Development of oral delivery systems for targeted gastro intestinal tract delivery of nutraceuticals using food grade polymers

Sampathkumar Kaarunya

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**NANYANG
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**DEVELOPMENT OF ORAL DELIVERY SYSTEMS FOR
TARGETED GASTRO INTESTINAL TRACT DELIVERY
OF NUTRACEUTICALS USING FOOD GRADE
POLYMERS**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

04/03/2019

Date

Sampathkumar Kaarunya

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

04/03/2019
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.....
Assoc. Prof. Joachim Loo

Authorship Attribution Statement

This thesis contains material from the paper published in the following peer-reviewed journal where I was the first author.

Chapter 4 is published as K. Sampathkumar and S.C.J. Loo. Targeted Gastrointestinal Delivery of Nutraceuticals with Polysaccharide-Based Coatings. *Macromolecular Bioscience*. 2018, 18, 1700363. DOI: 10.1002/mabi.201700363

The contributions of the co-authors are as follows:

- Assoc Prof Loo provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts.
- I co-designed the study with Assoc Prof Loo and performed all the laboratory work at the School of Materials Science and Engineering and the Singapore Centre for Environmental Life Sciences Engineering. I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing and Simulation.

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Abstract

The focus on alternative therapeutic strategies to overcome the side effects of the drugs and to act in synergy with drugs to maximize recovery is on the rise. Nutraceuticals are one of the candidates being explored as alternative or adjunctive therapy. Being of plant origin, most of the nutraceuticals are sensitive to degradation and suffer from the disadvantage of loss of bioactivity before they reach the target site. A suitable carrier would help maximize the benefits of these nutraceuticals. Oral route is one of the common methods for the consumption of such nutraceuticals or active ingredients (AI). Despite its numerous advantages, it faces many disadvantages also. Some of them being the poor bioavailability of the AI, degradation of the AI during transit of the gastro intestinal tract (GIT), poor absorption and lack of action specificity. In order to overcome this, a food grade carrier system that can encapsulate nutraceuticals of varying solubilities and can also be incorporated into food materials, as a direct food additive is required. In spite of the growing research in this field, a food grade carrier, that can successfully target the delivery of the AI to different parts of the GIT, while preserving its bioactivity, is still lacking commercially. In view of this, a food grade oral delivery system for nutraceuticals was developed using chitosan and starch as the base materials. Chitosan nanoparticles (chnp) were prepared by electrospraying and used for encapsulating the AI. A coating of starch on these particles ensured targeted delivery of the encapsulated AI. The performance of the coating layer was tested in different simulated GIT fluids and was found to be comparable to that of the commercial enteric polymers. The berries of the plant, *Withania coagulans*, chosen as the nutraceutical candidate, was used to extract the nutraceutical coagulans. The extracts, an aqueous fraction and an organic fraction, were characterized using various analytical techniques and was found to match with that of previously reported extracts from the same plant. Three different bioactive properties of the extract were tested. The aqueous fraction was found to possess wound healing and anti-diabetic effects while the organic fraction was found to have an anti-cancer effect. The small intestine targeting ability of the carrier was proven by encapsulating the aqueous extract into the food grade carrier and demonstrating the anti-diabetic effect both *in vitro* and *in vivo*. The large intestine targeting

ability of the carