed from lactic acid.[51] Nowadays, polymers are still the most used materials in this field of research mainly because of their ease of processing and also because of the possibility of researchers to control both their physical and chemical properties.[52]

Controlled release systems aim to achieve a delivery profile that would yield as high blood level concentration as required for the drug in question over an extended period of time. With conventional dosing formulations, the drug level in the blood often exceeds the toxic level immediately after each administration of the drug and then declines sharply below the minimum therapeutic level until the next administration, Figure 1-10.[53] This bolus administration of drugs is therefore far from ideal not least of all in pain relief therapies. Controlled drug delivery systems are designed for long term administration where the drug level in the blood remains constant, between the desired

maximum and minimum, for an extended period of time. Hydrogels due to their attractive physicochemical and biological characteristics have attracted a lot of attention as they are excellent candidates for delivery systems of therapeutic agents.[21a, 54] The delivery of drugs for pharmaceutical and medical applications is usually achieved through a variety of drug delivery systems such as injections, tablets, ointments, suppositories and sprays.[55] Hydrogel based delivery devices can be used for ocular, transdermal, subcutaneous, rectal and oral delivery, Table 1-1.[56]

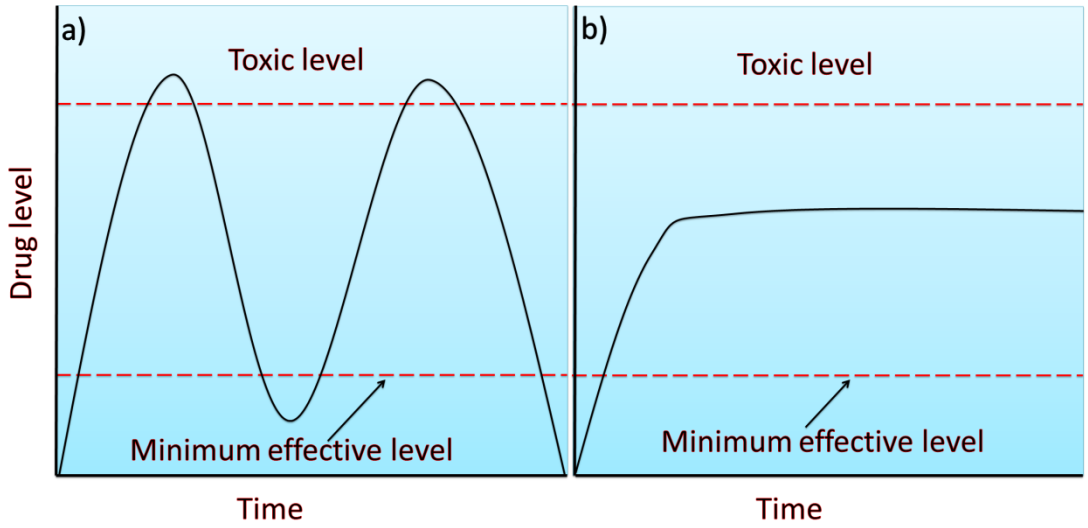


Figure 1-10 Drug level in blood with a) traditional drug administration and b) controlled drug delivery**.**

1.3.3.1. Ocular drug delivery

Ocular drug delivery has been a major challenge to pharmaceutical scientists due to the unique anatomy and physiology of the eye. The physiological barriers imposed by the protective mechanism of the eye such as effective tear drainage, blinking and low permeability of the cornea, lead to the low absorption of drugs and results in a short duration of therapeutic action.[57] Therefore, conventional eye drops containing a drug solution tend to be eliminated rapidly from the eye, and drugs administered exhibit limited absorption, leading to poor ophthalmic bioavailability.[58] Additionally, due to the short retention time, a frequent dosing regimen is necessary for required therapeutic efficacy. These challenges have initiated the development of new ophthalmic drug delivery systems that provide prolonged residence time.[59] Hydrogels have sparked

particular interest in their use in ocular drug delivery applications because of their facility of liquid dosing and long term retention properties of the gels.[60] Thermosensitive hydrogels such as Cts/β-glycerophosphate, ion sensitive hydrogels such as gellan gum and pH sensitive hydrogels such as cellulose acetate hydrogen phthalate have been examined for their potential as vehicles for ocular drugs.[61]

1.3.3.2. Rectal drug delivery

The rectum has been studied as a favourable site of drug delivery for treatment of diseases associated with the rectum such as haemorrhoids. This route is also an ideal way to administer drugs suffering heavy first pass metabolism. Furthermore, the use of rectal administration allows for drugs to be effectively administered whilst the patient is undertaking bouts of vomiting, nausea or unconscious convulsions.[62] Conventional suppositories often cause discomfort during insertion and also suppositories are unable to be sufficiently retained at a specific position in the rectum.[63] Sometimes they can migrate upward to the colon and therefore undergo the undesirable first pass effect. Recently, hydrogels have been investigated as substitutes to overcome the problems of conventional suppositories.[64] A number of studies have been carried out on diclofenac sodium loaded into Cts as the matrix. The release of indomethacin from a PVA hydrogel has also investigated for rectal delivery.[65]

1.3.3.3. Subcutaneous delivery

Subcutaneous delivery has been extensively utilised as a delivery route. All implantable materials must be compatible with the body.[66] Subcutaneously inserted materials can be prone to undesirable immune responses of the body leading to inflammation, immunogenicity and carcinogenicity in the extreme. Many hydrogels are considered as biocompatible therefore one of the most substantial applications of hydrogels is probably in implantable therapeutics.[67] Development of biodegradable systems such as biodegradable PEG based hydrogels, which do not require surgical removal once the drug has been administered have attracted great attention as excellent candidates for delivery of therapeutic agents.

1.3.3.4. Transdermal delivery

Transdermal delivery refers to the delivery of drugs through the skin which requires penetration through the two sublayers of the epidermis to reach the microcirculation of the dermis. Ease of application, reduced systematic side effects and avoidance of drug gastrointestinal decomposition of drugs are considered as benefits of transdermal route.[68] Researchers have developed several natural and synthetic hydrogel-based systems to enhance the drug permeation across the skin and some have been successfully commercialized.[67a, 69]

1.3.3.5. Oral drug delivery

Administering drugs orally is by far the most widely used route of administration which will help eliminate the pain caused by injection, psychological barriers associated with multiple daily injections and possible infection from injection sites.[70] A large surface area and the ability to absorb material into the blood stream makes the gastrointestinal system (GIS) the most popular target for many types of drug delivery.[70-71] Almost 90% of all medicines are oral formulations. However, it is important for oral drug administration to overcome several different obstacles during the delivery through the gastrointestinal tract (GIT). The barriers can be morphological barriers such as mucus layers, microvilli and physiological factors such as a wide range of pH and enzymatic activities.[72]

Table 1-1 Example of various environmentally stimulated hydrogel systems used for drug delivery.

Hydrogel	Stimuli	Application	Drug
Poloxamers[73]	Temperature	Ocular delivery	Liposomes
Xyloglucan[74]	Temperature	Ocular delivery	Timolol/pilocarpine
Polyacrylic acid/hydroxypropylmethylcellulose[75]	pH	Ocular delivery	Ofloxacin
Chitosan[76]	pH	Ocular delivery	Ofloxacin
Ethylene-co-vinyl acetate[77]	Magnetic field	Oral drug delivery	Insulin
Poly(2-hydroxyethyl methacrylate)[78]	Electric field	Oral drug delivery	Propranolol hydrochloride
Poly(2-hydroxyethyl methacrylate-co-*N*,*N*-dimethylaminoethyl methacrylate)[27b]	Glucose	Oral drug delivery	Insulin
Polyethylene glycol[79]	Temperature	Nasal drug delivery	Mucin
Carboxymethyl Chitosan[80]	pH	Intestinal drug delivery	Methyl prednisolone

1.4. Site specific drug delivery

Drug discovery and development involves highly challenging, laborious, and expensive processes which take an average of 15 years and a cost of about US $1 billion for a drug to travel from the research lab to the patient. However, most of the drugs fail to achieve favourable clinical outcomes because they do not have the ability to reach the intended targets.[81] This limits their efficacy and creates issues associated with off-target and systemic side effects and repetitive and complex administration regimens and costs. The major advantages of site-specific drug delivery system are as follows:[82]

- Less side effects
- Reduced dosing frequency
- Better patient convenience and compliance
- Less fluctuating plasma drug levels
- More uniform drug effect
- Lower total dose

Therefore aggressive research efforts have recently focused on development of new strategies for delivering drugs to the site of action. This technology is based on interdisciplinary approaches that combine polymer science, pharmaceutics, bioconjugate chemistry, and molecular biology.[83]

1.5. Human gastrointestinal physiology

The human GIT, (Figure 1-11) also called the alimentary canal is a selective barrier between the environment and the systemic circulation, which functions to digest dietary food, to absorb nutrients, electrolytes and fluid, and to prevent the absorption of potentially harmful substances. The GIT is approximately 9 metres long and consists of the oral (buccal) cavity, pharynx, oesophagus, stomach, small intestine and large intestine.[84]

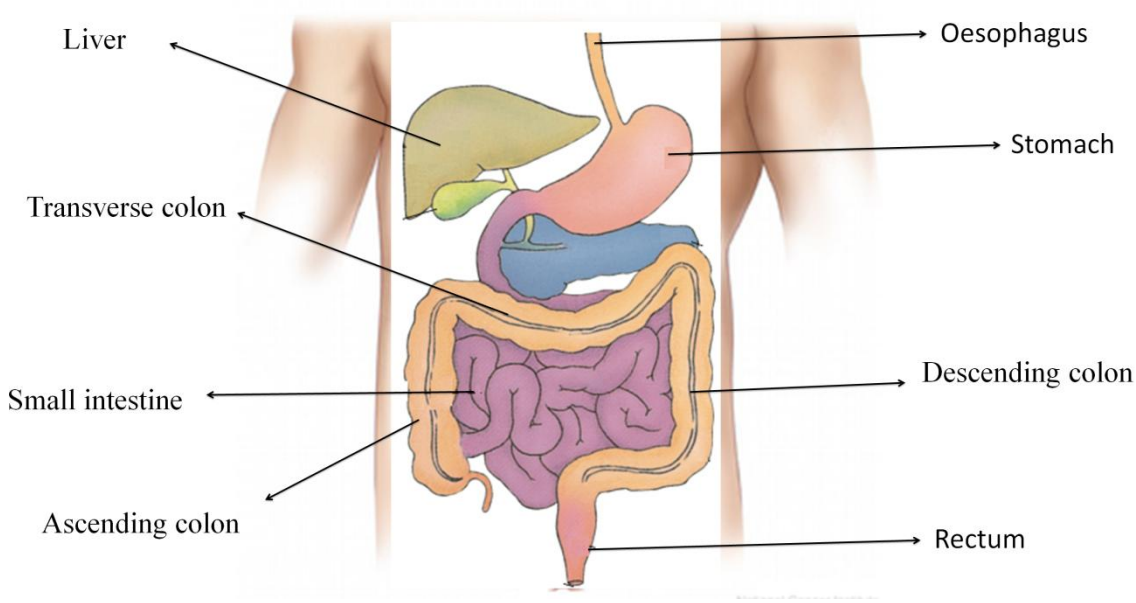


Figure 1-11 Anatomy of gastrointestinal tract.

1.5.1. Anatomy and physiology of the stomach

The oral dosage initially comes in contact with saliva. Most drugs are not absorbed to any significant extent in the mouth. The oral cavity or mouth aids in digestion of food. The oral dosage once swallowed rapidly goes to the oesophagus. The function of the oesophagus is to pass food material into the stomach by wavelike muscular contractions called peristalsis (20-30 seconds transit time).[85] The drug is barely dissolved here before going to the stomach. the stomach is a J-shaped organ in the GIT connected to the oesophagus at the upper portion and small intestine at the lower portion.[86] When the oral dosage reaches the stomach (fluid volume ≈ 1.5 L), the acidic milieu will dissolve drugs with the propensity for such at low pH. The parietal cells of the gastric glands are responsible for secretion of hydrochloric acid (0.15 M) and proteases such as pepsin which lead to denaturation and proteolytic degradation with a loss of drug efficacy.[87] The pH of the stomach ranges from 1.5 to 3 and the residence time is highly variable depending on the type of food and the amount eaten. As a result of all the *in vivo* measurements the British Pharmacoepoeia recommends a pH of between 1-1.5, 0.1 M HCl to be used to simulate gastric media when assessing delayed release dosage forms

(British Pharmacoepoeia 2010, Appendix XII A309). The exit of food from the stomach is controlled by the pyloric sphincter which relaxes to release the stomach contents into the small intestine. This release will only occur for smaller objects within the fed stomach (<10mm) whilst larger objects (>20 mm) will be retained and processed until broken into smaller particles. The transit time within the stomach is in the range 1-2 hours.

1.5.2. Anatomy and physiology of the small intestine

The disaggregated dose forms enter the small intestine from the stomach. The small intestine is the longest part of the GIT and is located below the stomach. The small intestine contains villi and microvilli that increase the absorptive surface area.[84] Due to the large surface area and presence of intestinal enzymes, most of the enzymatic metabolism and absorption in the GIT takes place in the small intestine. The small intestine is approximately 5 m long and 2-5 cm in diameter. The first 20-30 cm is the duodenum, followed by the longest section (jejunum) and the ileum is the last section which connects to the large intestine.[88]

1.5.3. Anatomy and physiology of the large intestine

The remaining undigested contents are then emptied into the large intestine through ileo-caecal junction where the entry of digested food is controlled by the ileo-caecal valve. The large intestine is structurally very similar to the small intestine but without the villi. The large intestine consists of the caecum and appendix, colon and rectum and is approximately 1.5 metres long, with a diameter of 6-6.3 cm and a pH range of 5.5-7.0. The transit time through the large intestine varies from 12 to 72 hours. The major function of the large intestine is to absorb water and salts from partially undigested food then send waste out of the body through the anus.[89]

Table 1-2 Anatomical and physiological features of the gastrointestinal tract.

Region of gastro intestinal tract		Length (cm)	Surface area (m^2)	Transit time	pH
Entire gastrointestinal tract		500-700			
Oral Cavity		15-20	-	-	7
Oesophagus		20-30	0.02	3.5 s	6-7
Stomach		~20	0.1	Up to 2 h fasted 2-8 h fed	1.5-3
Small intestine	Duodenum	20-30	0.1	> 5 mins	5.5-6.5
	Jejunum	150-250	60	1-2 h	6.1-7.1
	Ileum	200-350 cm	60	2-3 h	7-8
Large intestine	Cecum	6-7	0.05	≤ 48h	~6.4
	Ascending colon	20	0.25		~6.4
	Transverse colon	45			~6.6
	Descending colon	30			~7
	Sigmoid Colon	40			5-7
	Rectum	12	-		7

1.6. Colon specific drug delivery

Absorption and storage are two main functions of colon which lead to a lower water content and fluid mobility than other areas of the GI tract. These conditions mean drugs can have higher residency times which will allow for the maximum possible drug uptake efficiency in patients. Therefore the colon as a site for drug delivery has received a large amount of interest for the treatment of localised diseases such as irritable bowel syndrome, colon cancer, and inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis.[90] As an example, 5-aminosalicylic acid (5-ASA) is a first-line therapy for mild to moderate ulcerative colitis (UC).[91] UC is a chronic inflammatory bowel disease characterized by mucosal inflammation in the colon. However in oral formulations about 75% of 5-ASA absorbs in the upper GIT tract.[92] Due to the topical nature of the drug administration of 5-ASA, it is necessary to get the highest efficiency of delivery to the site of infection which is the large intestine. Therefore the use of enemas and suppositories have proven the most effective method for treating ulcerative colitis.[93]

Oral colon specific drug delivery of protein and peptide drugs has also attracted the attention of worldwide drug delivery scientists due to the relatively low proteolytic enzyme activity in the colon compared to the small intestine.[94] The main benefits of colonic delivery as a site for drug delivery are listed below:

a) Proteolytic activity of colon mucosa is less than that observed in the small intestine, thus the colon may be helpful in achieving a reasonable absorption of certain drugs that are enzymatically labile in the small intestine.[95]
b) The colon has a longer retention time and appears highly responsive to agents that enhance the absorption of generally poorly absorbed drugs.[96]
c) The colon is rich in lymphoid tissue which can take absorbed antigens into the mast cells of the colonic mucosa. This produces rapid local production of antibodies which can help in efficient vaccine delivery.[97]
d) The colon continues to attract interest as a site where poorly absorbed drug molecule may have an improved bioavailability.[98]

e) The colonic region has a somewhat less hostile environment with less diversity and less intensity of activity as compared to the stomach and small intestine.[99]

To achieve successful oral colonic delivery, a drug needs to be protected from the absorption and degradation pathways of the upper GIT and then achieve abrupt released into the colon.

1.6.1. Anatomy and physiology of colon

The colon is a cylindrical tube that is lined with a moist, soft pink lining called the mucosa. In general, the colon consists of four sections: (1) Ascending colon, (2) Transverse colon (3), Descending colon, and (4) Sigmoid colon.[100] The average size of the colon is 1.5 m, the pathway is called the lumen and is approximately 5-7.5 cm in diameter. The transverse colon is the longest and most mobile part and has an average diameter of about 6.5 cm. The physical properties of the luminal content of the colon also changes, from liquid in the cecum to semisolid in the distal colon. The major functions of the colon are the consolidation of the intestinal contents into faeces by the absorption of the water and electrolytes and to store the faeces until excretion.[101]

1.6.2. Factors affecting in the design of colon-specific drug delivery system

1.6.2.1. pH of the colon

The pH of the GIT is subject to both inter and intra subject variations. The highest pH levels were measured radiotelemetry and were found to be 7.5 ± 0.5 in the terminal ileum. On entry into the colon, the pH drops to 6.4 ± 0.6. The pH in the transverse colon was measured at 6.6 ± 0.8. Polysaccharide drugs and diet can affect the colonic pH.[102] For example, lactose is fermented by colonic bacteria to produce large amounts of lactic acid resulting in a drop of pH to about 5.0. Disease also can change the colon pH, for example some patients with untreated ulcerative colitis have a mean pH in the proximal colon of 4.7 ± 0.7.[103]

1.6.2.2. Transit time to colon

Arrival time of a drug or drug composite in the colon depends on the rate of gastric emptying and intestinal transit time. The movement of materials through the colon is slow, tends to be highly variable and influenced by a number of factors such as diet, stress, disease state and presence of drugs.[95]

1.6.2.3. Colonic micro flora and their enzymes

The sluggish movement of material through colon provides perfect conditions for bacterial growth with over 400 resident species and a range of 1011-1012 CFU/g in comparison to the stomach (102 CFU/g) and the small intestine (104-107 CFU/g).[99] The most important anaerobic bacteria found in colon are bacterocides, bifidobacteria, eubacteria as well as species such as peptococcus, peptostreptococcus and ruminococcus.[104] The colonic microflora are able to break down polysaccharides by producing a large number of reductases and carbohydrases.[105]

1.6.3. Strategies for targeting drugs to the colon

1.6.3.1. Covalent linkage of drug with a carrier

1.6.3.1.1. Prodrug approaches

Prodrug as a pertinent term was introduced for the first time in the 1950s.[106] Prodrugs are pharmacologically inactive, bioreversible derivatives of active drug molecules that must undergo an enzymatic and/or chemical transformation in the biological environment to release the active parent drug, which can then elicit its desired pharmacological effect in the body. This approach generally involves a covalent linkage between the drug and its carrier in such a manner that upon oral administration the moiety remains intact in the stomach and small intestine, and after reaching the colon, enzymatic cleavage occurs which releases the drug.[98, 107]

1.6.3.1.1.1. Azo bond conjugate

Azo compounds have the potential to act as drug carriers that facilitate the selective release of therapeutic agents to the colon. In this approach the drug is attached via an azo bond to a carrier. This azo bond is stable in the upper GIT and is cleaved in the colon by the azo-reductases produced by the microflora. Sulfasalazine, is an example of a prodrug which is made by attaching 5-ASA to sulfapyridine via an azo bond, Figure 1-11. In the colon, the azoreductases cleave the azo bond releasing the drug, 5-ASA and the sulfapyridine.[108]

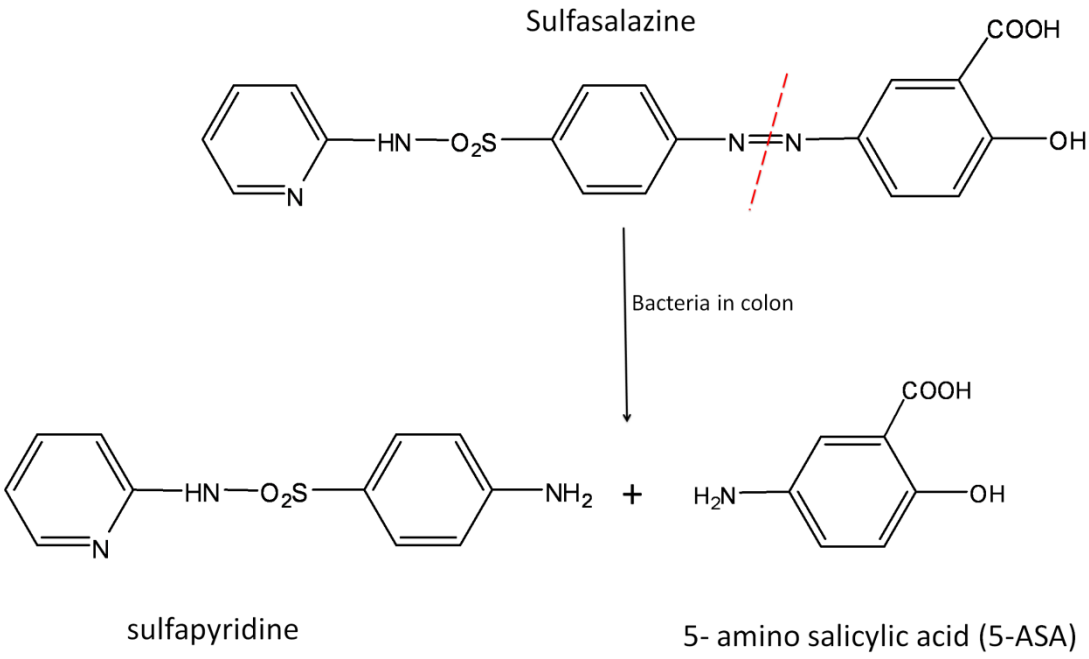


Figure 1-12 Pathway of colonic reduction of sulfasalazine.

1.6.3.1.1.2. Glycoside conjugation

This approach has been based upon the unique glycosidease activity of the colonic microflora. The major glycosidases produced by the intestinal microflora are β-D-galactosidase, α-L-arabinofuranosidase, β-D-xylopyranosidase, and β–D-glucosidase. Certain drugs can be conjugated to different sugar moieties to form glycosides.[109] Because they are bulky and hydrophilic, these glycosides do not penetrate the biological membranes upon ingestion. Breakdown into monomeric units by the action of

glycosidic enzymes leads to release of the drug from the carbohydrate. Friend *et al*. (1984) prepared dexamethason-21-β-glucoside and prednisolone-21-β-glucoside for delivery of these steroids to the colon, Figure 1-13.[110]

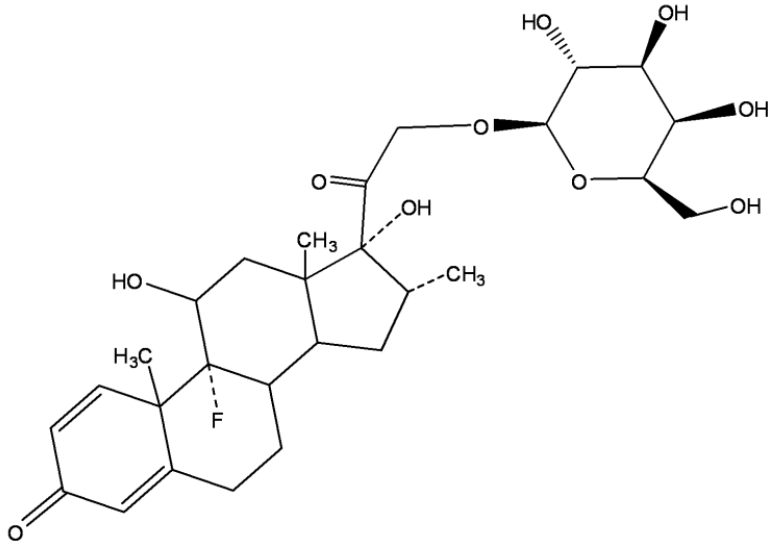


Figure 1-13 Dexamethasone-2-β-D-glucoside prodrug.

1.6.3.1.1.3. Glucoronide conjugates:

This approach has been based upon the conjugation of a drug with glucuronates. Bacteria of the lower GIT secrete glucuronidases which lead to deglucuronidation processing and release of the active drug, Figure 1-14.[111]

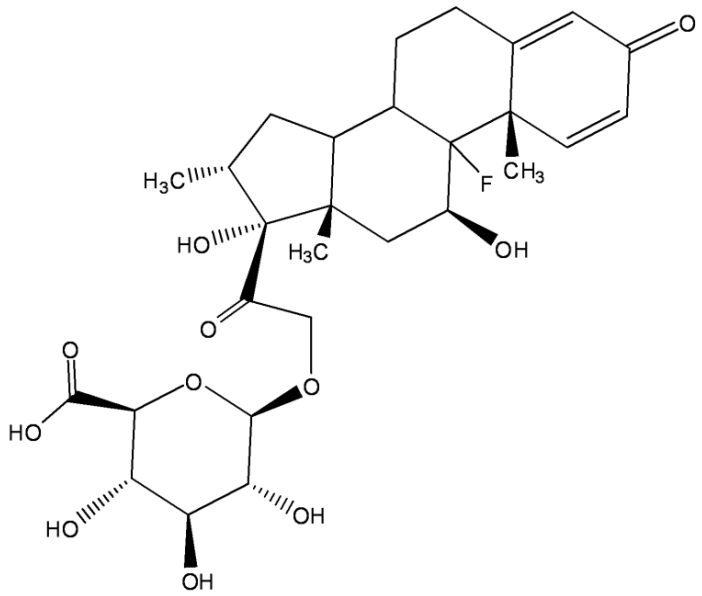


Figure 1-14 Dexamethasone β-D-Glucuronide prodrug.

1.6.3.1.1.4. Amino acid conjugation

Various prodrugs have been prepared by the conjugation of drug molecules to amino acids such as tyrosine, glycine, methionine and glutamic acid, Figure 1-15. Increase in hydrophilicity and length of a chain amino acids decrease the permeability of amino acids, peptides and proteins. Various prodrugs have been prepared by the conjugation of drug molecules to these polar amino acids. Non-essential amino acids such as tyrosine, glycine, methionine and glutamic acid were conjugated to ASA.[108]

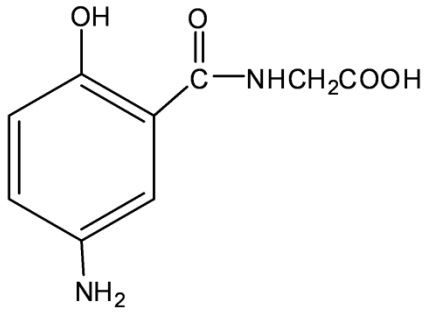


Figure 1-15 Structure of 5-aminosalicyl-glycine prodrug.

1.6.3.2. Approaches to intact molecule delivery to the colon

1.6.3.2.1. Bioadhesive systems

Bioadhesion has been proposed as a means of improving the performance and extending the mean residence time of colonic drug delivery systems. Bioadhesive systems are based upon the principle of adhesion between drug and the biological membrane by virtue of which the drug molecule remains in contact with a particular organ for an extended period of time. This strategy can be applied to the formulation of colonic drug delivery systems. Various polymers including polycarbophils and polyurethanes have been investigated as materials for bioadhesive systems.[98]

1.6.3.2.2. Pressure controlled systems

This approach relies on the strong peristaltic waves in the colon. These strong peristaltic waves in the colon are of short duration, occurring only three to four times a day. However, they temporarily increase the luminal pressure within the colon, which leads to the concept of pressure-controlled systems. In pressure controlled system drug release occurs following disintegration of a polymer capsule as a result of pressure in the lumen of the colon.[99]

1.6.3.2.3. Time dependent delivery

In this approach, drug release from the system occurs after a predetermined lag time according to the transit time from mouth to colon. The lag time depends upon the gastric motility and size of the dosage form. However, large variations in gastric emptying times exist in humans. In these approaches, colon arrival time of dosage forms cannot be accurately predicted, resulting in poor colonic availability in many cases.[112]

1.6.3.2.4. pH dependent approach

In these systems, drugs can be formulated as solid dosage forms such as tablets, capsules, pellets and coated with pH sensitive polymers as enteric coatings, Table 1-3. The pH dependent systems can be achieved by means of a coating that is able to withstand the lower pH values of the stomach and be able to dissolve at neutral pH of the colon. The most commonly used pH dependent polymers are derivatives of acrylic acid and cellulose. The problem with this approach is that the intestinal pH may not be stable because it is affected by diet, disease and presence of fatty acids, carbon dioxide, and other fermentation products. Moreover, there is considerable difference in inter and intra individual GIT pH, and this causes a major problem in reproducible drug delivery to the large intestine.[113]

Table 1-3 Commonly used pH responsive coating polymers in oral drug delivery.

Brand name	Polymer	Threshold pH
Eudragit® L 100-55	Poly (methacylic acid-co-ethylacrylate) 1:1	5.5
Eudragit L 100	Poly (methacylic acid-co-methylmethacrylate) 1:1	6.0
Eudragit® FS 30D	Poly (methylacrylate-co-methyl methacrylate-co-methacrylic acid) 7:3:1	6.8
Eudragit® S 100	Poly (methacylic acid-co-methylmethacrylate) 1:2	7.0
Eudragi® L 12,5	Poly (methacylic acid-co-methylmethacrylate) 1:1	6.0
HPMCP	Hydroxypropyl methylcellulose phthalate	4.5-4.8
HPMCP 50	Hydroxypropylmethylcellulose phthalate 50	5.2
HPMCP 55	Hydroxypropylmethylcellulose phthalate 55	5.4
CAT	Cellulose acetate trimellate	4.8
PVAP	Polyvinyl acetate phthalate	5.0
CAP	Cellulose acetate phthalate	6.0

1.6.3.2.5. Microbially triggered drug delivery to the colon

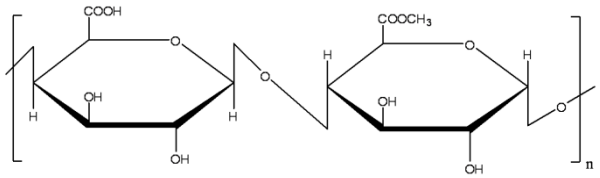
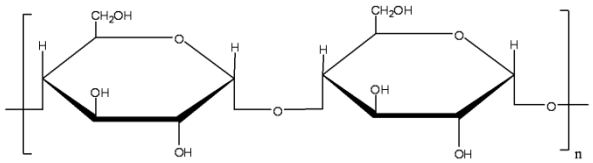
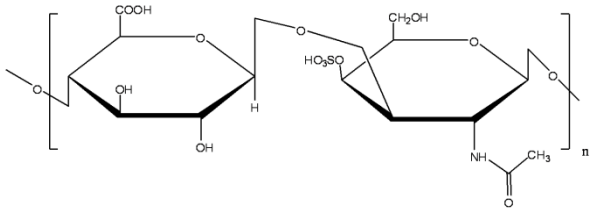
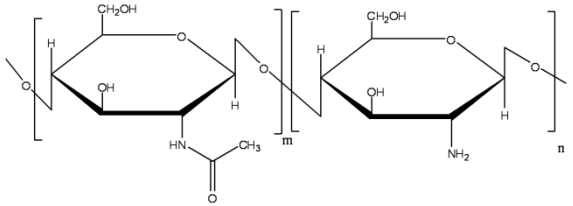
Amongst all the approaches used for colon targeting, a microbially controlled delivery system is the most appealing as it relies on the unique enzymatic ability of the colonic micro flora and enables a more specific targeting, independent of pH variations along the GIT. Thus by using the biodegradable polymers such as Cts, colonic targeting can be achieved.[114]

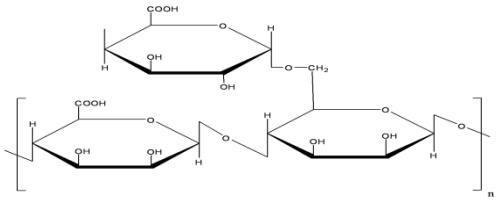
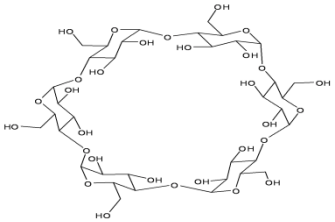
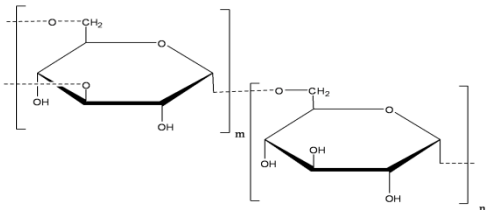
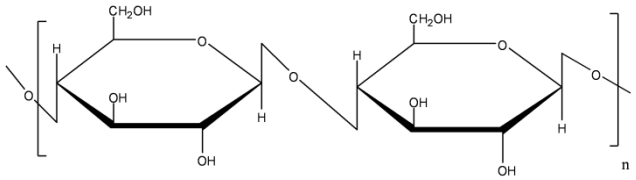
1.7. Polysaccharide-based colon targeted drug delivery systems

Due to the toxicity associated with the synthetic polymeric systems, a wide variety of natural polymers are being studied for the design and development of colon targeted delivery systems, Table 1-4.[95, 115]

Polysaccharides have gained much attention in developing colon specific drug release systems because of their flexibility in obtaining a desirable drug release profile, cost effectiveness, ease of modification, biocompatibility, biodegradability and ability to form hydrogels. Polysaccharides are widely distributed natural polymers. They are formed by condensation reactions of mono saccharides that result in glycosidic linkages. Hydrolysis of the glycosidic linkages on arrival in the colon triggers the release of the entrapped bioactive. The main saccharolytic species responsible for this biodegradation are bacterocides and bifidobacteria.[116] Natural polysaccharides occurring in plants (eg. pectin, guar gum, inulin), animals (eg. Cts, chondroitin sulfate), algae (eg. alginates), or microbes (eg. dextran, xanthan gum) have been studied for colon targeting.[117]

Table 1-4 Polysaccharide based colon targeted delivery systems.

Polysaccharide	Structure	Source	Bacterial species that degrade polysaccharide	Model Drugs
Pectin		Citrus peel and apple pomace[118]	Bacteroids, Bifidobacterium, Eubacterium[119]	Indomethacin, Insulin, Paracetamol, Sulfamethoxazole, Theophylline[120]
Amylose		Plant[121]	Bacteroids, Bifidobacterium[122]	5-Aminosalicylic acid (5-ASA),[123] Glucose[124]
Chondroitin sulfate		Animals and humans[125]	Bacteroids[126]	Indomethacin[127]
Chitosan		Exoskeleton of crustacean and insects or cell walls of bacteria and fungi[128]	Bacteroids[129]	Insulin,[130] Sodium diclofenac,[131] Acetaminophen[132]

Polysaccharide	Structure	Source	Bacterial species that degrade polysaccharide	Drug
Guar gum		Seeds of plants[133]	Bacteroids, Ruminococcus[134]	Dexamethasone,[135] Indomethacin,[136] 5-aminosalicylic acid (5-ASA)[137]
Cyclodextrin		Plant[138]	Bacteroids[139]	Aminosalicylic acid (5-ASA)[140]
Dextran		Microbial (bacterium Leuconostoc mesenteroides)[141]	Bacteroids[142]	Hydrocortisone[143], Theophylline[144]
Cellulose		Plant, microbial (Acetobacter xylinum)[145]	Bacteroids[146]	5-Aminosalicylic acid (5-ASA),[147] Curcumin,[148]

1.7.1. Chitin

In 1811, Professor Henri Braconnot, director of the botanical garden in Nancy, France, first described and named chitin in mushrooms, He originally named it fungine.[149] In 1823, Odier found a compound with the same general properties as fungine in the cuticle of beetles and named it the chitin, which comes from Greek etymology meaning "A coat of mail".[150] The chemical structure of chitin was confirmed by X-ray diffraction by Meyer in 1935.[151]

Chitin is the most abundant nitrogen-bearing organic compound found in nature.[152] Chitin is the world's second most abundant naturally polysaccharide after cellulose and is composed of unbranched chains of β-(1→4) linked-2-acetamido-2-deoxy-D-glucose.[153] Chitin is identical to cellulose in structure, except that it has acetamide groups (-$NHCOCH_3$) at the C-2 position in place of the hydroxyl group.[154] Chitin is a white, hard, inelastic, nitrogenous polysaccharide found in the outer exoskeleton of crustaceans, cartilages of molluscs, cuticles of insects, and cell walls of micro-organisms. Shell waste of crabs and shrimps is the most important industrial source of chitin.[155] It is estimated that worldwide production of chitin is approximately 10^{11} tons per year, mainly from marine sources. On a dry basis, shells consist of chitin, proteins, calcium carbonate, lipids, and astaxanthin. Several methods have been used for the preparation of chitin free of the other components.[156]

For isolation of chitin, the ground up shells are initially treated with 5% NaOH for removing protein then 30% HCl is needed to hydrolyze the lipids and dissolve calcium salts, Figure 1-16.[157] The crystalline structure of chitin originates from extensive hydrogen bonding that occurs between the hydroxyl groups and the *N*-acetamido groups in the repeating units. This in turn prevents the polymer's complete dissolution in common organic and aqueous solvents.[158]

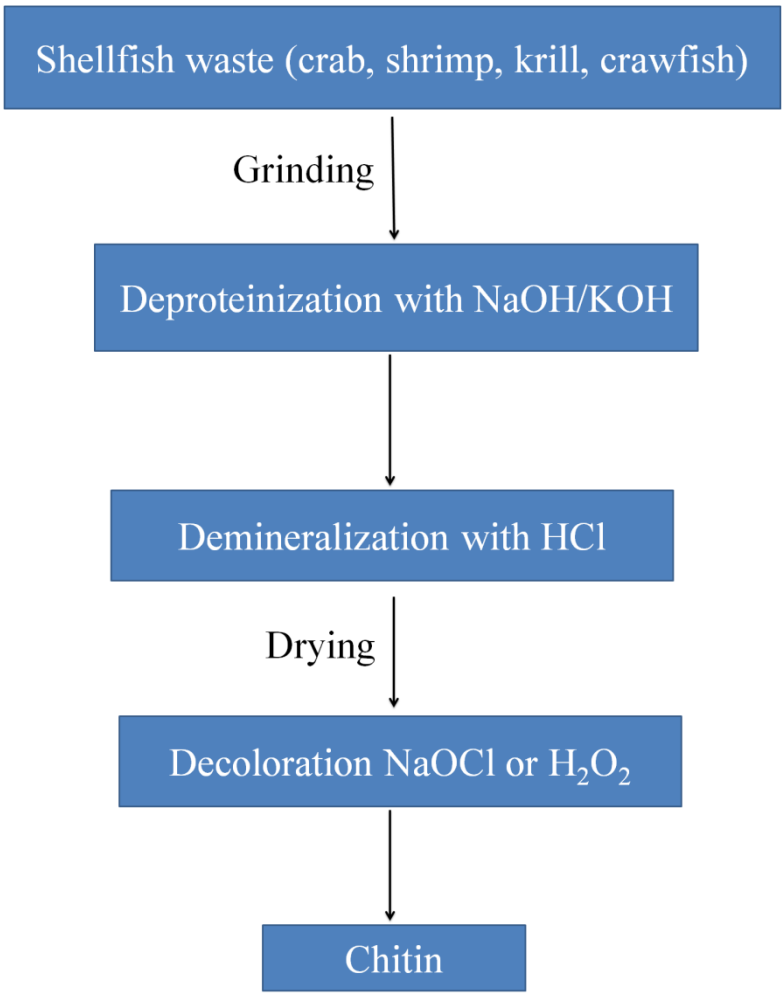


Figure 1-16 Chemical manufacturing processes for chitin.

1.7.2. Chitosan

In 1859 Rouget observed that boiling chitin in potassium hydroxide converted it into a form that was soluble in organic acids,[159] Then in 1894, Hoppe-Seyler named this material chitosan.[160] The structure of Cts was determined in 1950.[161] Interest in Cts has increased in recent years due to its unique properties such as biodegradability, biocompatibility, solubility in dilute acid (as weak as 2% acetic acid), amenablity to chemical modifications and hydrophilicity.[162]

1.7.2.1. Production of chitosan

Cts exists naturally in the cell walls of fungi, but it is mainly derived from chitin by treating chitin flakes with aqueous alkali, Figure 1-17. The most frequently method used requires 30-60% NaOH at 80-120 ^{o}C.[163]

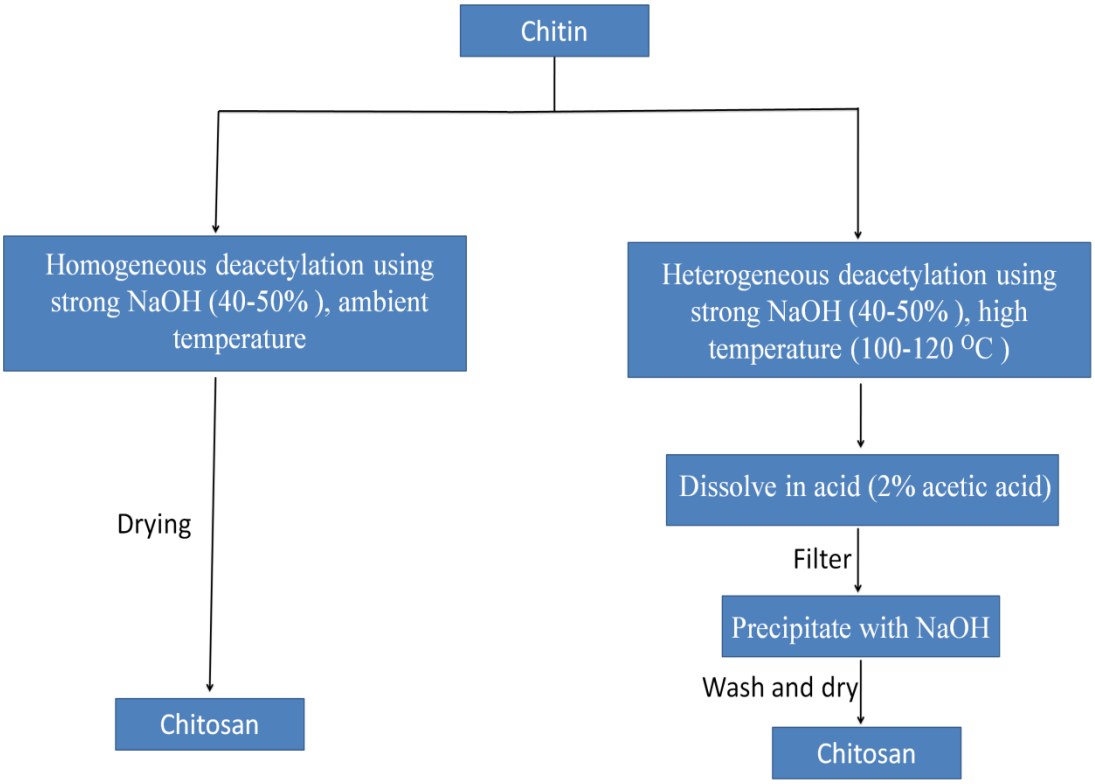


Figure 1-17 Chemical manufacturing processes for chitosan.

1.7.2.2. Structure of chitosan

Cts is linear heteropolysaccharide composed of randomly (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose linked by (1→4)-β-glycosidic bonds. Similar to cellulose, the monomeric units are linked by (1→4)-β-glycosidic linkages, Figure 1-18, and the polymer chains are linear due to the configuration of the anomeric carbon atoms in Cts. Cts have no melting point because they decompose before melting.[164]

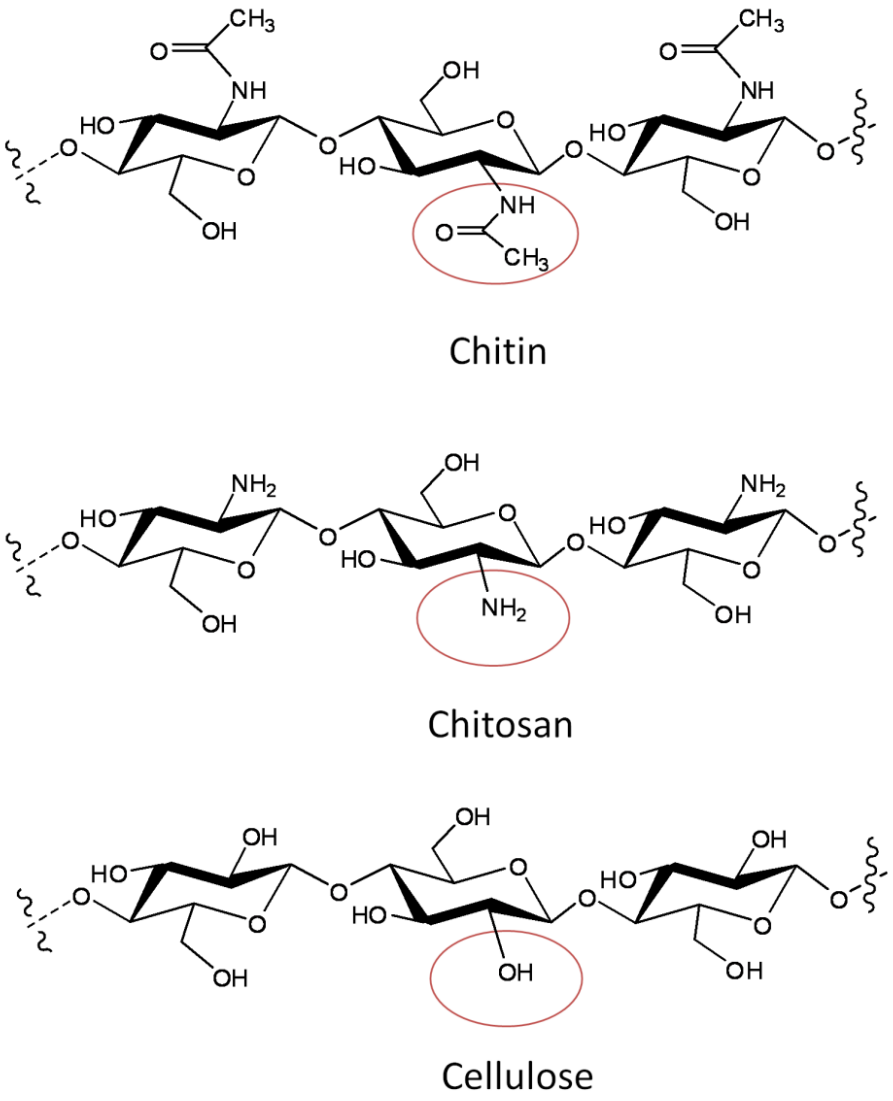


Figure 1-18 Structure of chitin, chitisan and cellulose.

1.7.2.3. Properties of chitosan

Most of the naturally occurring polysaccharides such as cellulose, dextran, pectin, alginic acid, agar, agarose and carragenans, are neutral or acidic in nature, whereas Cts is an examples of basic polysaccharides.

1.7.2.4. Physiochemical characteristics of chitosan

Chemical and physical priperties of Cts can be summarized as follow:

- high molecular weight linear polyelectrolyte.
- cationic polyamine.
- chelate certain transitional metals.
- amenable to chemical modifications.
- high positive charge density at lower pHs.
- forms hydrogels with polyanions.
- hydrophobic at neutral and higher pHs.
- forms highly viscous solutions.
- degrades before melting.
- good optical properties.
- forms films and polyoxysalts.

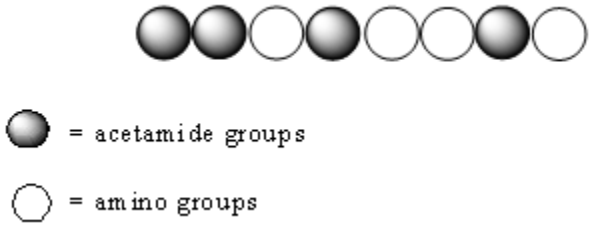
Crystallinity, degree of acetylation, and average molecular weights are the main parameters affecting the Cts properties.[165]

1.7.2.4.1. Degree of deacetylation

Cts is produced through a process called deacetylation Figure (1-19). The deacetylation process is based on removing of acetyl groups from chitin chains and leaving behind amino groups. Therefore, the degree of deacetylation (DD) can be used as a means of specification between chitin and Cts. Homogenous or heterogeneous deacetylation can affect sequencing arrangements of the 2-acetamido-2-deoxy-D-glucopyranose units and 2-amino-2-deoxy-D-glucopyranose units. Block and random are two types of sequencing arrangements of the monosaccharide units of Cts.[158, 166]

Alkaline media, low temperature, and prolonged reaction time are the parameters that influence homogenous experimental conditions.[158] These conditions result in random sequencing of the 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-

glucopyranose units along the polymer chain. In the random copolymers, monomers have no specific sequence and appear in a random fashion:



Heterogeneous experimental conditions include an alkaline solvent system, high temperatures, and short reaction times that result in blocks of *N*-amino units joined to *N*-acetamido units. In theory, block copolymers contain a block of one monomeric unit connected to a block of another monomeric unit.

DD is managed by the alkali concentration, the temperature and the contact time. Enhancing the reaction time or strength of sodium hydroxide solution to achieve full deacetylation causes degradation of polymer at the (1→4)-β-glycosidic linkages in the first instance and considerable decrease in solution viscosity as the chain lengths are reduced.

DD is the ratio of the 2-acetamido-2-deoxy-D-glucopyranose to the 2-amino-2-deoxy-D-glucopyranose units. DD is one of the most important factors in the physicochemical properties of Cts. Therefore, it is critical to use an accurate and rapid method for determining the degree of deacetylation. Many methods have been reported for assessing the DD including the ninhydrin test, linear potentiometric titration, near infrared spectroscopy, nuclear magnetic resonance spectroscopy, hydrogen bromide titrimetry, infrared spectroscopy (IR), and first derivative ultraviolet spectrophotometry.[167] Some of these methods are not suitable because of the cost of

facilities and sophistication for routine purposes. The most convenient way compared to other methods for determining the DD is IR spectroscopy and the following equation:

$$\% \text{ N-deacetylation} = 100\,[1 - (A_{1655}/A_{3450})\,(1/1.33)]$$

where A_{1655} and A_{3450} were the absorbance at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band. The factor "1.33" denotes the value of the ratio of A_{1655}/A_{3450} for fully N-acetylated Cts.[168] It is assumed that the value of this ratio was zero for fully deacetylated Cts and there was a rectilinear relationship between the N-acetyl group content and the absorbance of the amide-I band.

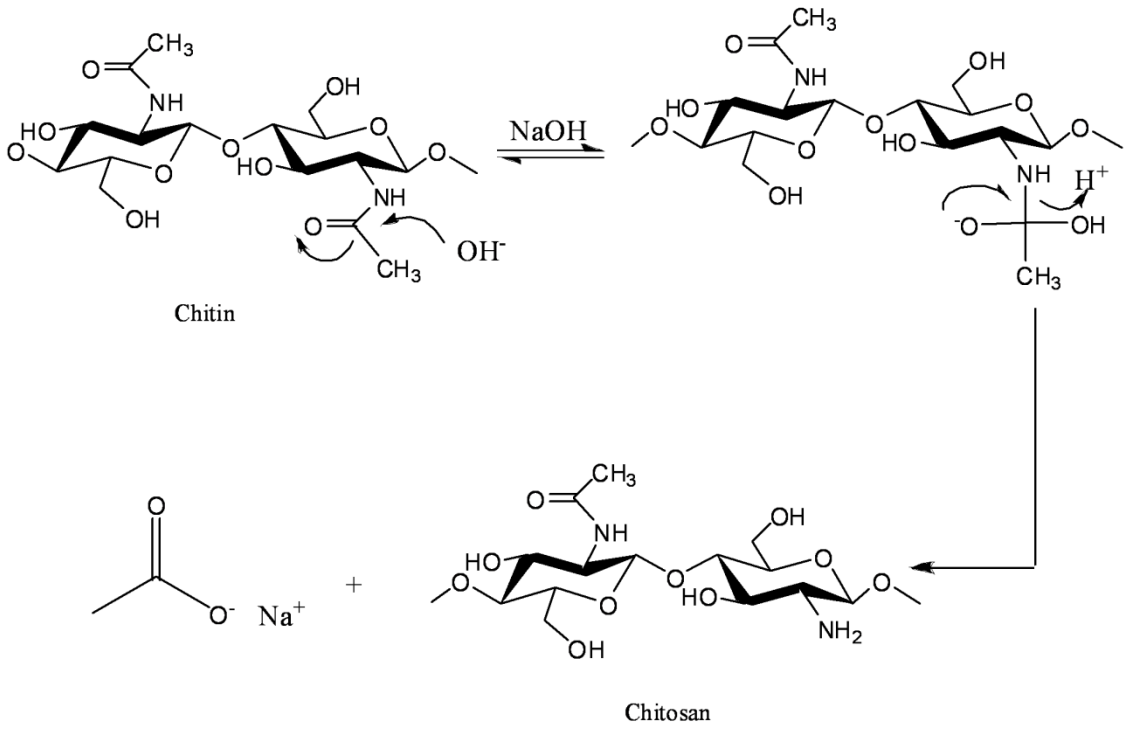


Figure 1-19 Deactylation mechanism of chitin.

1.7.2.4.2. Molecular weight

Molecular weight (Mw) is another important characteristic of Cts, but precise determination of the average Mw of Cts is difficult due to its poor solubility and structural uncertainty. Several techniques have been applied to estimate the Mw of Cts. Viscometry is claimed to be the simplest, most rapid and probably the most precise

determination method for the determination of the average Mw of Cts.[169] This is an important issue for Cts as commercially available Cts have average Mw ranging between 3800 and 20,000 daltons and can be 66 to 95% deacetylated.[170] In other words, Cts can have a wide range of viscosites. One of the simplest and most rapid methods for determining the average Mw of Cts is the Mark-Houwink viscometry method[169, 171]:

$$[\eta] = K M_v^a$$

where [η] is the intrinsic viscosity and M is the molecular weight. a and K are experimental values which can be determined in several solvents.

1.7.2.5. Biological properties of chitosan

Cts is natural polysaccharide with unique biological properties. Its oral LD_{50} in mice was reported of around 16g/kg.[172] Some of the major biological properties of Cts are summarized as follow:[172]

- Biodegradability
- Biocompatibility
- Mucoadhesion
- Hemostatic
- Analgestic
- Adsorption enhancer
- Antimicrobial
- Antioxidant
- Anticholesterolemic

Effects of DD and Mw on biological properties of Cts are summarized on Table 1-5.[172]

Table 1-5 Relationships between Cts biological properties and its degree of deacetylation (DD) and molecular weight (Mw)

Biological peroperty	Factors
Biodegradability	DD, M_w
Biocompatibility	DD,
Mucoadhesion	DD, M_w
Hemostatic	DD, M_w
Analgestic	DD,
Adsorption enhancer	M_w
Antimicrobial	DD, M_w
Antioxidant	DD, M_w

1.7.2.6. Derivatives of chitosan

There has been intensive interest in Cts and its potential use in various biological and clinical applications, including wound dressing, tissue engineering and drug delivery, because of its ready availibility, low production costs, biodegradability and biocompatibility. Reactive groups in the Cts backbone make it easy to adapt this material for biomedical applications by physical and chemical modifications.[164b]

1.7.2.6.1. Physical modification

Various methods have been used to physically modify Cts for varied fields of application. The material can readily be modified physically to obtain conditioned polymer forms such as powders, nano particles, gel beads, membranes, sponges, honeycombs and fibers.[173]

1.7.2.6.2. Chemical modification

Chemical modification of Cts is feasible because it has reactive amino and hydroxyl groups that can be readily modified, Table 1-6.[174] Cts contains 5 to 8% nitrogen depending on the DD.[155] The free amino groups in Cts are suitable for chemical modification, Figure 1-20. It is important to know that this kind of substitution changes the bioactivity and solubility behaviour of Cts.

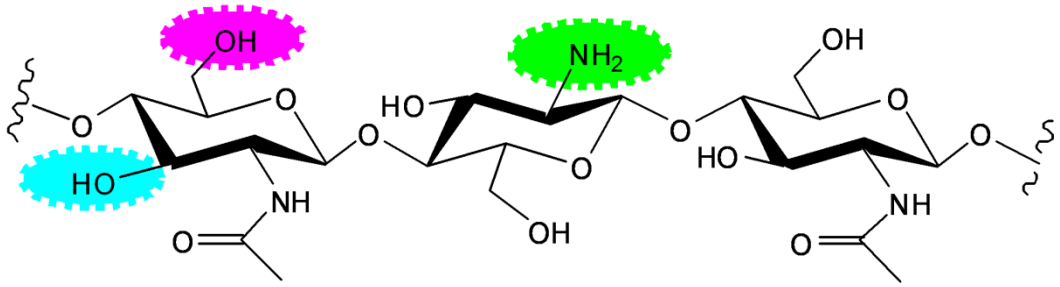
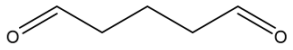
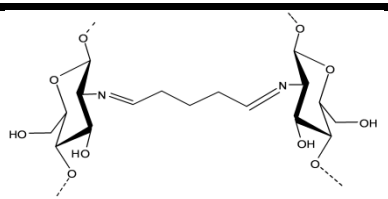
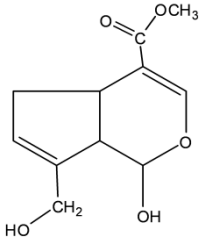
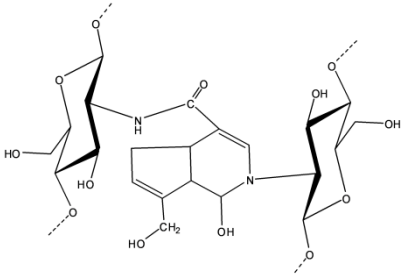
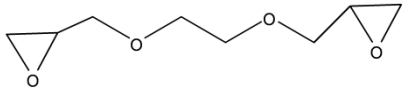
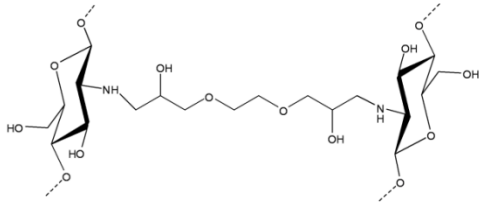
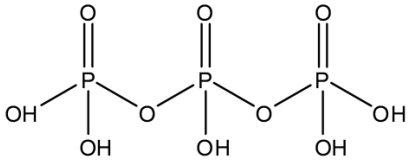
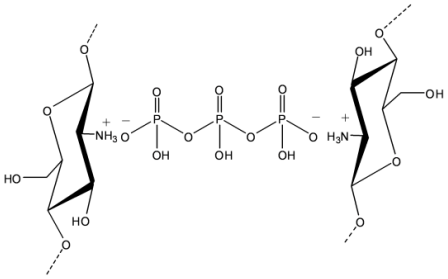
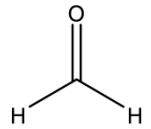
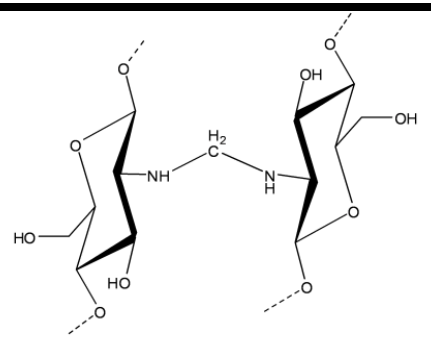
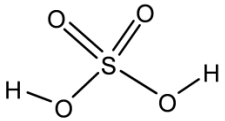
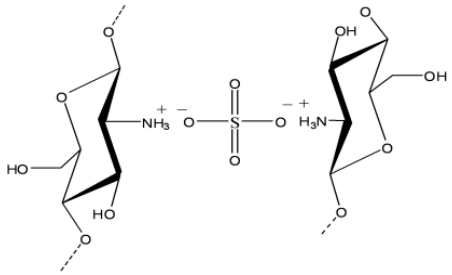
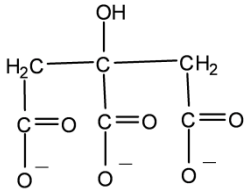
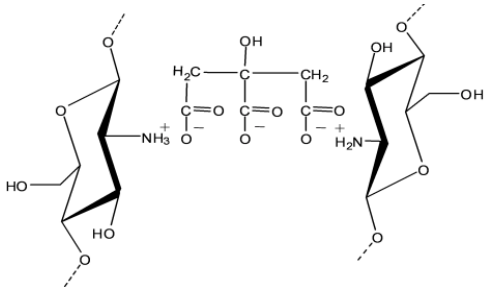


Figure 1-20 Chitosan active sites

After substitution of the *N*-amino groups, strong intramolecular and intermolecular hydrogen bonding between the chains and sheets of Cts is reduced. Spaces are created for water molecules to fill. Therefore the water solubilility of the polymer can increase in suitable cases.[175]

Table 1-6 Examples of various crosslinked chitosan

Agent	Type of modification	Chemical structure of Cts derivatives	References
 Gluteraldehyde	Chemical		176
 Genipin	Chemical		177
 Ethylene glycol diglycidyl ether	Chemical		178
 Tripolyphosphate	Physical		179

Agent	Type of modification	Chemical structure of Cts derivatives	References
 Formaldehyde	Chemical		180
 Sulfuric acid	Physical		181
 Citrate	Physical		182

1.8. Chitosan based colon drug delivery systems

Recently, it was found that Cts is degraded by the microflora that are available in the colon.[164a] Therefore, Cts based delivery systems should be able to be designed to protect bioactives from the hostile conditions of the upper GIT and release the entrapped bioactive specifically in the colon through degradation of the glycosidic linkages of Cts by microflora present in colon. As a result of this concept, the interest in Cts and its derivatives as colon specific drug delivery vehicles has increased in recent years. As a result different pharmaceutical dosage forms are being developed for colon specific drug delivery systems.[128, 183]

1.8.1. Microspheres

Microspheres have many applications in medicine. In most cases, microspheres are used as drug carriers to deliver drugs to the areas of interest and slowly release encapsulated drugs over a desired period of time to maintain an effective local drug concentration. The size of microsphere depends on the method of preparation, types of Cts and/or crosslinking methods.[184] Lorenzo *et al* prepared Cts microspheres of sodium diclofenac by a spray drying technique. These microspheres were enteric coated with Eudragit L-100 or Eudragit S-100. No release was seen in acidic pH for three hours, but at higher pH, Eudragit dissolved and swelling of Cts started leading to continuous drug diffusion which was completed in four hours.[185] Berthold *et al*. prepared Cts microparticles by a precipitation/coacervation technique using sodium sulfate for delivery of prednisolone to the colon. The highest loading of 30.5% was achieved. The drug release profile in 0.2 M phosphate buffered salines (PBS) (pH 7.0) was investigated.[186] Cts crosslinked with sodium cellulose sulfate (NaCS) and sodium polyphosphate (PPS) was also prepared and loaded with 5-ASA as a model drug. The microcapsules prepared had an average diameter of 1.90 mm with a loading efficiency of 61% and encapsulation efficiency of 90%. The amount of 5-ASA released was determined in simulated colonic fluid (SCF) at pH 6.4.[187] Mura *et al.* designed N-succinyl-Cts microparticles using spray drying for colon specific delivery. 5-ASA release was investigated for up to 24 hours in three different solutions: pH 2.0, 5.5 and 7.4. In simulated gastric fluid (SGF) (pH 2.0), during the first two hours, 5-ASA release was less than 10% while at pH 7.4 the drug release after four hours reached 51%.[188] 5-ASA loaded Cts-Ca-alginate microparticles were also prepared using a spray drying method followed by ionotropic gelation/polyelectrolyte complexation and were tested for colon targeted drug delivery.[189] Albendazole was also delivered specifically into the colon, in another

study, using microspheres of Cts hydrochloride. Drug release in 24 hours was 49% and 77% in colonic fluid without and with rat caecal contents, respectively.[190]

1.8.2. Nanoparticles

Nanoparticles have been widely applied in therapeutics, diagnostics and imaging in medical and pharmaceutical fields.[191] In therapeutics, surface-modified or multifunctional nanoparticles have been used to delivery various therapeutic drugs such as cancer therapeutics, vaccines and nucleic acids. They increase the effective drug concentration at desired diseased sites and decreased undesirable side effects of current therapies.[192] For the preparation of Cts nanoparticles, several techniques are available such as emulsion, ionotropic gelation, reverse micellar, solvent evaporation, coacervation, and sieving methods.[193] For cancer therapy, hydrophilic 5-FU was successfully loaded into Cts nanoparticles (250-300 nm diameter) using the water-in-oil emulsion method, followed by chemical crosslinking of the Cts with glutaraldehyde.[194]

HA coupled with Cts nanoparticles were prepared using an ionotropic gelation method for colon targeted delivery of 5-FU.[195]

Tozaki *et al*. developed colon specific insulin delivery with Cts capsules. *In vitro* drug release experiments using Cts capsules containing 5(6)-carboxyfluorescein (CF) were carried out. Small release of CF from the capsules was observed in an artificial gastric fluid (pH 1) and in an artificial intestinal fluid (pH 7). However, the release of CF was markedly increased in the presence of rat caecal contents.[130] Enteric coated Cts capsules, loaded with acetaminophen, were found to protect the drug in the upper GIT. The drug was postulated to be released in the colon by biodegradation of Cts.[196]

1.8.3. Beads

Alginate-Cts blended gel beads were prepared based on Ca^{2+} or dual crosslinking with various proportions of alginate and Cts by Xu *et al*. The sustained release of a model protein drug, was

investigated in SGF, SIF and SCF. The bovine serum albumin (BSA) cumulative release from alginate-Cts blended gel beads with mass ratios 9:1, 7:3 and 5:5 were reported to be 2.35, 1.96 and 1.76% (in SGF, 4 h), 82.86, 78.83 and 52.91% (in SIF, 3 h) and 97.84, 96.81, 87.26% (in SCF, 3 h) respectively.[197]

Cts beads loaded with ciprofloxacin hydrochloride were also fabricated by ionic crosslinking with sodium tripolyphosphate. The number of coatings or thickness of coat on beads influenced the release rate of ciprofloxacin.[198]

1.8.4. Tablets

The compressed tablet is the most popular dosage form in use today. Little research has been done so far to develop colon specific drug delivery systems using compression coated Cts tablets. Nunthanid *et al* reported spray dried Cts acetate (CSA) and ethylcellulose (EC) as compression coats for 5-ASA tablets.[199] Various% weight ratios of EC:CSA were investigated at 0.1N HCl (SGF, pH1.2), Tris.HCl (SIF, pH 6.8) and acetate buffer (SCF, pH 5.0). EC:CSA of 87.5:12.5% weight ratio provided the lag time needed for the tablets to reach SCF. The drug was released over 90% within 12 hours. The effect of enzymes was not tested in this study.

Cts-chondroitin sulfate based matrixed tablets of indomethacin were investigated by Amrutkar *et al*. In this report the *in vitro* drug release studies were conducted in 0.1 N HCl (pH1.2) for 2 h, phosphate buffer pH 7.4 for three hours and finally in phosphate buffer pH 6.8 up to 24 hours with and without rat cecal contents. They found the drug release rate of the tablet depended on the concentration of polysaccharide used as binder, the matrix composition, and the time of crosslinking.[200]

Omwancha *et al*. investigated caffeine containing core tablets. EC and Cts were used in the compression coated tablets in an attempt to achieve colon specific drug delivery.[103a]

1.9. Thesis objective

There are approximately 15,000 people in New Zealand affected by inflammatory bowel disease and according to New Zealand ministry of health. Bowel cancer is one of New Zealand's most common cancers and the second highest cause of cancer deaths. The death rate from bowel cancer in New Zealand is one of the highest in the developed world. At least 2,700 people are diagnosed with bowel cancer every year and more than 1,200 die each year as a result. That is equivalent to more than 100 New Zealanders every month. Because a high intracolonic drug concentration is required for the treatment of diseases associated within colon, a considerable amount of research work has been carried out to develop colon targeted drug delivery systems.

In the present research, two strategies were applied to develop an efficient colon targeted drug delivery system.

In the first approach, the aim was to design and construct a carrier with high mechanical and chemical stability to reach the colon and to protect the drug from the obstacles of gastrointestinal tract.

In the second approach, the aim was to design pH sensitive and bacterially degradable carriers to protect the drug from the harsh acidic environment of the stomach and the rigorous enzymic activity of the small intestine and deliver the drug to the colon to provide increased effective therapy for diseases associated within colon.

1.10. The gap in knowledge that this research aims to fill

Despite the scientific progress being made in the application of Cts in colon drug delivery, variations of pH lead to unpredictable drug release throughout the GIT.[201] No chitosan based drug delivery systems have been launched on the market to date. My project has delivered the successful synthesis of new bioacceptable polymer systems which are pleasingly stable in acidic and basic media. In particular, the tablets are designed for enzymic disintegration and drug release in colon. This polysaccharide based polymer system with both acid and base stability is a unique result that carries with it distinct patenting possibilities.

1.11. Thesis structure

This thesis presents the developments of novel chitosan based systems that would be able to protect a drug from degradation and release in the stomach and small intestine and yet allow the targeted drug release in the colon.

Chapter 2

Describes the Cts based films with improved physical properties by introduction of the cyclic imide moiety into the Cts matrices. The results show that the heterocyclic imide linkage imparts excellent thermal, mechanical and chemical stability to the Cts film.

Chapter 3

Investigates the potential of a Cts microsphere crosslinked with pyromellitic dianhydride (PMDA) for controled drug release. In this chapter, bovine serum albumin (BSA) as a model protein drug was used to investigate the drug release behaviour of spray dried Cts-PMDA microspheres.

Chapter 4

A novel generation of Cts based amphoteric pH sensitive hydrogels was designed and characterized. In Chapter 2, Cts crosslinked with dianhydride derivatives were employed to insert an imide ring on to the Cts backbone. In this chapter, Cts was crosslinked with dianhydride derivatives to obtain amic acid derivatives of Cts.

Chapter 5

In this chapter, films were formulated with a view toward colonic delivery of 5-fluorouracil (5-FU) using a novel amphoteric crosslinked Cts matrix. In order to improve the pH sensitivity of the crosslinked Cts, sodium alginate, which has a high concentration of carboxylic groups, was incorporated into the crosslinked Cts hydrogel.

Chapter 6

A combination of time, pH, and enzyme control using a novel amphoteric Cts based tablet was developed. The major objective of the this study was to develop colon specific tablets that would be

able to protect a drug from degradation and release in the stomach and small intestine and yet allow targeted drug release in the colon.

Chapter 7

Ronidazole (RDZ) is currently the most widely used drug to treat *Tritrichomonas foetus* intestinal infection in cats. Despite its efficacy in the treatment of *T. foetus*, RDZ has been reported to cause neurotoxicity in some cats due to rapid absorption in the small intestine. Therefore a compression coated tablet containing an amphoteric derivative of Cts was developed to protect RDZ from release in the stomach and small intestine.

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Chapter Two

Preparation and characterization of crosslinked chitosan based films with excellent physiochemical properties

2.0. Introduction

The development of functional biodegradable polymeric materials has received considerable attention for diagnostic and medical applications such as wound healing, hypocholesterolemic activity, anticoagulation, antithrombogenic activity, tissue reconstruction and as drug delivery vehicles with effective release capability.[1] Among these materials, there has been a growing interest in Cts for biomedical and drug delivery applications due to its several favorable biological properties such as biodegradability, biocompatibility, nontoxicity, antimicrobial and mucoadhesive properties.[2] Cts is a hydrophilic polyelectrolyte polysaccharide composed of glucosamine and N-acetylglucosamine units linked by β(1–4) glycosidic bonds, that is produced by alkaline N-deacetylation of chitin. Chitin is the main component of the shells of crabs, shrimps, and krill.[3] The polycationic nature of Cts allows excellent bioadhesivity which in turn allows it to readily bind to negatively charged surfaces of mucosal membranes. The excellent film-forming properties of Cts have led it to be recognized as a promising biomaterial for controlled drug delivery systems.[4] For any film-based controlled release system, having a comprehensive understanding of film characteristics is crucial. As an alternative to some surgical applications, a film exhibiting high mechanical strength is considered as a favourable feature for controlled release devices.[5] Therefore, improving mechanical properties and the thermal stability of Cts composites through blending with other polymers or cross-linking has lately sparked much attention in academia and industry.[6] Due to extensive intra-and intermolecular hydrogen bonding in Cts, most crosslinking attempts lead to destruction of Cts crystallinity and reduction in the puncture and tensile strength of the film.[7]

Glutaraldehyde (GA), epoxy compounds and genipin are the most common cross-linking reagents used as cross-linkers for Cts.[8] Significant efforts have been devoted to developing materials with high thermal and mechanical properties. Aromatic polyimides are a major component of an important class of high performance polymers which have received extensive attention for their exceptional characteristics including high thermal and mechanical stability and chemical resistance.[9] These properties are influenced by the nature of the five-membered heterocyclic imide originating from the aromatic dianhydride.[10]

The aim of this study is to develop Cts based films with improved physical properties by introducing a cyclic imide moiety into the Cts matrices. In this chapter a protocol for the crosslinking of dianhydride derivatives with Cts is developed. The results show that the heterocyclic imide linkage imparts excellent thermal, mechanical and chemical stability to the Cts film.

2.1. Materials and Methods

2.1.1. Materials

Cts was purchased from Acros Organics, (Geel, Belgium, batch number: A0319220). The molecular weight of Cts was determined to be 1.62×10^5 D (determined by the Mark-Houwink viscometry method in a solvent of 0.1M acetic acid/0.2M NaCl maintained at 25^{o}C). The degree of deacetylation of the Cts was 70%, which was determined by elemental analysis. Pyromellitic dianhydride (PMDA), benzophenone-3,3′,4,4′-tetracarboxylic dianhydride (BTDA), 4,4′-oxydiphthalic dianhydride (ODPA), 4,4′-(hexafluoroisopropylidene) diphthalic anhydride (FDA) and 1,4,5,8-naphthalenetetracarboxylic dianhydride (NTDA) were obtained from Sigma-Aldrich (Auckland, New Zealand). Lysozyme (LYZ) was from Boehringer Mannheim GmbH (Mannheim, Germany).

2.1.2. Preparation and crosslinking of the polymeric films

The Cts films used in the study were produced by a casting/solvent evaporation technique. Cts powder (5 g) was dissolved in Milli-Q water (500 ml) containing 2.0% (v/v) acetic acid at room temperature. The resulting viscous Cts solution was filtered to remove any undissolved Cts and then sonicated for 15 min to remove trapped air bubbles. The solution (30 g) was then poured into a glass Petri dish and allowed to air dry at room temperature. The resulting film was peeled off and neutralized by immersion into an aqueous NaOH solution (1 M) and subsequently oven dried at 40^{o}C for 24 h. The cross-linked films were prepared by soaking the Cts containing film (2 g) in glacial acetic acid (25 ml) for 1 hr then 3 eq of the respective dianhydride derivatives dissolved in DMF (50 ml) were added. The dianhydrides used were PMDA, BTDA, FDA, OPDA and NTDA. The mixture was heated to 130^{o}C for 24 h. The films were removed from the solutions, rinsed with methanol and dried in an oven at 40^{o}C for 24 h.

2.2. Characterization of the crosslinked films

2.2.1. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the films were recorded using Nicolet 5700 FTIR spectrometer in the range of 4000–400 cm^{-1}.

2.2.2. Nuclear magnetic resonance (NMR) spectroscopy

Solid-state carbon-13 (^{13}C) magic angle spinning (MAS) NMR spectra were obtained at a ^{13}C frequency on a 50.3 MHz on a Bruker (Rheinstetten, Germany) DRX 200 MHz spectrometer. Samples were packed in 7-mm diameter cylindrical zirconia rotors with Kel-F end-caps and spun at 5.0 ± 0.2 kHz in a Doty Scientific MAS probe. During acquisition, the sample temperature was maintained at 20^{o}C. Cross-polarised (CP-MAS) ^{13}C spectra free induction decays (FIDs) were acquired with a ^{1}H 90^{o} pulse of 5.5 µs, a sweep width of 16 kHz and 960 data points were collected over an acquisition time of

28 ms, a relaxation time of 1 sec, 2 k scans and a cross-polarisation contact time of 1000 µs. All spectra were zero filled to 4k data points and processed with a 0.005 s Gaussian broadening. Chemical shifts were externally referenced with glycine.

2.2.3. Determination of the degree of substitution

Degree of substitution (DS) was determined by elemental analysis. DS was calculated on the basis of the percentage of nitrogen in the product by the following Equation:[11]

$$\frac{(\mathrm{N}-7.7)}{\left(\frac{938}{143+\mathrm{M}}+\frac{462}{185+\mathrm{M}-7.7}\right)}\times 100$$

where N is the percentage of nitrogen content and M is the molecular weight of cross-linking agents.

2.2.4. Swelling studies

The swelling characteristics of Cts and crosslinked Cts films were determined by immersing dried films in buffer solutions ranging from pH 1.2 to 10 at 37 °C. After 24 h, the films were taken out and were blotted with a filter paper to remove surface absorbed water, and weighed immediately. The swelling percentages S (%) of samples were calculated from the following expression:

$$\mathrm{S}\ (\%)=\left[\frac{(\mathrm{W_s}-\mathrm{W_d})}{\mathrm{W_d}}\right]\times 100$$

where W_s and W_d are the weights of swollen and dried films.

2.2.5. Film thickness

Film thickness was measured using a digital micrometer. Measurements were taken at 6 different locations on each film sample and were averaged.

2.2.6. CHN elemental analysis

Elemental analysis was performed using a Carlo Erba Elemental Analyser EA 1108 using a flash combustion technique. The analyses were carried out at the Campbell Microanalytical Laboratory, Otago University, Dunedin,New Zealand.

2.2.7. Mechanical test

The mechanical properties of the dry films were determined using a TA-XT.plus Texture Analyzer (Stable Micro Systems, UK). A 5-mm-diameter spherical puncturing probe was driven through the dry film (square piece of 1×1 cm^2) with a speed of 1 mm/min. The load at break and the maximum displacement of the film samples were measured, and then converted to puncture strength (MPa) and elongation. The puncture strength (S) and % elongation were calculated using the following Equations:[12]

$$\text{Puncture strength} = \frac{F_{max}}{A_{cs}}$$

where F_{max} is the maximum applied force, A_{cs} is the cross-sectional area of the edge of the film located in the path of the cylindrical hole of the film holder.

$$\text{Percentage of elongation} = \left[\frac{\left\{(r^2 + d^2)^{1/2} - r\right\}}{r}\right]$$

where r is the radius of the film exposed in the cylindrical hole of the film holder and d represents the displacement of the probe from the point of contact to the point of puncture.

2.2.8. Thermal gravimetric analysis (TGA) and differential thermal gravimetric analysis (DTG)

A TA Instruments SDT Q600 instrument was used for simultaneous DTG and TGA data acquisition. Data were analyzed using TA Universal Analysis software. Samples were loaded into an aluminium oxide crucible and heated at a rate of 5^{o}C/min to 600^{o}C under a dynamic nitrogen atmosphere up.

2.2.9. *In vitro* biodegradation study

A ninhydrin assay was used to determine the *in vitro* degradability of each film using LYZ[8c] with an activity of 87900 U/mg. The membranes (0.01 mg) were completely immersed in a 1000 U/ml LYZ solution and incubated at 37^{o}C in a pH 7.4 buffer solution for 5 days. After degradation, the formation of oligomers containing N-glucosamine units, due to the cleaved β-glycosidic bonds of Cts, was determined by the ninhydrin assay.

2.2.10. Contact angle analysis

Water contact angles of the films were measured using a CAM-200 instrument (KSV instruments, Finland). The reported angle is expressed as the mean standard deviation from the average of five measurements on different areas of each sample.

2.3. Results and discussion

2.3.1. The degree of substitution

In an attempt to improve the mechanical and thermal stability of the Cts matrices, crosslinking was carried out with the dianhydride derivatives: PMDA, BTDA, ODPA, FDA and NTDA. Addition of the dianhydrides to Cts led to the formation of the cyclic imides. This heterocyclic imide linkage offers the potential for excellent improved

thermal, mechanical and chemical stability to the Cts. The imidization reaction mechanism involves a reversible nucleophilic substitution reaction where the Cts amino group attacks one of the carbonyl carbons in the anhydride moiety and displaces a carboxylate functionality, followed by proton transfer. Thermally induced imidization results in cyclization of the *o*-carboxyamide intermediate through nucleophilic attack of the amide nitrogen on the acid carbonyl carbon followed by elimination of water. Degrees of substitution calculated from elemental analysis were 80.4, 69.5, 68.1, 69.1 and 40.2% for Cts-PMDA, Cts-BTDA, Cts-ODPA, Cts-FDA and Cts-NTDA respectively.

Dianhydride reactivity is governed by electron affinity (Ea)[13] and thus PMDA with the highest Ea, shows the highest reactivity (DS = 80.4%). The lower DS of Cts with NTDA could be related to the poor solubility of NTDA in DMF. Their reactivity of BTDA and ODPA is influenced by the bridging group. BTDA is bridged by an electron withdrawing carbonyl group, which by withdrawing electron density through the π-orbitals facilitates attack by the nitrogen atom of the Cts. ODPA on the other hand is linked by an oxygen atom which by donating electrons into the ring reduces the reactivity of the anhydride toward nucleophilic nitrogen attack.

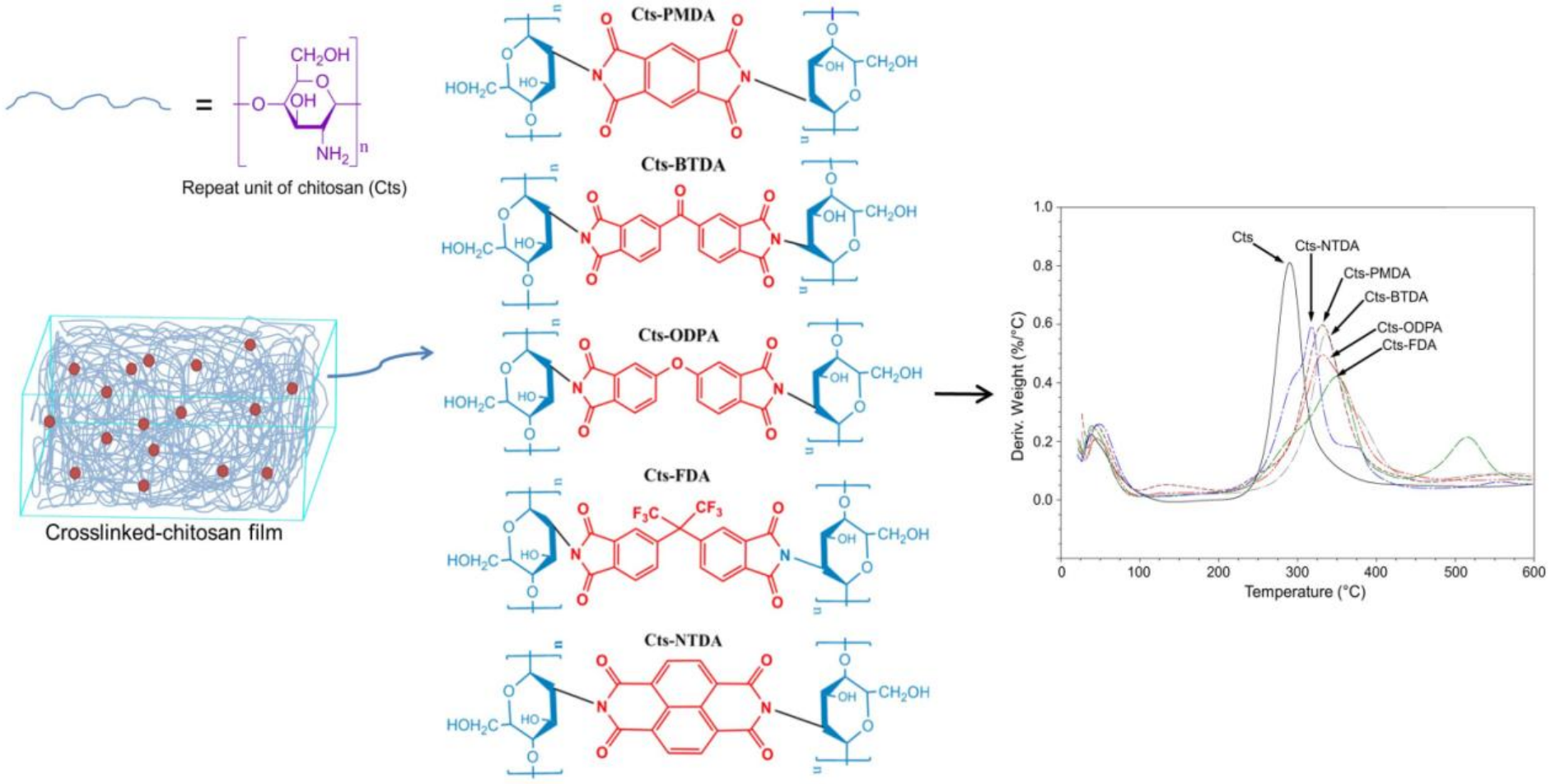

Figure 2-1 Representation of crosslinked chitosan based films.

2.3.2. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of Cts and crosslinked Cts based films (Cts-PMDA, Cts-BTDA, Cts-FDA, Cts-ODPA, Cts- NTDA) are shown in Figure 2-2. The major peaks for the Cts can be attributed as follows: the peak at 3288.9 cm^{-1} is assigned to the -OH and -NH stretching vibrations, while the peak at 2877.2 cm^{-1} is assigned to the aliphatic C-H stretching vibration in the -CH and -CH_2 groups. The amide frequencies consisting of the -C=O bond stretch of the remaining acetamido groups and the N-H bending vibrations of the -NH_2 groups are observed at 1659.9 and 1595.2 cm^{-1} respectively. The peak at 1423.0 cm^{-1} is assigned to the -NH_2 deformation. Further bending vibrations are observed at 1381.2 cm^{-1} for the C-C-H symmetrical bending vibration in the alcohol.

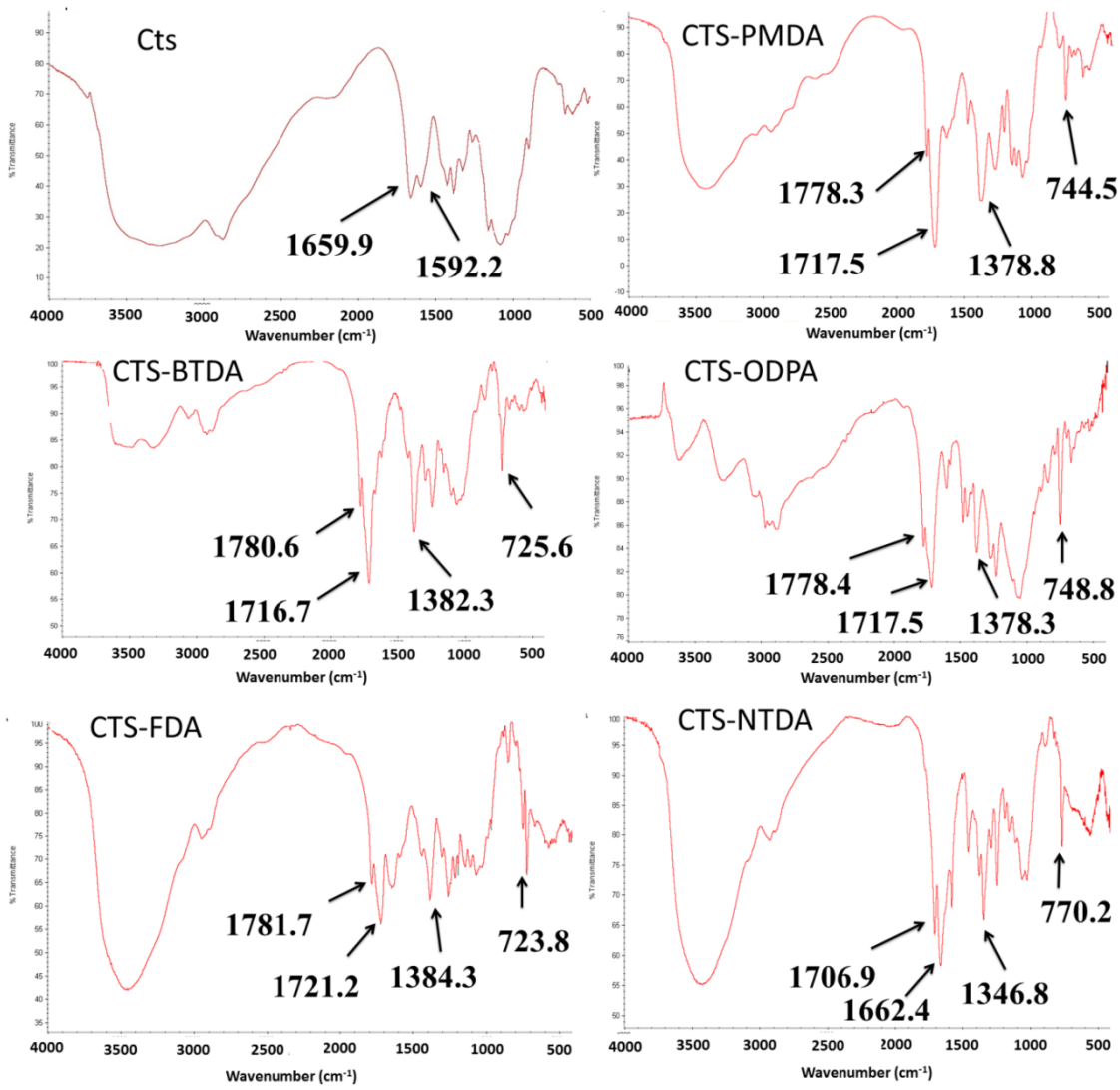

Figure 2-2 FTIR spectra of chitosan, and dianhydride-crosslinked chitosan.

Stretching vibrations are also observed at 1323.4 and 1155.6 cm^{-1} for the C-N stretching vibration and at 1078.3 and 1033.4 cm^{-1} for the -CO stretching vibration of the alcohol groups. All the spectra of crosslinked Cts-based films exhibit the intense characteristic bands of the imide which demonstrates a high degree of imidization. After modification of Cts, the spectra showed some major changes. The three bands around 1780, 1720, and 730 cm^{-1} are assigned to the characteristic bands of the symmetrical and unsymmetrical C=O stretches of the cyclic imide, and the bending vibrations of the C=O, respectively.[14] The band around 1380 cm^{-1} is assigned to the stretching vibration of the C-N group in the five-membered cyclic imide. In the case of Cts-NTDA (six-membered cyclic imide) the asymmetric and symmetric stretching, and the bending vibrations of C=O were shifted to 1706, 1662.4, and 770.2 cm^{-1}, respectively. The C-N stretching band had shifted to 1346.8 cm^{-1}.[15]

2.3.3. Mechanical testing

Poor mechanical properties limit applications of Cts for some biomedical applications. The enhancement of mechanical properties comprises a critical step for expanding the biomedical applications of Cts. Many attempts have been carried out to improve the mechanical properties of Cts, such as modification of Cts through blending with other polymers and cross-linking.[6a] Due to extensive intra- and intermolecular hydrogen bonding interactions of Cts, most modification attempts lead to destruction of Cts crystallinity and reduction in the puncture and tensile strength. Alexeev *et al*. reported that for the Cts/PEO blends containing 16.7 wt% of PEO, a doubling of the tensile strength was observed but dissolution of this blended film limited their application.[16] Long Mi *et al.* state that the ultimate tensile strength values of the Cts membranes increased after crosslinking of GA or genipin, while tensile strength decreased when the degree of substitution reached 30%.[17] Leceta *et al*. prepared Cts-based films plasticized with glycerol. The mechanical test data showed a reduction in tensile strength when the glycerol content was increased from 0 to 30%.[18] Chen *et al.* studied the mechanical properties of *N*-phthaloyl acylated Cts, and this showed a reduction in tensile strength compared to unmodified Cts.[7] The results for the PS of Cts and dianhydride cross-linked Cts based films in this study are shown in Table 1. To our knowledge, these films are the only crosslinked Cts films that show significant PS at high levels of substitution.

This property can be attributed to the cyclic imide moiety which plays a key role, imparting the excellent mechanical properties to these polymers. Additionally, participation of the carbonyls in the cyclic imides in the formation of hydrogen bonding with the N-H groups of Cts could be a further crucial factor in the high PS of the studied films. Based on the data in this study, Cts-PMDA was shown to have an almost three fold improvement in PS. Table 2-1 also shows that the dried crosslinked Cts films led to a decrease in their elongation capability.

Table 2-1 Puncture strength (PS) and elongation at break (% E) of the chitosan and crosslinked chitosan films.

Film	Film thickness (µm ± SD)	Puncture strength (MPa ± SD)	Elongation (% ± SD)
Cts	250.2 ± 0.6	7.6 ± 0.1	7.2 ± 0.1
Cts-PMDA	270.2 ± 0.2	19.8 ± 0.3	3.2 ± 0.2
Cts-BTDA	280.2 ± 0.7	17.9 ± 0.1	3.3 ± 0.1
Cts-ODPA	270.2 ± 0.5	14.9 ± 0.4	3.1 ± 0.3
Cts-FDA	280.2 ± 0.6	19.5 ± 0.2	3.0 ± 0.1
Cts- NTDA	280.2 ± 0.8	18.4 ± 0.3	3.0 ± 0.1

2.3.4. Swelling studies

The swelling capability of mucoadhesive polymers is a very important parameter to consider as it contributes to the adhesive and cohesive properties.[19] Figure 2-3 shows that all crosslinked Cts matrices produced the highest degree of swelling at pH 4 and 6. This can be explained by the fact that below the pKa value of the Cts amine groups (pH 6.5), the swelling is controlled mainly by the amino group (NH_2) on the C-2 carbon of the Cts component. The protonation of the Cts amino groups leads to repulsion of the

polymeric chains.[20] The Cts derivatives show greater swelling compared to unmodified Cts at pH 2, due to dissolution of unmodified Cts under very acidic conditions. Swelling of dianhydride derivatives (about 60%) at pH 2 confirms the chemical stability of these novel Cts-based hydrogels. Cts derivatives shows higher swelling percentage at pH 7.4 compared to pH 4. This trend can be explained due to higher amount of NH_3^+ groups at pH 4. Crosslinked networks shows higher swelling compared to unmodified Cts at pH above 6 due to an increase of the hydrophilicity of the matrix after crosslinking with the dianhydride derivative (Figure 2-6). Cts-FDA shows the lowest water absorption compared to the other derivatives. This results from the increase in hydrophobicity of this polymer due to the two trifluoromethyl groups which lead to a reduction in the adsorption of water. These results indicate that the dianhydride crosslinked Cts matrices have applicability as insoluble films, at least in the range from pH 2-10.

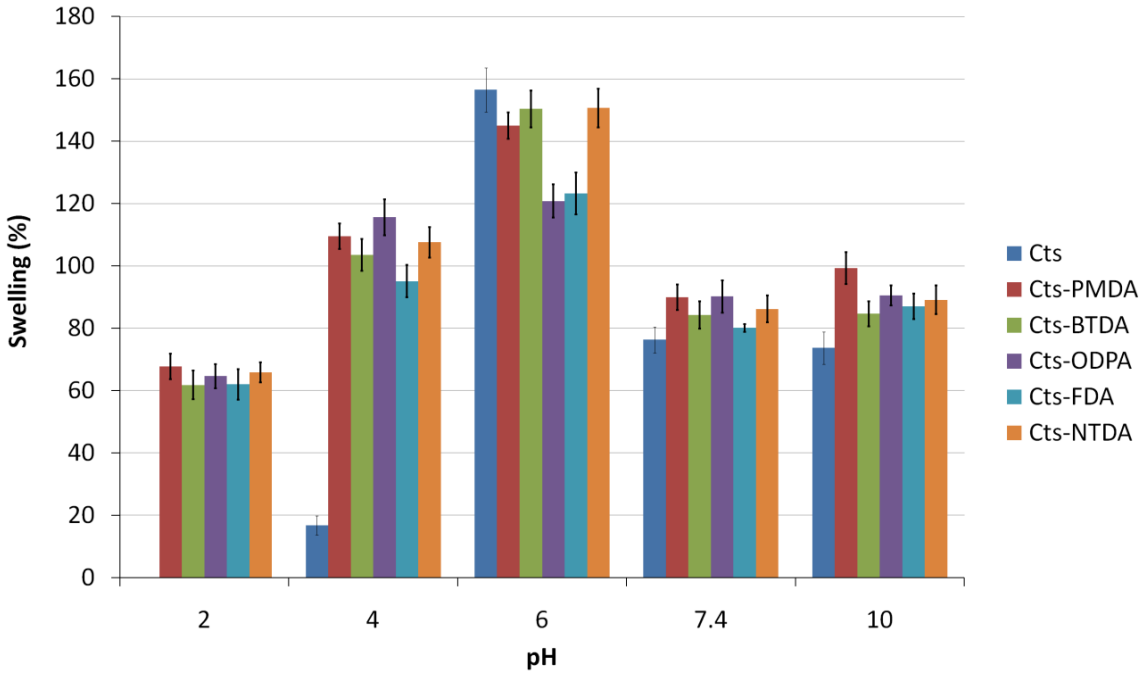

Figure 2-3 Swelling behaviour of chitosan films at different pH.

2.3.5. Solid state ^{13}C NMR analysis

The analysis of the ^{13}C DP-MAS spectrum of Cts and the dianhydride crosslinked Cts hydrogels are shown in Figure 2-4. The ^{13}C DP-MAS spectrum for Cts is very similar to that reported in the literature.[21] Signals at 51.2 and 76.2 ppm are attributed to the C2/C6 and C4 carbons respectively. A well-defined signal is observed at 68.8 ppm, which is assigned to overlapping C3 and C5 carbons.

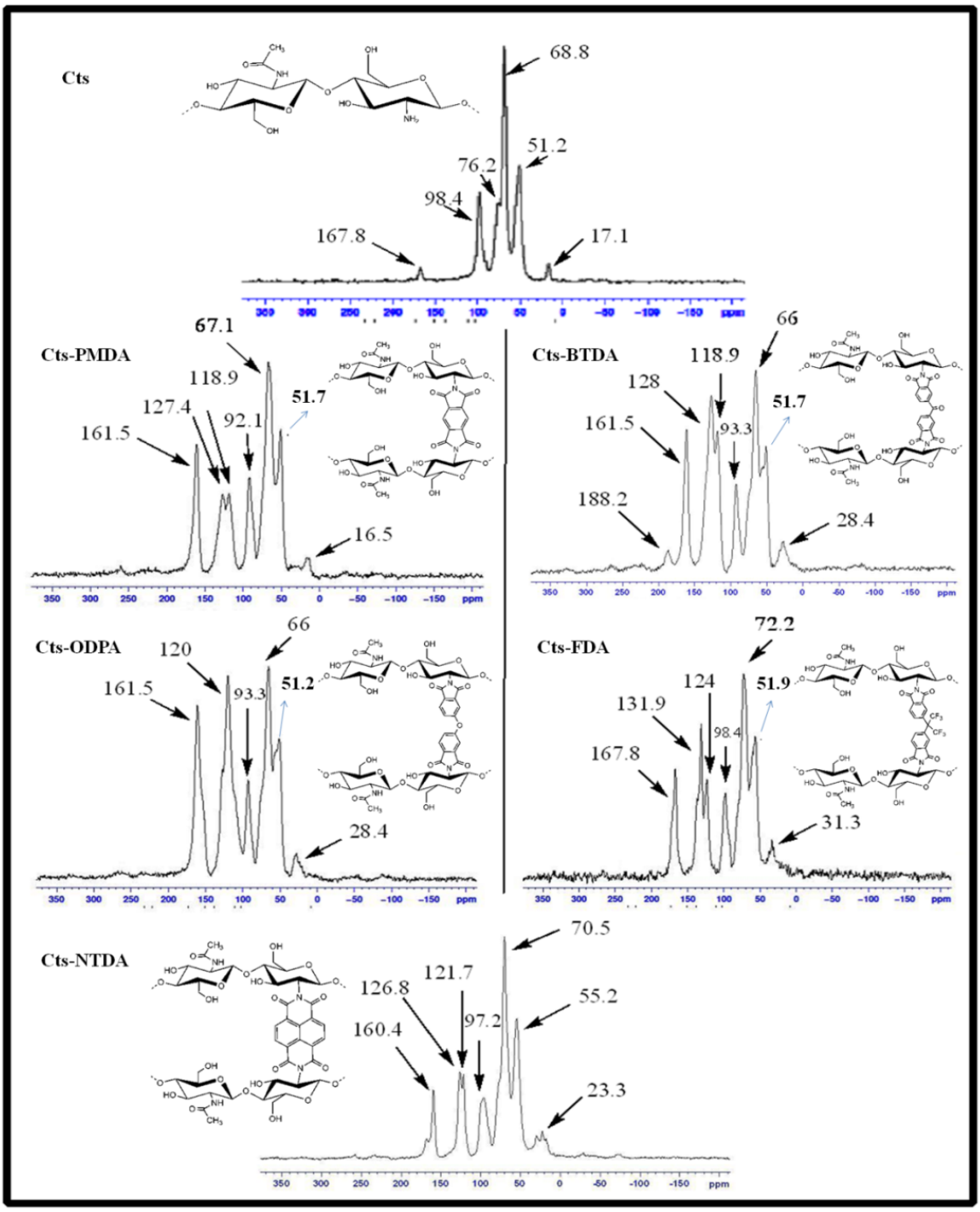

Figure 2-4 13C DP-MAS spectra of chitosan and dianhydride-crosslinked chitosan.

The signal due to the C1 carbon, which is directly attached to two oxygen atoms, was found at 98.4 ppm which is at a significantly lower magnetic field value compared with the signals of the remaining five carbons. The signal related to the carbonyl peak (acetyl group) is also observed at 167.8 ppm. After grafting the dianhydride derivatives to the Cts backbone, the resonance due to C4 (76.2 ppm) has disappeared. This could be related to transferring conformations of Cts from a linear structure to crosslinked Cts.[22] Two new peaks can be distinguished in the spectra of all the dianhydride crosslinked Cts: the peak related to the carbonyl group carbon of the cyclic imide around 161 ppm and aromatic peaks in the region between 118 and 130 ppm. In case of Cts-BTDA, a peak at 188.2 ppm corresponding to the carbonyl bridge of BTDA is also observed.

2.3.6. *In vitro* biodegradability

Cts is considered a biodegradable polymer because of its susceptibility to various enzymes.[23] To evaluate the biodegradability of the Cts films they were incubated in a LYZ solution and the formation of oligomers containing N-glucosamine units were monitored by a ninhydrin assay. The degradability of the test films in a LYZ solution are presented in Figure 2-5. As shown, the increments in the free amino groups in the LYZ solution for all crosslinked Cts membranes were greater than for the unmodified Cts membrane. This indicates that crosslinked Cts matrices have a faster enzymatic degradation rate as compared to its unmodified Cts at pH 7.4. This observation is probably related to the higher swelling ratios of the crosslinked Cts films compared to unmodified Cts. Therefore, the LYZ approach to the glycosidic bonds is facilitated due to a higher swelling rate of these modified films which causes less steric hindrance for penetration of LYZ. The higher degradation of crosslinked Cts over unmodified Cts over time should make these new matrices good candidates for implantation.

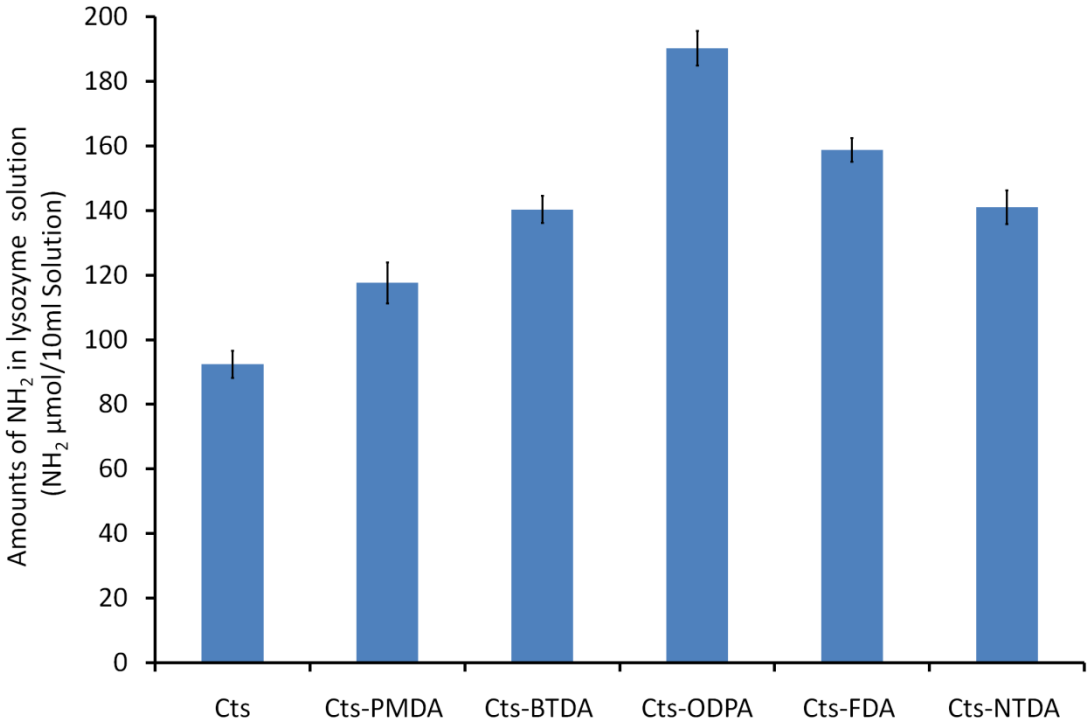

Figure 2-5 Results of the degradability of the chitosan and crosslinked chitosan in a LYZ solution.

2.3.7. Thermal gravimetric analysis (TGA) and differential thermal gravimetric analysis (DTG)

The degradation and thermal stability behaviors of Cts and these dianhydride derivatives of Cts were evaluated by TGA under a nitrogen atmosphere, Figure 2-6a. The TGA of Cts showed a weight loss in two distinct stages. Cts showed an initial weight loss of 10% when the temperature reached 100^{o}C due to loss of water which are bound to macromolecule. Thermal decomposition is more marked in the region between 240-340^{o}C with a weight loss of 54%. Cts-PMDA, Cts-BTDA, Cts-ODPA, Cts-FDA, Cts-NTDA all showed weight loss of about 25, 16, 20, 22 and 29% at 300^{o}C, respectively.

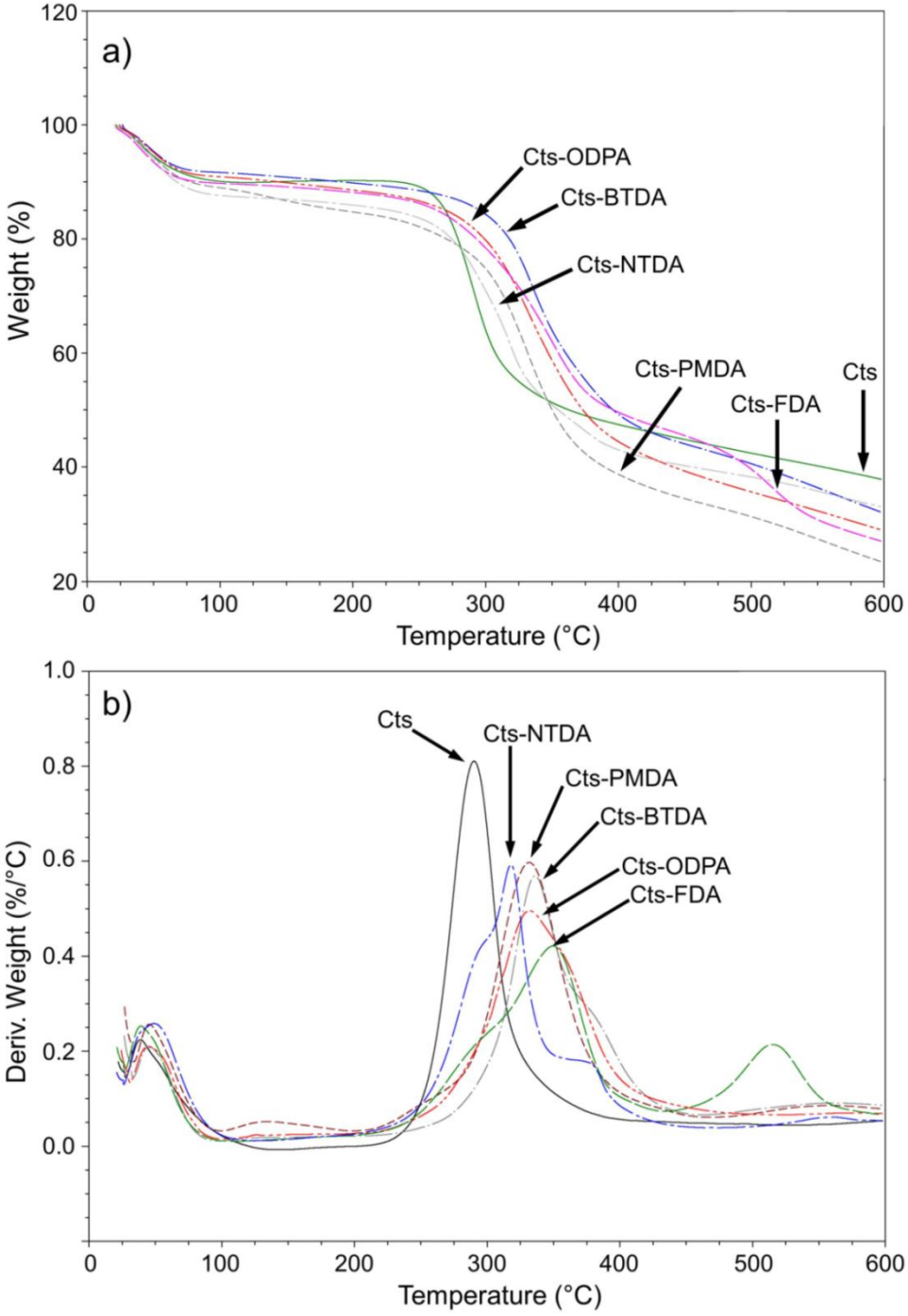

Figure 2-6 a) TGA and b) DTG thermograms of chitosan and crosslinked chitosan.

The second weight loss of Cts begins at about 250^{o}C relating to the degradation of Cts. Cts showed 40% weight loss at the same temperature (300^{o}C) providing evidence that crosslinking of Cts with dianhydride derivatives had decreased the rate of degradation and achieved significant increased thermal stability. More accurate differences of the

thermal behavior of the Cts and Cts-dianhydride derivatives can be noted from the DTG curves, Figure 2-6b. The DTG peak of Cts showed a maximum value at ~289 ^{o}C, while the maximum value of this peak for the Cts-dianhydride derivatives curve are observed at 332, 336, 332, 349 and 319^{o}C for Cts-PMDA, Cts-BTDA, Cts-ODPA, Cts-FDA, Cts-NTDA, respectively. These results show that all dianhydride derivatives significantly increase the thermal stability of the Cts.

2.3.8. Water Contact Angle Measurements

A water contact angle study was applied to all matrices to illustrate the influence of crosslinking on the hydrophilic properties of the Cts films, Figure 2-7. Water contact angles are most frequently used to determine the hydrophilic properties of films.[24] The results of the membrane's water contact angles are as follows: Cts (98.7 ± 1.3^{o}), Cts-PMDA (59.8 ± 3.2^{o}), Cts-BTDA (61.2 ± 3.6^{o}), Cts-ODPA (62.2 ± 2.9^{o}), Cts-FDA (65.0 ± 4.1^{o}), Cts-NTDA (62.7 ± 3.8^{o}). The results show that cross-linking resulted in an increase in the wettability of the film surfaces.

This observation could be related to a reduction in crystallinity of the Cts chain after crosslinking which leads to an increase in the amount of accessible amino groups and as a result an increase in hydrophilicity. The higher contact angles of Cts-FDA compared to other crosslinked films could be attributed to the hydrophobic nature of trifluoromethyl groups in FDA. From the water contact angle results, we can conclude that the Cts-dianhydride network shows better hydrophilicity than Cts. On the whole, the contact angle analysis was helpful in aiding the understanding of the surface properties of the networks.

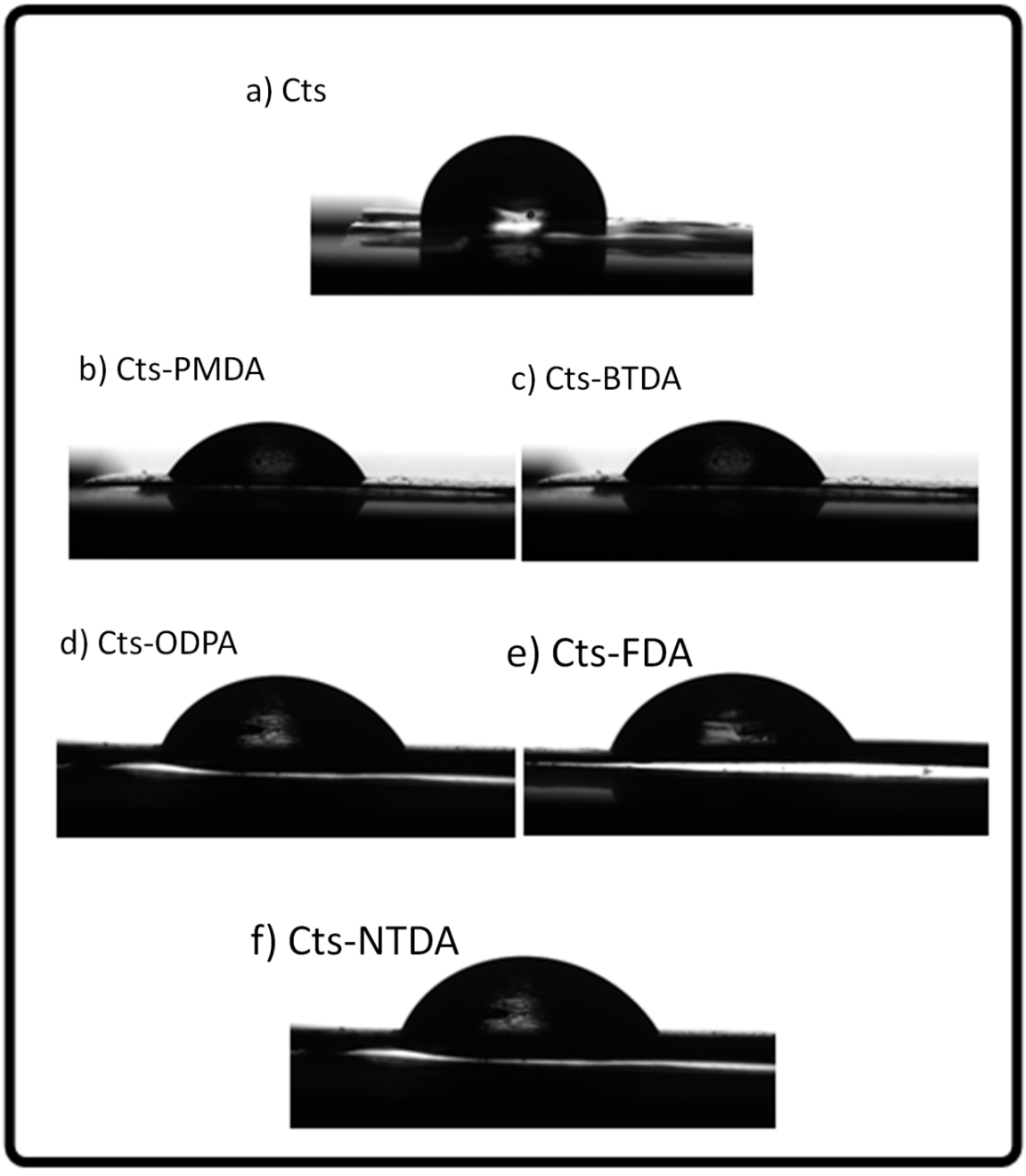

Figure 2-7 Appearances of water drops on Cts and crosslinked Cts film surfaces.

2.4. Conclusion

Facility of preparation, low cost and excellent physical properties are major advantages of this new generation of Cts based films. The results of this study indicate that using

dianhydride derivatives as crosslinking agents significantly improve the thermal and PS of the studied films. In terms of wettability, the crosslinked Cts were more hydrophilic than the uncrosslinked ones. These highly desirable results both in terms of the swelling properties and the high stability in acidic media of the new crosslinked films hold great promise in their application as stimuli responsive controlled release systems. In conclusion, these results point to a promising pathway to tougher bio-hydrogel matrices for medical and industrial applications.

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Chapter Three

In vitro evaluation of spray-dried chitosan microspheres cross-linked with pyromellitic dianhydride for oral colon-specific delivery of protein drugs

3.0. Introduction

Therapeutic peptides and proteins have attracted the attention of scientists due to their great potential for treatment of numerous diseases as a result of their exquisite selectivity and their ability to provide effective and potent action and fewer side effects.[1] Oral delivery is by far the most widely used route of administration of drugs because of its simplicity and convenience, although injection remains the most common means for administering therapeutic protein and peptide drugs.[2] Overcoming obstacles such as acid-catalyzed degradation and extensive hydrolysis of protein and peptide drugs in the gastrointestinal tract requires the efficient delivery of these drugs into the systemic circulation.[3] Pharmaceutical researchers have tried various strategies to overcome such obstacles and to develop effective peptide and protein drug delivery systems. In recent years, delivery of therapeutic peptides and proteins into systemic circulation through colonic absorption has attracted great interest due to the relatively low activity of proteolytic enzymes in the colon.[4] Among the various approaches for colon targeted drug delivery, using biodegradable polymers such as Cts, which is degraded by the colonic microflora, holds great promise.[5] Chitosan microspheres as drug carriers to deliver drugs to the areas of interest have been prepared using different techniques such as spray-drying, solvent evaporation and ionic/covalent crosslinking.[6] Lorenzo *et al*. prepared Cts microspheres of sodium diclofenac by a spray drying technique. These microspheres were enteric coated with Eudragit L-100 or Eudragit S-100. No release was seen in acidic pH for three hours, but at higher pH Eudragit dissolved and swelling of Cts started leading to continuous drug diffusion which was completed in after four hours.[7] Chitosan crosslinked with sodium cellulose sulfate and sodium polyphosphate was also prepared

and loaded with 5-ASA as a model drug.[8] Mura *et al*. designed spray dried N-succinyl-chitosan microparticles for colon specific delivery. In SGF (pH 2.0), during the first two hours, 5-ASA release was less than 10% while at pH 7.4 the drug release after four hours reached 51%.[9] Modification of Cts is a common method to prevent rapid release of drugs that results from dissolution and swelling of unmodified Cts.[10] Therefore many investigations have been pursued to develop new reagents to crosslink Cts.[5a]

This study has developed a new generation of crosslinked Cts using dianhydrides as the cross linking agent.[11] Herein, we examined for the first time the ability of a Cts microsphere crosslinked with PMDA for controlled drug release. In this study, BSA as a model protein drug was used to investigate the drug release behaviour of spray-dried Cts-PMDA microspheres. The effect of initial BSA concentration in encapsulation efficiency and loading capacity was also investigated.

3.1. Material and methods

With the exception of the following details, the same reagents and instruments as per **Chapter 2**, was used throughout this chapter.

3.1.1. Material

β-glucosidase from almonds (8.1 U/mg) were purchased from Sigma-Aldrich (Auckland, New Zealand).

3.1.2. Preparation of the spray-dried chitosan microspheres

Chitosan solution (1.5%, w/w) was prepared by dissolving Cts (10 g) in an aqueous solution of acetic acid (2%, v/v) at room temperature. Subsequently, the completely dissolved Cts solution and air were passed separately to the nozzle of a spray dryer at a feed rate of 9 ml/min in a Büchi-B190 spray dryer (Büchi, Switzerland). The inlet temperature was controlled at 140 ºC. The Cts microspheres were collected from the cyclone of the spray dryer.

3.1.3. Crosslinking of the spray-dried chitosan microspheres

The spray dried Cts microspheres (1.5 g) were dispersed in glacial acetic acid (40 ml) for 10 min then dimethylformamide (DMF) containing 4 eq PMDA (8.12 g) was added and heated at 130^{o}C. The crosslinked Cts microspheres were washed with methanol, then 0.1 N NaOH followed by rinsing with ethanol to remove NaOH and vacuum dried to evaporate ethanol.

3.2. Characterization

The average molecular weight (M) of the Cts was determined by the viscometric method. Chitosan solution was prepared in 0.1 M acetic acid/0.2 M sodium chloride aqueous solutions. The relative viscosity (η) of Cts solutions were measured using a U Cannon-Fenske routine viscometer (Cannon Instrument Co., State College, PA) at 25^{o}C.
The average molecular weight (Mw) of Cts was calculated from the intrinsic viscosity [η] by Mark–Houwink–Sakurada's empirical equation:[12]

$$[\eta]=kM^{a}$$

where k = 1.81 × 10^{-3} and a = 0.93 in the prepared solution at 25^{o}C.

Elemental analysis was performed using a Carlo Erba Elemental Analyser EA 1108 using a flash combustion technique. The analyses were carried out at the Campbell Microanalytical Laboratory, Otago University, Dunedin, New Zealand.
The degree of substitution (DS) was determined by elemental analysis and was calculated on the basis of the percentage of nitrogen in the product.[13]
Crosslinked Cts microspheres were subjected to particle-size distribution analysis using a particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd, Malvern, Worcestershire, UK). The morphological features of the microspheres were examined by an FEI Quanta 200 Scanning Electron Microscope (Eindhoven, The Netherlands) at an accelerating voltage of 20 kV. The samples were mounted onto stubs using double sided adhesive tape and sputter coated with gold.

3.3. Determination of the swelling behavior of the microparticles

The swelling characteristics of Cts and cross-linked Cts microspheres in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) was investigated. In addition, a test was carried out for 2 h in SGF, followed by 6 hrs in SIF and then 12 h in simulated colonic fluid (SCF, pH 7) to simulate the swelling behavior of microspheres in the GIT. At a predetermined time, the swollen samples were collected with a centrifuge, blotted with filter paper for the removal of the absorbed water on the surface, and then weighed immediately. The percentage of swelling S (%) of microspheres in the media was then calculated from the formula: [14]

$$S\ (\%) = \left[\frac{(W_s - W_d)}{W_d}\right] \times 100$$

where W_s and W_d are the weights of swollen and dried microspheres.

3.4. BSA loading and release experiments

Dried particles (500mg) were soaked for 6 h in 5ml of phosphate-buffer saline solution (PBS, 5ml, pH 7.4) containing various concentrations of BSA (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) for remote loading. Subsequently, excess surface BSA solution, if any, was removed using filter paper and the protein-loaded particles were dried at 4 °C.

3.4.1. Protein encapsulation efficiency and loading capacity

Protein content inside the microparticles was determined by suspending particles (50 mg) in 5 ml of PBS (pH 7.4) for 24 h. BSA-loaded microspheres were separated from the solution by centrifugation at 4000 rpm for 10 min. Then, the supernatant from the centrifugation was decanted carefully and the protein content in the supernatant was analyzed by HPLC. All samples were analyzed in triplicate. The BSA loading capacity

(LC) and encapsulation efficiency (EE) of Cts-PMDA microspheres were calculated according to the following formula:[15]

$$\mathrm{LC} = \frac{\text{Total amount BSA} - \text{Free amount BSA}}{\text{Microparticle weight}} \times 100$$

$$\mathrm{EE} = \frac{\text{Total amount BSA} - \text{Free amount BSA}}{\text{Total amount BSA}} \times 100$$

3.4.2. *In vitro* drug-release study

For the BSA release experiments, drug-loaded microparticles (50 mg) were immersed SGF (5ml, pH 1.2) and SIF (5ml, pH 7.4) at 37^{o}C. In addition, a test was carried out to simulate passage through the GIT. At first, the drug release was determined in SGF for 2 h, followed by 6 h in SIF, and then the matrix was transferred to SCF (pH 7) with and without β-glucosidase for 12 h. At the desired times, an aliquot of sample (100 µl) was withdrawn and protein content was estimated by HPLC. The dissolution medium was replaced with fresh buffer to maintain total volume after each withdrawal.

3.5. HPLC protein analysis

The quantitative determination of BSA was determined using a HPLC system consisting of a Waters 2690 separation module and a Waters 996 photodiode array detector, equipped with a Phenomenex® C18 reverse-phase column (250×4.6 mm, 5 µm). The separation was performed using a gradient between mobile phase A (water/acetonitrile, 90/10 v/v, 0.05% TFA) and mobile phase B (100% acetonitrile, 0.04% TFA).[16] The injection volume was 50 µl and elution was performed at a flow rate of 1 ml/min. The wavelength of the detector set at 280 nm. The peak corresponding to BSA has the retention time of 19.62 min. The observed linear calibration curves with correlation had coefficients of 0.999.

3.6. Statistics

All analyses were expressed as means ± SD. Analysis of variance (ANOVA) was used to determine statistical significance. A difference was considered statistically significant if the P value is less than 0.05.

3.7. Results and discussion

3.7.1. Preparation of the spray-dried chitosan microspheres

The degree of deacetylation of Cts used in the present study was calculated at 70% by elemental analysis (C, 43.8; N, 8.02; and H, 6.98). The approximate molecular weight of Cts was also determined to be 162 kDa by the Mark-Houwink viscometry method.

The Cts microspheres were crosslinked with PMDA to improve the stability of Cts in the acidic medium of the stomach. Addition of dianhydride to Cts led to the formation of the cyclic imide (Figure 3-1). The heterocyclic imide linkage offers excellent thermal, mechanical and chemical stability to the Cts.[13] The degree of substitution (DS) calculated from elemental analysis was 73%. This high DS is related to the electron affinity of PMDA (Ea = 1.90 eV) which governs the reactivity of the dianhydride.[17]

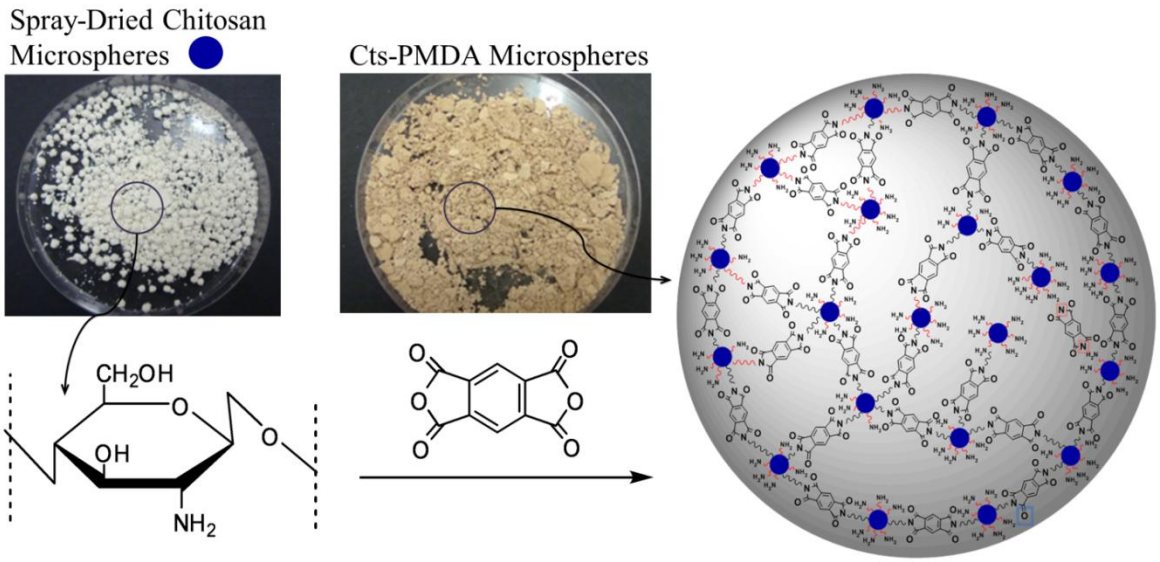


Figure 3-1 Schematic representation of a Cts-PMDA microsphere.

The IR spectra for Cts and Cts-PMDA microspheres are compared in Figure 3-2. After crosslinking Cts with PMDA, two new peaks appear at 1778 and 1721 cm^{-1} which correspond to the characteristic symmetrical and unsymmetrical C=O stretching bands of the cyclic imide respectively.

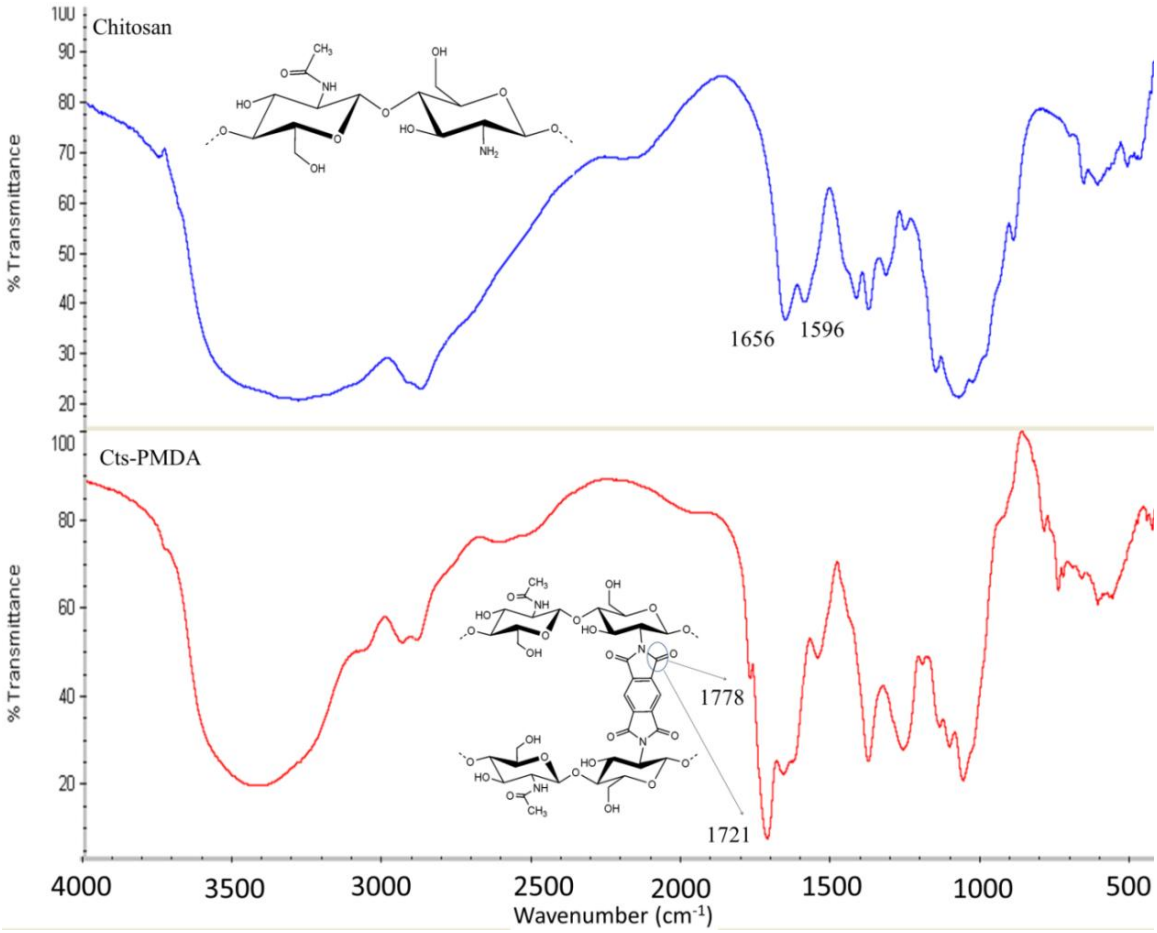


Figure 3-2 FTIR spectra of Cts and Cts-PMDA microspheres.

The disappearance of the vibrational band corresponding to the primary amino group at 1596 cm^{-1} further confirms that the Cts had been modified.

To further validate the structure, the ^{13}C DP-MAS spectrum of dianhydride crosslinked Cts microsphere was obtained (Figure 3-2). The Cts backbone related signals appear at 78.7 (C4), 52.9 (C3/C5) and 38.2 ppm (C2/C6). The signal at 146.2 ppm is attributed to

the carbonyl group carbon of the cyclic imide and signals at 112.5 and 103 ppm are attributed to the phenyl ring.

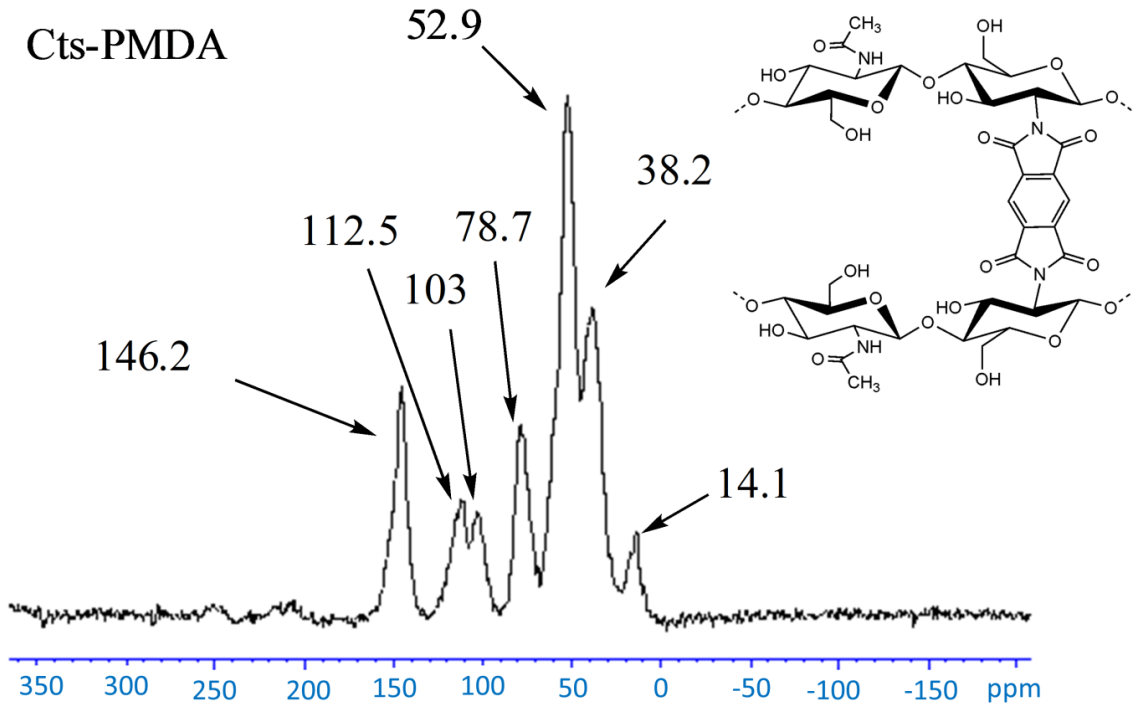
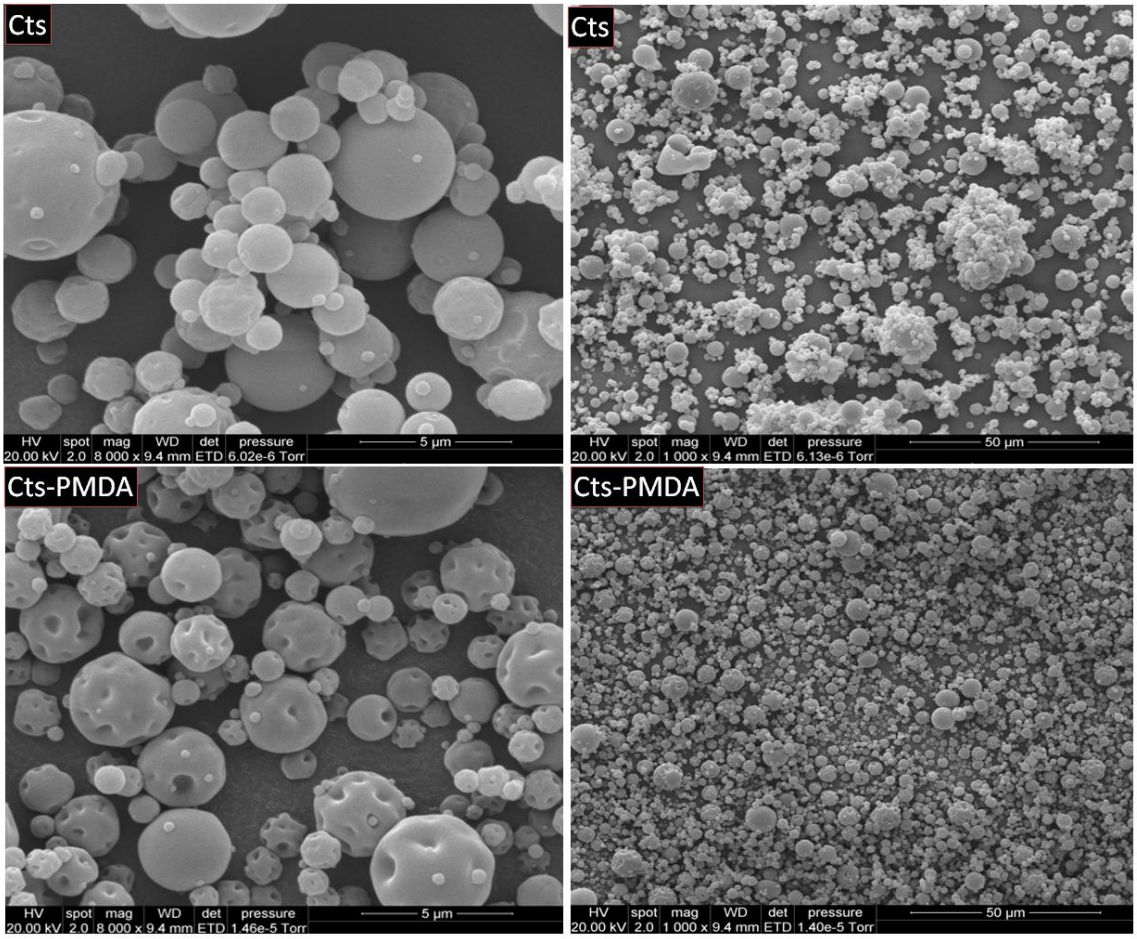


Figure 3-3 ^{13}C DP-MAS spectra of chitosan-PMDA.

A scanning electron microscopy (SEM) study by Wei *et al*. (2008) indicated that microspheres with a size of below 7.2 µm can be adsorbed in the gastrointestinal tract.[18] The particle-size distribution analysis of Cts-PMDA microspheres prepared in this study showed the average size of 3.55 µm, Figure 3-4.

SEM was used to visualize the surface morphology of the spray-dried microspheres as shown in Figure 3-4. The non-crosslinked microspheres were almost spherical in shape with a smooth surface. After crosslinking Cts with PMDA, a well-defined change in the surface morphology of the spray dried Cts microspheres was observed. The SEM photographs show that the surface roughness increased after crosslinking, however the particles were still nearly spherical in shape.



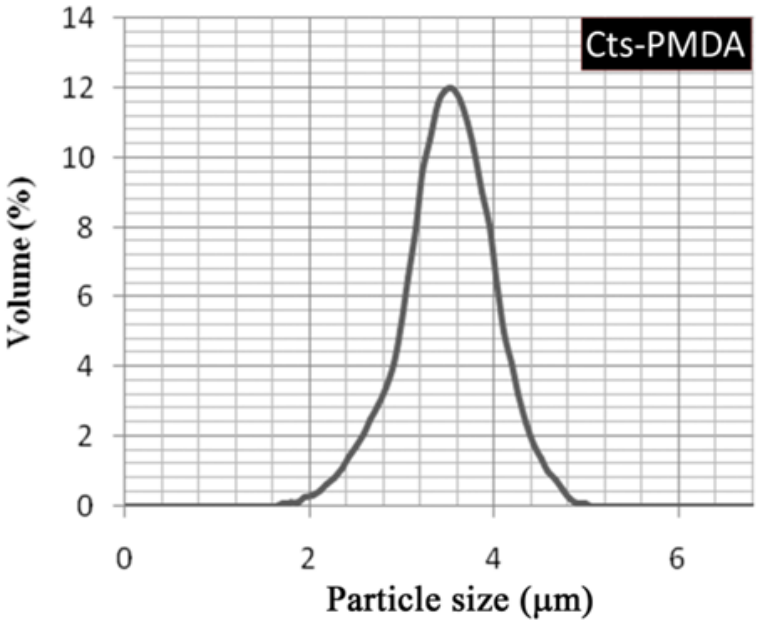


Figure 3-4 SEM images and size distribution of chitosan and Cts-PMDA microparticles.

3.7.2. Swelling studies

The swelling behavior of a hydrogel plays a crucial rule in controlling the rate of drug release from hydrogel networks. The swelling behavior of the Cts-PMDA microspheres was analyzed at pH 1.2 and 7.4. As shown in Figure 3-5a, the crosslinked Cts matrix produced a higher degree of swelling at pH 7.4 (671.7 ± 2.9%) than at pH 1.2 (643.5 ± 3.2%). This can be explained by the fact that at pH 1.2, the swelling is controlled mainly by repulsion of the protonated amino group on the C-2 carbon of the Cts component. Therefore, reducing the number of amino groups after crosslinking has led to a reduction in the swelling percentage of the matrix. Partial dissolution of the matrix at pH 1.2 also has an effect on this trend.

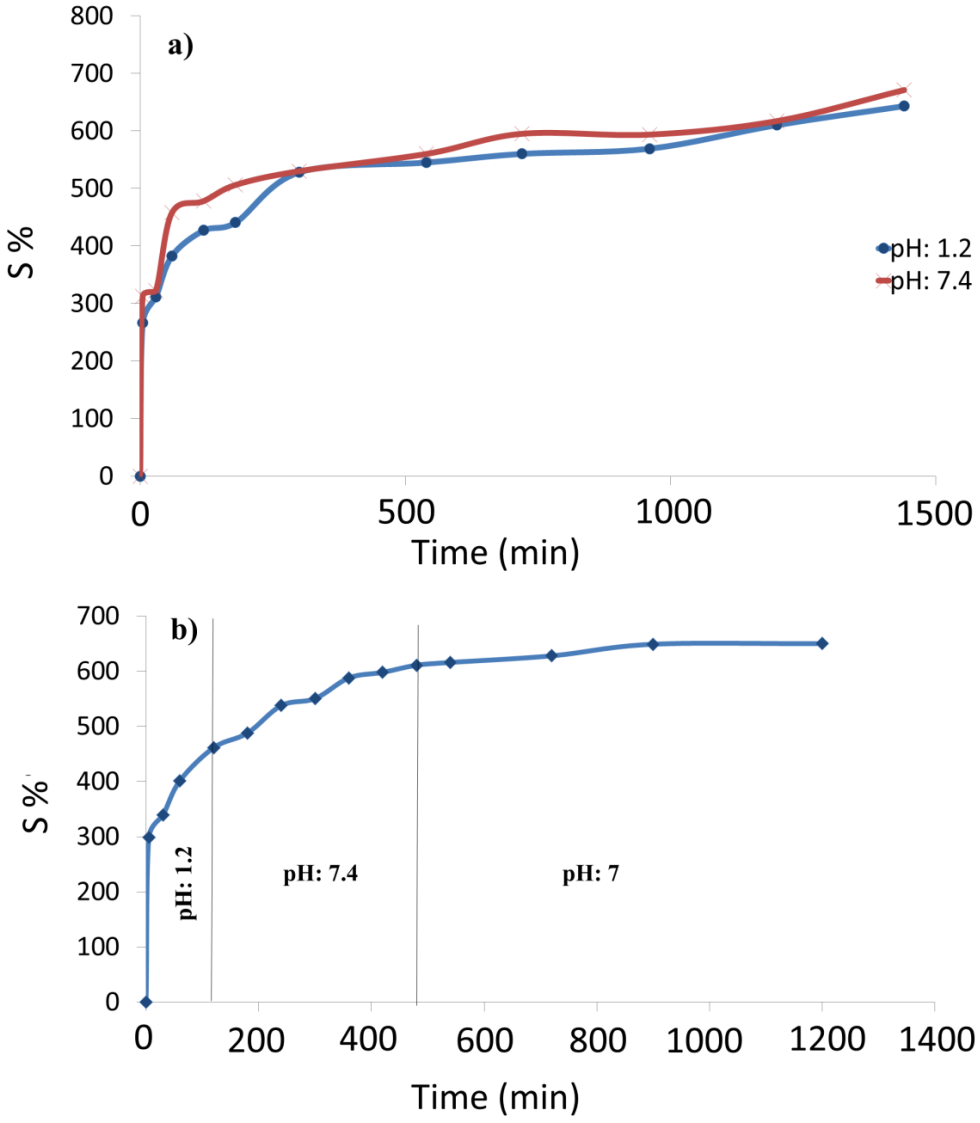


Figure 3-5 Swelling behaviour of Cts-PMDA microspheres in a) SGF and SIF b) simulated gastrointestinal tract (2 h in pH 1.2, 6 h in pH 7.4, and 12 h in pH 7).

For further simulating GIT, the swelling characteristics of the Cts-PMDA microsphere were determined by immersing dried test samples to swell in a solution at pH 1.2 for two hours and subsequently in another solution at pH 7.4 for six hours then 12 h in pH 7, Figure 3-5b. Results also indicated that matrix swelled 650 ± 4.2% at the end of third step (Figure3-5b).

3.7.3. Microsphere encapsulation efficiency (EE) and loading capacity (LC) study

The drug encapsulation efficiency (EE) and loading capacity (LC) was found to be dependent upon the initial BSA concentration. Cts-PMDA microspheres were loaded with different concentrations of BSA. It was observed that a higher initial concentration of BSA led to lower encapsulation efficiency. Results showed that the protein EE decreased from 88.4 ± 3.1% to 62.8 ± 2.9%, Figure 3-6a, when the initial BSA concentration was increased. Similarly, the LC was also affected by the initial BSA concentration. However the results indicate that the protein LC was enhanced dramatically from 6.3 ± 0.3% to 41.8 ± 4.1% by increasing the initial BSA concentration from 0.1 to 1 mg/mL (Figure 3-6).

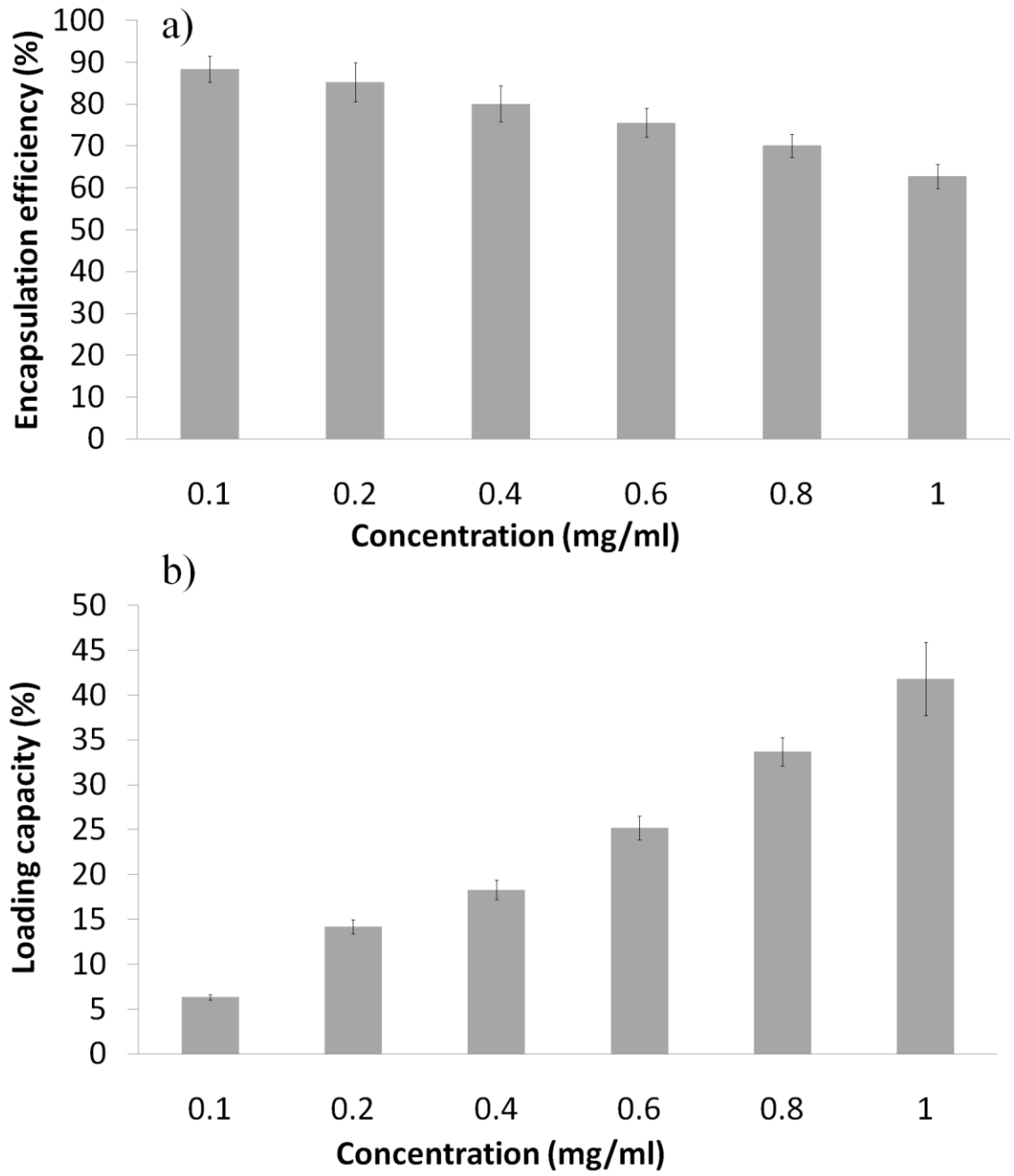


Figure 3-6 The influence of BSA initial concentration on a) encapsulation efficiency and b) loading capacity of Cts-PMDA microspheres.

3.7.4. *In vitro* release study

In vitro BSA release tests in SGF and SIF were performed to provide a comparison between the release profile of the spray-dried microsphere with and without PMDA cross-linking. As shown in Figure 3-7, within the first two hours, 93.4 ± 3.2% of loaded BSA was released at SGF (pH 1.2) from the unmodified Cts microsphere. This observation can be attributed to the dissolution of the Cts in the acidic media. These results indicate that the uncrosslinked Cts fails to provide adequate retention of encapsulated proteins at stomach pH. On the other hand, the amount of BSA released from the cross-linked microspheres at SGF was relatively low; 36.4 ± 3.1% was released after two hours. This delayed release characteristic could be explained as being due to the network of the cross-linked structures of Cts-PMDA controlling the release of BSA from the Cts microspheres. Acid stability of the chemically modified Cts is also another factor in the slow release of the drug in SGF.

In SIF (pH 7.4) the amount of BSA released from Cts and Cts-PMDA was 82.3 ± 2.1% and 64.8 ± 3.4% after six hours, respectively. These results also indicated that 36.4 ± 3.1% of BSA was released from Cts-PMDA in SGF (pH 1.2) within two hours, while 42.1 ± 3.8% of BSA was released in SIF (pH 7.4) during the same length of time. This result can be attributed to the higher swelling percentage of the Cts-PMDA microspheres in pH 7.4 than at pH 1.2, which leads to the opening up of the pores and channels of the matrix. Within an hour approximately 30% of the protein is released from the crosslinked Cts microspheres at either pH 1.2 or 7.4. This initial burst is attributed to the release of BSA on or near the surface of the microspheres.

It is clear from Figure 3-7 that after 14 hours less than 80% of BSA has been released in either acidic or basic media from the Cts-PMDA microspheres. This observation can be related to the entanglement of the BSA molecules within the hydrogel network which prevent release until the polymer matrixes are degraded.

To simulate GIT conditions, the Cts-PMDA microspheres were incubated in an acidic pH (1.2) environment to simulate the retention time in the stomach, then subsequently in another solution at pH (7.4) to simulate the retention time in the small intestine over six hours, followed finally by 12 hours in SCF (pH 7). The results of this study are shown in Figure 3-8 which indicates that cumulative release from the Cts-PMDA microspheres was found to be 37.1 ± 2.8% after two hours in SGF and 73.1 ± 4.8% at the end of 8 hours (2

h in SGF + 6 h in SIF). The results reveal that after 12 hours in SCF 80.9 ± 4.1% of the drug was released.

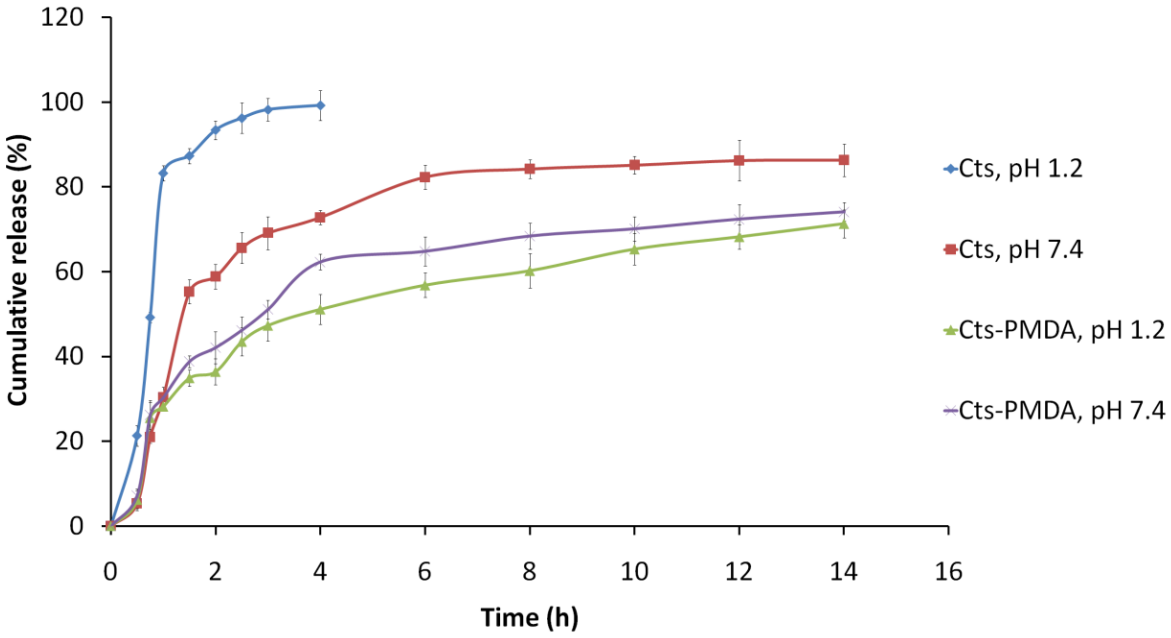


Figure 3-7. Effect of pH on cumulative release of BSA from Cts and Cts-PMDA microspheres.

The drug release in SCF was also evaluated in the presence of an enzyme. Since a similar degradation function of commercially available almond β-glucosidase occurs with Cts as that of colonic enzymes, β-glucosidase was used to simulate the colonic medium.[19] When compared with the results where there was an absence of the enzyme at SCF (pH 7), after 12 hours, the release of BSA was increased by almost 6% in the presence of the β-glucosidase, indicating that the enzyme had catalysed the hydrolysis of the crosslinked Cts.

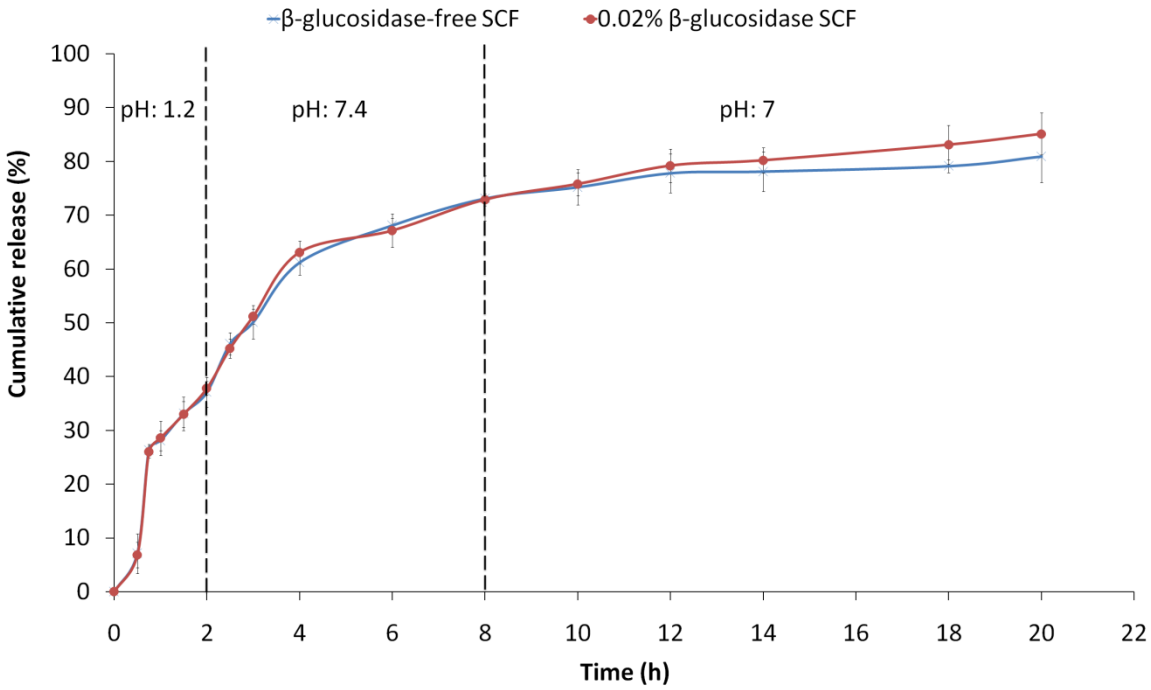


Figure 3-8 The BSA release profile in simulated gastrointestinal fluid for 2 h, followed by 6 h in simulated intestinal fluid and then 12 h in simulated colonic fluid.

Ganza-Gonzalez *et al*. prepared Cts and chondroitin sulfate microspheres for controlled release of the drug metoclopramide hydrochloride.[20] Chondroitin sulfate microspheres prepared without formaldehyde as the crosslinking agent showed very rapid drug release in dissolution assays (50% released in less than 1 h) at pH 1.2. They also prepared Cts microspheres by a spray drying method employing different amounts of formaldehyde as the crosslinker. Based on their study, 50% of drug was released in approximately 180 minutes. The study in this chapter shows that Cts-PMDA microspheres have a slower drug release at the same pH (50% released in approximately 4 h).

Lorenzo-Lamosa *et al*. prepared micro-encapsulated Cts microspheres for delivery of the drug sodium diclofenac.[7] Based on their study, Cts acetate showed a rapid drug release profile (50% released in approximately 30 min), whereas Cts-glutamate provided a delayed release (50% released approximately in 2 h) at pH 7.4.

He *et al*. prepared microparticles of both uncrosslinked and Cts crosslinked with glutaraldehyde and formaldehyde by using a spray drying method for the delivery of the drugs cimetidine, famotidine and nizatidine.[21] Their study revealed that more than 50% of

the drug was released within a few minutes at pH 7.4.The study in this chapter revealed that a slower drug release is possible by using Cts- PMDA microspheres at pH 7.4 (50% released approximately in 3 h).

Based on a survey of the literature, there are to date no Cts based microspheres tested in a simulated GIT (2 h in SGF followed by 6 h in SIF, then 24 h in SCF) with or without an enzyme. There is slower drug release from Cts-PMDA microspheres at pH 1.2 and pH 7.4 as compared with the literature studies noted above. Also the ability of these microspheres to protect approximately 30% of the protein drug from the harsh environment of upper GIT presents these microspheres as good candidates for colon targeted drug delivery application.

3.8. Conclusion

Chitosan microspheres crosslinked with the PMDA intended for delivery of protein to the colon were successfully prepared. BSA as a model protein was then loaded into the microsphere by the diffusion filling method (remote loading). Altering the concentration of BSA from 0.1 to 1 mg/mL enhanced significantly the loading capacity from 6.3 ± 0.3% to 41.8 ± 4.1% and decreased the encapsulation efficiency from 88.4 ± 3.1% to 62.8 ± 2.9%. A number of factors such as time, pH, and enzyme availability were investigated for their influence on the release of the BSA from the microspheres. The results from this study clearly show the potential of Cts-PMDA microspheres for drug delivery purposes.

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Chapter Four

Synthesis and characterization of a novel generation of amphoteric pH sensitive hydrogels

4.0. Introduction

Stimuli responsive or smart hydrogels are substances that are able to respond to small environmental changes resulting in abrupt changes in their swelling behavior, network structure, permeability and/or mechanical strength.[1] The utility of smart hydrogels has become increasingly important not only because of their unique properties, but also because of their potential for significant technological and biomedical applications.[2] Among the various hydrogels, including natural, synthetic and natural/synthetic hybrid hydrogels, Cts has attracted significant attention in a broad range of pharmaceutical and biomedical applications.[3] Chitosan is a hydrophilic polyelectrolyte polysaccharide composed of glucosamine and N-acetyl glucosamine units linked by β(1–4) glycosidic bonds.[4] Unlike most known bioadhesive polymers, Cts displays unique pharmaceutical and biomedical applications due to the large number of hydroxy and amino groups on the backbone of Cts that can be modified.[3b, 5] A number of reagents have been used for crosslinking of Cts, such as glutaraldehyde,[6] genipin,[7] tripolyphosphate,[8] ethylene glycol,[9] diglycidyl ether and diisocyanate[10] to modulate the properties of Cts. In recent years, particular interest has been devoted to the fabrication of amphoteric pH-sensitive hydrogels. The reason is that amphoteric Cts, containing both anionic and cationic groups are believed to be more effective for a wider range of applicability.[11] However, in spite of the growing interest in Cts based hydrogels due to its several favorable biological properties, only a few amphoteric Cts based hydrogels has been studied so far.[11-12] Currently, the most widely studied amphoteric Cts based hydrogels are composed of carboxymethyl Cts.[13]

In this research a novel generation of Cts based amphoteric pH sensitive hydrogels was designed and characterized. In a previous study (Chapter 2), a crosslinked Cts with dianhydride derivatives were employed to insert an imide ring on to the Cts backbone.[14]

In this present study, Cts was crosslinked with dianhydride derivatives to obtain amic acid derivatives of Cts for the first time. The films obtained from these derivatives were characterized by their thermal, mechanical, swelling and hydrophilic properties. Biodegradation behaviour and enzyme inhibitory effects of these hydrogels were also investigated. Due to the importance of amphoteric polymers coupled with the low price of some of the dianhydride derivatives and the facile preparation method as compared to other amphoteric Cts based biomaterials, this new generation of amphoteric polymers should attract considerable significant scientific and commercial interest.

4.1. Materials and Methods

With the exception of the following details, the same reagents, instruments and methods as per **Chapter 2**, was used throughout this chapter.

4.1.1. Materials

N-α-benzoyl-l-arginine p-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester solution (BTEE), α-chymotrypsin and trypsin were purchased from Sigma-Aldrich (Auckland, New Zealand).

4.1.2. Preparation of chitosan films

The Cts films were produced by a casting/solvent evaporation technique. Chitosan powder (5 g) was dissolved in deionized water (500 ml) containing 2.0% (v/v) acetic acid at room temperature. The resulting viscous Cts solution was filtered to remove any undissolved Cts and followed by sonication for 15 min to remove trapped air bubbles. The solution (30 g) was then poured into a glass Petri dish and allowed to dry at room temperature. The resulting film was peeled off and neutralized by immersion into an aqueous NaOH solution (1 M) and subsequently oven dried at 40^{o}C for 24 h.

4.1.3. Preparation of amic acid derivatives

Chitosan (2g, 0.0124 mol of glucosamine residues) were first suspended in 50ml glacial acetic acid under vigorous stirring at room temperature. 1eq of the respective dianhydride derivatives dissolved in DMF (50 ml) was subsequently added. The mixture was stirred at 25^{o}C for 24 h. The amic acid derivatives of the Cts powder were filtered off, washed with methanol and then dried in vacuum.

4.1.4. Preparation of crosslinked chitosan films

Chitosan film (2g, 0.0124 mol of glucosamine residues) was first suspended in glacial acetic acid (25 ml). After 1 h, 1 eq of the respective dianhydride derivatives dissolved in DMF (50 ml) was added and the mixture was shaken for 24 h at room temperature. The amic acid Cts films Cts crosslinked with PMDA (CPAA), Cts crosslinked with BTDA (CBAA), Cts crosslinked with ODPA (COAA), Cts crosslinked with FDA (CFAA), and Cts crosslinked with NTDA (CNAA), were removed from their solutions, rinsed with methanol and air dried.

4.2. Enzyme inhibitory effect

4.2.1. Trypsin inhibition study

The enzymatic inhibitory activity of Cts and crosslinked Cts matrices towards trypsin was evaluated with BAPNA as the substrate.[15] Polymer matrices were dispersed in Tris-buffer (1 ml, 0.05 M, pH 8.2) containing $CaCl_2$ (0.02M) to reach a final concentration of 0.1% w/v solutions. After adding 30 U of trypsin solution (0.3 mL in 10 mM HCl), the mixture was incubated at 37^{o}C. Thereafter, 2.1 mL of the BAPNA solution (dissolved in DMF) was added and incubation continued for 15 minutes at 37^{o}C. After stopping the enzymatic action with 1% trichloroacetic acid solution, the nitroaniline formed was analysed by measuring the absorbance at 405 nm using a UV/visible spectrometer.

4.2.2. α-Chymotrypsin inhibition study

The assay of α-chymotrypsin inhibition by Cts and crosslinked Cts matrices was performed with N-benzoyl-L-tyrosine ethyl ester solution (BTEE) as the substrate.[16] Polymer matrices were dispersed in Tris–HCl buffer (1 ml, pH 7.8) containing $CaCl_2$ (0.02 M) to get a final concentration of 0.1% w/v solutions. After 15 minutes incubation in 25^oC, chymotrypsin solution (0.3 ml, 2 mg/ml in 1 mM HCl) was added. Thereafter, BTEE (1.4 ml, 37 mg dissolved in 63 ml of methanol and 37 ml of demineralized water) was added and the mixture was incubated at 37^oC for 5 min. After stopping the enzymatic action with 1% trichloroacetic acid solution, absorbance at 256 nm (resulting from the hydrolysis of benzoyl-L-tyrosine ethyl ester) was recorded.

4.3. Results and discussion

4.3.1. Preparation of chitosan amic acid films

Reaction between dianhydrides and Cts in a dipolar aprotic solvent can takes place in two steps. The first step involves the formation of the intermediate amic acid due to the nucleophilic attack of the amino group on a carbonyl carbon of the anhydride group. Subsequently the intermediate amic acid converts into the final imide ring by the thermal imidization route, Figure 4-1. As reported in Chapter 2, a new generation of crosslinked Cts derivatives was developed by introducing heterocyclic imide linkages to improve the mechanical and thermal stability of the Cts matrices.

In this chapter a method was devised to synthesis amic acid derivatives of Cts due to the growing importance of amphoteric polymers. The molecular weight of Cts was also determined to be 198 kDa with the Mark-Houwink viscometry method in a solvent of 0.1 M acetic acid/0.2 M NaCl maintained at 25^oC.

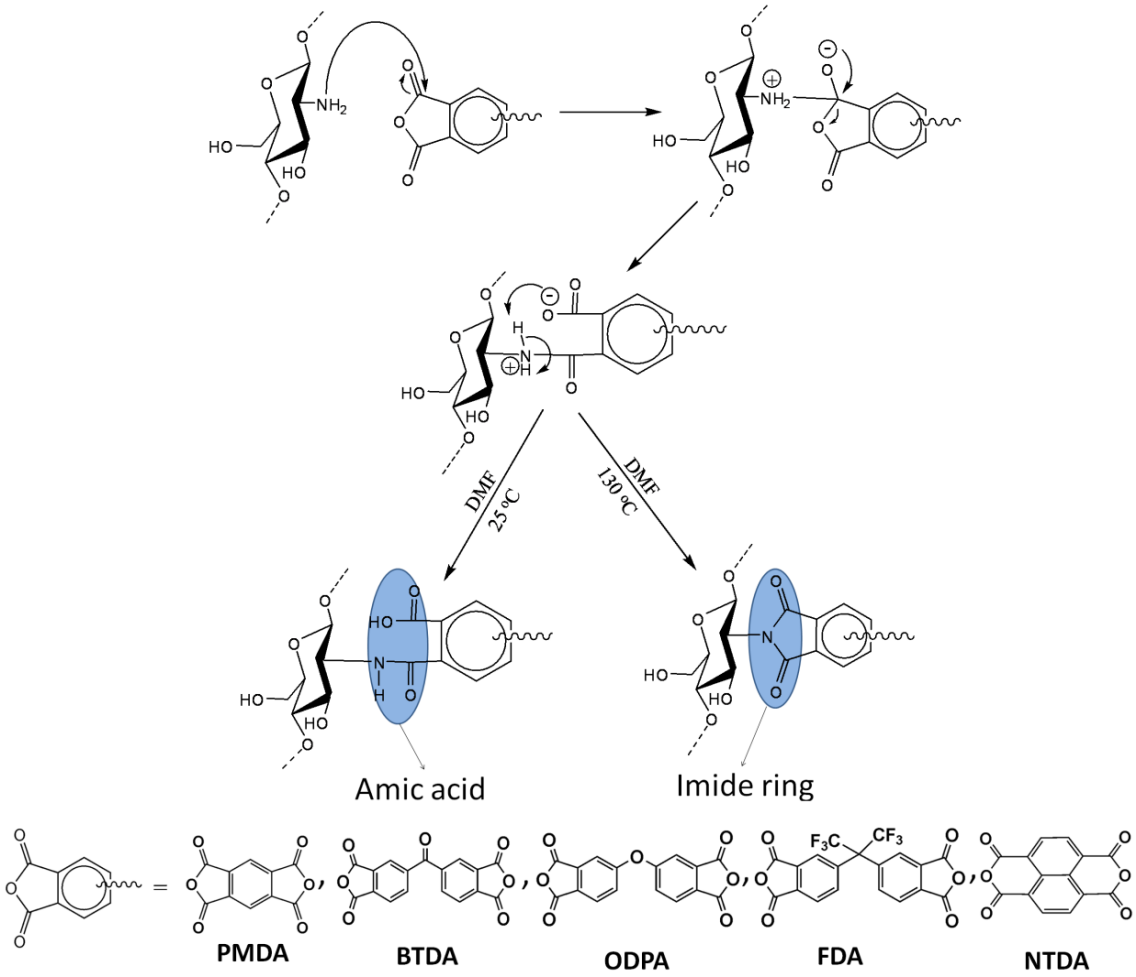

Figure 4-1 Schematic representation of the different possible reactions between the amino group of chitosan and dianhydride derivatives

The DD was 77% according to the following equation:

$$DD = \left(1 - \frac{\frac{C}{N} - 5.145}{6.186 - 5.145}\right) \times 100$$

where C/N is carbon-to-nitrogen ratio, 5.145 is related to the completely *N*-deacetylated Cts ($C_6H_{11}O_4N$ repeat unit) and 6.186 to the fully *N*-acetylated polymer ($C_8H_{13}O_5N$ repeat unit).

The degree of substitution (DS) was determined from the C/N value of elemental analysis on the basis of the following formula:

$$DS = \frac{\left(\frac{C}{N}\right)_f - \left(\frac{C}{N}\right)_i}{n}$$

where $(C/N)_f$ is the C/N ratio of the Cts derivative, $(C/N)_i$ is the C/N ratio of the unmodified Cts and n is the number of carbons introduced into the Cts derivatives.

Elemental analysis was used to determine the degree of deacetylation (DD) and the degree of substitution (DS) of Cts. Carbon, hydrogen and nitrogen contents of Cts and crosslinked Cts derivatives are listed in Table 4-1.

Table 4-1 The elemental analyses results and the substitution degree of chitosan and crosslinked chitosan.

Compounds	Elemental analyses (%)			Substitution degree
	C	N	H	
Cts	43.19	8.03	6.97	-
CPAA	45.5	3.79	4.45	0.66
CBAA	51.07	3.92	4.7	0.45
COAA	50.80	3.77	4.81	0.84
CFAA	45.50	3.33	4.21	0.44
CNAA	47.23	4.81	5.33	0.32

4.3.2. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was employed to investigate the characteristic chemical structure of the Cts crosslinked matrices, Figure 4-2. The major peaks for the Cts can be attributed as follows. The peak at 3374 cm^{-1} is assigned to the -H and -NH stretching vibrations, while the peak at 2878 cm^{-1} is assigned to the aliphatic C-H stretching vibration in the -CH and -CH_2 groups.

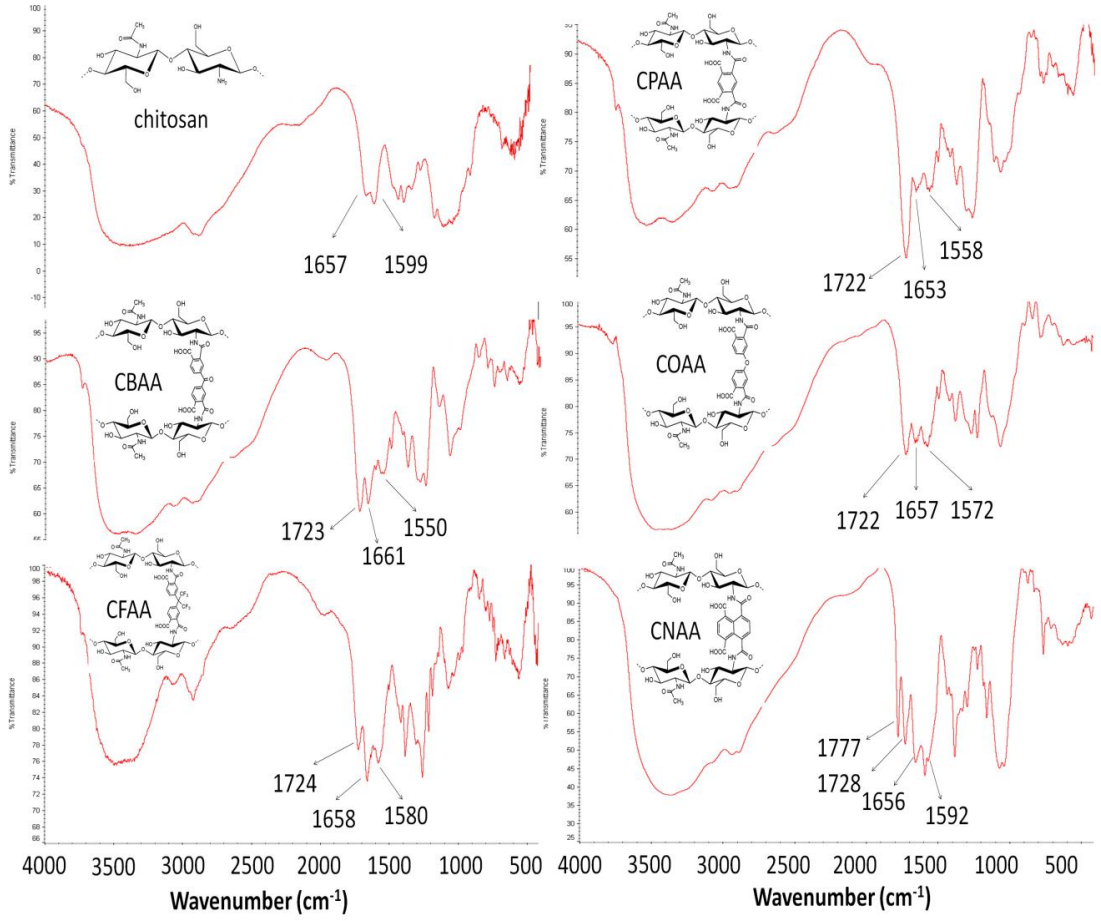

Figure 4-2 FTIR spectra of chitosan, and amic acid derivatives of chitosan

The amide frequencies consisting of the -C=O bond stretch of the remaining acetamido groups and the N-H bending vibrations of the -NH_2 groups are observed at 1657 and 1599 cm^{-1} respectively. After modification of Cts, the spectra showed some major changes.

The three bands around 1720-1725, 1655-1660, and 1550-1590 cm^{-1} are assigned to the characteristic bands of the C=O (COOH), C=O (CONH) and (C-NH), respectively.[17] In the case of CNAA the C=O (COOH) and C=O (CONH) were shifted to 1777 and 1728 cm^{-1}.

4.3.3. Solid state ^{13}C NMR analysis

The ^{13}C DP-MAS spectra of Cts and Cts derivatives are illustrated in Figure 4-3. The ^{13}C DP-MAS spectrum of Cts shows six peaks at 51.2 (C2/C6), 68.8 (C3/C5), 76.0 (C4) and 98.4 (C1) attributed to pyranose ring carbons of Cts. The methyl and carbonyl moieties of acetyl amine groups can be clearly observed at 17.1 and 167.8 ppm, respectively.[18]

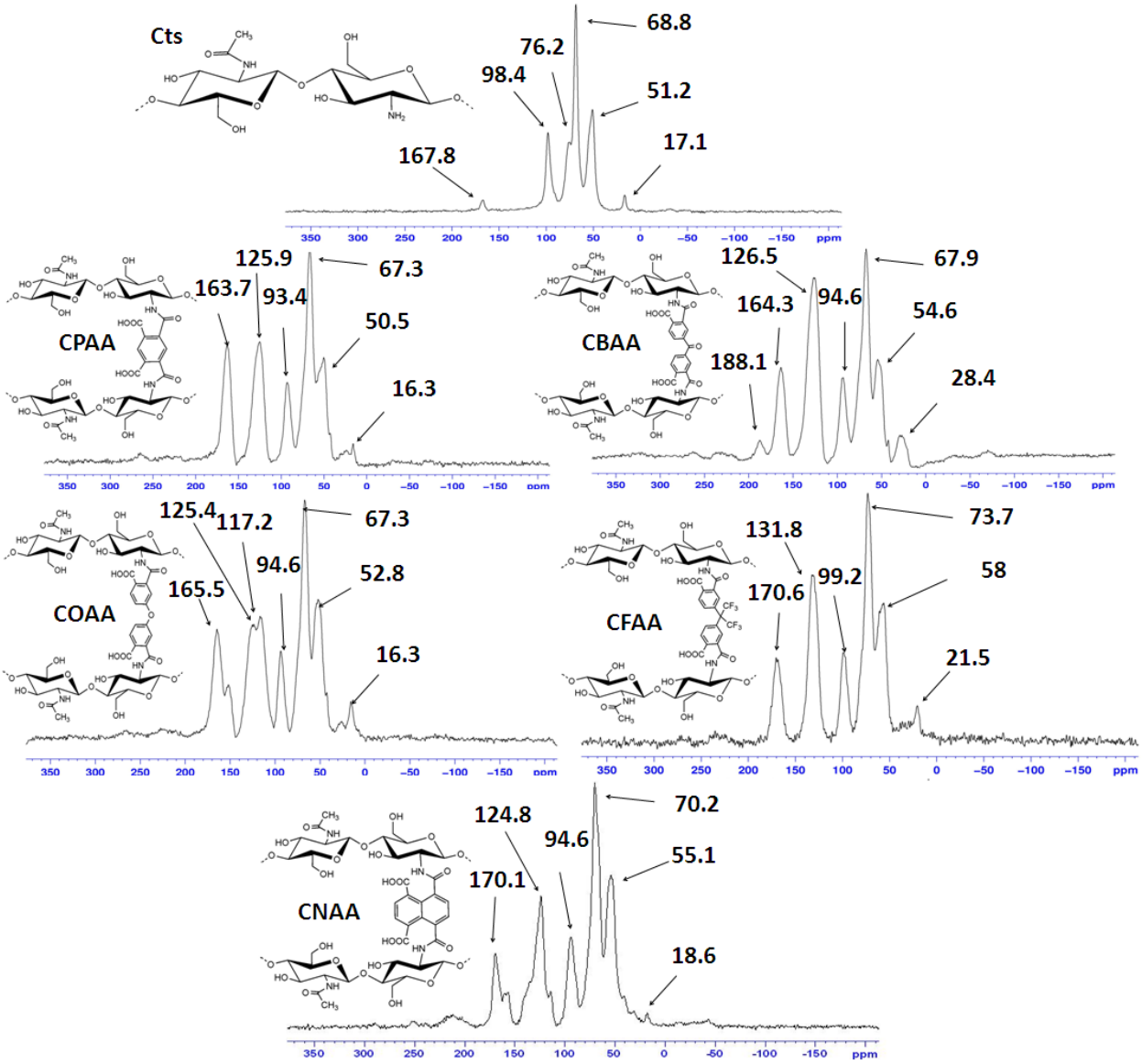

Figure 4-3 ^{13}C DP-MAS spectra of chitosan and amic crosslinked chitosan.

After grafting the dianhydride derivatives to the Cts backbone, two new peaks can be distinguished in the spectra of all the amic acid Cts derivatives. A new aromatic peak was observed between 118 and 130 ppm. The peak related to the amic acid groups was also observed around 170 ppm. The resonance due to C4 (76.2 ppm) has disappeared which could be related to a conformations change of Cts from a linear structure to the crosslinked form.[19] In the case of CBAA, a peak at 188.1 ppm corresponding to the carbonyl bridge of BTDA is also observed.

4.3.4. Mechanical test

The results for the puncture strength (PS) of Cts and crosslinked Cts-based films in this study are shown in Table 4-2. The mechanical test data showed a small reduction in puncture strength compared to unmodified Cts. This reduction in the puncture strength could be related to destruction of Cts crystallinity due to destruction of extensive intra- and inter-molecular hydrogen bonding interactions of Cts after modification. However more study is needed in this area to confirms this reduction.

Table 4-2 Puncture strength (PS) of the chitosan and crosslinked chitosan films.

Film	Film thickness (μm)	Puncture strength (MPa)
Cts	250.2 ± 10.3	7.6 ± 0.8
Cts-PMDA	260.8 ± 12.1	7.4 ± 0.6
Cts-BTDA	280.9 ± 9.7	6.6 ± 0.8
Cts-ODPA	280.6 ± 14.6	6.8 ± 0.9
Cts-FDA	270.6 ± 11.9	6.7 ± 0.7
Cts-NTDA	270.3 ± 13.2	7.0 ± 0.6

4.3.5. Thermal gravimetric analysis (TGA) and differential thermal gravimetric analysis (DTG)

To examine the thermal stability behavior of Cts and crosslinked Cts, thermogravimetric analysis (TGA) was carried out, Figure 4-4. The TGA of Cts showed a weight loss in two distinct stages.

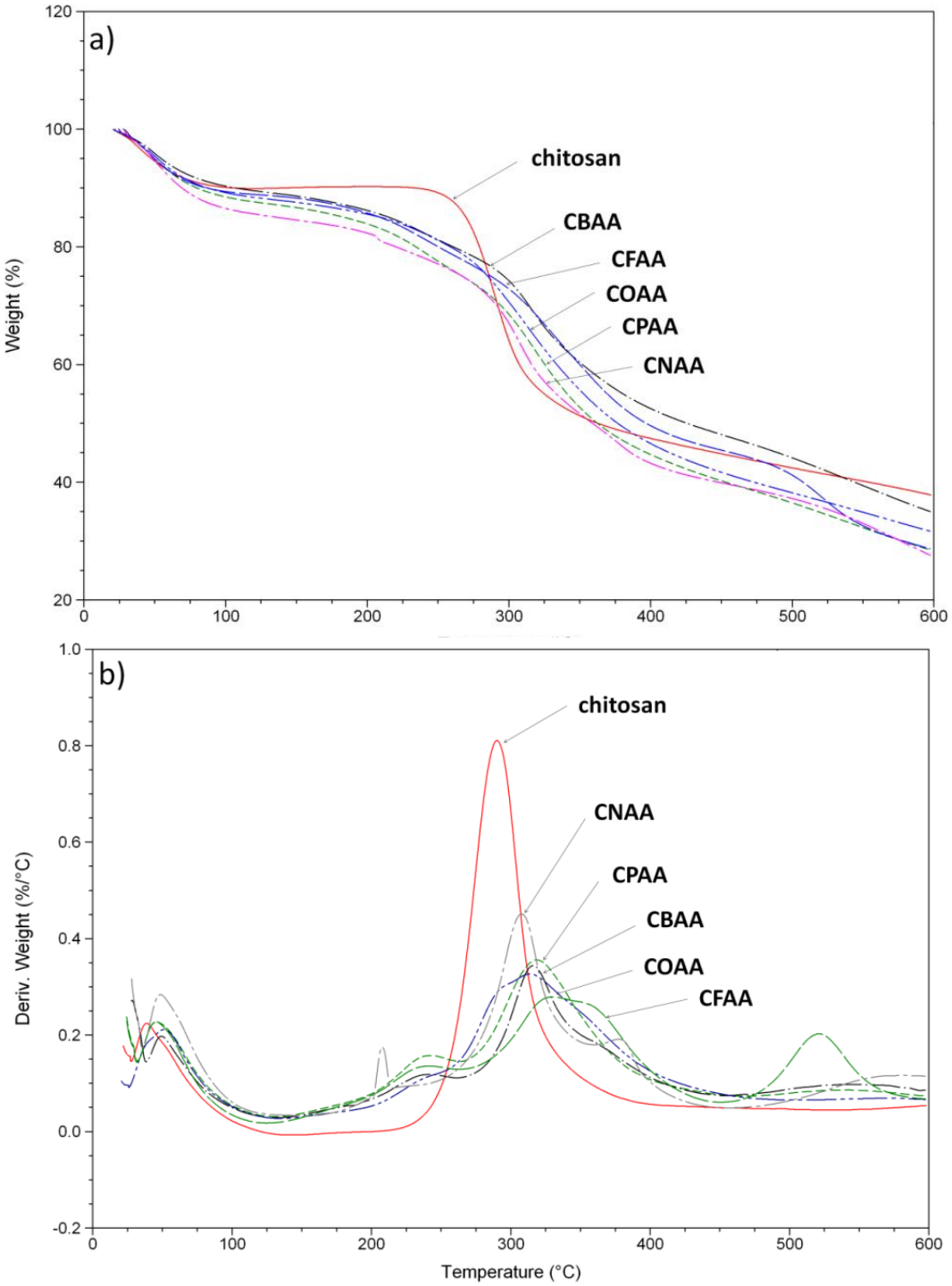

Figure 4-4 a) TGA and b) DTG thermograms of chitosan and crosslinked chitosan.

The loss of 10% in a temperature range between 20 and 240^{o}C relates to loss of adsorbed and bound water. The second weight loss of Cts was in the region between 240-340^{o}C with the sharp and considerable weight loss of 37%, which could be related to decomposition of Cts. CPAA, CBAA, COAA, CFAA and CNAA all showed weight loss of about 31, 25, 30, 28 and 34% at 300^{o}C, respectively. Unmodified Cts showed a 37% weight loss at the same temperature (300^{o}C) providing evidence the amic acid derivatives of Cts had decreased the rate of degradation and achieved increased thermal stability.

More accurate differences of the thermal behavior of the Cts and this novel amphoteric Cts derivative can be noted from the DTG curves, Fig. 4-4b. The DTG peak of Cts showed a maximum value at ~289^{o}C, while the maximum value of this peak for the amic acid derivatives are observed at 318, 316, 315, 325 and 307^{o}C for CPAA, CBAA, COAA, CFAA and CNAA, respectively. These results show that all amic acid derivatives increase the thermal stability of the Cts.

4.3.6. Swelling studies

In Figure 4-5 the swelling behaviour of Cts and amic acid derivatives of Cts was compared as a function of pH. The amic acid derivatives of Cts contained both amino groups and carboxylic acid group. The swelling behavior of these amphoteric hydrogels depends strongly on the ionization of these groups in various pH. From Figure 4-5 it can be seen that all amphoteric Cts derivatives produced a higher percentage of swelling in basic medium which may be due to the dissociation of the -COOH groups of amic acid. Below the pKa value of the Cts amine groups (pH 6.5), the swelling is controlled mainly by the amino group (NH_2) on the C-2 carbon of the Cts component. The protonation of the Cts amino groups leads to repulsion of the polymeric chains in acidic medium. In the case of unmodified Cts under very acidic conditions (pH < 4), protonation of the amino groups leads to full dissolution of the matrix. These results also indicate that the amic acid crosslinked Cts matrices have applicability as insoluble films, at least in the range from pH 2-10.

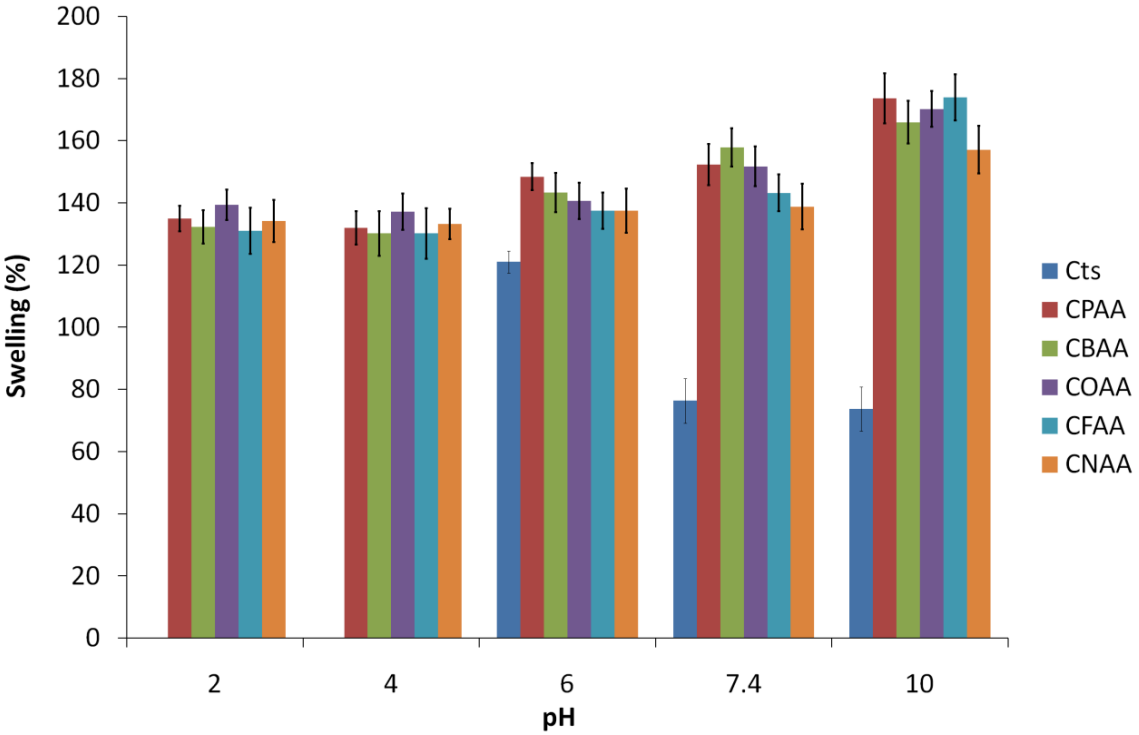

Figure 4-5 Swelling behaviour of chitosan films at different pH.

4.3.7. Water contact angle measurements

For better understanding of the surface properties of the networks and analysing the influence of crosslinking on the hydrophilic properties of the Cts films, a water contact angle study was carried out, Figure 4-6. Water contact angles are most frequently used to determine the hydrophilic properties of films.[20] Unmodified Cts has a water contact angles of 98.7 ± 1.3°. The amic acid derivatives showed lower contact angles as compared to the unmodified Cts which indicates an increase in hydrophilicity in the modified Cts films. The results of the amic acid derivatives of Cts film water contact angles are as follows: CPAA (46.5 ± 1.9°), CBAA (45.0 ± 2.0°), COAA (45.9 ± 1.2°), CFAA (46.9 ± 1.7°), and CNAA (40.0 ± 2.4°). From these results, it can be concluded that these amphoteric Cts based films show better hydrophilicity than Cts.

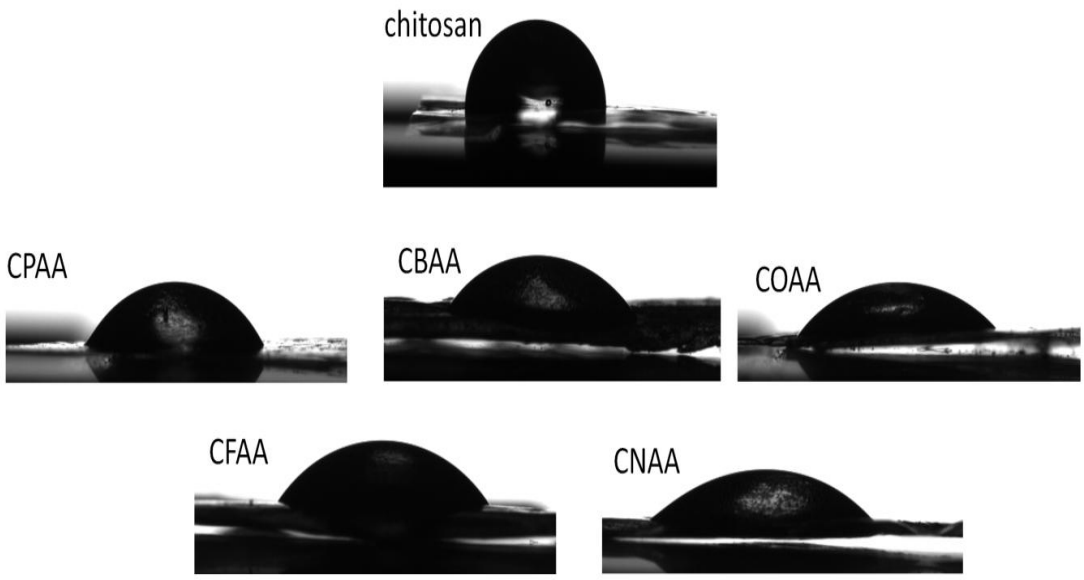

Figure 4-6 Visualisation of water contact angles on chitosan and crosslinked chitosan film surfaces.

4.3.8. *In vitro* biodegradability

The degradability of the test films in a lysozyme solution are presented in Figure 4-7. To evaluate biodegradability, the Cts films were incubated in a lysozyme solution and the formation of oligomers containing N-glucosamine units were monitored by a ninhydrin assay. The results indicate that crosslinked Cts matrices have a faster enzymatic degradation rate as compared to its unmodified Cts at pH 7.4. This observation is probably related to the higher swelling ratios of the crosslinked Cts films compared to unmodified Cts at pH 7.4. Therefore, the lysozyme's approach to the glycosidic bonds is facilitated due to higher swelling rate of these modified films which causes less steric hindrance allowing facile penetration of lysozyme. The higher degradation of crosslinked Cts over unmodified Cts over time should make these new matrices good candidates for therapeutic implantation.

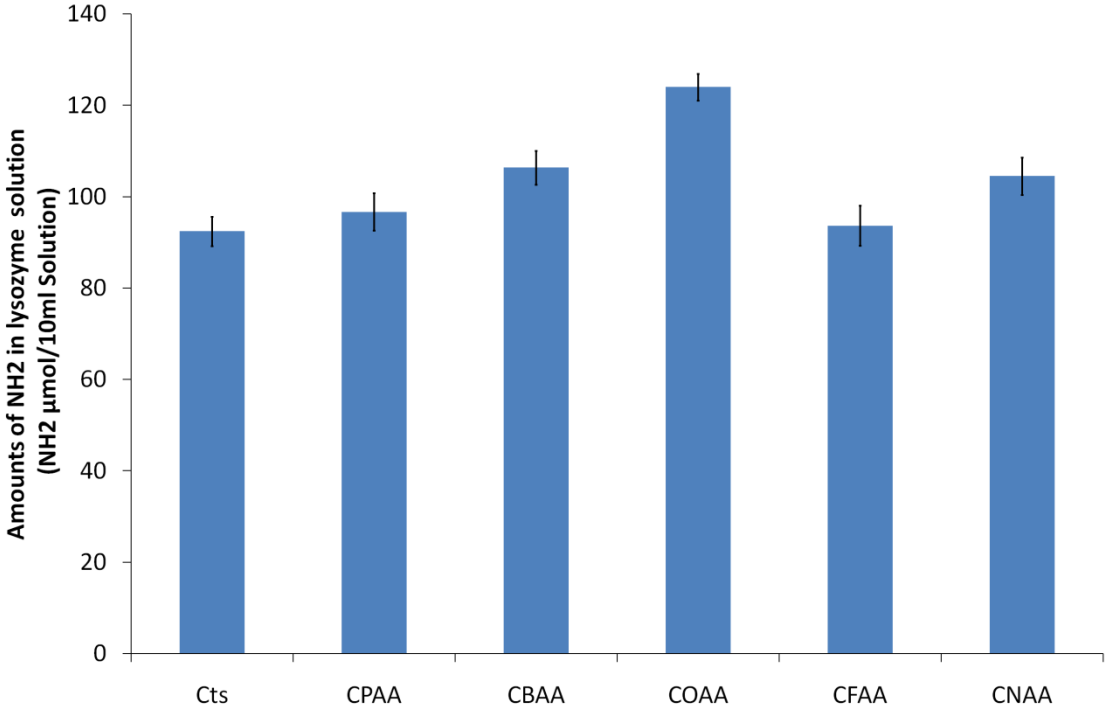

Figure 4-7 Results of the degradability of the chitosan and amic acid derivatives of chitosan in a lysozyme.

4.3.9. Enzyme inhibitory effect

To evaluate the protease inhibitory effects of Cts derivatives, trypsin and chymotrypsin were chosen as a representative of endopeptidases. The inhibition mechanism of amic acid derivatives of Cts is explained by the binding affinity of the polymer toward calcium, which leads to minimizing the proteolytic activity of calcium-dependent enzymes like trypsin and chymotrypsin.[21] Pure Cts showed 3.5 ± 0.3% and 8.2± 0.4% inhibition capability for trypsin and chymotrypsin respectively. The results of the enzymatic inhibitory effect of crosslinked Cts showed a much stronger inhibitory effect toward trypsin and chymotrypsin compared to pure Cts. CPAA, CBAA, COAA, CFAA and CNAA showed an inhibitory effect of 43.2 ± 1.2%, 40.2 ± 1.1 %, 44.2 ± 2.3%, 52.7 ± 2.0%, 45.3 ± 1.8% for trypsin, and 82.5 ± 2.9%, 80.6 ± 2.1%, 85.4 ± 1.8%, 90.3 ± 2.7%, 78.1 ± 2.4% for chymotrypsin. The higher inhibitory effect of modified Cts compared to pure Cts can be explained by the increased binding of calcium by the carboxylic acid groups on modified Cts, which may cause thermodynamic instability in the enzymes.

4.4. Conclusion

In spite of a particular interest to fabricate amphoteric pH-sensitive hydrogels, only a few amphoteric Cts based hydrogels have been studied so far. In this study, a new generation of crosslinked amphoteric Cts matrices has been synthesised. These amphoteric Cts derivatives which contain -COOH and -NH_2 groups exhibit many significant advantages such as improved thermal stability, an enzyme inhibitory effect and excellent stability in a wide range of pHs. In terms of wettability, the amic acid crosslinked Cts were more hydrophilic than uncrosslinked ones. The low cost of some of these dianhydride derivatives particularly PMDA and BTDA, which are extensively used in polyimide industry and the simplicity of preparation of these novel amphoteric biopolymers will draw considerable attention.

4.5. References

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Chapter Five

Development of a pH sensitive carrier system based on a novel water soluble chitosan and alginate for colon targeted drug delivery

5.0. Introduction

Colorectal cancer manifests as cancerous growths in the colon, rectum and appendix and is reported as one of the most frequent causes of cancer deaths.[1] Various drugs and combination of drugs have been exploited for the chemotherapy of colorectal cancer.[2] 5-Fluorouracil (5-FU) is a hydrophilic drug that has been the most widely used drug for the treatment of colorectal cancer for many decades.[3] It can be administered either orally or intravenously. To achieve successful oral colonic delivery, a drug needs to be protected from absorption and/or the environment of the upper gastrointestinal tract and then be abruptly released into the proximal colon, which is considered the optimum site for colon-targeted delivery of drugs. Oral colon-specific drug delivery of 5-FU can be mediated by means of prodrugs, azopolymers, time-, pH-, and pressure-sensitive, as well as microbial-activated approaches.[4] Among the various hydrogels, including natural, synthetic and natural/synthetic hybrid hydrogels, Cts has attracted significant attention in the field of colon delivery, due to its unique polymeric cationic character, biocompatibility and its degradation by anaerobic microflora present in the colon.[3a,5] Chitosan is a hydrophilic polyelectrolyte polysaccharide composed of glucosamine and N-acetyl glucosamine units linked by β(1–4) glycosidic bonds, produced by alkaline N-deacetylation of chitin, which is the main component of the shells of crabs, shrimps, and krill.[6] The poor solubility of Cts in water becomes the major limiting factor in its utilization.[7] Therefore a special emphasis has been placed on the chemical modifications employed to prepare Cts derivatives with improved solubility in water.[8]

In the present investigation, films have been formulated with a view toward colonic delivery using a novel amphoteric crosslinked Cts matrix. In order to improve the pH-

sensitivity of crosslinked Cts, sodium alginate, which has a high concentration of carboxylic groups, was incorporated into the crosslinked Cts hydrogel.

5.1. Experimental

With the exception of the following details, the same reagents, instruments and methods as per **Chapter 4**, were used throughout this chapter.

5.1.1. Materials

Trimellitic anhydride chloride (TMAC), 5-fluorouracil (5-FU), trypsin from porcine pancreas and α-chymotrypsin from bovine pancreas were purchased from Sigma-Aldrich (Auckland, New Zealand).

5.1.2. Preparation of Cts-TMAC amic acid (CTAA)

A total of Cts (2 g, 0.0124 mol of glucosamine residues) were first suspended in glacial acetic acid (50 ml) under vigorous stirring at room temperature. Then TMAC (2.61 g, 0.0124 mol anhydride) dissolved in DMF (50 ml) was added. The mixture was stirred at room temperature for 24 h. The crosslinked Cts powder was filtered off, washed with methanol and then dried in vacuum.

5.1.3. Preparation of CTAA amic acid (CTAA) film

CTAA film used in the study was produced by a casting/solvent evaporation technique. The CTAA powder was dissolved in deionized water to obtain 1% polymer solution. The resulting solution was then sonicated for 15 min to remove trapped air bubbles. The CTAA solution (30 g) was then poured into a glass Petri dish and oven dried at 40^{o}C for 24 h to form a film.

5.2. Characterization of the CTAA amic acid (CTAA) film

5.2.1. NMR spectroscopy

^{1}H ^{1}D and ^{1}H-^{13}C HSQC spectra were recorded at 298 K on a Bruker Avance 700 MHz spectrometer equipped with a cryoprobe. HSQC spectra were recorded with ^{1}H and ^{13}C spectral widths of 12 ppm and 140 ppm respectively using a data matrix consisting of 2k x 400 points. Signals were averaged for 100 scans per row. The ^{1}H NMR spectra for the crosslinked Cts was recorded using a Bruker Avance 500 MHz NMR spectrometer. The concentration of the sample was about 10 mg/ml in D_2O. Solid-state carbon-13 (^{13}C) magic angle spinning (MAS) NMR spectra were also obtained at a ^{13}C frequency of 50.3 MHz on a Bruker (Rheinstetten, Germany) DRX 200 MHz spectrometer.

5.2.2. X-ray diffraction (XRD)

The X-ray diffraction patterns were recorded on a Rigaku SPIDER curved image-plate detector using a multi-metal-layer Osmic confocal optic to monochromate and focus the Cu Kα radiation produced by a Rigaku MM007 micro-focus rotating-anode generator. Data were collected using Rapid XRD and integrated in Area Max.

5.2.3. Scanning electron microscope

The freeze dried powder particles of the copolymer and its hydrogel were mounted onto stubs using a double sided adhesive tape and sputter coated with gold. The surface morphology of the powders was studied using an FEI Quanta 200 Scanning Electron Microscope (Eindhoven, The Netherlands) at an accelerating voltage of 20 kV.

5.2.4. Solubility test

The sample (10 mg) was dispersed in the test solvent (5 ml) and the mixture was continuously shaken at room temperature for 3 days.

5.3. Preparation of CTAA/alginate film

The CTAA/alginate hydrogels were produced using a casting/solvent evaporation technique. CTAA solutions with different concentrations (0.25, 0.5, and 1% w/v) and an alginate stock solution (0.25% w/v) in water were prepared. Subsequently, 25 ml of CTAA solution (0.25, 0.5 and 1%) was added drop wise to 25 ml of the sodium alginate solution (0.25% w/v) under vigorous agitation for 60 min. The degassed and well mixed solutions were then cast into polyethylene petri dishes, and allowed to dry at ambient temperature. In the case of pure CTAA (1%) or alginate films (0.25%), 50 ml of each solution was used for film preparation. The thickness of the dried films ranged from 0.04 to 0.10 mm.

5.4. Swelling studies of CTAA/alginate films

The swelling characteristics of CTAA/alginate films were determined by immersing the dried films (0.100 g) in 50 ml buffer solutions of simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4) and simulated colonic fluid (SCF, pH 7) at 37^{o}C. At predetermined times, the films were taken out and blotted with a filter paper to remove surface absorbed water, and weighed immediately. The experiment was done in triplicate and the swelling ratio (S) was evaluated by the following equation:

$$S\ (\%) = \left[\frac{(W_s - W_d)}{W_d}\right] \times 100$$

where W_s and W_d are the weights of swollen and dried films.

5.5. Release profile of 5-fluorouracil (5-FU) from CTAA/alginate films

The drug-loaded CTAA/alginate films were prepared by adding 5-FU (50mg) to 100ml dissolved CTAA solution (100 ml, 1%). After dissolution of the drug, 25 ml of the blend was added drop wise to 25 ml of sodium alginate solution (0.25% w/v) under vigorous agitation for 60 min. The remainder of the procedure used was similar to those in the preparation of the CTAA/alginate films without loading 5-FU (section 5-3). To study the release profiles for the drug-loaded CTAA/alginate films, dried test samples were immersed in simulated gastrointestinal fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4) and simulated colonic fluid (SCF, pH 7). At predetermined time points, 100 µl of this solution was taken out and analyzed by the HPLC. In addition a test was carried out to simulate passage through the GI tract. At first, the drug release was determined in SGF for 2 h, followed by 6 h in SIF and then 24 h in SCF.

5.6. HPLC analysis

The quantitative determination of 5-FU released was determined using a HPLC system consisting of a Waters 2690 Separation module and a Waters 996 photodiode array detector, equipped with a Phenomenex® C18 reverse-phase column (250×4.6 mm, 5 µm). The mobile phase for HPLC analysis consisted of acetonitrile: acetate buffer pH 4.4, (15:85). The injection volume was 50 µl and elution was performed at a flow rate of 0.4 ml/min. The wavelength of the detector set at wavelengths of 260 nm.[9]

5.7. Cell cultures

Crandall Rees feline kidney (CRFK) cells were maintained in an advanced dulbecco modified (Adv DMEM, Invitrogen) supplemented with 2% (growth medium) or 1% (maintenance medium) fetal bovine serum (Thermo Scientific, MyClone), 1% Pen/Strep solution (Invitrogen), and 1% glutamax (Invitrogen). The cells were seeded into pre-selected wells of 96-well tissue culture plates (Thermo Scientific, Nunc) at a

concentration of 5 x 10^4 cells/well in a 100 µL volume. The plates were incubated at 37˚C in a humidified atmosphere with 5% CO_2 overnight.

5.8. Cytotoxicity test

An aliquot (2 mL) of the maintenance medium was added to each well with 20 mg of a sterilized compound. The compound suspensions (10 mg/mL) were then subjected to serial 2-fold dilutions in the maintenance medium. The growth medium was removed after approximately 24 hours, when the monolayers were 95% confluent, and replaced with 200 µL of each compound's dilution, from 0.16 mg/mL to 0.005 mg/mL in duplicate (test wells). For each compound dilution, an equal amount (200 µL) was also added in duplicate to wells that were not pre-seeded with cells (control wells). The growth medium was similarly replaced in four cell control wells (pre-seeded with cells, without addition of any compounds) and added to four medium control wells (empty wells not pre-seeded with cells). Plates were incubated at 37°C, 5% CO_2 humidified atmosphere for 24 hours. The cultures were inspected for signs of toxicity using an inverted microscope. In addition, the number of viable cells in each well was quantified using a colorimetric cell viability assay (WST-1 reagent, Roche), according to the manufacturer's instructions. Briefly, cell viability was assessed based on the optical density (OD) readings measured at 450 nm wavelength following a 2.5 hour incubation of cells in the presence of the WST-1 reagent. The % cell viability as compared to control cells was calculated separately for each dilution of each compound as follows:

$$\%\ \text{cell viability} = \frac{(\mathrm{OD}_{450}\ \mathrm{TX} - \mathrm{OD}_{450}\ \mathrm{CX})}{(\mathrm{OD}_{450}\ \mathrm{CC} - \text{mean}\ \mathrm{OD}_{450}\mathrm{MC})} \times 100$$

OD_{450} TX: mean OD_{450} of duplicate wells with the same dilution of compound x following incubation with CRFK cells.

OD_{450} CX: mean OD_{450} of duplicate wells with the same dilution of compound x without cells.

OD_{450} CC: mean OD_{450} of quadruple wells with the CRFK cells (cell control - no compound).

OD_{450} MC: mean OD_{450} of quadruple wells without CRFK cells (media control).

5.9. Results and discussion

5.9.1. Preparation of CTAA

Up to now, TMAC as a crosslinker for chitin and Cts has been investigated in only a few publications.[10] In all these reports, the imide ring is formed after thermal imidization. The mechanism of this reaction involves the nucleophilic attack of the amino group of the Cts on the carbonyl carbon of the anhydride moiety of trimellitic anhydride followed by opening of the anhydride ring to form the intermediate amic acid group, as shown in Figure 5-1. Subsequently, cyclization of the amic acid intermediate will occur with elimination of water given a suitable temperature increase. In this study, a method was devised to yield the amic acid intermediate as a novel amphoteric Cts derivative.

The molecular weight of Cts was also determined to be 198 kDa with the Mark–Houwink viscometry method in a solvent of 0.1M acetic acid/0.2M NaCl maintained at 25^{o}C.

Carbon, hydrogen and nitrogen contents of Cts and modified Cts were found to be C, 43.19; H, 6.97; N, 8.03% and C, 38.5; H, 6.3; N, 5.11%, respectively. The DD and DS were obtained 77% and 0.24, respectively.

5.9.2. Characterization of the chitosan derivative

FTIR analysis was employed to investigate the characteristic chemical structure of the Cts crosslinked with TMAC. The FTIR results for the Cts and CTAA are shown in Figure 5-1. After crosslinking Cts with TMAC at 130^{o}C, two peaks appear at 1778 and 1720 cm^{-1} which correspond to the characteristic symmetrical and unsymmetrical C=O stretching bands of the cyclic imide respectively, Figure 5-1a. However after crosslinking Cts with TMAC at ambient temperature, two peaks were observed at 1721

cm^{-1} and 1663 cm^{-1} which were assigned to the C=O stretch of –COOH and amide group respectively, Figure 5-1b.

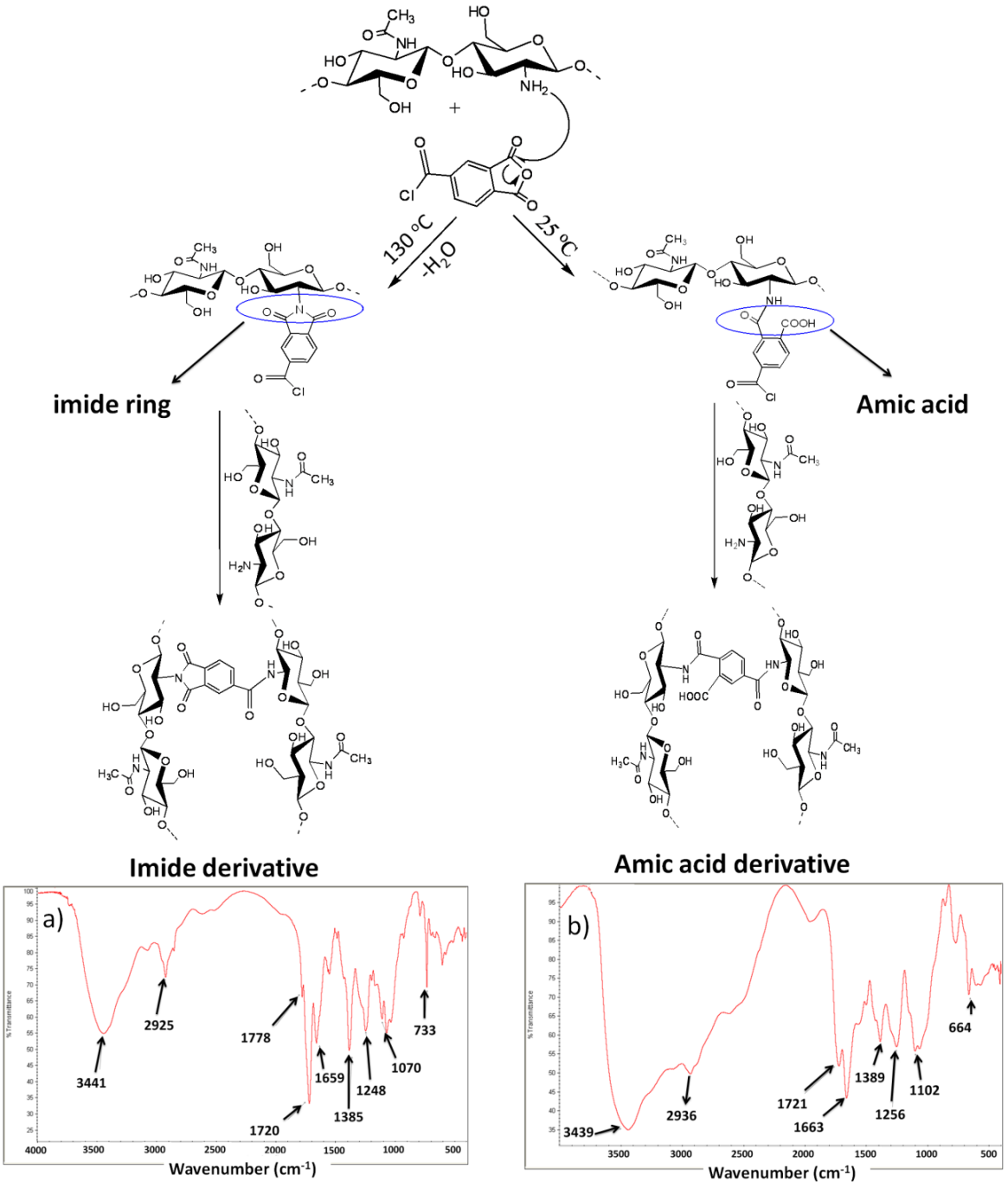

Figure 5-1 Reaction schemes and FTIR spectra of crosslinked chitosan with TMAC a) at 130 °C b) at room temperature.

The ^{1}H NMR (D_2O) spectrum of CTAA is given in Figure 5-2a. This spectrum shows all the signals typical of Cts.[11] The signal at 1.98 ppm corresponds to the $–CH_3$ groups of the acetamido residue. The signal at 3.10 ppm corresponds to H2 of glucosamine an N-acetyl glucosamine. The multiplets from 3.6-3.9 ppm correspond to H3-H6 of glucosamine and N-acetyl glucosamine. The C-1 proton is not observable in most instances as it is known to appear near the signal due to water. The chemical shifts at δ = 7.40-8.34 ppm are assigned to the aromatic protons.

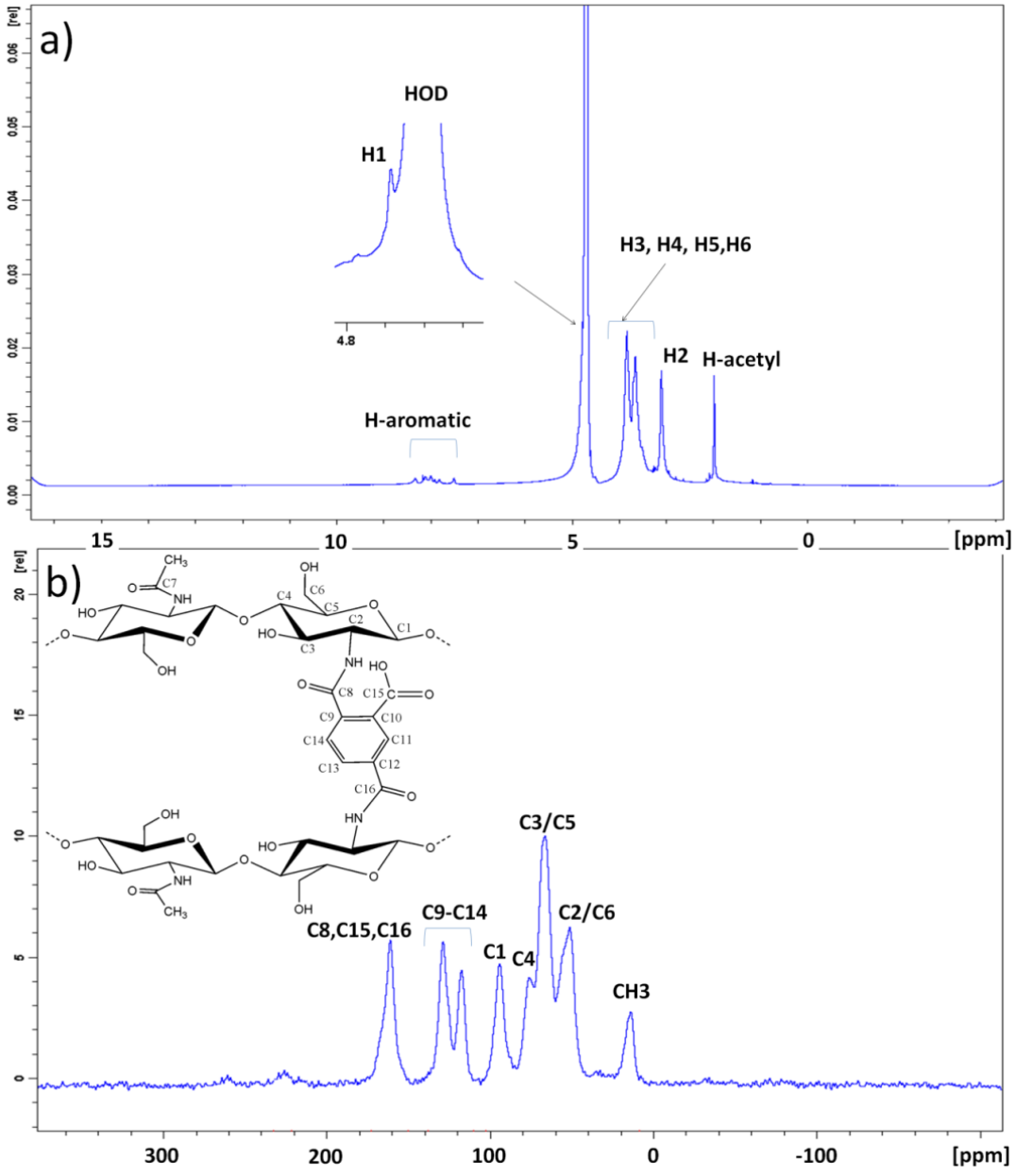

Figure 5-2 The (a) ^{1}H NMR (500 MHz) and (b) ^{13}C DP-MAS spectrum of CTAA.

The solid state ^{13}C NMR spectrum of crosslinked Cts is shown in Figure 5-2b. The signal at 13.9 ppm is attributed to the methyl of the acetamido group. Signals at 51.7 and 66.2 ppm are attributed to overlapping C2/C6 and C3/C5 of the pyranose ring, respectively.[12] The signal related to C4 is also observed at 74.0 ppm.[13]

The signal due to the C1 carbon, which is directly attached to two oxygen atoms, was assigned to the peak at 94.0 ppm which is at a significantly lower magnetic field value compared with the signals of the pyranose ring carbons. The signals at 17-128 ppm are attributed to the phenylene ring (C9-C14). A well defined signal is observed at 161 ppm, which can be assigned to the overlapping carbonyl group carbon of the amic acid and acetyl amine (C8, C15, C16).

The chemical structures of the CTAA were further investigated by ^{1}H-^{13}C HSQC (in D_2O) spectroscopy, as shown in Figure 5-3.This data confirms my assignment in 1H NMR study.

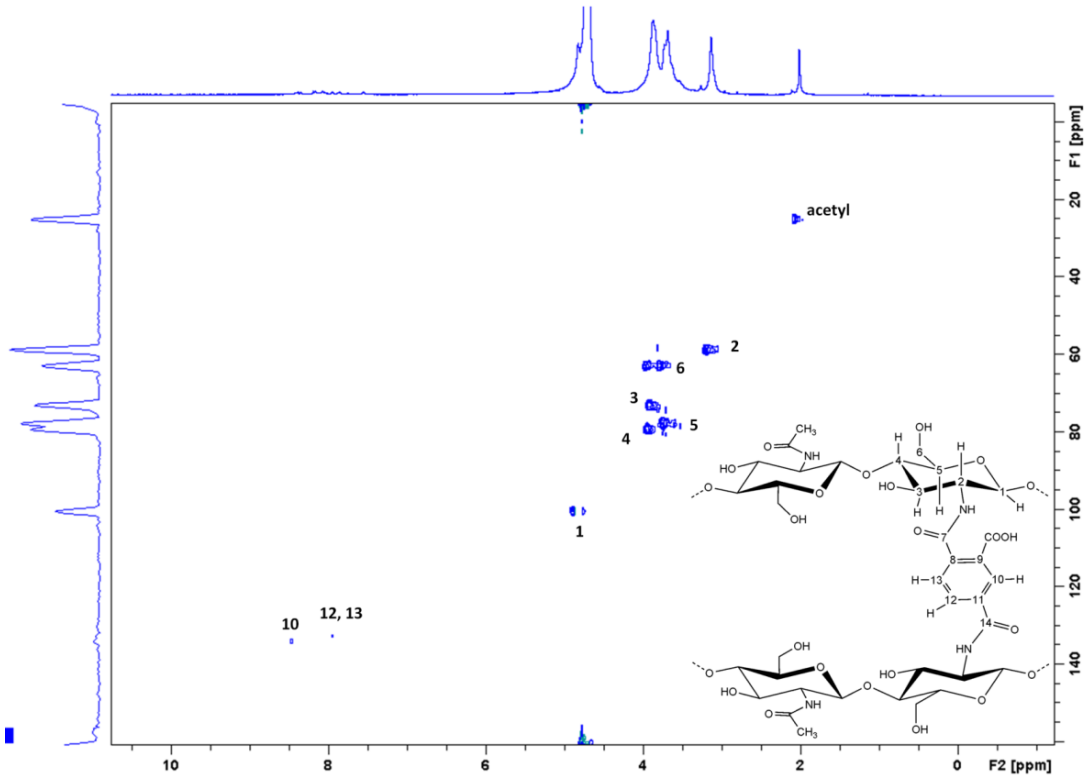

Figure 5.3 ^{1}H-^{13}C HSQC of CTAA (700 MHz)

The 2D-XRD patterns of Cts and its derivative are shown in Figure 5-4. The diffractogram of Cts, Figure 5-4a, exhibits two main crystalline peaks at 2θ values of 9.80^{o} and 19.8^{o}, which are generally assigned to weak chain-chain ordering of the Cts in addition to several weak crystalline peaks which are in agreement with the characteristic diffractogram of the unmodified Cts. However, these diffraction signals at $2\theta = 9.8^{o}$ and 19.8^{o} were not observed in the XRD patterns of CTAA which indicates that the chemical crosslinking between Cts and TMAC destroys the crystallinity of Cts and increases the amorphous volume in the modified Cts hydrogel (Figure 5-4b). This result indicates that the intermolecular hydrogen bonding (H-bonds) in CTAA has been greatly decreased relative to that of Cts.

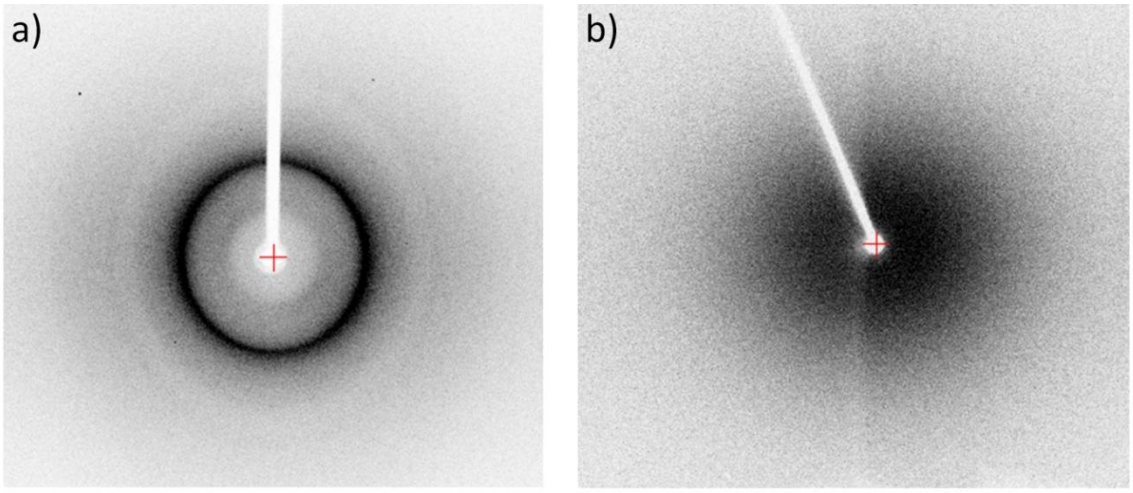

Figure 5-4 2D-XRD of (a) Cts and (b) CTAA.

Scanning electron microscopy (SEM) was used to visualize the surface morphology of the native Cts and the Cts derivative (Figure 5-5). As noted from this figure, the surface roughness increased after crosslinking (Figure 5-5b). The scale of 10 and 50 µm was used in this study. These noticeable changes in the surface morphology of Cts are related to chemical modification of Cts.

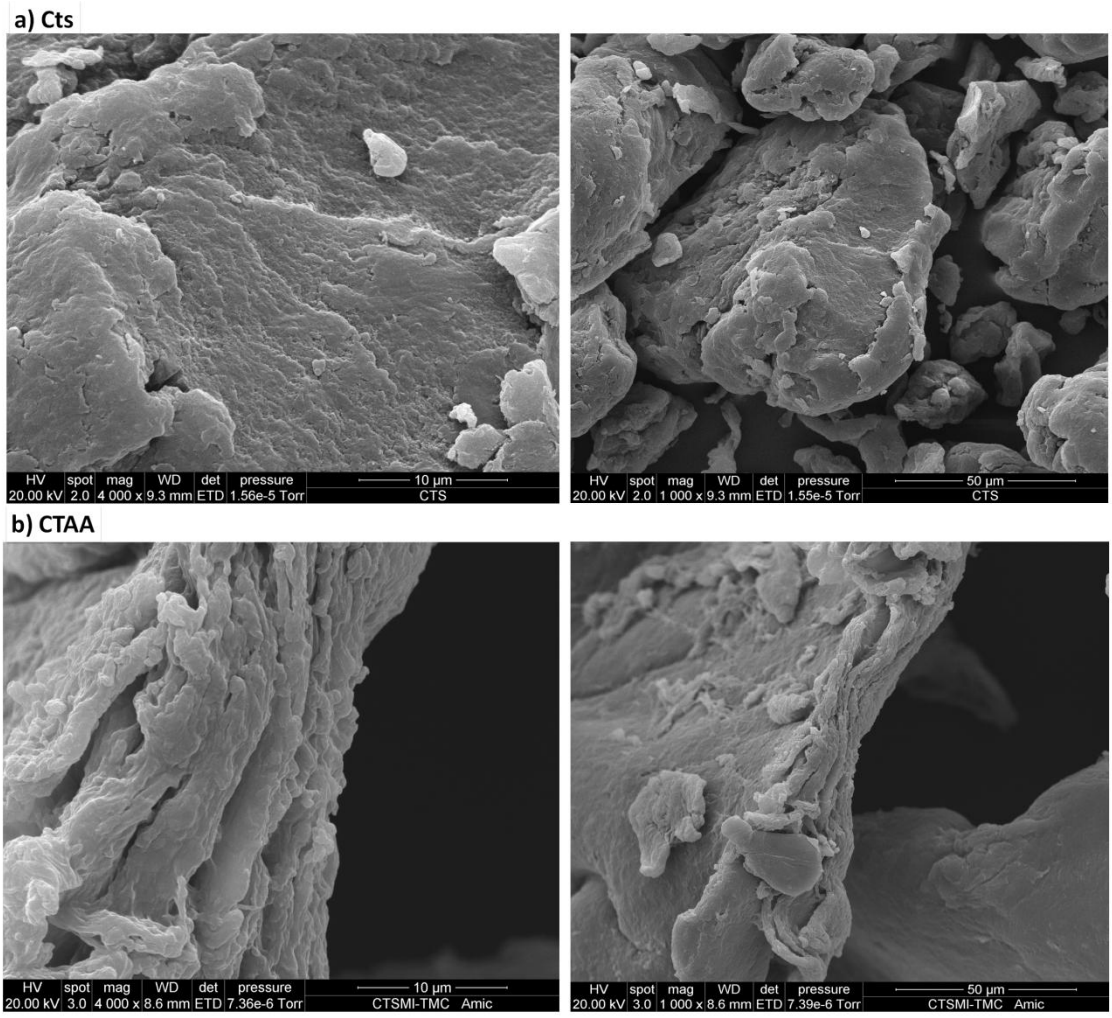

Figure 5-5 SEM images of a) Cts (at 10 µm and 50 µm) and b) CTAA (at10 µm and 50 µm).

5.9.3. Thermal gravimetric analysis (TGA) and differential thermal gravimetric analysis (DTG)

To examine the thermal stability behavior of Cts and CTAA, thermogravimetric analysis (TGA) was carried out, Figure 5-6. The TGA of Cts showed a weight loss in two distinct stages. Cts showed an initial weight loss of 10% in a temperature range between 20 and 240^{o}C due to the loss of adsorbed and bound water. The second weight loss of Cts was in the region between 240-340^{o}C shown as a clear drop on the graph and indicating a weight loss of 37.3%. This loss can be related to the decomposition of Cts.

CTAA showed a faster weight loss starting from 170°C, which shows the weaker thermal stability of CTAA compared to unmodified Cts.

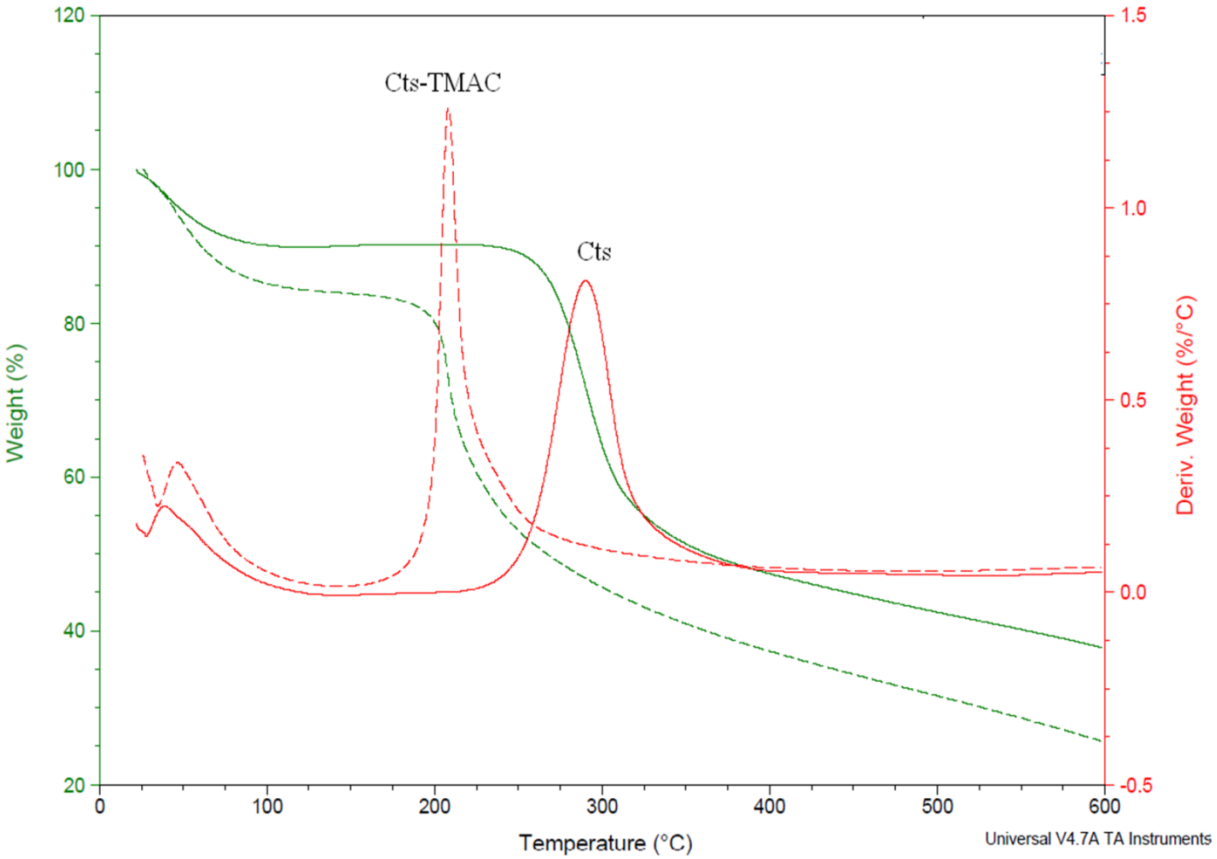

Figure 5-6 TGA and DTG thermograms of Cts and CTAA

More accurate differences of the thermal behavior of the CTs and CTAA can be noted from the DTG curves. The DTG peak of Cts showed a maximum value of about 290°C, while the maximum value of this peak for the CTAA curve is observed at about 208°C which indicates the decreased thermal stability of the CTAA relative to Cts.

5.9.4. Enzyme inhibitory effect of CTAA

To evaluate the protease inhibitory effects of the CTAA, trypsin and chymotrypsin were chosen as representative of GIT endopeptidases. The peptidase inhibition mechanism of CTAA can be explained by the binding affinity of the polymer towards calcium, which leads to the lowering of the proteolytic activity of calcium-dependent enzymes like

trypsin and chymotrypsin. The results of this enzymatic inhibitory study revealed CTAA showed a much stronger inhibitory effect toward trypsin and chymotrypsin as compared to the pure Cts. Unmodified Cts showed 3.5 ± 0.3% and 8.2 ± 0.4% inhibition capability for trypsin and chymotrypsin, respectively. CTAA showed a considerably increased inhibitory effect of 21.2 ± 2.1% and 46.6 ± 2.9% for trypsin and chymotrypsin, respectively.

5.9.5. Solubility test

Table 5-1 lists the solubility of Cts and CTAA in distilled water, organic solvents, acid and alkali media. As can be seen, Cts becomes water soluble after the introduction of the hydrophilic carboxylic acid groups after modification with TMAC.

Table 5-1 Solubility test results for Cts and CTAA

Solvent	Chitosan	CTAA
H_2O	insoluble	soluble
NaOH (1M)	insoluble	swelling
HCl (0.2 N)	soluble	soluble
Acetic acid (1%)	soluble	soluble
Dimethylformamide	insoluble	insoluble
Dimethylsulfoxide	insoluble	insoluble

5.9.6. Swelling studies of CTAA/alginate films

The swelling characteristics of CTAA/alginate films were determined under SGF or SIF. It can be seen from the swelling data shown in Figure 5-7, that in both pH 1.2 and 7.4 solutions as the amount of CTAA is increased, the amount swelling of the

CTAA/alginate films increased. This phenomenon may be attributed to the increasing number of amino and carboxylic acid groups, which led to stronger electrostatic repulsion between the positively charged groups at pH 1.2 and negatively charged groups at pH 7.4. As shown in Figure 5-7, all test groups with the same ratio showed smaller swelling percentages in acidic media than in mildly basic media.

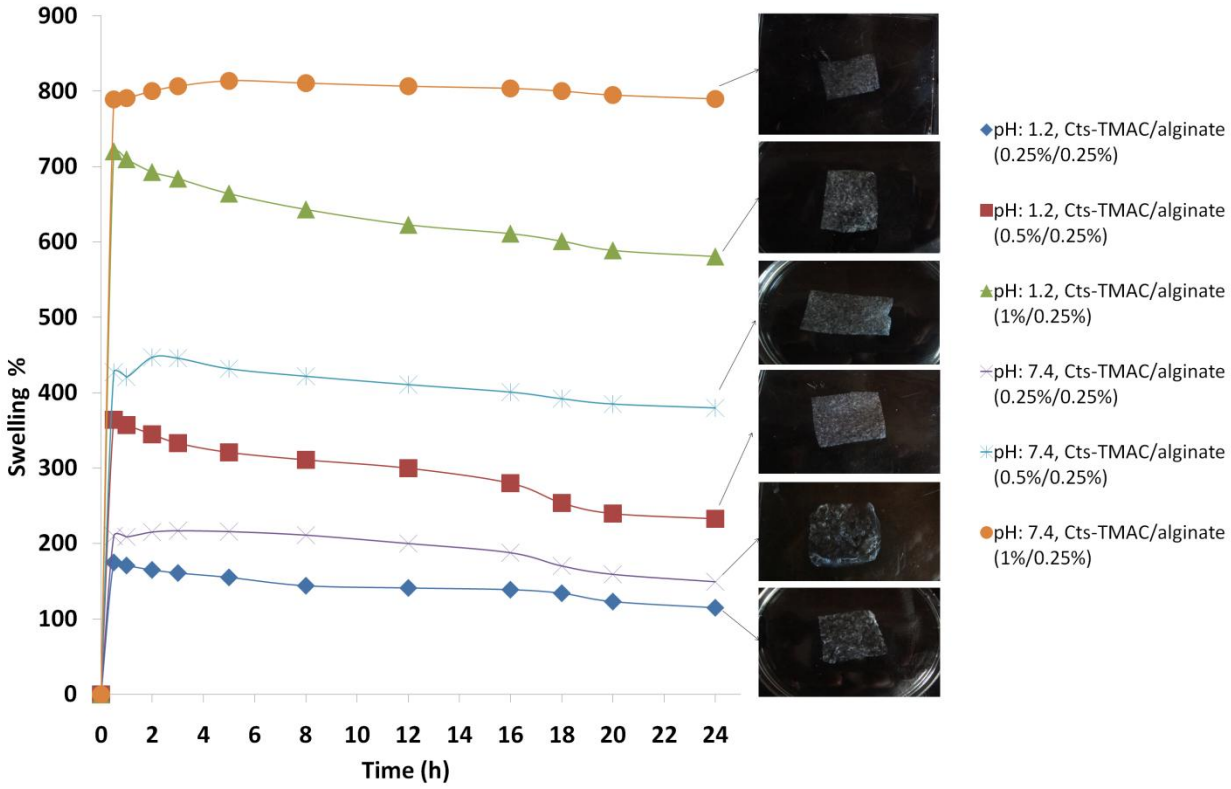

Figure 5-7 Swelling behaviour of CTAA/alginate films at SIF and SGF.

This phenomenon can be attributed to the large swelling force created by the electrostatic repulsion between the ionized carboxylic acid groups in the CTAA/alginate films at pH 7.4.

5.9.7. *In vitro* drug release study

In an attempt to decrease the leakage of the encapsulated drugs and improve the pH-sensitivity of the CTAA hydrogel, sodium alginate, a polyanionic polysaccharide, was incorporated into the CTAA hydrogel.

5.9.7.1. Effect of pH of media on release profile of 5-FU from CTAA/alginate films

The effect of pH on the release profiles of CTAA/alginate (0.5% : 0.25%) is shown in Figure 5-8. The time for 50% drug release ($T_{1/2}$) in acidic media (pH 1.2) was obtained after 10 hours, while the $T_{1/2}$ of those at pH 7.4 was obtained after eight hours. The amount of drug released after 24 hours was 81.6 ± 4.7% and 84.3 ± 4.3% at pH 1.2 and 7.4 respectively. This finding can be related to the higher degree of swelling of the CTAA/alginate film at pH 7.4 which shows a slightly enhanced diffusion of drug out of the film.

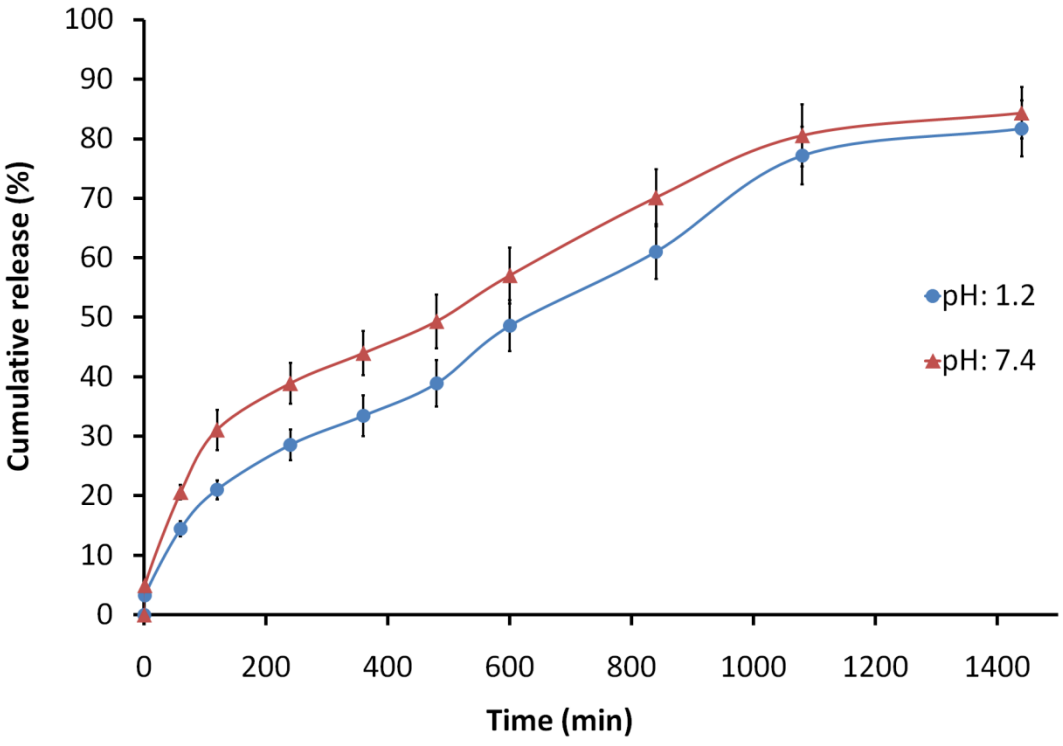

Figure 5-8 Effect of pH on the release profile of 5-FU from CTAA/alginate films.

5.9.7.2. Effect of enzyme on the release profile of 5-FU from CTAA/alginate films

To further simulate GIT conditions, selected formulations were assessed in the presence and absence enzymes, Figure 5-9. After two hours in SGF (stage I: pH 1.2, with and without pepsin), then in SIF (stage II: pH 7.4, with and without pancreatin) for six hours. Afterwards, the film was transferred to SCF (stage III: pH 7, with and without β-glucosidase) for 24 hours. Based on the results shown in Figure 5-9, the release of 5-FU from films at stage I showed no significant difference with or without pepsin. In all cases, about 33% of drug was released after two hours in acidic pH, 37^{o}C. These results are attributed to the optimal conditions needed for chitosanolytic activity of pepsin which are pH 5 at 45^{o}C. The optimal pepsin conditions are out of the pH and temperature ranges for the stomach (stage I).

At pH 7.4 (stage II), a total of 59.3 ± 3.7% of the drug has been released after 6 hours in a pancreatin free media, while in the presence of pancreatin 69.6 ± 2.8% of a drug had been released. This faster release of drug in presence of pancreatin may be related to depolymerization of Cts by the lipase in pancreatin. This study shows with using this novel formulation approximately 39 and 28% of the drug would arrive at the colon in the presence and absence of enzyme respectively. With respect to colonic enzymatic degradation of carbohydrates, in this case Cts, similar enzymatic degradation functionality exists in commercially available almond β-glucosidase. Hence, almond β-glucosidase was used as a model enzyme to evaluate the effect of colonic enzymes on CTAA. In the presence of this β-glucosidase, the drug released within two hours was 10.3 ± 2.1% more than without the enzyme, indicating that Cts crosslinking was still available for degradation by the enzyme.

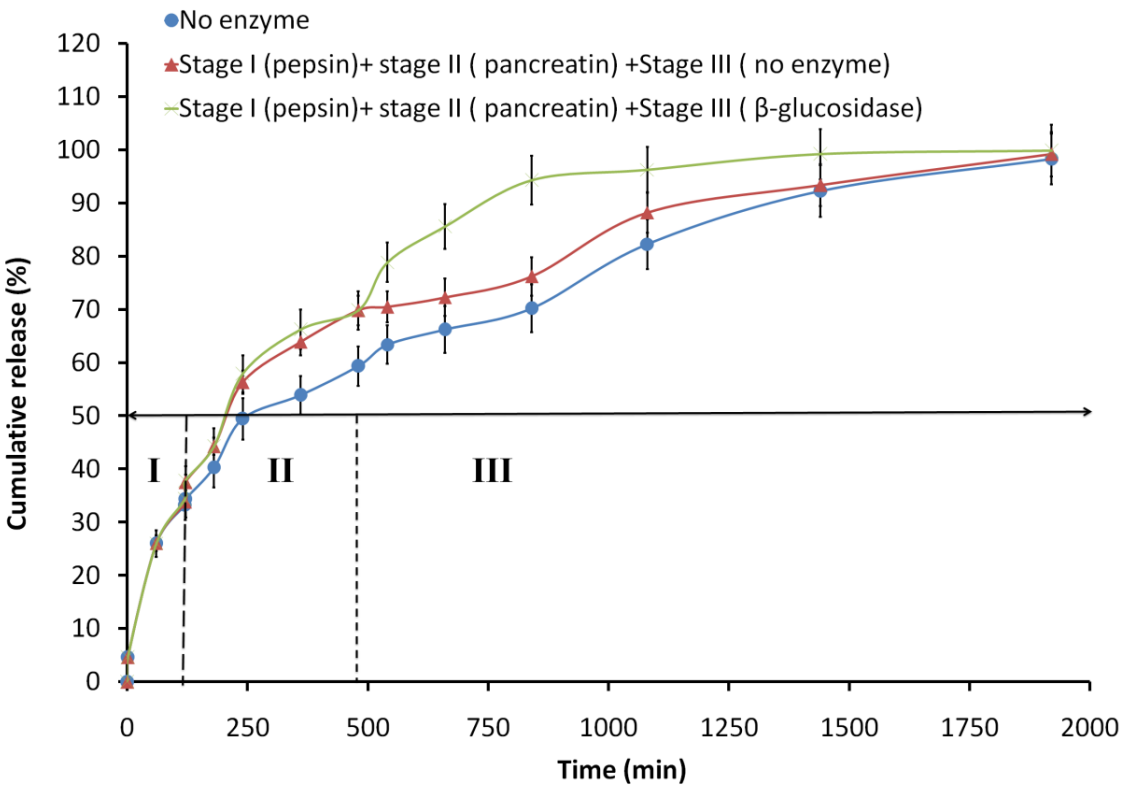

Figure 5-9 Effect of enzyme on the release profile of 5-FU from CTAA/alginate films

5.9.8. Cytotoxicity studies

Chitosan is widely regarded as being a non-toxic, biologically compatible polymer. To improve Cts properties for biomedical applications, several derivatives of Cts have been studied so far. However, the use of crosslinking agents may lead to toxic side effects. Therefore it is desirable to introduce a crosslinking reagent that has low cytotoxicity and would form biocompatible crosslinked products. The cytotoxicity of Cts and CTAA were evaluated against CRFK cells by WST-1 assay. The percentage of cell viability as compared to the control cells after 24 hours exposure to the polymers (concentration from 0.16 to 0.005 mg/mL) are shown in Figure 5-10. Over 100% cell viability was observed in the case of Cts at all concentrations, which indicates that Cts facilitated growth of the CRFK cell line. The viability of cells incubated with CTAA was greater than 87% for all studied concentrations (0.16 to 0.005 mg/mL).

CTAA shows 87.6 ± 2.5%, 99.0 ± 0.7%, 104.0 ± 2.9% and 94.5 ± 7.3% cell viabilities for concentrations of 0.62, 0.04, 0.02 and 0.005 mg/ml, respectively. These results

indicate that not only are these novel formulations able to be used as colon targeted drug delivery systems, but also they can be considered as very promising materials as safe drug vehicles.

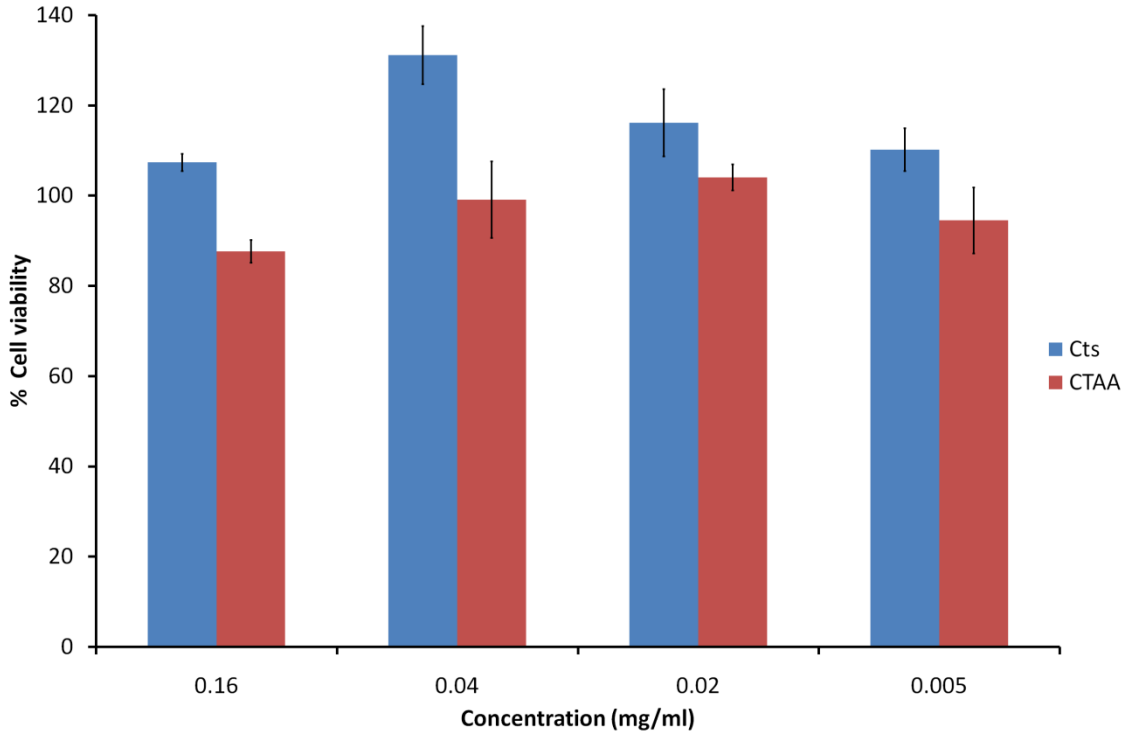

Figure 5-10 Percent viability of cells incubated with tested compounds as compared to control cells.

Based on a survey of literature few Cts based films have been developed for colon specific drug delivery applications. Ching Chen *et al*. prepared a pH-sensitive hydrogel system composed of a water-soluble Cts derivative (*N,O*-carboxymethyl Cts, NOCC) and alginate blended with genipin for controlling the release of a protein drug.[14] BSA release profiles from the genipin-cross-linked NOCC/alginate hydrogel were tested at pH 1.2 and 7.4. Their study showed about 20% of protein is released within five hours at pH 1.2. At pH 7.4, the amount of BSA released was about 82% after five hours. The release profiles were also investigated by immersing the film into a solution at pH 1.2 for two hours followed by another solution at pH 7.4 for an additional 14 hours. In this study about 70% of the drug was released after eight hours (2 h in SGF + 6 h in SIF).

Shu *et al*. developed Cts films cross-linked with sodium citrate in various pHs.[15] They showed that under acidic conditions (pH less than 3.5), the citrate-Cts film swelled and dissociated and the model drugs (brilliant blue and riboflavin) incorporated in the film were released quickly (within 2 h). The films developed in this chapter (CTAA/alginate films) revealed that about 48 and 57% drug release occurred within five hours at pH 1.2 and pH 7.4, respectively. Also in the simulated GIT solution (without any enzyme) approximately 60% of the drug was released after eight hours (2 h in SGF + 6 h in SIF). This result shows the significant potential of the CTAA/alginate films for controlled drug released.

5.10. Conclusion

The objective of this study was to design a pH-sensitive matrix system for specific 5-FU delivery to the colon. To this end a novel amphoteric crosslinked Cts was prepared and characterized. This amphoteric polymer was mixed with sodium alginate in order to improve the pH-sensitivity of the crosslinked Cts. The results indicate that the CTAA/alginate films showed a pH and enzyme sensitive drug release pattern. Therefore the CTAA/alginate films should provide a suitable polymeric carrier for colon-specific drug delivery.

5.11. References

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Chapter Six

Formulation and evaluation of a pH and enzyme controlled colon specific delivery system using novel amphoteric chitosan based matrix tablets.

6.0. Introduction

Lower water content and fluid mobility of the colon, which leads to longer retention time and also lower proteolytic activity of the colon compared to other areas of the GIT, make the colon an ideal site for both systemic and local delivery of drugs.[1] Treatment of large intestinal disorders such as irritable bowel syndrome, colon cancer, and inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis where a high concentration of active drug is required, can be improved with a colon-targeted drug delivery system.[2] To achieve successful oral colonic delivery, a drug needs to be protected from the absorption and degradation pathways of the upper GIT and then achieve abrupt released into the colon. Various drug delivery systems have been designed that deliver the drug quantitatively to the colon and then trigger the release of the drug.[3] Among the various polymers, including natural, synthetic and natural/synthetic hybrid polymers, Cts has attracted significant attention for the design and development of colon targeted delivery systems because of degradation of the glycosidic linkages of Cts by microflora present in the colon.[4] Chitosan is a hydrophilic polyelectrolyte polysaccharide composed of glucosamine and N-acetyl glucosamine units linked by β(1–4) glycosidic bonds, produced by alkaline N-deacetylation of chitin.[5] Several crosslinking reagents have been used for crosslinking of Cts, such as glutaraldehyde, genipin, tripolyphosphate, ethylene glycol, diglycidyl ether and diisocyanate to modulate general properties of Cts.[6] In recent years, particular interest has been devoted to fabricate amphoteric pH-sensitive hydrogels.

Different approaches have been designed based on prodrug formulation, pH-sensitivity, time dependency, and microbial degradation to formulate the different dosage forms

such as tablets, capsules, multiparticulates, microspheres, liposomes for colon targeting.[7] A tableted matrix is the simplest and most cost-effective method of fabricating an extended release solid oral dosage form. However, little research has been done so far to develop colon specific drug delivery systems using Cts based tablets. my literature review shows that a mixture of Cts and chondroitin,[8] a mixture of Cts and ethylcellulose,[9] chitosan succinate and chitosan phthalate,[10] a mixture of Cts and pectin,[11] and a combination of spray-dried Cts acetate and hydroxypropyl methylcellulose[12] are the only Cts based tableted systems reported so far for colon targeted drug delivery.

The major objective of the present study was to develop a novel colon specific matrix tablet that would be able to protect the drug from the harsh acidic environment of the stomach and the rigorous enzymic activity of the small intestine and yet allow targeted drug release in the colon.

6.1. Materials and methods

With the exception of the following details, the same reagents, instruments and methods as per **Chapter 5**, was used throughout this chapter.

6.1.1. Materials

5-aminosalicylic acid (5-ASA) was purchased from Sigma-Aldrich (Auckland, New Zealand) Bovine serum albumin (BSA) was purchased from Acros Organics (Geel, Belgium).

6.1.2. Preparation of amic acid derivatives

Chitosan (2g, 0.0124 mol of glucosamine residues) was first suspended in 50 ml glacial acetic acid under vigorous stirring at room temperature. 1eq of the respective dianhydride derivatives dissolved in DMF (50 ml) was subsequently added. The dianhydrides used were BTDA and ODPA. The mixture was stirred at 25^{o}C for 24 h. The amic acid derivatives of Cts powder (CBAA and COAA) was filtered off, washed with methanol and then dried in vacuum.

6.2. Characterization of the hydrogel

The X-ray diffraction patterns were recorded on a Rigaku SPIDER curved image-plate detector using a multi-metal-layer Osmic confocal optic to monochromate and focus the Cu Kα radiation produced by a Rigaku MM007 micro-focus rotating-anode generator. Data were collected using Rapid XRD and integrated in AreaMax.

The freeze dried powder particles of the copolymer and its hydrogel were mounted onto stubs using a double sided adhesive tape and sputter coated with gold. The surface morphology of the powders was studied using an FEI Quanta 200 Scanning Electron Microscope (Eindhoven, The Netherlands) at an accelerating voltage of 20 kV.

6.3. Preparation and evaluation of tablets

6.3.1. Preparation of compression-coated tablets

Cts:CBAA and Cts:COAA dry blends with a total weight of 400 mg at various % weight ratios were used to achieve compress-coated tablets with a 100 mg core (80 mg drug and 20mg α-lactose monohydrate) at a compression force of 50 kN using a hydraulic press and a 13-mm diameter die and flat-face punch set.

6.3.2. Tablet crushing strength

The thickness (H) and diameter (D) of the matrix tablets was determined using a caliper (Mitutoyo Dial Thickness Gauge, Mitutoyo, Japan). A TA.XT Plus Texture Analyzer (Stable Micro Systems, UK) was used to measure the hardness of tablets. The tablet tensile strength is the force required to break a tablet by compressing it in the radial direction. A 5-mm-diameter spherical puncturing probe was driven through the tablet with a speed of 1 mm/min. The load at break of the the point of break tablet was measured. Tablet crushing strength (TCS) was calculated by using the formula:

$$\mathrm{TCS} = \frac{2\mathrm{F}}{\pi \mathrm{dt}}$$

where F is the crushing load, and d and t are the diameter and thickness of the tablet, respectively. The data reported are the means of five individual determinations.

6.3.3. Swelling behaviour of tablets

Radial and axial swelling of tablets (400 mg, Cts:CBAA and Cts:COAA at 75:25%) in simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4), simulated colonic fluid in healthy human (SCF, pH 7) was investigated. Simulated IBD colonic fluid (SCF, pH 5) was also tested in the case of Cts:CBAA at 37^{o}C. In addition a test was carried out for 2 h in SGF, followed by 6 h in SIF and then 24 h in SCF to simulate the swelling behaviour tablet in gastrointestinal tract. The experiment was done in triplicate and the swelling rates were evaluated by the following equations:

$$\%\ \mathbf{Swelling_{axial}} = \frac{(\mathbf{h_t} - \mathbf{h_o})}{\mathbf{h_o}} \times \mathbf{100}$$

$$\%\ \mathbf{Swelling_{radial}} = \frac{(\mathbf{A_t} - \mathbf{A_o})}{\mathbf{A_o}} \times \mathbf{100}$$

where h_o and A_o are the thickness and upper surface area ($\pi\ [d_o/2]^2$, d_o is diameter) of the original dry tablet respectively, while h_t and A_t are measured from axial and radial swelling at time *t*, respectively.

6.3.4. Tablet erosion study

The measurement of the erosion rates of matrix tablets was carried out in the SGF, SIF and SCF media. Erosion studies were also carried out in simulated GT fluid (2 h in SGF, 6 hours in SIF and 24 h in SCF). After immersion of the tablets in the test medium, the wet samples were then dried in an oven at 60 °C for 24 h, allowed to cool in a desiccator and weighed. The experiment was performed in triplicate and the tablet erosion was calculated from the following equation:

$$\text{ES}\ (\%) = \frac{(W_i - W_f)}{W_i} \times 100$$

where W_i is the initial weight and W_f is final weight of the tablet.

The percentage tablet remaining (ES) after erosion was evaluated from the following equation:

$$\%\ \text{Remaining} = 100 - \text{ES}$$

6.3.5. Enzymatic degradation study of tablets

The enzymatic degradation behavior of the tablets was investigated at pH 7 in the presence of 0.02% (w/v) β-glucosidase enzyme at 37°C. After 24 h, tablets were dried at 60°C until constant weight and the erosion behaviour of the polymer tablets in the presence of the enzyme was evaluated by the following equation:

$$\text{Erosion}\ (\%) = \frac{(W_i - W_f)}{W_i} \times 100$$

where W_i is the initial weight and W_f is final weight of the tablet.

6.4. *In vitro* drug release study

Simulated gastrointestinal fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.4) and simulated colonic fluid (SCF, pH 7) were chosen to find out the best coating weight ratios of Cts and amic acid derivative for colon-specific drug delivery. The best coated formulation was first tested in enzyme free SGF (stage I, pH 1.2) for 2 h, then the tablet was transferred to SIF (stage II, pH 7.4) for 6 h. Afterwards, the matrix tablet was transferred to simulated healthy colonic fluid (SCF, pH 7) for 24 h. Simulated IBD colonic fluid (SCF, pH 5) was also investigated in the case of Cts:CBAA. Selected formulations were further assessed in the presence of enzymes. 2 h in SGF (pH 1.2,

pepsin present), then in SIF (pH 7.4, pancreatin present) for 6 h. Afterwards, the matrix tablet was transferred to the SCF (pH 5.5, β-glucosidase present) for 24 h. All tests were conducted in triplicate using 100 mL of dissolution media at 37 ℃ with a rotation speed of 100 rpm.

6.5. HPLC analysis

The quantitative determination of drug was determined using a HPLC system consisting of a Waters 2690 separation module and a Waters 996 photodiode array detector, equipped with a Phenomenex® C18 reverse-phase column (250×4.6 mm, 5 μm).

6.5.1. 5-ASA determination

The quantitative determination of 5-ASA was performed using the mobile phase consisting of 0.1 M acetic acid-acetonitrile-triethylamine (920:80:2, v/v).[13] The injection volume was 25 μl and elution was performed at a flow rate of 0.8 ml/min. The wavelength of the detector was set at starting and ending wavelengths of 300 and 315 nm, respectively.

6.5.2. BSA determination

The quantitative determination was performed using a gradient between mobile phase A (water/acetonitrile, 90/10 v/v and 0.05% TFA) and mobile phase B (100% acetonitrile, 0.04% TFA). The injection volume was 50 μl and elution was performed at a flow rate of 1 ml/min. The wavelength of the detector set at 280 nm.[14]

6.6. Results and discussion

6.6.1. Preparation of chitosan amic acid derivatives

Chapter 2 reports a study concerning how Cts is crosslinked directly with dianhydride derivatives to form an imide ring to improve the physiochemical properties of Cts.[15] The mechanism of this reaction involves the nucleophilic attack of the amino group of the Cts on the carbonyl carbon of the anhydride moiety of BTDA allowing an opening of the anhydride ring to form the intermediate amic acid group, followed by thermally cyclization of amic acid intermediate with elimination of water (Chapter 2). Addition of a dianhydride to Cts in an aprotic solvent (DMF) at ambient temperatures leads to the formation of the intermediate amic acid due to the nucleophilic attack of the amino group on the carbonyl carbon of the anhydride group (Chapter 4). The scheme that is illustrated in Figure 6-1 involves a reversible reaction leading to opening of the anhydride ring to form an amic acid group.

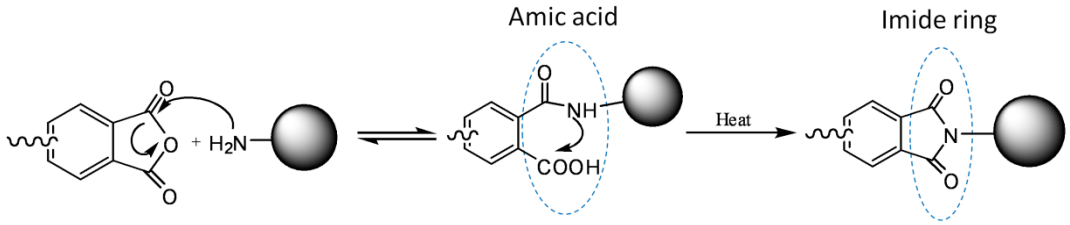

Figure 6-1 Generalized reaction mechanism of chitosan with dianhydride derivatives.

In this study, Cts was crosslinked with BTDA and ODPA to produce amic acid derivative (Figure 6-2).

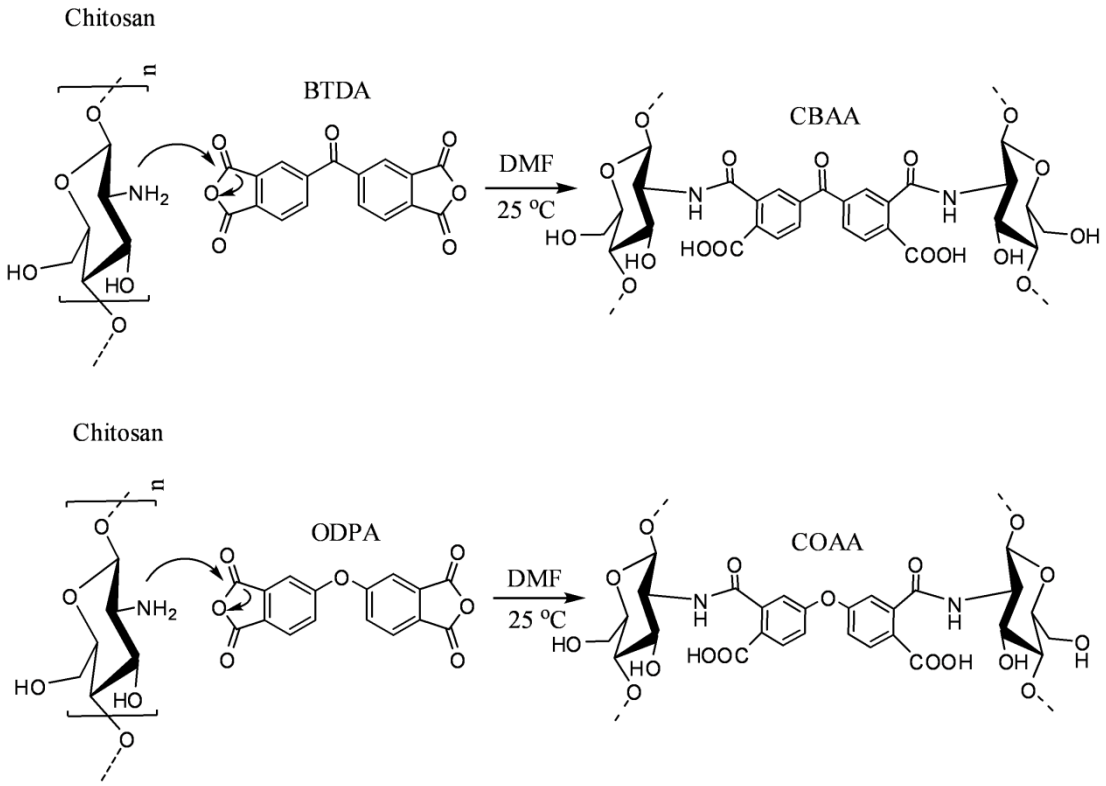

Figure 6-2 Schematic representation CBAA and COAA

The molecular weight of Cts was determined to be 198 kDa using the Mark-Houwink viscometry method in a solvent ratio of 0.1M acetic acid/0.2M NaCl maintained at 25°C.

Elemental analysis was used to determine degree of deacetylation (DD) and degree of substitution (DS) of Cts. Carbon, hydrogen and nitrogen contents of Cts, CBAA and COAA were found C, 43.19%; H, 6.97%; N, 8.03%, C, 51.07%; H, 4.7%; N, 3.92% and C, 50.80%; H, 4.81%; N, 3.77%, respectively.

The DD was 77% according to the following equation:

$$DD = \left(1 - \frac{\frac{C}{N} - 5.145}{6.186 - 5.145}\right) \times 100$$

where 5.145 is related to completely *N*-deacetylated Cts ($C_6H_{11}O_4N$ repeat unit) and 6.186 is the fully *N*-acetylated polymer ($C_8H_{13}O_5N$ repeat unit).

The degree of substitution (DS) of CBAA and COAA was determined to be 0.45 and 0.84 from the C/N value of elemental analysis on the basis of the following formula:

$$DS = \frac{\left(\frac{C}{N}\right)_f - \left(\frac{C}{N}\right)_i}{n}$$

where $(C/N)_f$ is the C/N ratio of the Cts derivative, $(C/N)_i$ is the C/N ratio of the pure Cts and n is the number of carbons introduced after derivative formation.

6.6.2. Characterization

The chemical structure of the Cts and crosslinked Cts were investigated by FTIR, Figure 6-3. The major peaks for the Cts can be attributed as follows. The peak at 3288.9 cm^{-1} is assigned to the -OH and -NH stretching vibrations, while the peak at 2877.2 cm^{-1} is assigned to the aliphatic C-H stretching vibration in the -CH and $-CH_2$ groups. The amide frequencies consisting of the -C=O bond stretch of the remaining acetamido groups and the N-H bending vibrations of the $-NH_2$ groups are observed at 1657 and 1599 cm^{-1} respectively. The peak at 1423.0 cm^{-1} is assigned to the $-NH_2$ deformation. Further bending vibrations are observed at 1381.2 cm^{-1} for the C-C-H symmetrical bending vibration in the alcohol. Stretching vibrations are also observed at 1323.4 and 1155.6 cm^{-1} for the C-N stretching vibration and at 1078.3 and 1033.4 cm^{-1} for the -O stretching vibration of the alcohol groups. After modification of Cts, the spectra showed some major changes. The IR spectra of CBAA and COAA show two peaks around 1720 cm^{-1} and 1660 cm^{-1} which are assigned to the C=O stretch of –COOH and amide group respectively.

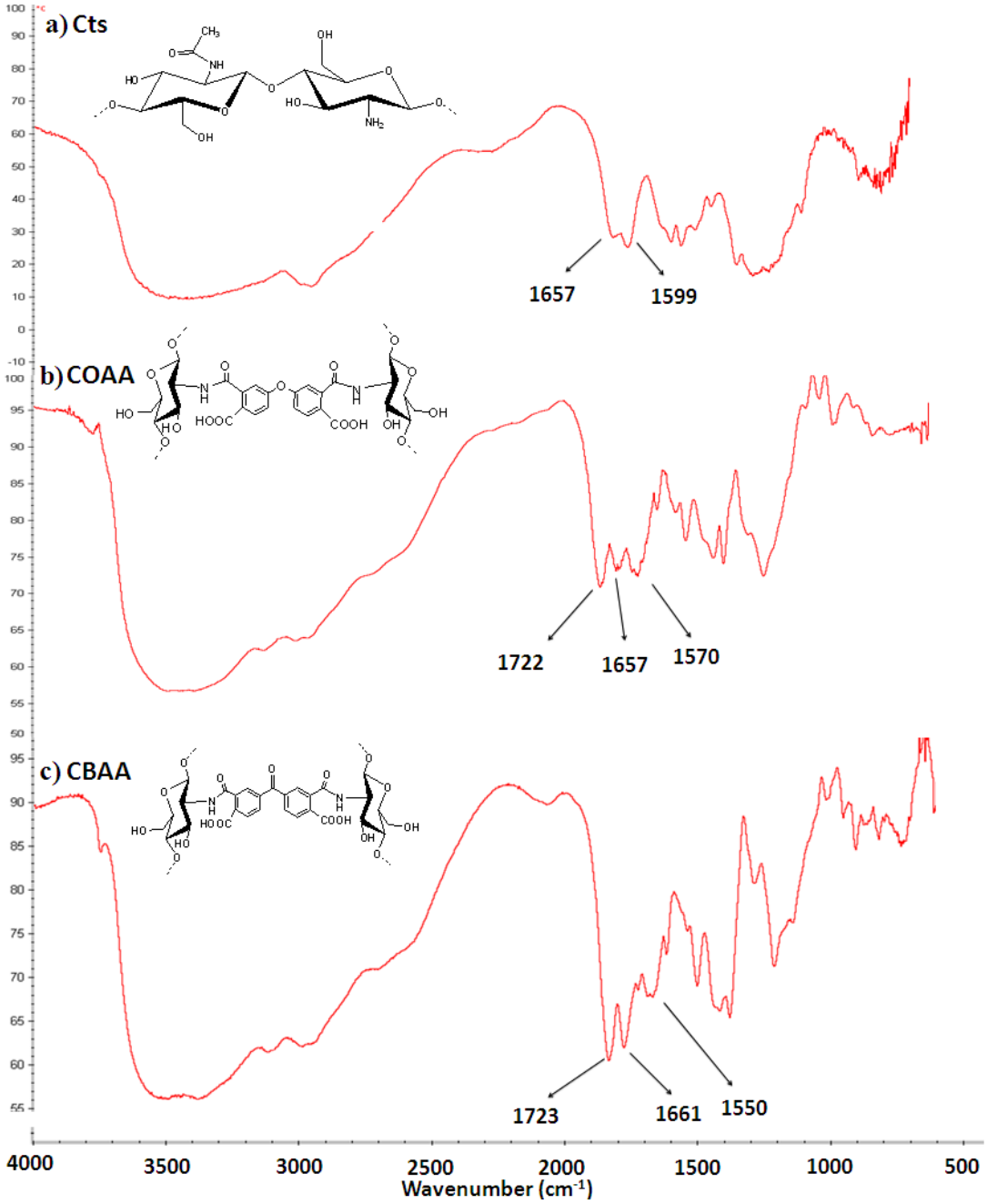

Figure 6-3 FTIR spectra of a) Cts, b) COAA and c) CBAA.

The analysis of the ^{13}C DP-MAS spectrum of Cts, CBAA and COAA are shown in Figure 6-4. The ^{13}C DP-MAS spectrum for Cts is very similar to that reported in the literature.[16]

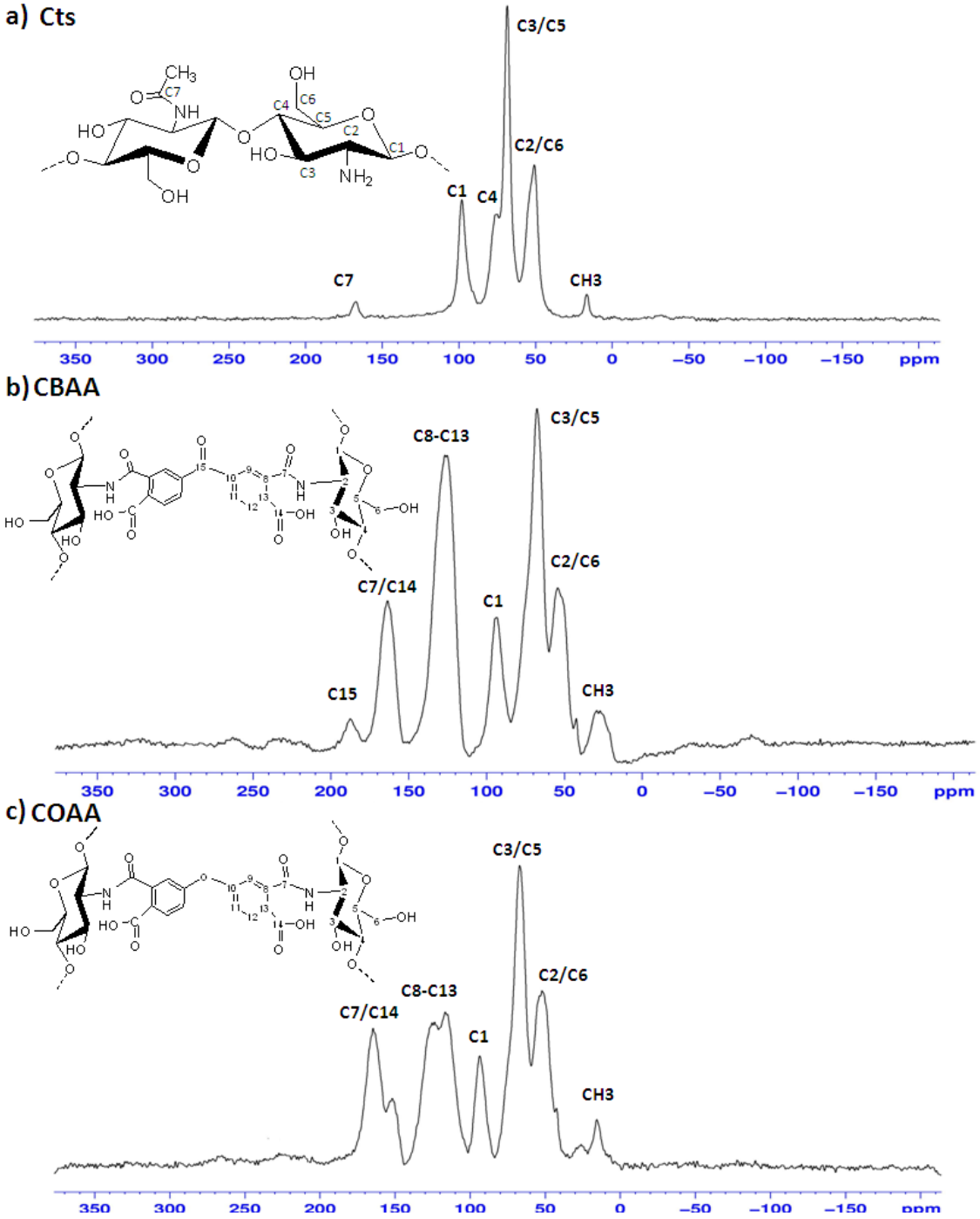

Figure 6-4 ^{13}C DP-MAS spectra of (a) Cts, (b) CBAA and c) COAA.

The signal at 17.4 ppm is attributed to the methyl of the acetamide group. Signals at 51.7 and 69.1 ppm are attributed to overlapping C2/C6 and C3/C5 respectively. The signal related to C4 is also observed at 76.0 ppm. The signal due to the C1, which is directly attached to two oxygen atoms, was found at 98.1 ppm which is at a significantly lower magnetic field value compared with the signals of the remaining five carbons. The signal related to the carbonyl peak (acetyl group) is also observed at 167.7 ppm. The ^{13}C DP-MAS spectrum of CBAA shows three new signals at 126.5 (C8-C13) and 164.3 ppm (C7 and C14) and 188.6 (C15), Figure 6-4b. The ^{13}C DP-MAS spectrum of COAA spectrum shows also three new signals at 117-126 (C8-C13) and 165.5 ppm (C7 and C14), Figure 6-4c.

The morphology of Cts, CBAA and COAA powders were evaluated by SEM imaging, Figure 6-5. As noted from this figure, the surface of unmodified Cts is smooth in comparison to crosslinked Cts derivatives. The surfaces of CBAA and COAA show a noticeable roughness and irregularity. These changes in the surface morphology of Cts are caused by chemical modification of Cts. The scales of 10, 20 and 200 µm were used in this study.

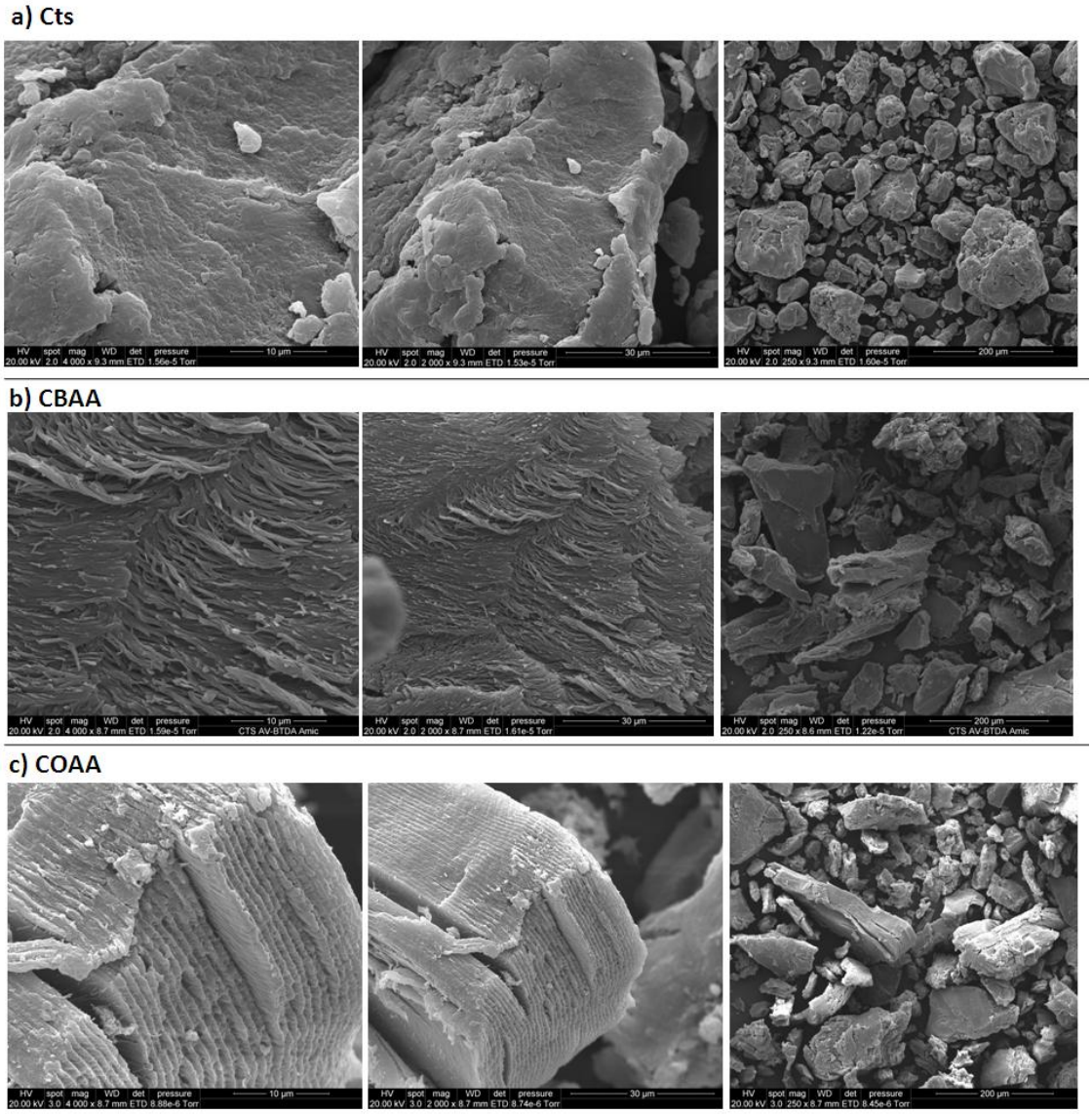

Figure 6-5 SEM image of (a) Cts, (b) CBAA and c) COAA.

The 2D-XRD patterns of Cts and crosslinked Cts are illustrated in Figure 6-6. The main crystalline peak at 2θ values of 20.8^{o} that are generally assigned to weak chain-chain ordering of the Cts chains, indicating the crystalline state of the unmodified Cts, Figure 6-6a. Figure 6-6b. indicates that the chemical crosslinking between Cts and BTDA destroys the crystallinity of Cts and increases the amorphous nature of the modified Cts hydrogel.

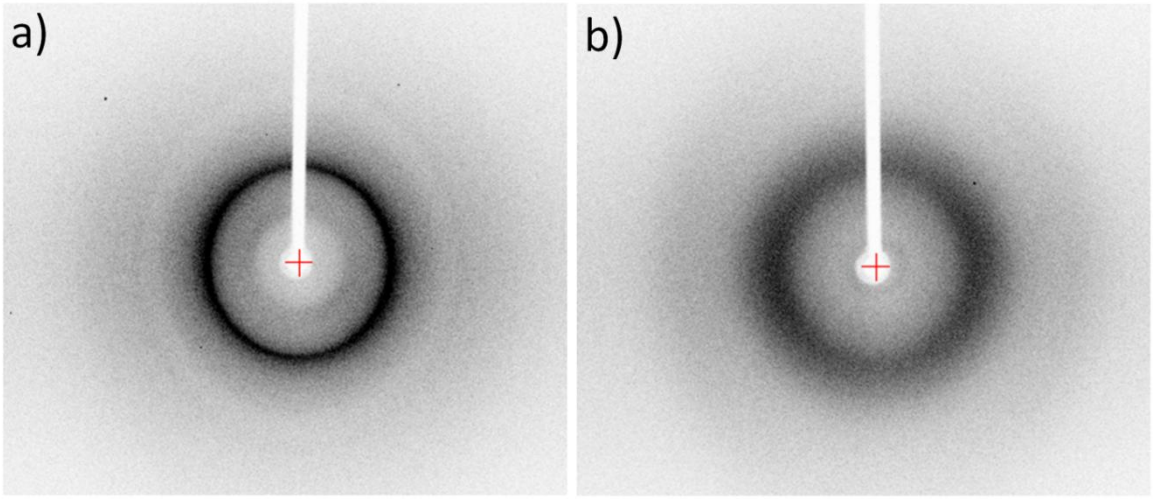

Figure 6-6 2D-XRD of (a) Cts and (b) CBAA.

As shown in Figure 6-7, chemical crosslinking between Cts and ODPA also destroys the crystallinity of Cts and increases the amorphous nature of the modified Cts hydrogel. This figure, shows a sharp peak at 2θ = 20.1^{o} which confirms the semicrystalline nature of pure Cts while the XRD spectrum of COAA shows a broad peak at 2θ=21.5, indicating that the sample has changed from the semicrystalline to the amorphous state presumably due to destruction of inter- and intra-molecular hydrogen bonding in the modified Cts.

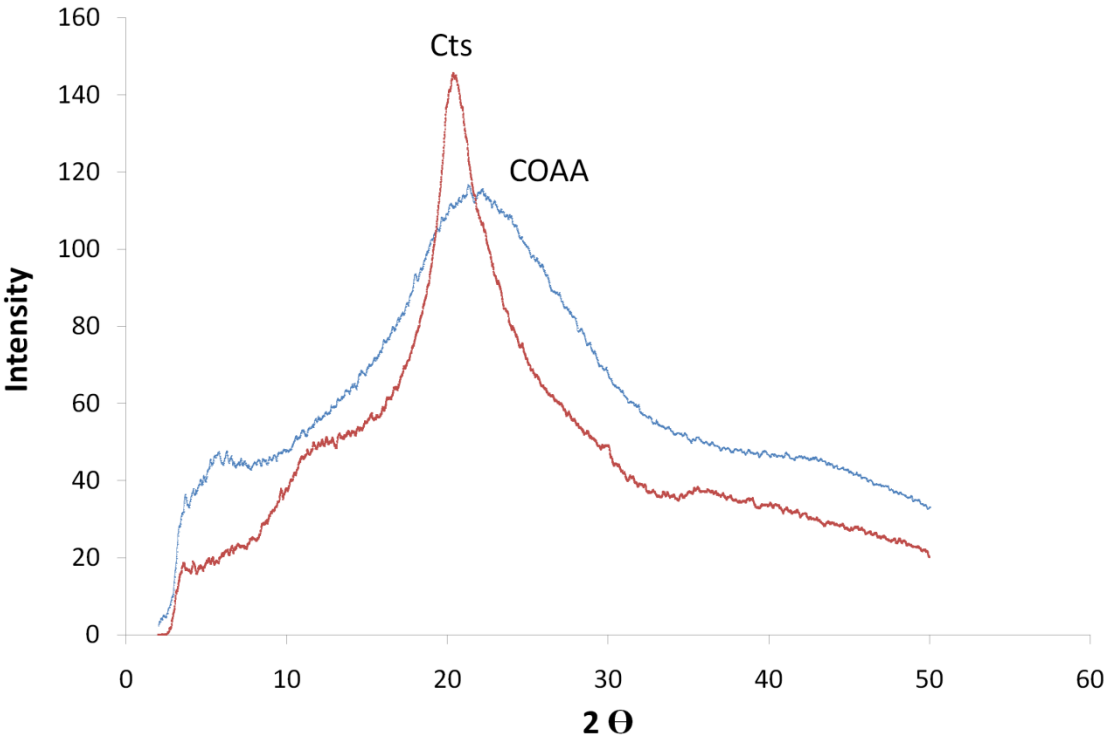

Figure 6-7 2D-XRD of (a) Cts and (b) COAA

Thermogravimetric analysis (TGA) was carried out to examine the thermal stability behavior of Cts and crosslinked Cts. Cts showed an initial weight loss of 10.1% in a temperature range between 20 and 240^{o}C due to loss of adsorbed and bound water. The second weight loss of Cts was in the region between 240-340^{o}C with the sharp and considerable weight loss of 37.3%, which could be related to decomposition of Cts.

CBAA showed a 17.6% weight loss between 20 and 240^{o}C which indicates a faster process of weight loss in this temperature range. Between 240-340^{o}C only 19.89% weight loss was observed (Figure 6-8).

More accurate differences in the thermal behavior of the Cts and CBAA can be noted from the DTG curves. The DTG peak of Cts shows a maximum value of about 290^{o}C, while the maximum value of this peak for the CBAA curve is observed at about 316 ^{o}C showing the increase in the thermal stability of the CBAA over Cts.

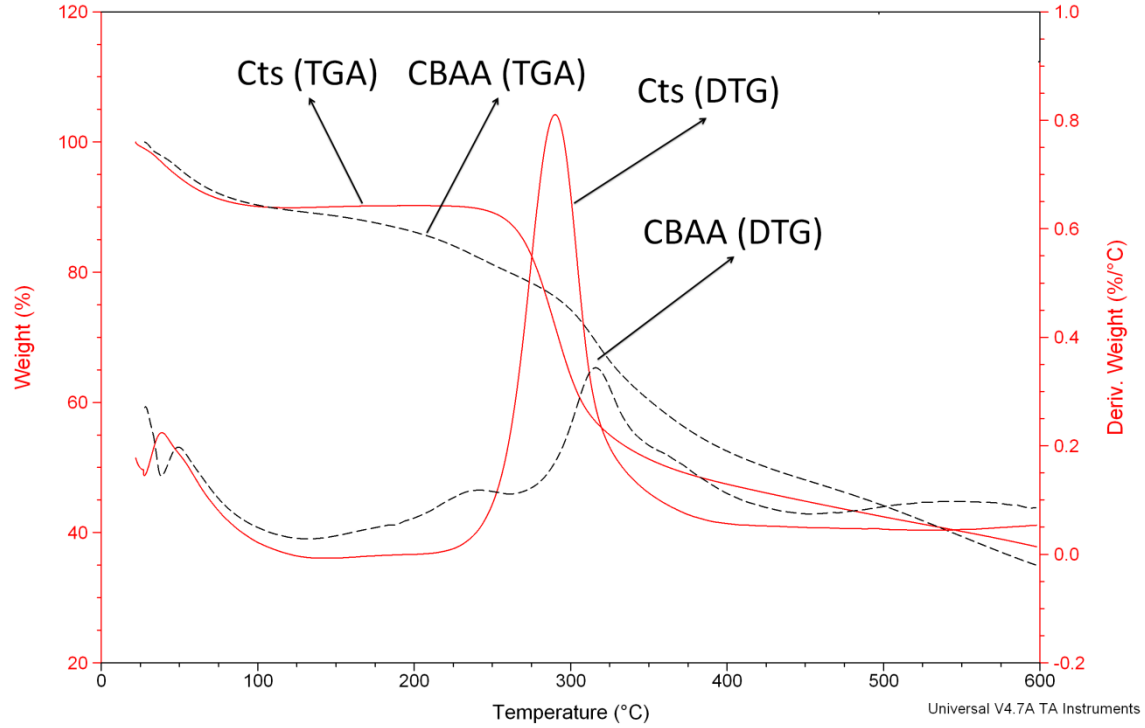

Figure 6-8 TGA and DTG thermograms of Cts and CBAA.

In the case of COAA 17.2% weight loss was observed between 20 and 240^{o}C which indicates a faster weight loss in this temperature range compared to Cts. A 24.1% weight loss was observed between 240-340^{o}C. The DTG peak of COAA showed a maximum value of about 315^{o}C showing the increase in the thermal stability of the COAA over Cts (Figure 6-9).

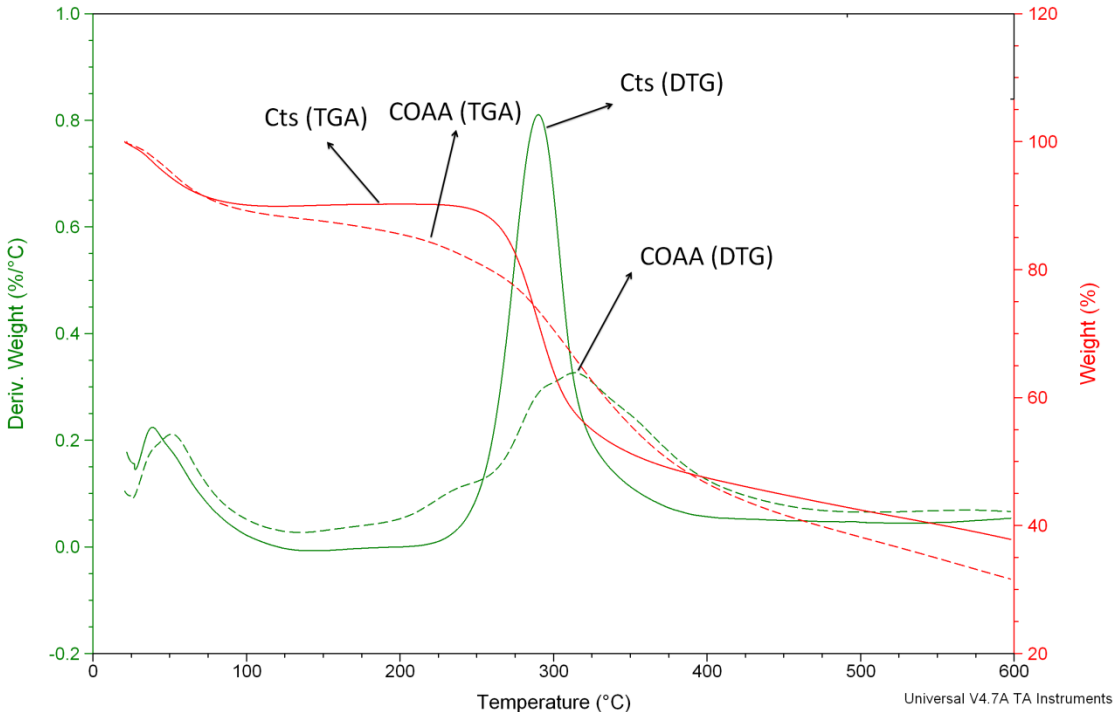

Figure 6-9 TGA and DTG thermograms of Cts and COAA.

6.6.3. Enzyme inhibitory effect of CBAA

To evaluate the protease inhibitory effects of the CBAA and COAA particles, trypsin and chymotrypsin were chosen as representatives of small intestinal endopeptidases. The inhibition mechanism of CBAA and COAA is explained by the binding affinity of the polymer toward calcium, which leads to minimizing the proteolytic activity of calcium-dependent enzymes like trypsin and chymotrypsin. Unmodified Cts showed 3.5 ± 0.3% and 8.2 ± 0.4% inhibition capability for trypsin and chymotrypsin respectively. CBAA and COAA displayed excellent inhibitory effects against trypsin and chymotrypsin. CBAA and COAA showed an inhibitory effect of 40.2 ± 1.1and 44.2 ± 2.3% for trypsin respectively. Inhibition effects of 80.6 ±2.1% and 85.4 ± 1.8% were observed for chymotrypsin in the presence of CBAA and COAA respectively.

6.6.4. Swelling and erosion behaviour tablets

Swelling and erosion of polymer plays a crucial role in controlling the release of drug. Due to potential errors incurred during weighing and from removing excess solvent from the samples, radial and axial swelling studies were used in the present study.[17]

6.6.4.1. Cts-CBAA (75:25%)

Swelling and erosion trends of the selected tablet Cts-CBAA (75:25%) matrices were investigated in different media, pH 1.2, 5, 7 and 7.4. Swelling behaviors of the Cts-CBAA (75:25%) tablets in different media are illustrated in Figure 6-10.

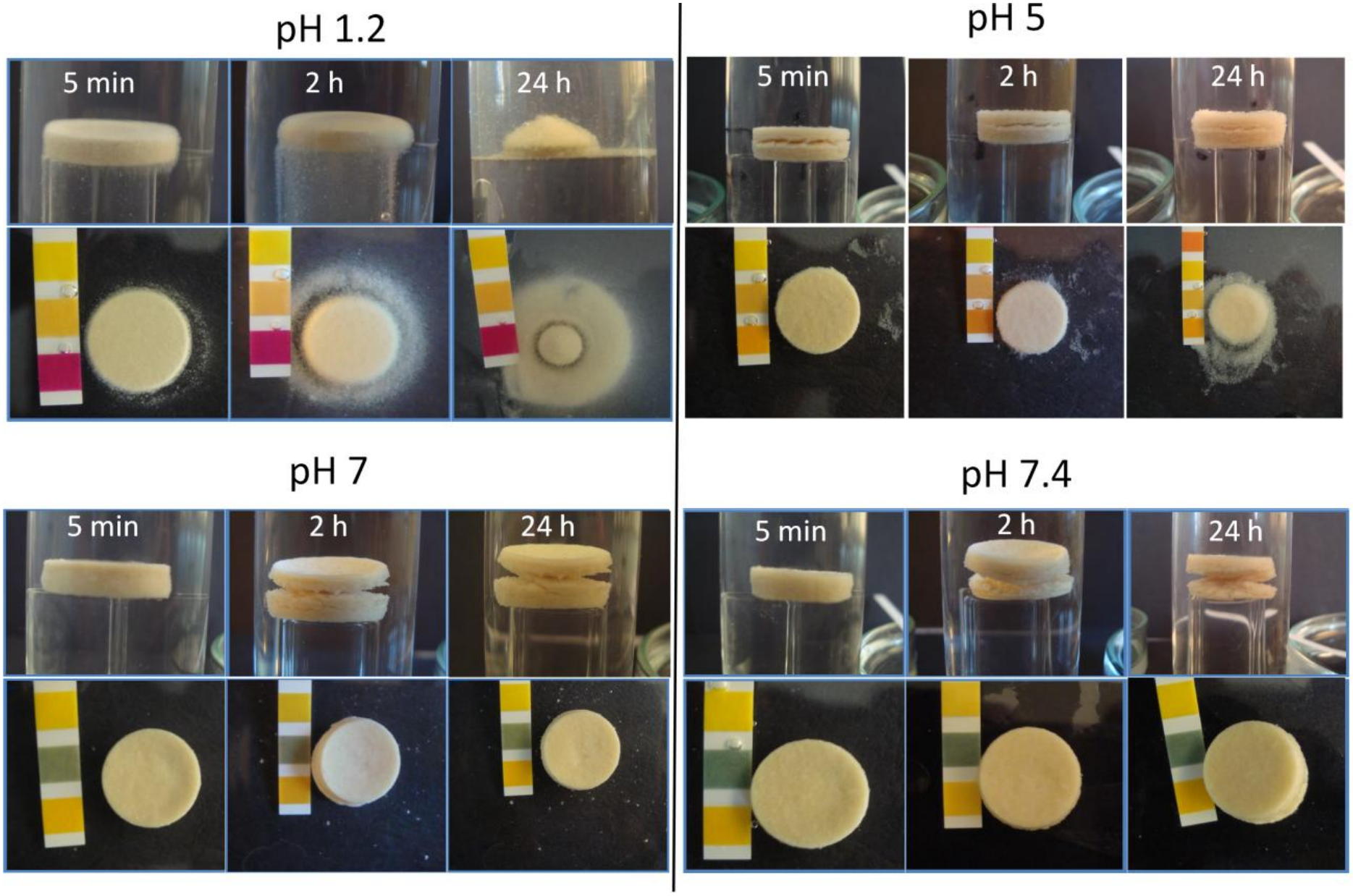

Figure 6-10 Photographs of radial and axial swelling behaviour of Cts-CBAA (75:25 %) tablets in various media.

The highest axial and radial swelling was observed at pH 7.4 with 209.4 ± 6.2% and 21.7 ± 5.1% respectively, Figure 6-11. This can be attributed to a swelling force created by the electrostatic repulsion between the ionized acidic groups.

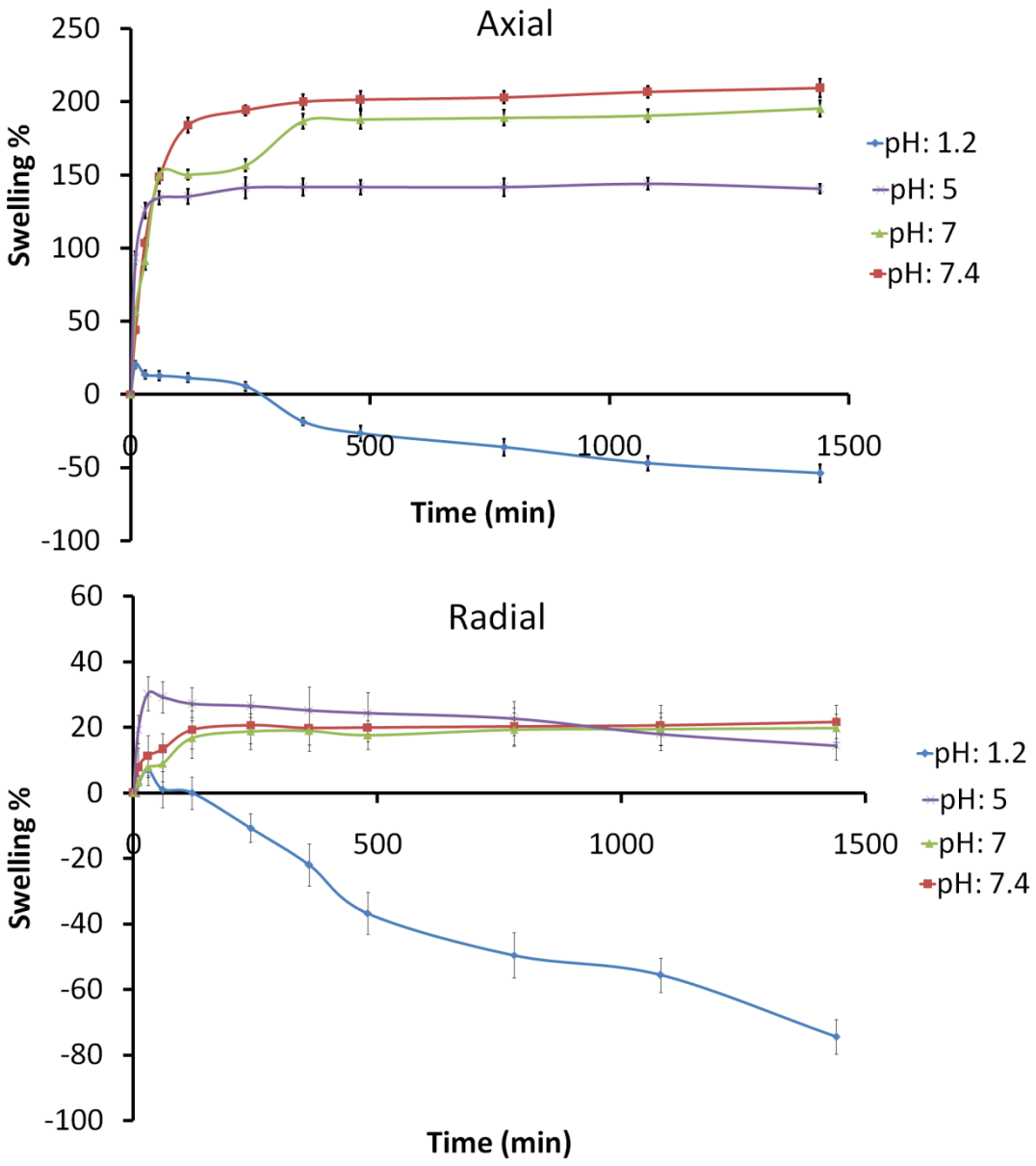

Figure 6-11 Swelling behaviour of Cts-CBAA tablets in various media.

The swelling behaviour of the tablet in the simulated healthy and simulated IBD colonic fluid is also illustrated in Figure 6-12.

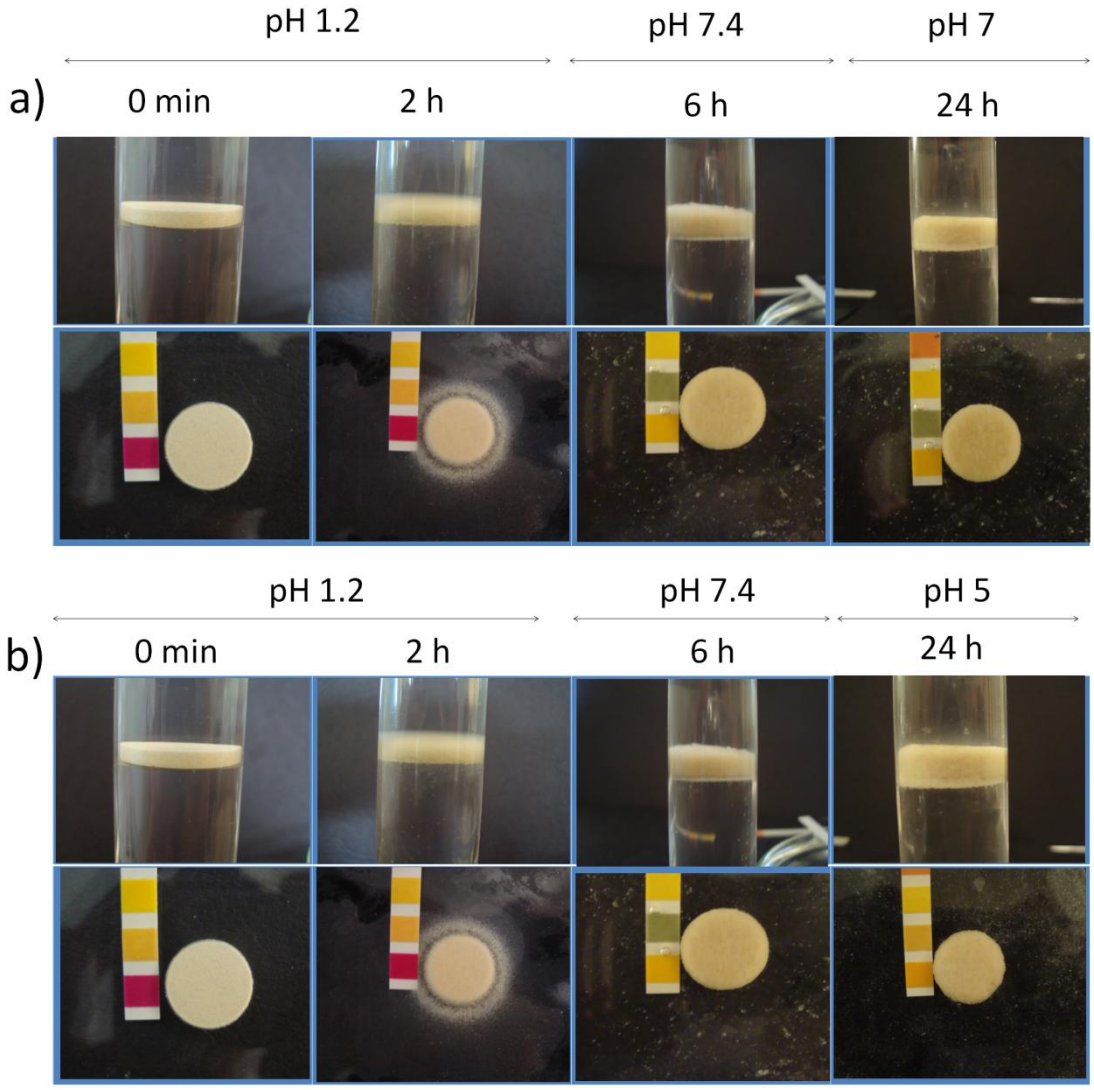

Figure 6-12. Photograph of the radial and axial swelling behaviour of tablets in a) healthy b) an IBD simulated gastrointestinal tract condition.

After two hours in the SGF, the Cts-CBAA tablets showed about 14.9 ± 4.5 and 1.8 ± 3.9% axial and radial swelling, respectively (Figure 6-13). Swelling percentage reached a maximum of 81.4 ± 5.3 and 18.9 ± 6.1% for the axial and radial after six hours in SIF. As can be seen from Figure 6-13, a higher axial and radial swelling percentage was observed after transferring of the tablet into a pH 7 buffer compared to pH 5. For instance, 136.2 ± 5.1% axial swelling was observed after 24 hours at pH 7 while at pH 5, 110.8 ± 5.5% axial swelling was observed. In the case of the radial swelling 29.8 ± 4.3 and 9.9 ± 5.8% was obtained after 24 hours at pH 7 and pH 5 respectively.

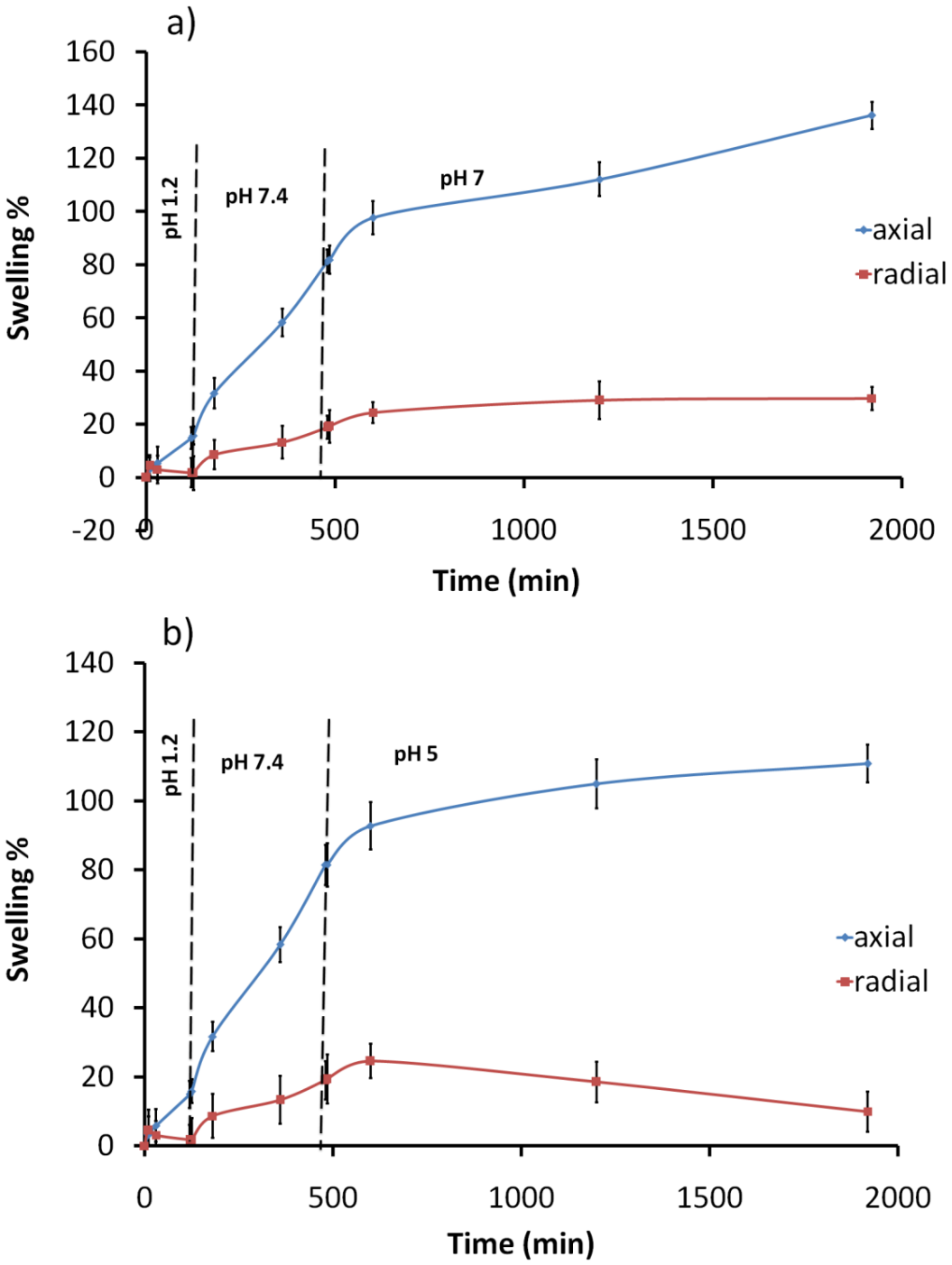

Figure 6-13 Swelling behaviour of the Cts-CBAA tablets in a) healthy b) an IBD simulated gastrointestinal tract condition.

The percentage of the remaining matrices is depicted in Figure 6-14. Tablets showed the highest erosion at pH 1.2 after 24 hours with 7.6 ± 0.8% remaining. Remaining percentages of 94.3 ± 5.2% and 91.1 ± 6.4% and 81.2 ± 4.7% were also observed at pH 7.4, 7 and 5 respectively. To simulate the erosion behavior of the tablets in the GIT, the tablets were left in the SGF for two hours, followed by six hours at SIF and then SCF

for 24 hours. The results indicate about 78.8 ± 5.3% of the tablet remained at the end of this process for simulated healthy gastrointestinal tract. The study also showed 61.9 ± 4.9% of the tablet remained at the end of the simulated GIT for IBD simulated conditions.

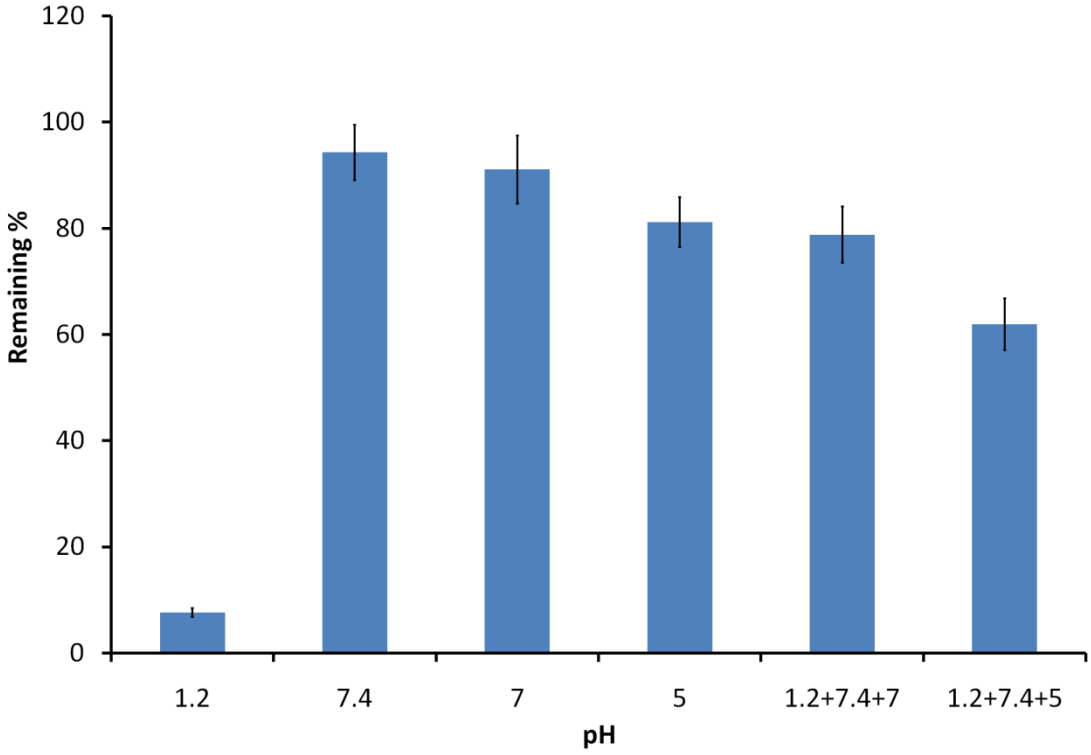

Figure 6-14 Percentages remaining of Cts-CBAA matrix tablets.

6.6.4.2. Cts-COAA (75:25%)

Swelling trends of the selected tablet Cts-COAA (75:25%) matrices in different media, pH 1.2, 7.4 and 7 are illustrated in Figure 6-15.

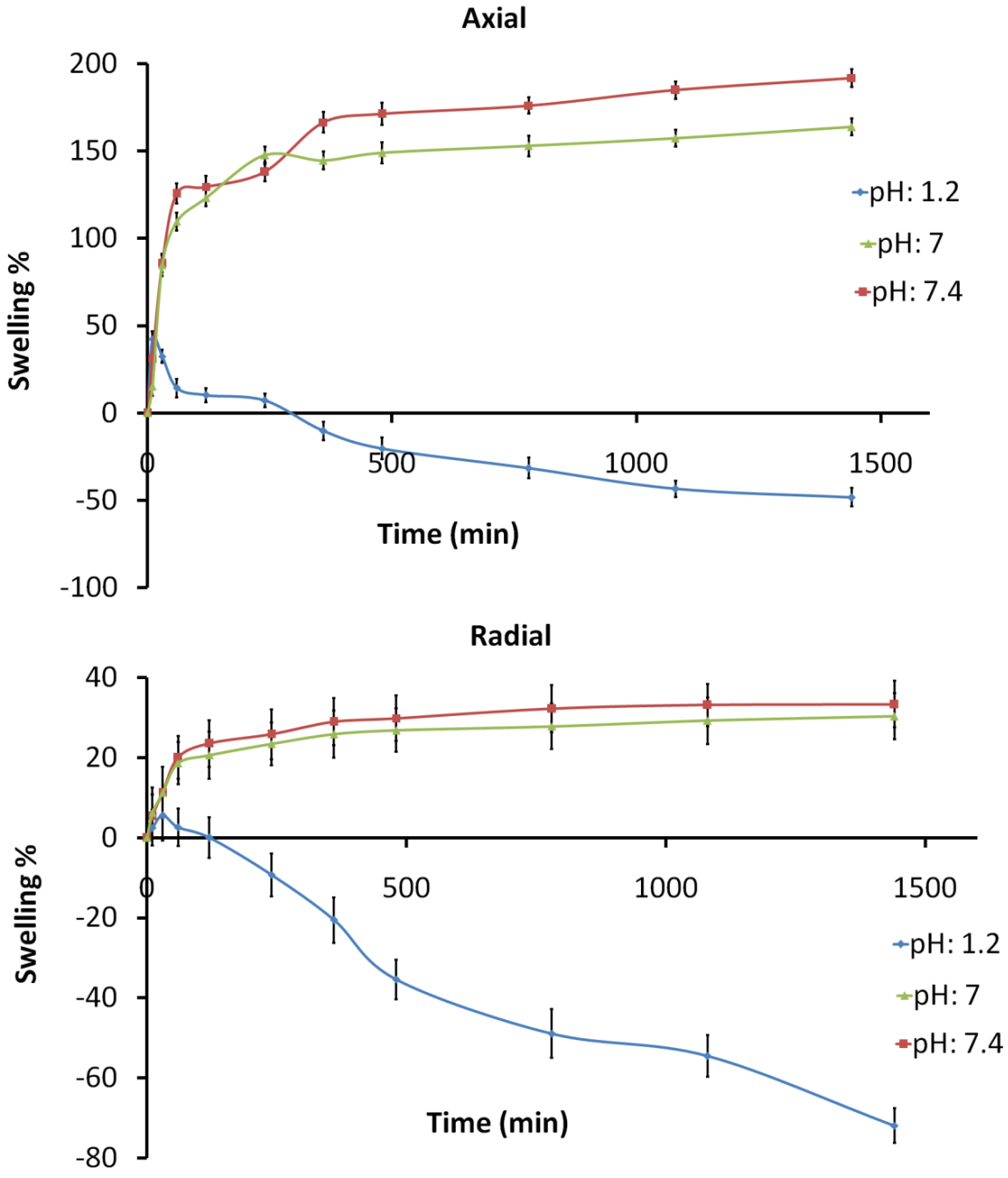

Figure 6-15 Swelling behaviour of Cts-COAA tablets in various media.

The highest axial and radial swelling was observed at pH 7.4 with 191.7 ± 5.5 and 33.3 ± 5.8% respectively, which is attributed to the swelling force created by the electrostatic repulsion between the ionized acid groups.

The swelling behaviour of the tablet in a simulated GIT is illustrated in Figure 6-16. After two hours in SGF, Cts-COAA tablets showed about 24.3 ± 5.4% axial and 2.1 ± 5.9% radial swelling. The swelling percentage reached a maximum of 94.7 ± 5.6% and 19.3 ± 5.9% for axial and radial swelling after six hours in SIF. As can be seen from Figure 6-8, the tablet shows an axial swelling percentage of 109.1 ± 5.8% and a radial swelling percentage of 24.9 ± 4.9% after 24 hours in SCF (pH 7).

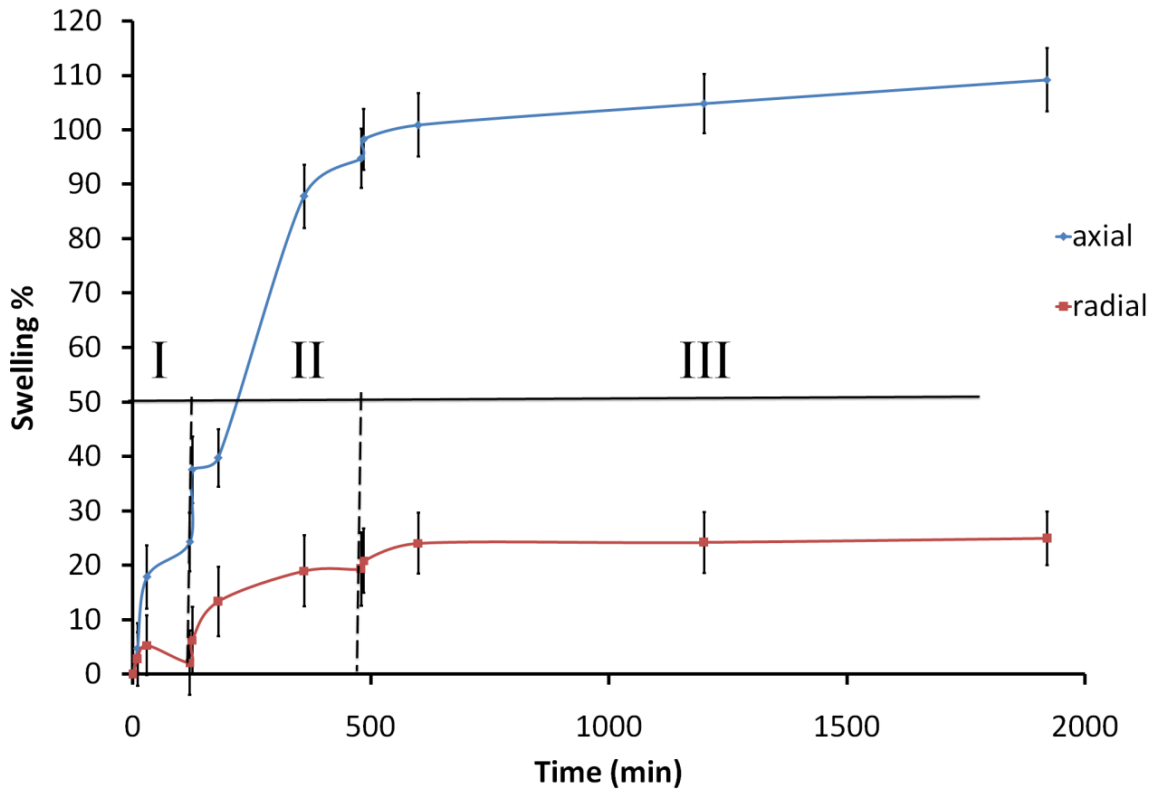

Figure 6-16 Swelling behaviour of Cts-COAA tablets in a simulated gastrointestinal tract.

Photographs of radial and axial swelling behaviour of the tablets in various media are illustrated in Figure 6-17.

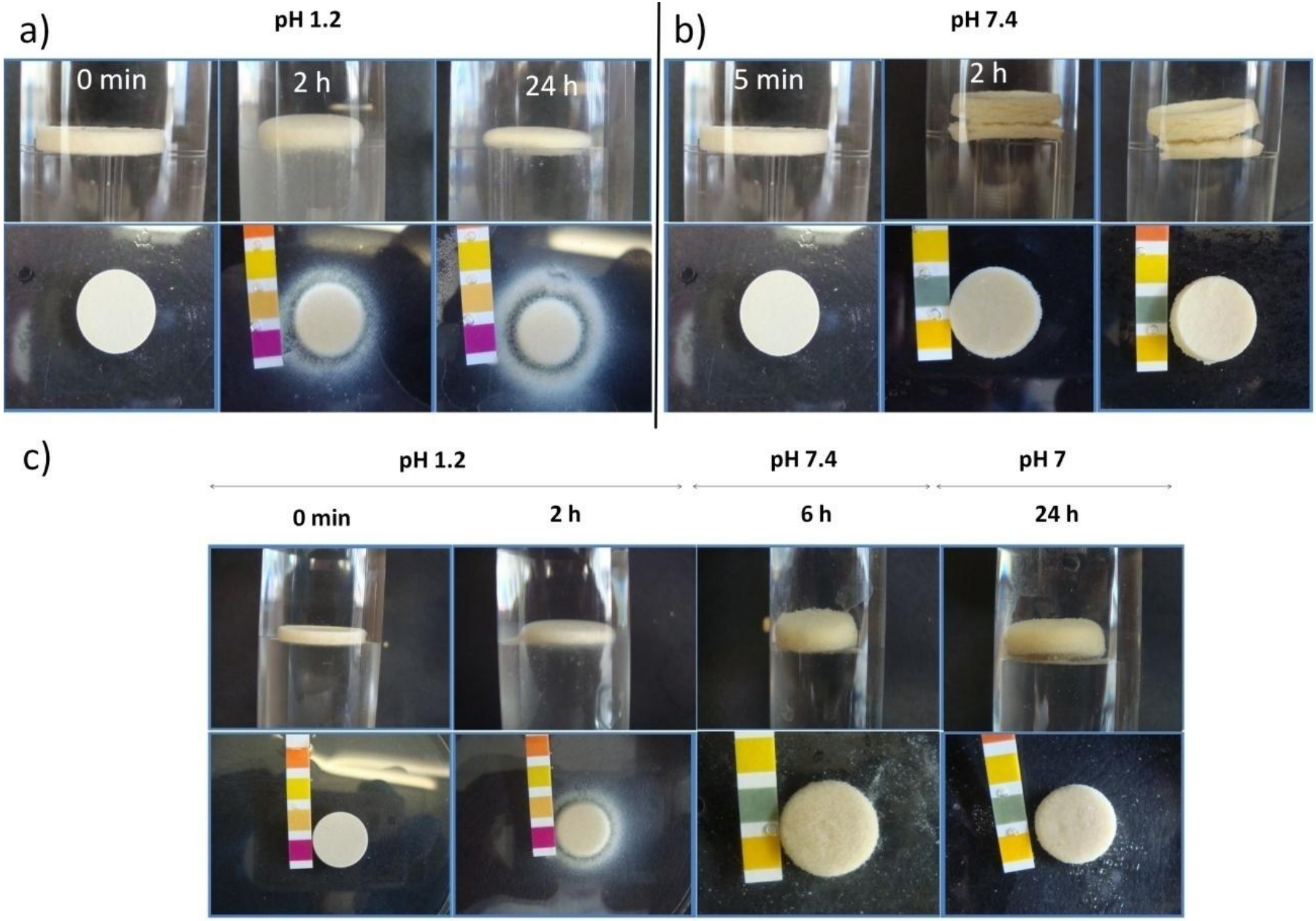

Figure 6-17 Photograph of radial and axial swelling behaviour of tablets in a) pH 1.2 b) 7.4 and c) simulated gastrointestinal tract pH protocol.

The percentage of the remaining matrices is depicted in Figure 6-18. The tablets show the highest erosion at pH 1.2 after 24 hours with 6.8 ± 0.4% remaining. Remaining percentages of 89.7 ± 5.3% and 88.3 ± 4.7% were also observed at pH 7.4 and pH 7 respectively. For the simulation of the erosion behavior of the tablets in the gastrointestinal tract, the tablets were left in SGF for two hours, followed by six hours in SIF and then in SCF for 24 hours. The results indicated 74.6 ± 4.9% of the tablet remained at the end of this process.

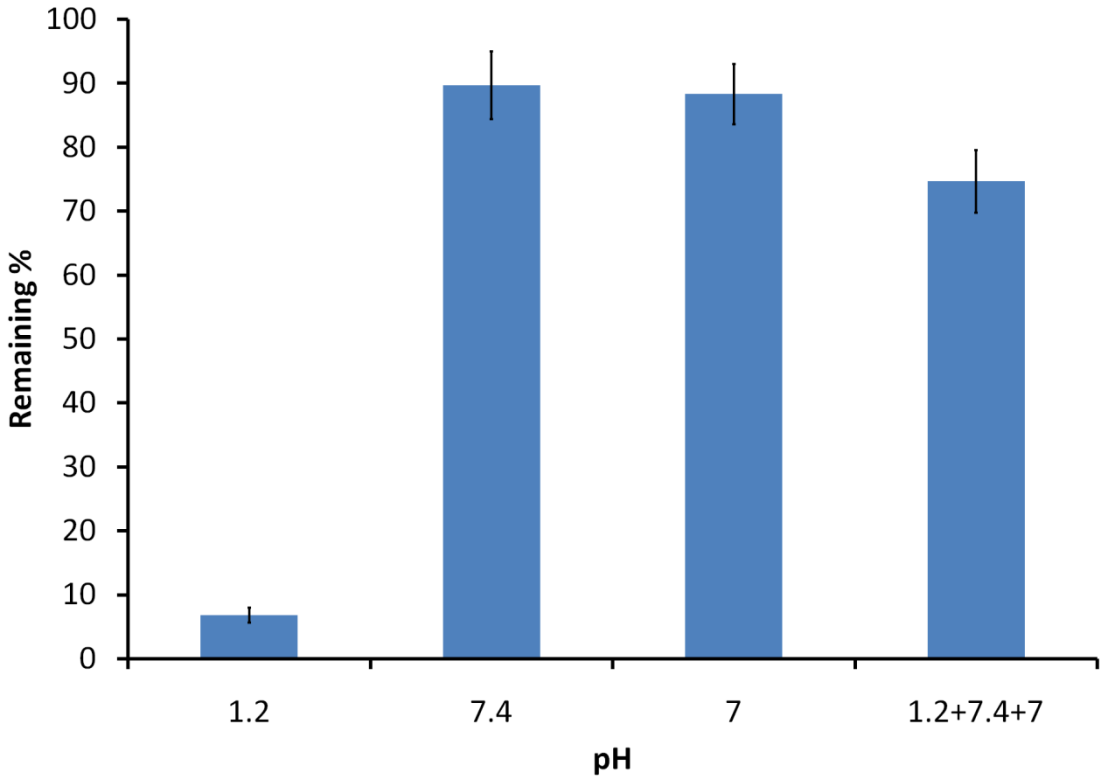

Figure 6-18 Percentages remaining of Cts-CBAA matrix tablets.

6.6.5. Enzymatic degradation of tablet

The degradation effect of almond emulsin *β*-glucosidase on Cts based tablets was investigated at pH 7. The erosion of the Cts:CBAA (75:25%) and Cts:COAA (75:25%) tablets in enzyme free media was about 8.3 ± 1.4 and 9.1± 0.8% within 24 hours, respectively. While in presence of *β*-glucosidase, this increased to 14.2 ± 2.8% and 15.9 ± 1.5% for Cts:CBAA and Cts:COAA, respectively. This finding supported the fact that

the enzyme controlled system could help increase the rate of drug release in this colon specific delivery system.

6.6.6. *In vitro* drug release study

6.6.6.1. Controlled colon specific delivery system of 5-ASA using Cts:CBAA tablet

6.6.6.1.1. Effect of coating polymer ratio

The *in vitro* release study of 5-ASA coated with various mixing ratios of Cts and CBAA is illustrated in Figure 6-19. The data indicates that the different ratio of coating layers present cause different release behaviors. Based on these results, tablets composed of 0:100, 25:75 and 40:60% of Cts: CBAA were easily degraded and released the drug within two hours (SGF), while the drug release reached about 100% for ratios 50:50, 60:40 and 100:0% of Cts:CBAA within four hours (2h in SGF followed by 2 h in SIF). As depicted in Figure 6-19, a tablet with a ratio of 75:25% of Cts:CBAA was able to pass through the stomach and small intestine intact.

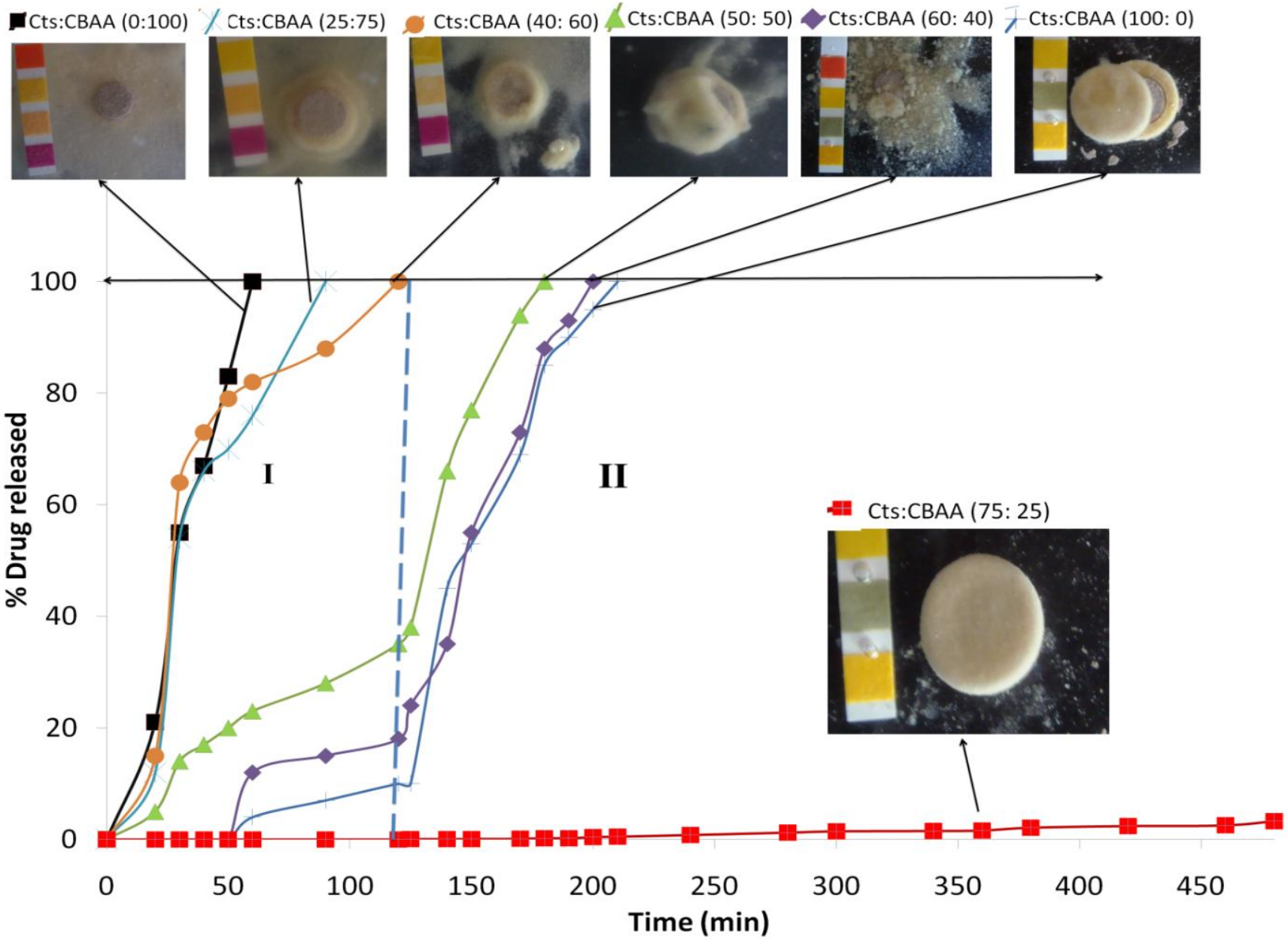

Figure 6-19 Effect of coating ratio on cumulative release of 5-ASA from matrix tablets.

Other formulations could also be used for delivery to other parts of the gastrointestinal tract. Tablets composed of 0:100, 25:75 and 40:60% of Cts:CBAA could be used for stomach specific drug delivery due to degradation of these tablets within two hours in SGF, in particular the tablet made from only CBAA which degraded and released the drug within 50 minutes. Tablets with ratios 50:50, 60:40 and 100:0% of Cts:CBAA were able to transfer the drug to the small intestine. Therefore these formulations have potential as a carrier for small intestine delivery. Based on these results, these novel biodegradable amphoteric Cts-based tablets could become suitable polymeric carriers for colon-specific drug delivery.

6.6.6.1.2. Effect of pH and enzyme

In this part of the study, tablets with weight ratios of 75:25% (Cts:CBAA) and a crushing strength of 428.1 ± 0.87 (N/cm^2) were studied for their ability to provide the appropriate lag time to release the drug at the colon. As it can be seen from Figure 6-20, the release profile of the selected tablet after two hours in SGF (pH 1.2) shows no significant difference with or without pepsin (less than 0.1%), which can be attributed to the optimal conditions needed for chitosanolytic activity of pepsin which are pH 5 at 45°C.[18] This study shows that less than 1% of 5-ASA was released after eight hours (2 h in SGF followed by 6 h in SIF) with or without the enzyme. This novel Cts based formulation shows considerable ability to protect the drug from the harsh environment of the upper GIT. As depicted in Figure 6-21, after arrival of the tablet to the colonic media (Stage III), the amount of 5-ASA released increased significantly. For instance 78.2 ± 5.2% of drug was released after 24 hours in the SCF.

The effect of β-glucosidase on the increase in drug release from the compression coated tablets was also studied, due to the similarity of the effect of almond β-glucosidase on Cts to that of colonic enzymes. In the presence of β-glucosidase (stage III, simulated colonic fluid in healthy human), the time for the $T_{1/2}$ of drug release was about 26 hours while the $T_{1/2}$, of those with no enzyme was about 28 hours. This finding indicates that colonic enzymic activity could affect a controlled release system for drugs in the colon.

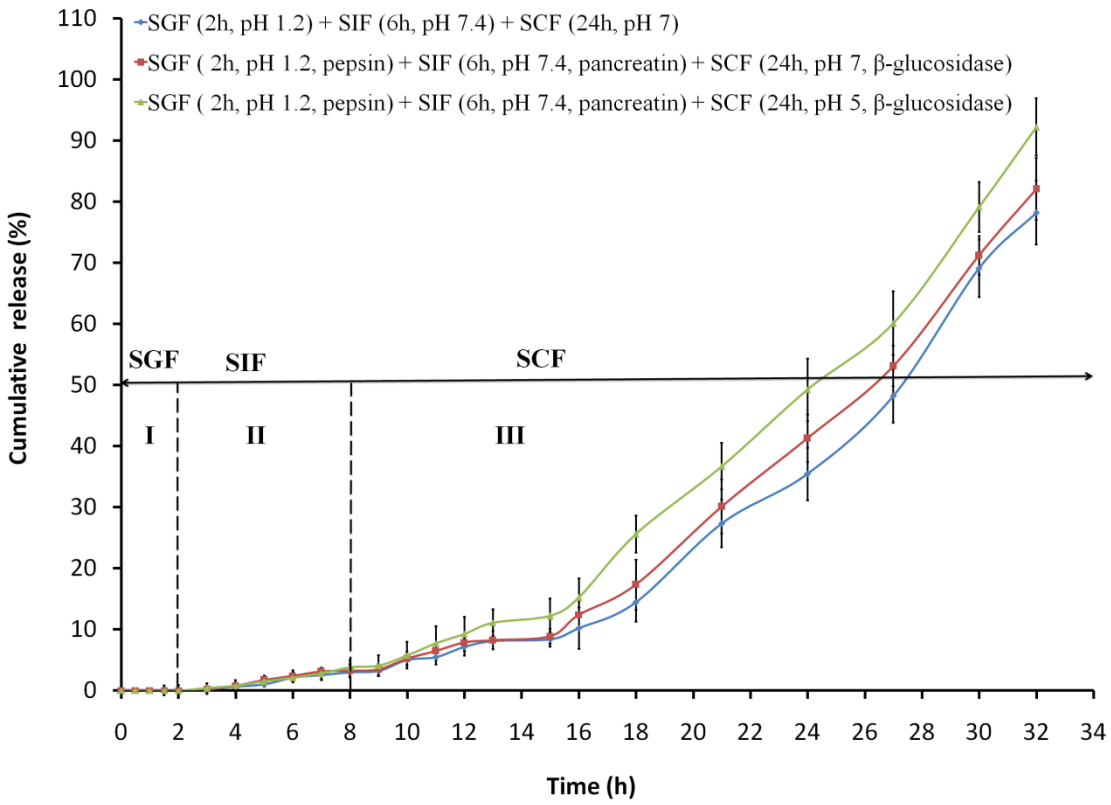

Figure 6-20 Effect of enzyme on cumulative release of 5-ASA from the selected tablet (Cts:CBAA 75:25%)

Based on Figure 6-20, the faster release of the drug was observed in the simulated IBD colonic fluid (pH 5.0) compared to that in the simulated healthy colonic fluid (pH 7.0). $T_{1/2}$ was reached at about 24 hours at pH 5.0. This faster release of drug in the IBD colonic fluid may be related to the higher erosion percentage of the selected tablet at the pH 5.0.

6.6.6.2. Colon specific delivery of protein therapeutics using a Cts:COAA tablet

6.6.6.2.1. Effect of coating polymer ratio

The *in vitro* release study of BSA coated with various mixing ratios of Cts and COAA is illustrated in Figure 6-21. The data indicates that different ratios of coating layers present cause different release behaviors. Based on the results, the tablets composed of

0:100, 25:75 and 40:60% of Cts:COAA were easily broken and released the drug within two hours (SGF). The time for the 50% of drug release ($T_{1/2}$) from tablets with coating ratios of 50:50, 60:40 and 100:0 of Cts: COAA were obtained in 140, 170 and 190 minutes respectively. Tablets composed of 0:100, 25:75 and 40:60% of Cts:COAA can be used for stomach specific drug delivery due to degradation of these tablets within two hours in SGF. Tablets with ratios of 50:50, 60:40 and 100:0% of Cts:CBAA were able to transfer the drug to the small intestine, therefore these formulations have the ability to be used as using as a carrier for small intestine delivery. As depicted in Figure 6-21, the tablet with the ratio of 75:25% of Cts:COAA was able to protect the protein drug from the harsh conditions of the stomach and small intestine. Based on the results, these novel biodegradable amphoteric Cts based tablets could be suitable carriers for colon-specific drug delivery.

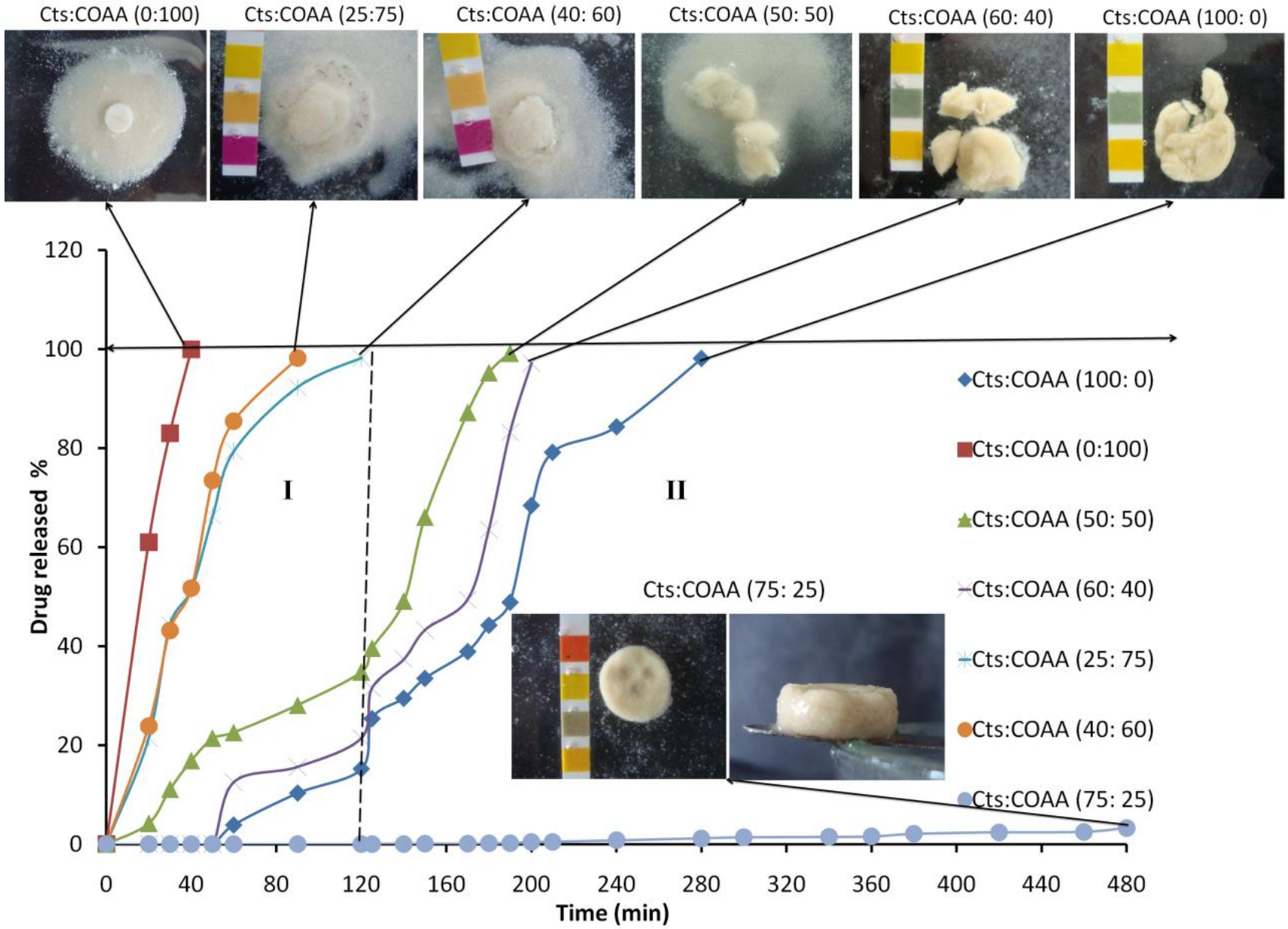

Figure 6-21 Effect of coating ratio on cumulative release of BSA from matrix tablets.

6.6.6.2.2. Effect of pH and enzyme

Figure 6-22 shows the percentage of BSA released as a function of time from Cts:COAA (75:25%) tablets with a crushing strength of 398.1 ± 1.42 (N/cm^2) during conditions mimicking the pH and times likely to be encountered during intestinal transit to the colon. The release profiles reveal that there was only 3.1 ± 0.4% of BSA released during the first eight hours (2h in SGF followed by 6h in SIF) indicating that this novel formulation is able to protect the protein drug from the harsh environment of the stomach and enzymic activity in the small intestine and deliver therapeutic molecules to the colon.

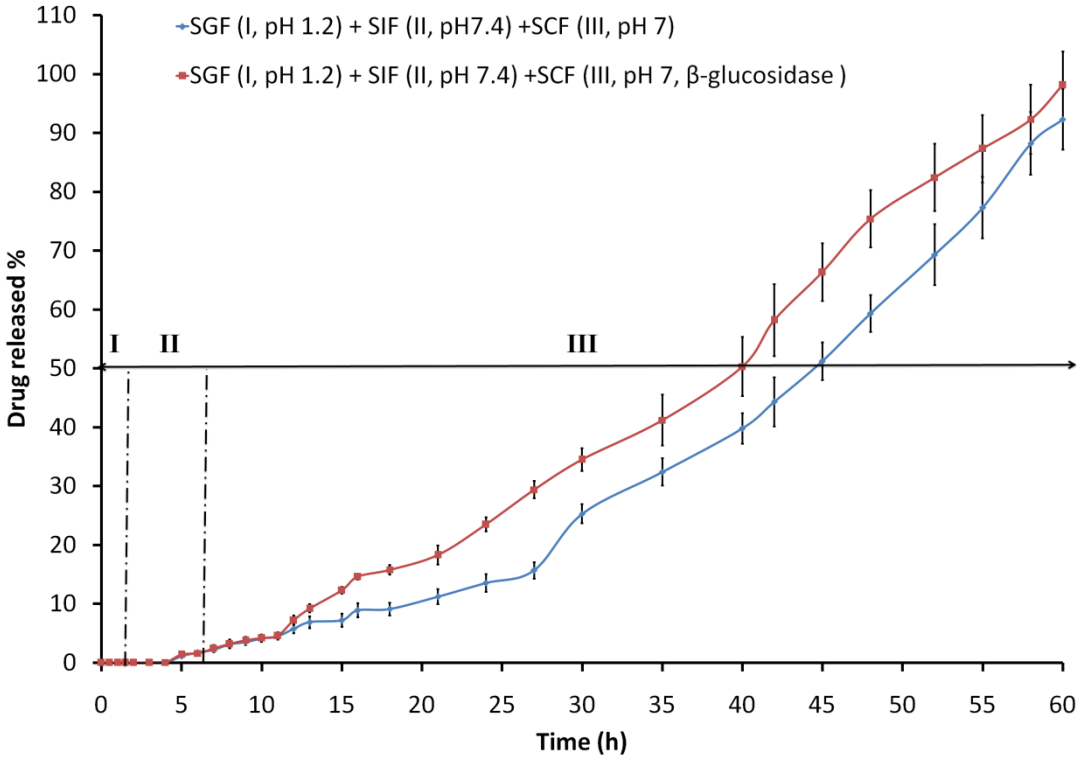

Figure 6-22 Effect of enzyme on cumulative release of BSA from selected tablet (Cts:COAA 75:25%).

The effect of β-glucosidase on the increase in drug release from the compression coated tablets was also studied, due to the similarity of the effect of almond β-glucosidase on Cts to that of colonic enzymes. Based on the results, the presence of β-glucosidase further increased the amount of BSA released. The time for 50% of the drug released ($T_{1/2}$) was about 40 hours while the $T_{1/2}$ of those with no enzyme was 45 hours. This finding supported the enzyme-controlled system could help increase the rate of drug release in this colon specific delivery system.

A literature review showed few Cts based tablets have been developed for colon specific drug delivery. Amrutkar *et al*.[8] prepared matrix tablets by wet granulation using crosslinked Cts and chondroitin sulfate. Indomethacin release studies were conducted in 0.1 N HCl for two hours without an enzyme, followed by PBS (pH 7.4, without enzyme) for three hours and finally in PBS (pH 6.8 up to 24 h). Selected formulation shows 45.6% release after 24 hours. Omwancha *et al*.[9] used a mixture of Cts and ethylcellulose for the colon targeted delivery of caffeine as the model drug. Release studies were conducted in SIF fluid (SGF, no enzyme or SIF, pH 6.8, no enzyme). Approximately 30% of the drug was released after eight hours (2 h in SGF + 6 h in SIF). Aiedeh *et al*. used Cts succinate and Cts phthalate tablets for diclofenac sodium release.[10] Three buffered dissolution media were used over three subsequent stages: pH 2 (2 h), then pH 6.4 (1 h) and pH 7.4 (3 h). $T_{1/2}$ of drug release was reached at about 200 minute.A combination of Cts and hydroxypropyl methylcellulose was used as compression-coats for 5-ASA tablets.[12] Drug dissolution was determined in pH 1.2 (2 h) Afterwards, each tablet was transferred to pH 6.8 (for 3 h) and then in, pH 5.0 (24 h). The time for 50% of the drug released was reached in 9.3 hours.

6.6.7. Cytotoxicity studies

The cytotoxicity of Cts, CBAA and COAA was evaluated against (CRFK) cells by the WST-1 assay. The percentage of cell viability as compared to the control cells after 24 hours exposure to the polymers (concentration from 1.25 to 0.005 mg/mL) are shown in Figure 6-23. Over 100% cell viabilities were observed in the case of Cts at all concentration, which indicates that Cts facilitated growth of the CRFK cell line. The

viability of cells incubated with CBAA and COAA was greater than 85% for all studied concentrations (1.25 to 0.005 mg/mL).

As shown in Figure 6-23, CBAA shows lower cytotoxicity compared to COAA in all concentrations. For instance CBAA shows 97 ± 1.65% cell viability, while 88.2 ± 5.0% cell viability was observed in the case of COAA with the concentration of 0.62 mg/ml. These results indicate that not only are these novel formulations able to be used as colon targeted drug delivery systems, but also they can be considered as a very promising material as safe drug vehicles.

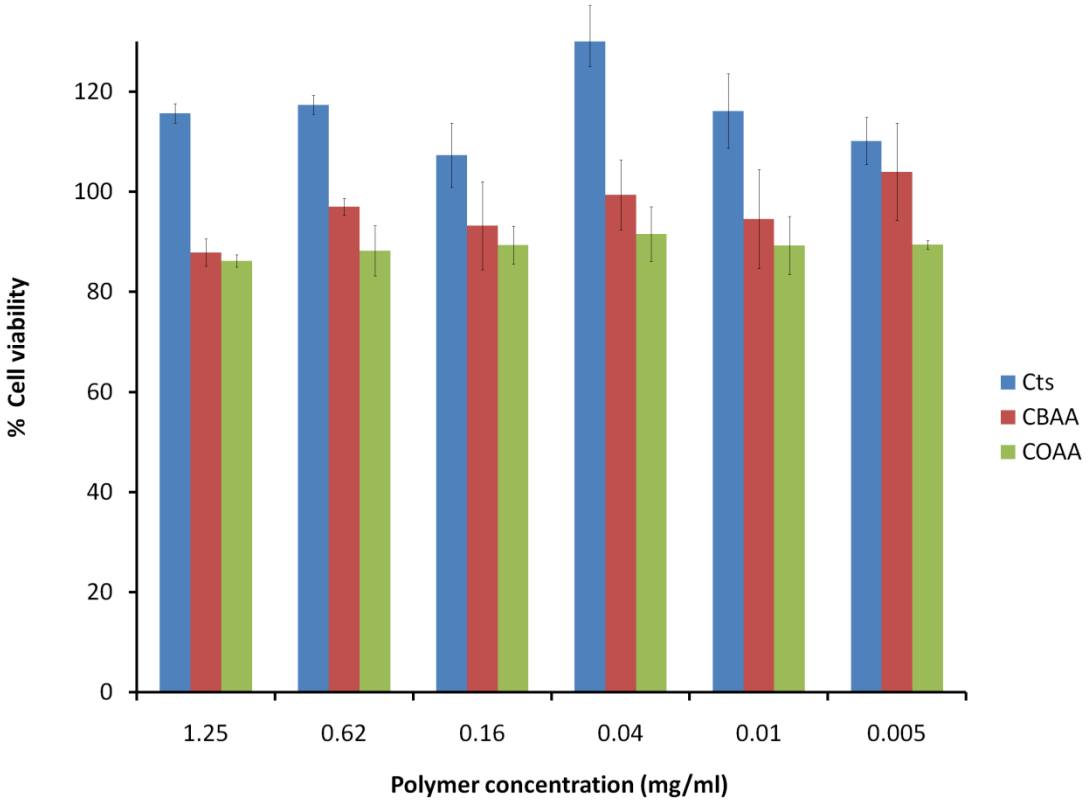

Figure 6-23 Percent viability of cells incubated with tested compounds as compared to control cells**.**

6.7. Conclusion

The aim of this chapter was to design a novel Cts-based carrier to protect the drug from the harsh environment of the stomach and enzymic activity of the small intestine and deliver therapeutic molecules to the colon. Based on the results of the present investigation, these novel amphoteric Cts based carrier was successful in protecting more than 90% of the coated drug and release in the colon. This finding supports the

facts that selected tablets (75:25%) could be used as a suitable polymeric carrier for colon-specific drug delivery. Other formulations from this study hold promise for drug delivery to other parts of the gastrointestinal tract.

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Chapter Seven

Development and evaluation of a novel colon targeting drug delivery system for the treatment of Tritrichomonas foetus intestinal infection in cats

7.0. Introduction

The treatment of gastrointestinal tract diseases has the advantage that orally administered drugs should present in much higher concentrations in the gastrointestinal tract (GIT) than those administered via systemic circulation. However, absorption of drugs in the proximal intestine may prevent therapeutic concentrations more distally. In other words drug delivery to the various regions of the GIT must be tailored accordingly. This is especially true of colonic diseases. *Tritrichomonas foetus (T. Foetus)* is a flagellated protozoan parasite that colonizes the feline colon and distal ileum, causing colitis and a chronic foul smelling diarrhea.[1] Ronidazole (RDZ) is currently the most widely used drug to treat *T. foetus* intestinal infection in cats.[1b, 2] RDZ is a nitroimidazole which is reduced by anaerobic organisms such as *T. foetus* to autotoxic free radicals and causes destabilization of the organism's DNA and subsequent death.[1b,4] Despite its efficacy in the treatment of *T. foetus*, RDZ has been reported to cause neurotoxicity in some cats due to rapid absorption in the small intestine which is thought to cause high plasma concentrations of the drug.[1b, 2a] Therefore reducing systemic absorption by colonic delivery would be hugely beneficial. Polysaccharides have gained much attention in developing colon specific drug release systems because of their flexibility in obtaining a desirable drug release profile, cost effectiveness, ease of modification, biocompatibility, biodegradability and ability to form hydrogels.[5] Among the various polysaccharides, Cts has attracted significant attention for the design and development of colon targeted delivery systems due to its potential degradation by the enzymes present in the colon.[6] Chitosan is a hydrophilic polyelectrolyte polysaccharide composed of glucosamine and N-acetyl glucosamine

units linked by β(1–4) glycosidic bonds, produced by alkaline N-deacetylation of chitin.[7]

The present study describes the development and evaluation of a novel colon targeting drug delivery system for ronidazole using an amphoteric Cts based matrix as a compression coat.

7.1. Materials and methods

With the exception of the following details, the same reagents, instruments and methods as per **Chapter 6**, was used throughout this chapter.

7.1.1. Materials

Ronidazole (RDZ) was obtained from Sigma-Aldrich (Auckland, New Zealand).

7.1.2. Preparation of Cts-PMDA amic acid (CPAA)

To a 200 mL round bottomed flask were added Cts (2g, 0.0124 mol of glucosamine residues) and glacial acetic acid (50 ml). The mixture was stirred at room temperature for 1 h. PMDA (2.71 g, 0.0124 mol), completely dissolved in DMF (100ml) was added to the flask. The mixture was stirred for 24 h at room temperature. The crosslinked hydrogel that formed was filtered off, washed with DMF and methanol and then freeze dried.

7.1.3. Swelling behaviour of the tablets

Radial and axial swelling of the tablet (400 mg, Cts: CPAA at 75:25%) in simulated cat gastric fluid (stage I, SGF, pH 1.5), simulated cat intestinal fluid (stage II, SIF, pH 6.5) and simulated cat colonic fluid (stage III, SCF, pH 5.5) at 37^{o}C was investigated. In addition a test was carried out for 30 min in SGF, followed by 2 h in SIF and then 24 h

in SCF to simulate the swelling behaviour tablet in a cat gastrointestinal tract. The experiment was carried out in triplicate and the swelling rate was evaluated by the following equations:

$$\%\ \mathbf{Swelling_{axial}} = \frac{(\mathbf{h_t} - \mathbf{h_o})}{\mathbf{h_o}} \times \mathbf{100}$$

$$\%\ \mathbf{Swelling_{radial}} = \frac{(\mathbf{A_t} - \mathbf{A_o})}{\mathbf{A_o}} \times \mathbf{100}$$

where h_o and A_o are the thickness and upper surface area ($\pi[d_o/2]^2$, d_o is diameter) of the original dry tablet respectively. The thicknesses, h_t and A_t, were measured for axial and radial swelling at time *t*.

7.1.4. Tablets erosion study

The measurement of the erosion rates of the matrix tablet, Cts:CPAA, 75:25% (400mg,) was carried out in SGF, SIF and SCF medium. An erosion study was carried out in simulated GIT fluid (30 min in SGF, 2 h in SIF and 24 h in SCF). After completion of the immersion studies in the test media, the tablets were dried in an oven at 60^{o}C for 24 h, allowed to cool in desiccator and finally weighed until a constant weight was achieved. The experiment was performed in triplicate and the tablet erosion was calculated from the following equation:

$$\text{Matrix erosion}\ (\%) = \frac{(W_i - W_f)}{W_i} \times 100$$

where W_i is the initial weight and W_f are final weight of the tablet.

The percentage remaining of the tablets (ES) after erosion was evaluated with the following equation:

$$\% \text{ Remaining} = 100 - ES$$

7.1.5. In vitro drug release study

Simulated cat gastrointestinal fluid (SGF, pH 1.5), simulated cat intestinal fluid (SIF, pH 6.5) and simulated cat colonic fluid (SCF, pH 5.5) were chosen to find the best coating weight ratios of Cts:CPAA for colon-specific drug delivery. The best coated formulation was first tested in enzyme-free SIF (stage I, pH 1.5) for 30 min, then the tablet was transferred to SGF (stage II, pH 6.5) for two hours. Afterwards, the matrix tablet was transferred to SCF (stage III, pH 5.5) for 24 hours. Selected formulations were further assessed in the presence of enzymes: 30 min in SGF (pH 1.5, pepsin present), then in SIF (pH 6, pancreatin present) for 2 h. Afterwards, the matrix tablet was transferred to SCF (pH 5.5, β-glucosidase present) for 24 h. For analysing the amount of drug released into the medium, the volume was made up to 100 ml with methanol, filtered through a 0.2-μm membrane and analysed by HPLC. The amount of drug in the tablet at the end of each test was also analysed by crushing the tablet in 50ml of methanol, filtering it through a 0.2-μm membrane and analysing it by HPLC. All tests were conducted in triplicate using 50 mL of dissolution media at 37 ± 0.5^{o}C with agitation speed of 100 rpm.

7.1.6. HPLC analysis

The quantitative determination of RDZ was performed using the mobile phase consisted of 0.01 M potassium phosphate-acetonitrile (90:10, pH 4). The injection volume was 25 μl and elution was performed at a flow rate of 0.5 ml/min. The wavelength of the detector was set at starting and ending wavelengths of 300 and 320 nm, respectively

7.2. Results and discussion

7.2.1. Preparation of CPAA

Cts:CPAA was readily formed at ambient temperatures by a nucleophilic substitution reaction whereby the Cts amine attacks one of the carbonyl carbons in the anhydride moiety and displaces a carboxylate functionality, followed by proton transfer Figure 7-1.

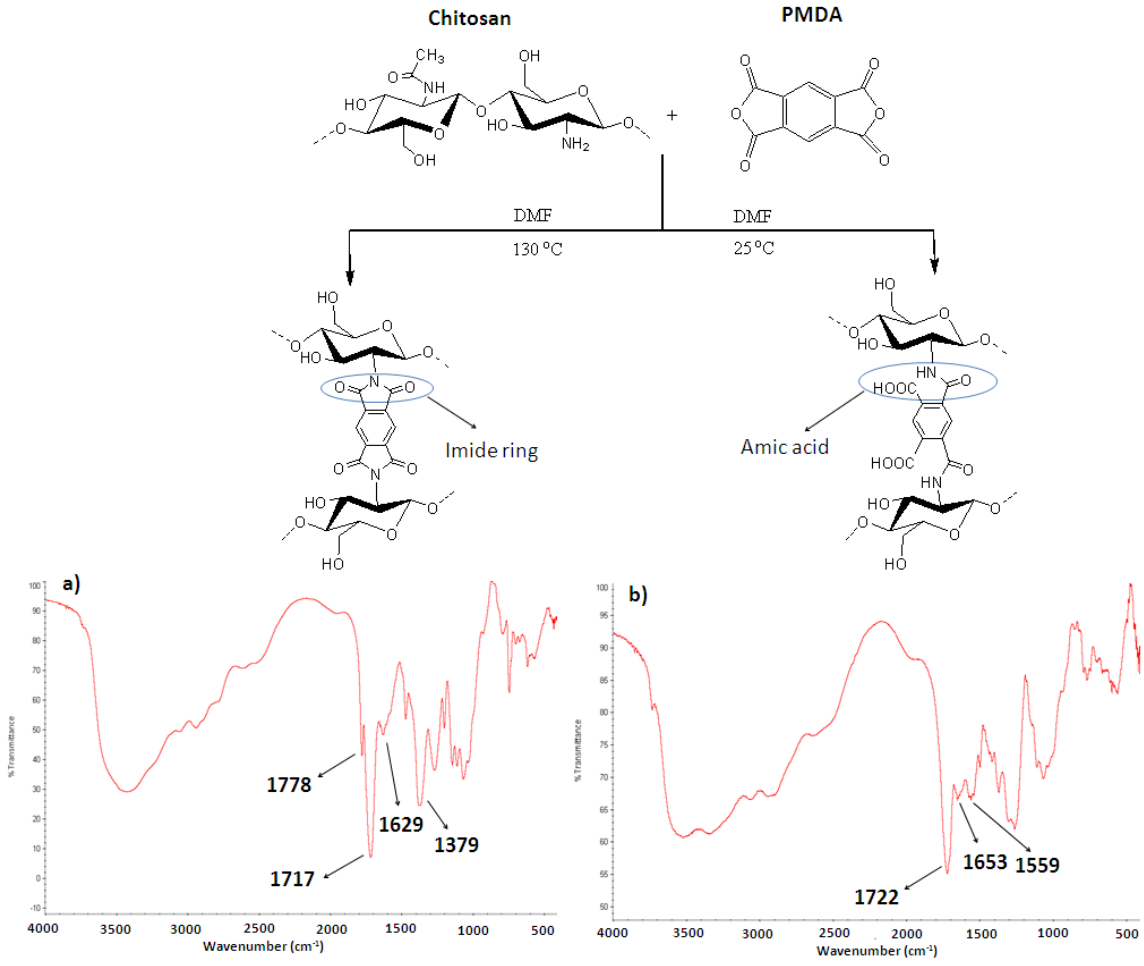

Figure 7-1 Reaction illustration and FTIR spectra of crosslinked chitosan with PMDA a) at 130 °C[3] b) at room temperature

Carbon, hydrogen and nitrogen contents of Cts and modified Cts were found C, 43.19%; H, 6.97%; N, 8.03% and C, 45.5%; H, 4.45%; N, 3.79% respectively. The DD of Cts was determined 77%.[8] The degree of substitution (DS) was determined to be 0.66.

7.2.2. Characterization

7.2.2.1. Fourier transform infrared (FTIR) spectroscopy

The chemical structure of the CPAA was investigated by FTIR (Figure 7-1a). The IR spectra CPAA show two peaks around 1722 cm^{-1} and 1653 cm^{-1} which are assigned to the C=O stretch of –COOH and amide group respectively.

7.2.2.2. Nuclear magnetic resonance (NMR) spectroscopy

The analysis of the ^{13}C DP-MAS spectrum of Cts and CPAA is shown in Figure 7-2. The ^{13}C DP-MAS spectrum for Cts is very similar to that reported in the literature.[9] Signals at 51.2 and 76.2 ppm are attributed to the C2/C6 and C4 respectively.

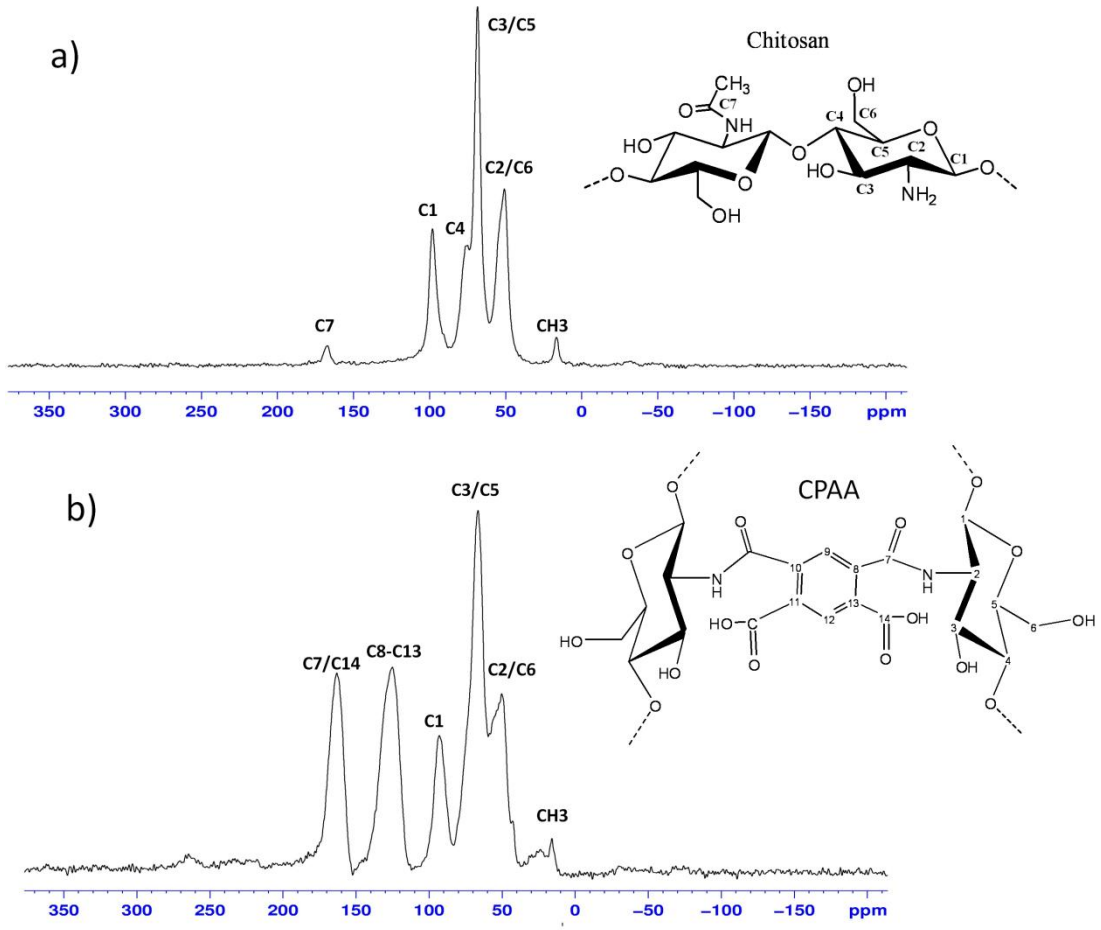

Figure 7-2 ^{13}C DP-MAS spectra of (a) Cts and (b) CPAA.

A well defined signal is observed at 68.8 ppm, which is assigned to overlapping signals attributed to C3 and C5. The signal due to the C1 carbon, which is directly attached to two oxygen atoms, was found at 98.4 ppm which is at a significantly lower magnetic field value compared with the signals of the remaining five carbons.
The signal related to the carbonyl peak of the acetyl group (C7) is also observed at 167.8 ppm. After the reaction between Cts and PMDA the ^{13}C DP-MAS spectrum displayed two signals in the carbonyl region for the amic acid moiety 163.2 ppm (C7, C14) and also a well defined signal is observed at 125.7 ppm which is related to aromatic moieties (C8-C13).

7.2.2.3. Scanning electron microscopy (SEM)

Figure 7-3 illustrates scanning electron micrographs of the surface of (a) Cts and (b) CPAA respectively. Figure 7-3a represents pure Cts powder, which displays a smooth surface.

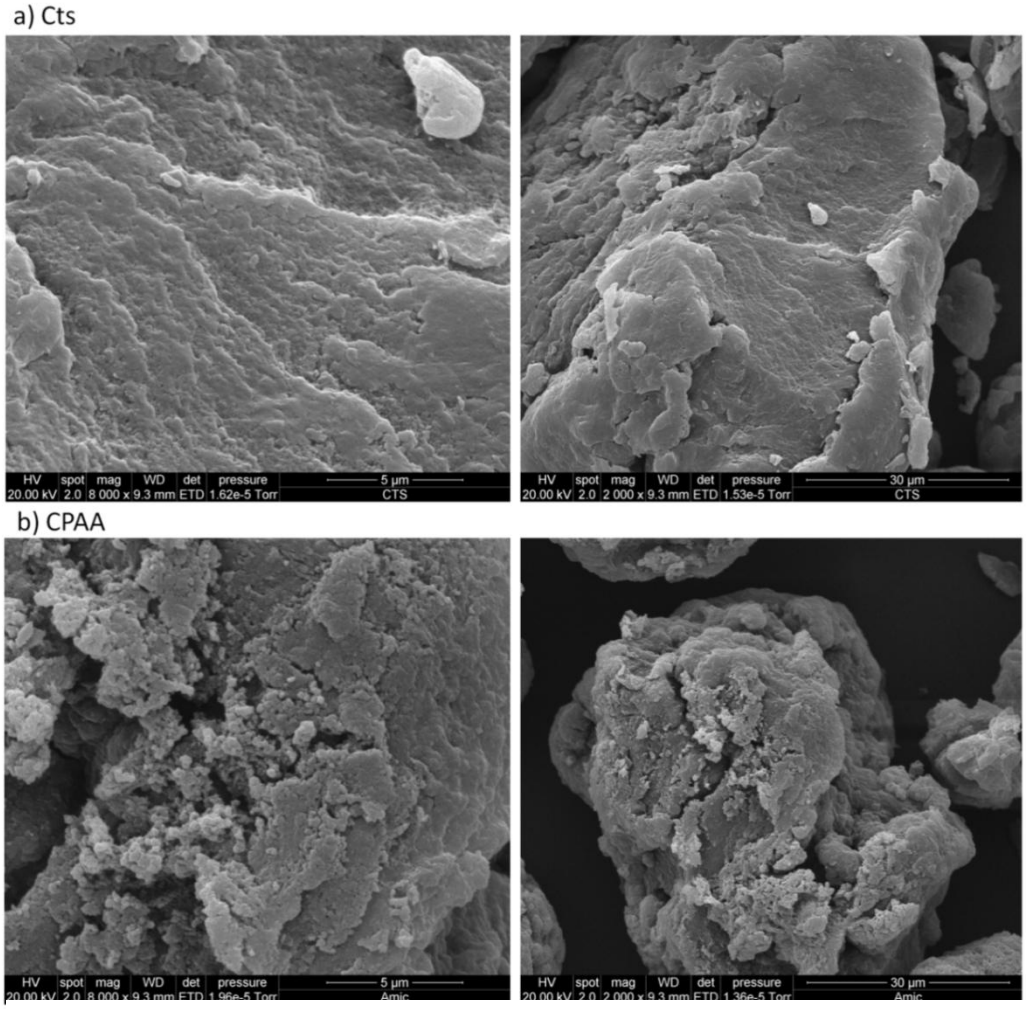

Figure 7-3 SEM image of (a) Cts and (b) CPAA

Figure 7-3b shows that significant morphological changes have occurred after modification of Cts with PMDA. The agglomerated and irregular surface structure of the modified Cts shows that the Cts has been chemically modified.

7.2.2.4. Powder X-ray diffraction study

The 2D-XRD patterns of Cts powder and modified Cts are illustrated in Figure 7-4. The main crystalline peaks at 2θ values of 20.8^{o} are generally assigned to weak chain-chain ordering of the Cts chains, indicating the crystalline state of the unmodified Cts (Figure 7-4a). Figure 7-4b indicates that the chemical crosslinking between Cts and PMDA destroys the crystallinity of Cts and increases the amorphous nature of the modified Cts hydrogel. The reduction in the crystallinity of Cts after modification is evidence that irregular packing of Cts subunits has occurred a result of the crosslinking. The substitution of amino groups by PMDA in addition causes a reduction of the strong hydrogen bonding that is found in the unmodified Cts.

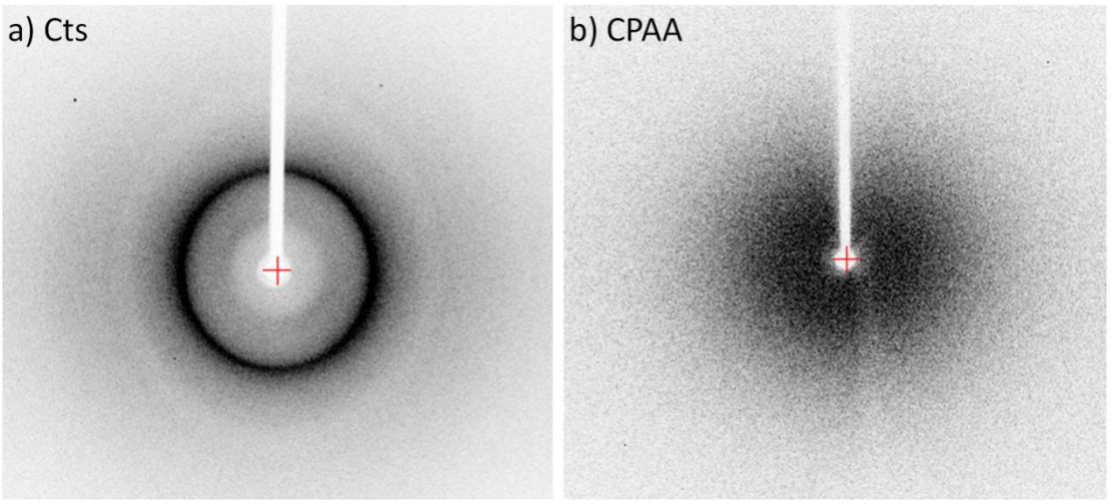

Figure 7-4. 2D-XRD of (a) Cts, (b) CPAA

7.2.2.5. Thermogravimetric analysis (TGA)

To examine the thermal stability behavior of Cts and crosslinked Cts, thermogravimetric analysis (TGA) was carried out, Figure 7-5. Cts showed an initial

weight loss of 10% in the temperature range between 20 and 240°C due to loss of adsorbed and bound water. CPAA showed a 20.8% weight loss between 20 and 240°C which indicated a faster process of weight loss in this temperature range. The second weight loss of Cts was in the region between 240-340°C with a sharp and considerable weight loss of 37%, which could be related to the decomposition of Cts. Over the equivalent temperature range a weight loss of 24% was observed in the case of CPAA, again must likely due to decomposition of Cts. More accurate differences of the thermal behaviour of the Cts and CPAA can be noted from the DTG curves. The DTG peak of Cts showed a maximum value of about 289°C, while the maximum value of this peak for the CPAA curve was observed at about 318°C showing the increase in the thermal stability of the CPAA, over Cts

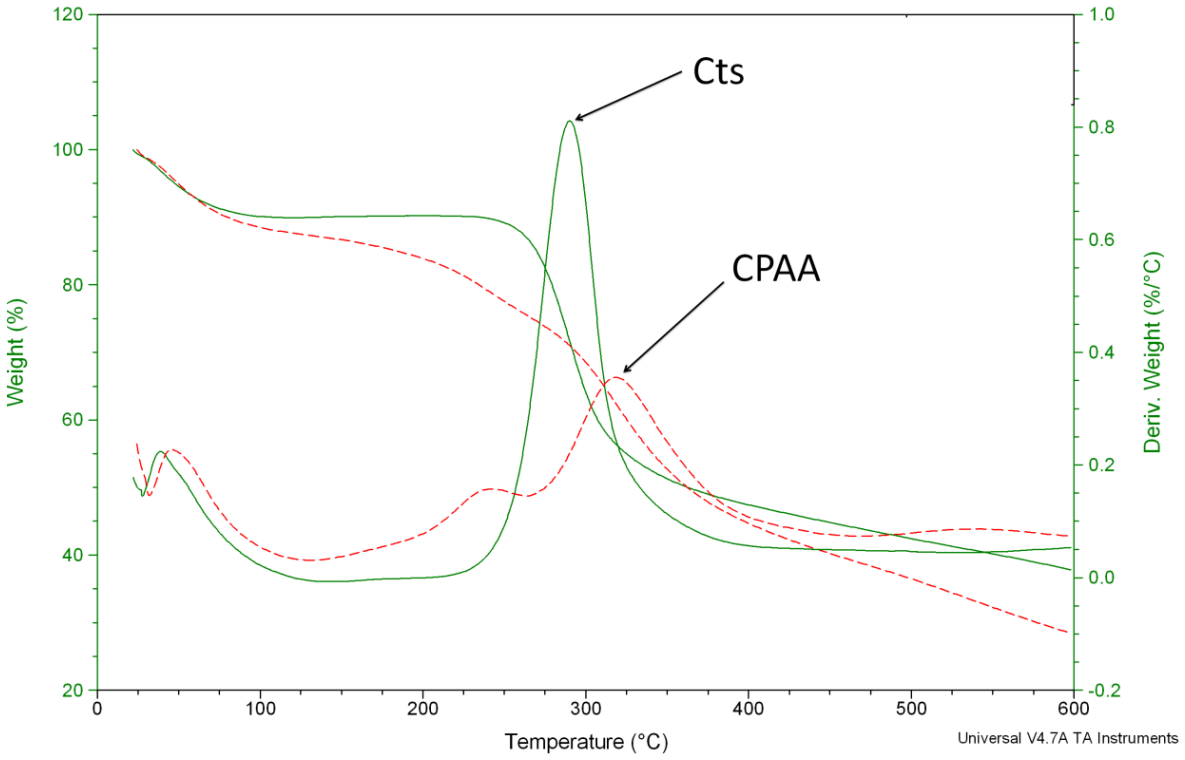

Figure 7-5 TGA and DTG thermograms of Cts and CPAA.

7.2.2.6. Enzyme inhibitory effect of CPAA

Unmodified Cts showed 3.5 ± 0.3% and 8.2 ± 0.4% inhibition capability for trypsin and chymotrypsin respectively. CPAA was able to exhibit an inhibitory effect of 43.2 ± 1.2% and 82.5 ± 2.9% for trypsin and chymotrypsin, respectively. The higher inhibitory

effect of CPAA as compared to pure Cts is explained by the binding of calcium by the carboxylic acid groups of the CPAA which may cause thermodynamic instability in the enzymes.[10]

7.2.2.7. Swelling and erosion behaviour of Cts:CPAA tablets

Swelling behavior of the Cts-CPAA (75:25%) tablet in pH 1.5, 6.5 and 5.5 are illustrated in Figure 7-6. Tablet swelling plays a crucial role in controlling the drug release from the hydrophilic matrices.[11]

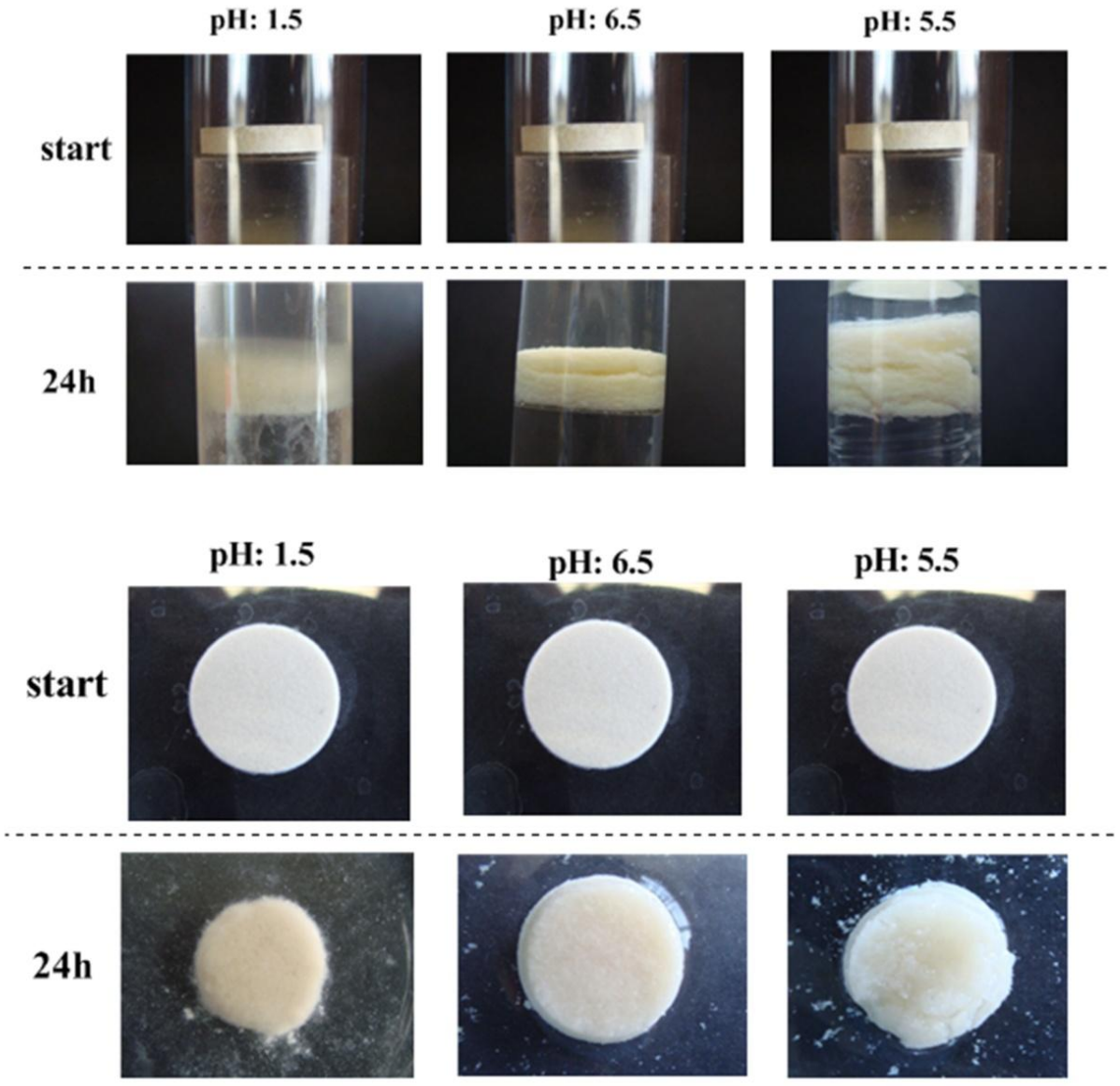

Figure 7-6 Photographs of the radial and axial swelling behaviour of the Cts:CPAA (75:25% **)** tablet in different pH media.

Swelling percentages of Cts:CPAA (75:25%) tablets in different media are shown in Figure 7-7. The results shows highest axial and radial swelling occurs at pH 5.5 with value of 371 ± 6.8% and 70.8 ± 5.1%, respectively. At pH 6.5, the swelling values are 181.4 ± 5.7% and 34.0 ± 3.1% for axial and radial swelling protocols (figure 7-7).

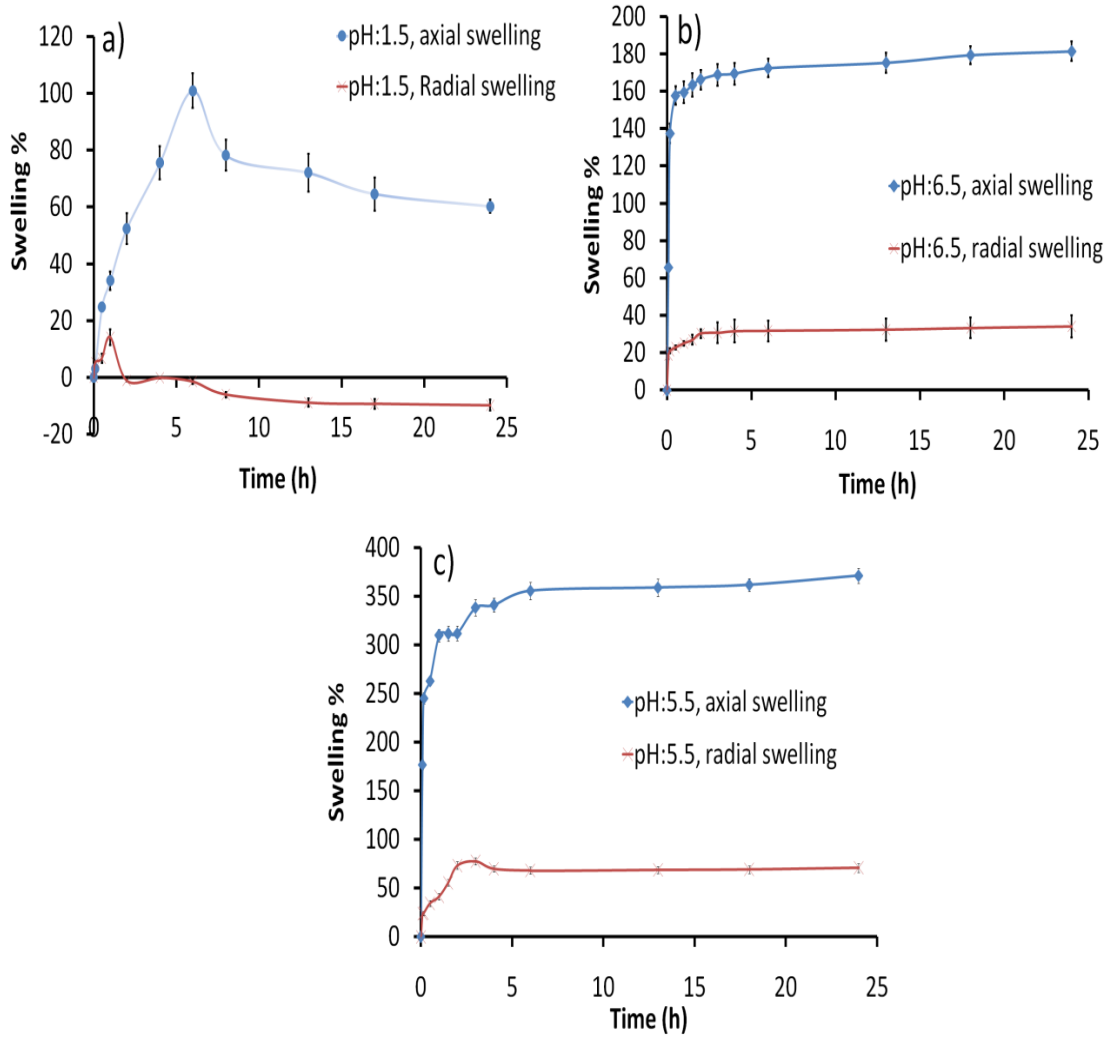

Figure 7-7 Swelling behaviour of Cts:CPAA (75:25%) tablets in a) pH 1.5, b) pH 6.5 and c) pH 5.5.

The swelling behaviour of the tablet in simulated feline GIT solution is illustrated in Figure 7-8.

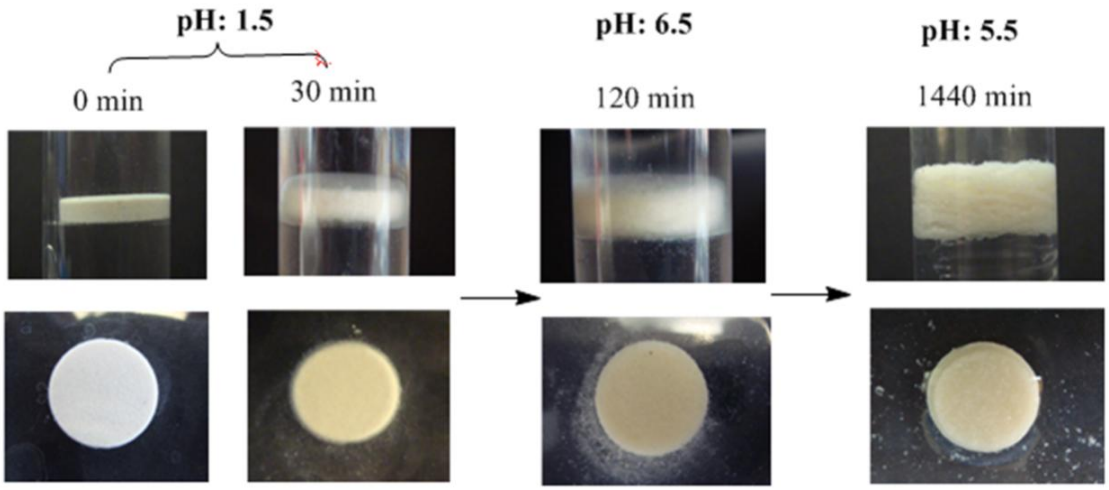

Figure 7-8 Photographs of radial and axial swelling behaviour of tablets in a simulated GIT of a cat.

Figure 7-9 shows the swelling percentage of Cts:CPAA (75:25%) tablets in a simulated gastrointestinal tract solution. After 30 min in SGF (I), the selected tablet showed about 28.1 ± 3.7% and 8.9 ± 0.8% axial and radial swelling, respectively. The swelling percentage reached a of maximum 120.7 ± 4.4% and 25.6 ± 3.2% for the axial and radial values after two hours in SIF (II). The tablet increases in swelling to 267.6 ± 7.3% and 71.3 ± 4.9% for the axial and radial after 24 hours in SCF (III).

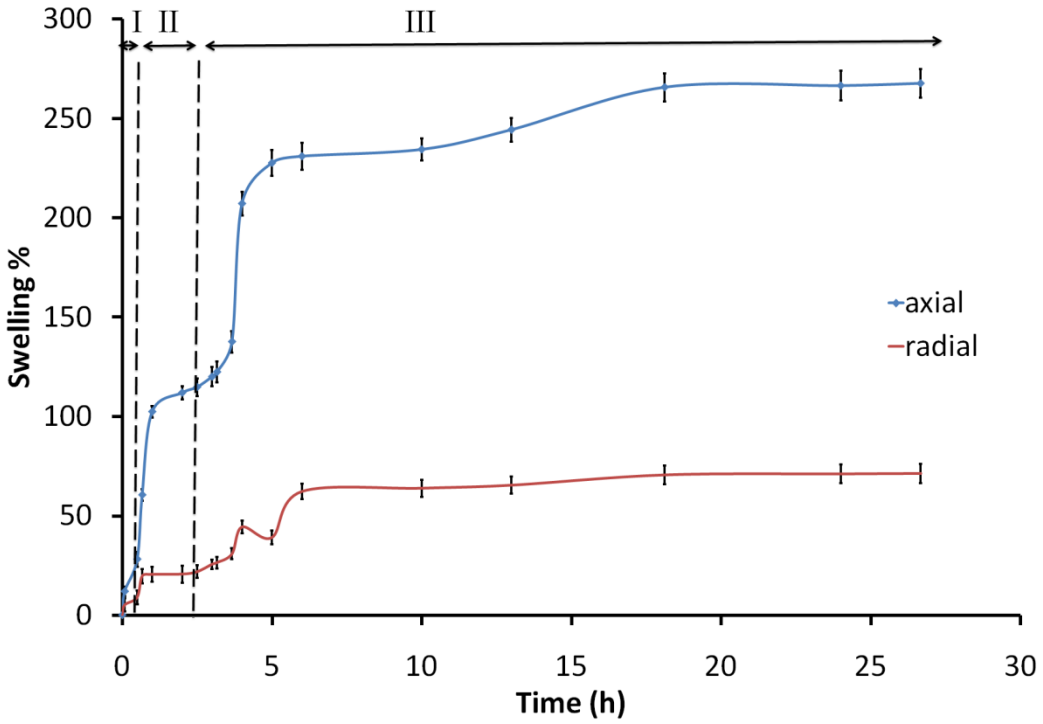

Figure 7-9 Swelling behaviour of Cts:CPAA (75:25 %) tablets in a simulated gastrointestinal tract.

Percentages of tablet remaining as a function of time are shown in Figure 7-10. Tablets show the highest erosion at pH 1.5 (25.6 ± 2.7%). Remaining tablet percentages of 90.1 ± 3.5% and 54.5 ± 2.9% were also observed at pH 6.6 and pH 5.5 respectively. For the simulated erosion behaviour of the tablets in a GIT of a cat, the tablets were left at pH 1.5 for 30 minute, followed by two hours at pH 6.5 and then pH 5.5 for 24 h. The results indicate about 48.8 ± 4.7% of the tablet remained at the end of this process.

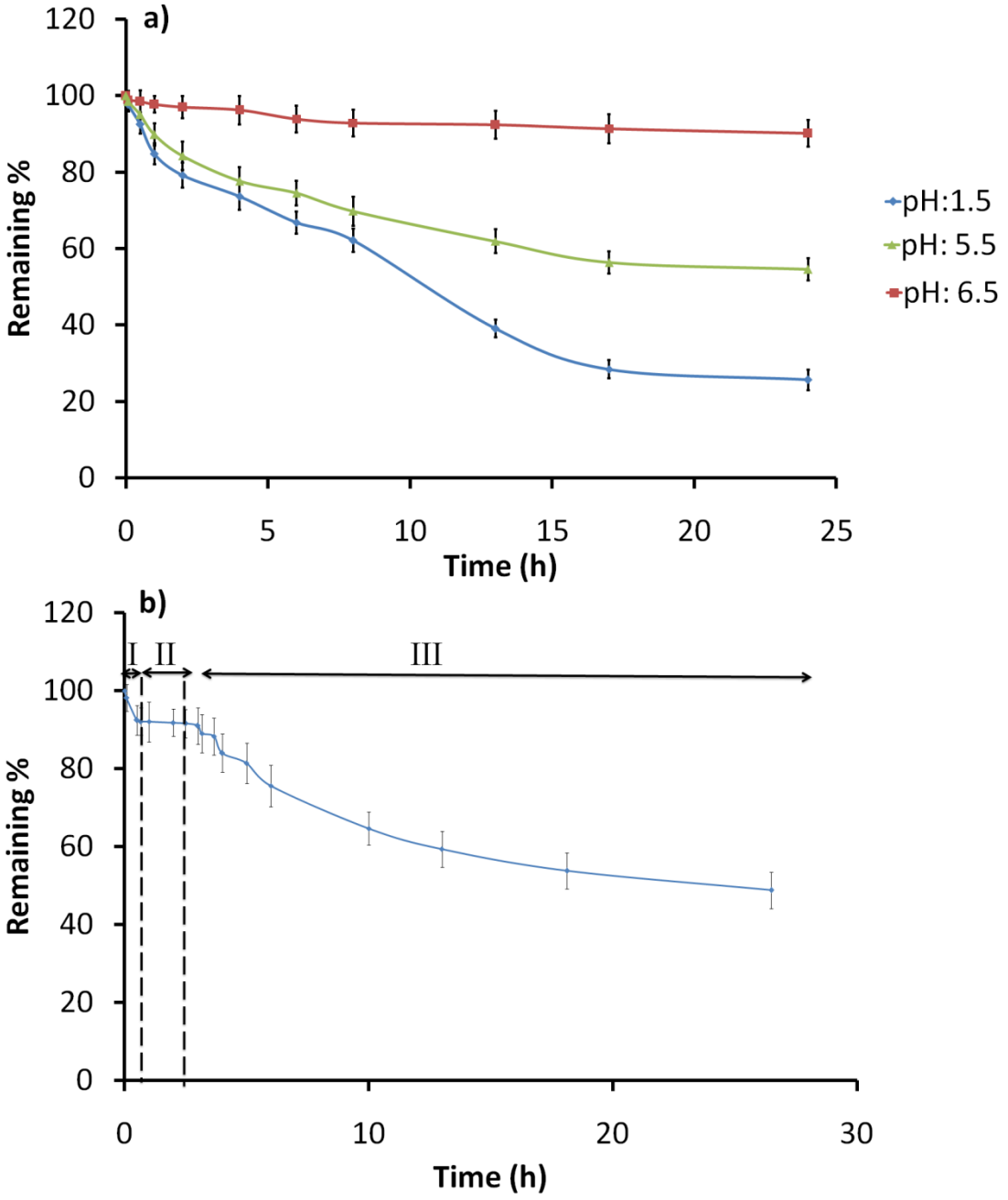

Figure 7-10 Percentages remaining of Cts:CPAA (75:25 %) matrix tablets a) 24 h in pH 1.2, 6.5, 5.5 b) in a simulated gastrointestinal tract (30 min in pH 1.5 followed by 2 h in pH 6.5 then 24 h in pH 5.5).

7.2.2.8. Enzymatic degradation study

The degradation effect of almond emulsin *β*-glucosidase on Cts based tablet is illustrated in Table 7-1. In the presence of β-glucosidase, the erosion of a Cts:CPAA (75:25%) tablet within 24 hours was about 6% higher than that with no enzyme. This finding supported the fact that the enzyme controlled system could help increase the rate of drug release in this colon specific delivery system.

Table 7-1. Effect of β-glucosidase enzyme on the Cts:CPAA (75:25% **)** polymer degradation in pH 5.5

	% Erosion (n = 3)	
	No enzyme	**With β-glucosidase**
Cts:CPPA, (75:25%)	49.2 ± 1.5	55.4 ± 2.6

7.2.3. *In vitro* drug release study

7.2.3.1. Effect of coating polymer ratio

The *in vitro* release study of ronidazole coated with various mixing ratios of Cts and CPAA is summarized in Table 7-2. Based on these results, ronidazole is rapidly released from the tablets composed of 20:80% and 25:75% Cts:CPAA. Due to rapid hydration and dissolution of Cts in SGF (pH 1.5), ronidazole coated with pure Cts was released within 40 min. the drug coated with just CPAA showed complete release of the drug within 5 minute. Tablets with ratios of 80:20% and 75:25% Cts:CPAA showed the highest lag time (>2 h) which is considered sufficient for colonic arrival.

Table 7-2. Effect of different coating weight ratio (%) on in-vitro release of ronidazole

Weight ratio (%)	Lag Time (min)
Cts:CPAA (100:0)	35
Cts:CPAA (0:100)	5
Cts:CPAA (50:50)	30
Cts:CPAA (80:20)	160
Cts:CPAA (75:25)	180
Cts:CPAA (20:80)	5
Cts:CPAA (25:75)	10

7.2.3.2. Effect of pH and enzyme

The Cts:CPPA tablet with a weight ratio of 75:25% and crushing strength of 418.1 ± 5.2 (N/cm^2) was chosen for further study. As it can be seen from Figure 7-11, the release profile of the selected tablet after 30 min in SGF (pH 1.5) showed no significant difference with or without pepsin, which can be attributed to the optimal condition needed for chitosanolytic activity of pepsin which is pH 5 at 45°C. This study shows that less than 2% of RDZ was released after 30 min in SGF followed by 2 h in SIF, with or without the enzyme being present.

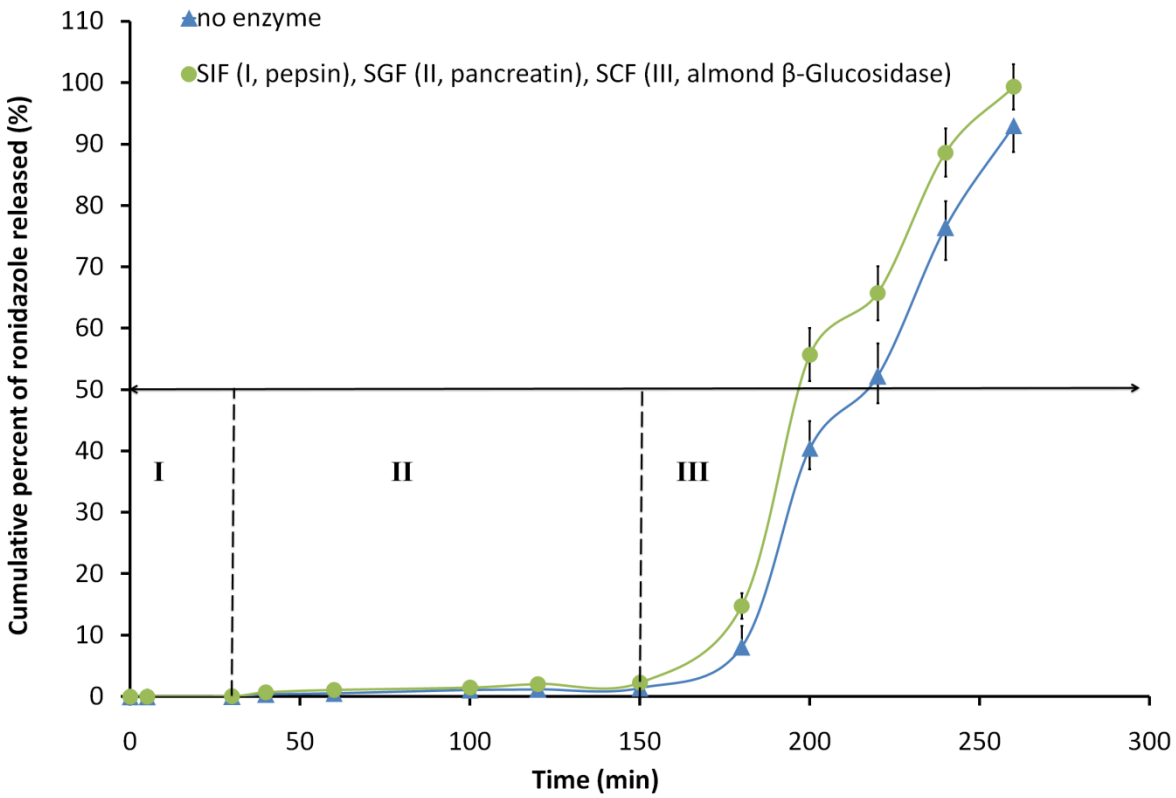

Figure 7-11 Effect of enzyme on cumulative release of ronidazole from the Cts:CPAA (75:25 %).

The potential effect of β-glucosidase to increase drug release from the compression-coated tablets was also studied. The presence of β-glucosidase further increased the amount of ronidazole released and the time for 50% of the drug be released ($T_{1/2}$) was about 200 min while the $T_{1/2}$, of those with no enzyme was about 220 min. This finding supported the enzyme-controlled system could help increase the rate of drug release in this colon specific delivery system. This novel amphoteric Cts based carrier successfully protected more than 98% of the coated drug and subsequently released the RDZ drug in the colon.

This finding supports that the selected tablet could be a suitable polymeric carrier for the colon-specific drug delivery of RDZ. Thus this formulation can lead to avoidance of the neurotoxic response seen in some cats due to small intestine absorption of this drug.

7.2.4. Cytotoxicity studies

The cytotoxicity of CPAA was evaluated against CRFK cells by WST-1 assay. The percentage cell viability as compared to the control cells after 24 hours exposure to the polymers (concentration 0.62 to 0.005 mg/mL) are shown in Figure 7-12. Over 100% cell viabilities were observed in the case of Cts at all concentrations, which indicates that Cts facilitated growth of the CRFK cell line. The viability of cells incubated with CPAA was greater than 80% for all studied concentration (0.62 to 0.005 mg/mL). CPAA shows 82.6 ± 1.9, 86.2 ± 4.2, 89.7 ± 3.0 and 96.2 ± 1.0% cell viability for concentrations of 0.62, 0.019, 0.01 and 0.005 mg/ml, respectively. These results indicate that not only are these novel formulations able to be used as colon targeted drug delivery systems, but also they can be considered as a very promising material for safe drug vehicles.

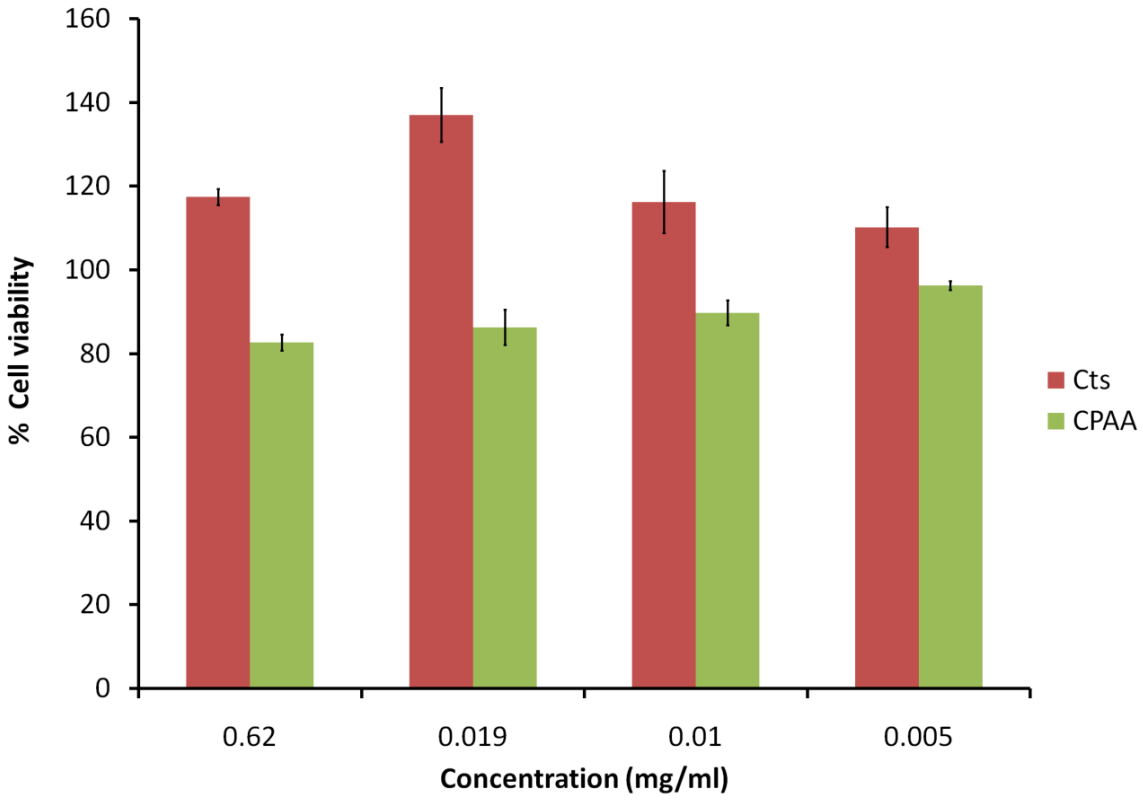

Figure 7-12 Percent viability of cells incubated with tested compounds as compared to control cells.

7.3. Conclusion

Because *T. foetus* resides chronically in the colon of infected cats, the aim of this study was to develop colon targeted drug delivery systems for RDZ, a known drug for colonic disorders. The results of this study showed that compression coated tablet containing Cts and CPAA (75:25%) was capable of protecting RDZ from the harsh environment of stomach and enzymes of the small intestine. The results show that less than 2% of RDZ was released in the physiological environment of stomach and small intestine. Therefore this study clearly shows that this novel tableting procedure was capable of retarding the release of tablet core materials until the colon was reached.

7.4. References

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Chapter Eight

Summary and possible future directions

8.0 Summary of results

Administering drugs orally is by far the most widely used route of administration which bypasses pain caused by injection, psychological barriers associated with multiple daily injections and possible infection from injection sites.

Hydrogels due to their attractive physicochemical and biological characteristics have attracted tremendous research interest as they are excellent candidates for the delivery systems of therapeutic agents. Hydrogels are defined as three-dimensional polymeric networks which can absorb from 10% up to thousands of times their dry weight of water or biological fluids without dissolving.

Chitosan has been extensively used to synthesize hydrogels because of its cost effectiveness, ease of modification, biocompatibility and biodegradability.

Chitosan is a linear heteropolysaccharide composed of randomly (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose linked by (1→4)-β-glycosidic bonds. It is easily obtained by the deacetylation of chitin, the second most abundant polysaccharide found in nature as a component of exoskeletons of crustaceans and insects.

Chitosan is structurally similar to cellulose, except for an acetamido or amine group at the C-2 position instead of a hydroxyl group. Physical and chemical modifications have been used to prepare chitosan derivatives with enhanced biological and physicochemical properties. Chitosan derivatives have been prepared by specific reactions involving the $–NH_2$ group at the C-2 position or –OH groups at the C-3 and C-6 positions.

This study sought to design chitosan based systems for drug delivery to the colon.

Drug delivery to the colon via the GIT presents a significant challenge. Lower water content and fluid mobility of the colon, which leads to longer retention times and also lower proteolytic activity of the colon compared to other areas of the gastrointestinal tract, make the colon an ideal site for both systemic and local delivery of drugs.

Therefore aggressive research efforts have recently focused on development of new strategies for delivering drugs to the colon.

To achieve successful oral colonic delivery, a drug needs to be protected from the absorption and degradation pathways of the upper gastrointestinal tract and then achieve abrupt released into the colon.

In the present research, two strategies was applied to develop an efficient colon targeted drug delivery system.

In the first approach, the aim was to design and construct a carrier with high mechanical and chemical stability to reach the colon and to protect the drug from the obstacles of the upper gastrointestinal tract.

In the second approach, the aim was to design pH sensitive and bacterially degradable carriers to protect the drug from the harsh acidic environment of the stomach and the rigorous enzymic activity of the small intestine and deliver the drug to the colon to provide increased effective therapy for diseases associated within colon.

Chapter 2, describes chitosan based films with improved physical properties. Polyimides have received much attention for their excellent characteristics including thermal stability, chemical resistance, excellent electrical properties, high glass transition temperature, high modulus and mechanical integrity. These properties are influenced by the nature of moiety originating from the aromatic dianhydride. Therefore in this chapter, chitosan was crosslinked with dianhydride derivatives. I hypothesized that inserting the imide ring into the chitosan backbone would increase the physiochemical stability of chitosan. The results show that the heterocyclic imide linkage imparts excellent thermal, mechanical and chemical stability to the chitosan film. Based on the results presented in this chapter, the chitosan film puncture strength was increased more than 200% after modification. Thermal study also showed a significant improvement in thermal stability. A swelling study also confirmed the stability of these matrices in the range from pH 2-10.

In chapter 3**,** the potential of a spray dried chitosan microsphere crosslinked with pyromellitic dianhydride for controlled drug release was investigated. In this chapter, BSA as a model protein drug was used to investigate the drug release behaviour of a PMDA modified chitosan with improved physical properties. This study showed that altering the concentration of BSA from 0.1 to 1 mg/mL enhanced significantly the

loading capacity from 6.3 ± 0.3% to 41.8 ± 4.1% and decreased the encapsulation efficiency from 88.4 ± 3.1% to 62.8 ± 2.9%. A number of factors such as time, pH, and enzyme availability were investigated for their influence on the release of the BSA from the microspheres. Slower drug release was observed from the Cts-PMDA microsphere at pH 1.2 and pH 7.4 compared with earlier studies. These novel microspheres also showed an ability to protect at least 30% of the protein drug from the harsh environment of the upper gastrointestinal tract.

In chapter 4, dianhydride derivatives of chitosan with improved physical properties did not show a promising pathway for colon targeted delivery. Therefore in this chapter modifications of chitosan were aimed at designing amphoteric chitosan based hydrogels.

A novel generation of chitosan-based amphoteric pH sensitive hydrogels were designed and characterized. Chitosan was crosslinked with dianhydride derivatives to obtain amic acid derivatives of chitosan for the first time. The results of the enzymatic inhibitory effect of crosslinked chitosan showed a much stronger inhibitory effect toward trypsin and chymotrypsin as compared to pure chitosan. The higher degradation of crosslinked chitosan over unmodified chitosan was also observed. An improvement in both thermal stability and hydrophilicity was also observed.

In chapter 5, films were formulated with a view toward colonic delivery of 5-FU using a novel amphoteric crosslinked chitosan matrix (CTAA). In order to improve the pH sensitivity of the crosslinked chitosan, sodium alginate, which has a high concentration of carboxylic groups, was incorporated into the crosslinked chitosan hydrogel. In a simulated gastrointestinal tract fluid (without any enzyme) approximately 30% of the drug reached the colon. The cytotoxicity studies of CTAA showed more than 85% of the cells were viable, which indicates that not only are these novel formulations able to be used as a colon targeted drug delivery system, but also that they can be considered as a very promising materials for safe drug vehicles.

In chapter 6, a tabletted matrix is the simplest and most cost-effective method of fabricating an extended release solid oral dosage form. However, little research has been done so far to develop colon specific drug delivery systems using chitosan based tablets. The major objective of the chapter was to develop a combination of time, pH, and

enzyme control tablets that would be able to protect the drug from the harsh acidic environment of the stomach and the rigorous enzymic activity of the small intestine and yet allow targeted drug release in the colon. Amic acid derivatives of chitosan and unmodified chitosan were used to prepare a compressed coated tablet. 5-ASA as an anti-inflammatory drug model and BSA as a protein drug was tested in this study. Based on the results of the investigation, these novel amphoteric chitosan based carriers were successful in protecting more than 90% of the coated drug and still allow release in the colon. This findings support the idea that selected tablets could be used as a suitable polymeric carrier for colon-specific drug delivery

In chapter 7, Ronidazole (RDZ) is currently the most widely used drug to treat *T. foetus* intestinal infection in cats. Despite its efficacy in the treatment of *T. foetus*, RDZ has been reported to cause neurotoxicity in some cats due to rapid absorption in the small intestine. Therefore in this chapter a compression coated tablet containing an amphoteric derivative of chitosan was developed to protect RDZ from release in the stomach and small intestine. The results show that less than 2% of RDZ was released in the physiological environment of the stomach and small intestine. Therefore this study clearly shows that this novel formulation has the capability to retard the release of tablet core materials until the colon was reached.

As an overall summary of the study presented in this thesis:

- I have established a tougher family of chitosan derivatives.
- Studies on one member of this family (Cts-PMDA) showed an ability toward colon targeted drug delivery
- I have established an amphoteric family of chitosan derivatives.
- Studies on one member of this family (CTAA) also showed an ability toward colon targeted drug delivery.
- Colon specific targeting studies were extended to coated drugs via amphoteric tablets. This tablet study showed considerable promise for delivery of drug (RDZ).
- My work shows that tablets, films or microspheres show promise for future development in the world of drug delivery to the colon.

8.1. Recommendations for future studies

The initial study explored the possibility of using dianhydride derivatives of chitosan as drug delivery vehicles; however this study could be further enhanced by investing the following:

a) **Effect of chitosan molecular weight and degree of substitution on drug release profiles of studied tablets should be investigated.**

   Chitosan with high molecular weight was studied throughout this thesis. Due to influence of molecular weight and degree of substitution on the physical and biological properties of chitosan, chitosans with various molecular weights and degrees of substitution need to be investigated.

b) **A comparative study of crosslinkers should be done.**

For example, the comparative toughness of amic acid and imide ring chitosan derivatives could be compared with other crosslinkers prepared under identical conditions as used in this study. Such a study will lead to a better understanding of the toughness and wetability illustrated by the chitosan derivatives in this study.

c) **The mucoadhesive properties of the chitosan derivatives should be investigated.**

   Chitosan has been shown to have mucoadhesive properties, probably mediated by ionic interaction between the positively charged amino groups in chitosan and the negatively charged sialic acid residues in mucus. Due to the potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption, mucoadhesive properties of the studied chitosan derivatives in this thesis need to be addressed.

d) **An *in vivo* study needs to be done to determine the blood levels of the drug over an extended period of time.**

e) **The release kinetics study should also be undertaken.**

   Mathematical modelling of drug release from the polymeric matrix has considerable academic and industrial importance. The composition of the hydrogel,

its preparation and its environmental considerations during drug release has affect on the drug release kinetics. Therefore determinations of kinetics of drug release from studied hydrogels are need to be investigated.

Appendices

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Iman Kavianinia

Name/Title of Principal Supervisor: Prof. David R.K. Harding

Name of Published Research Output and full reference:

I. Kavianinia, P. G. Plieger, N. G. Kandileand D. R. Harding,Fixed-bed column studies on a modified chitosan hydrogel for detoxification of aqueous solutions from copper(II). Carbohydrate Polymers, 90, 875-886 (2012).

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Name of Published Research Output and full reference:

I. Kavianinia, P. G. Plieger, N. G. Kandile and D. R. Harding,In Vitro Evaluation of Spray-Dried Chitosan Microspheres Crosslinked with Pyromellitic Dianhydride for Oral Colon-Specific Delivery of Protein Drugs. Journal of Applied Polymer Science, 131(2014).

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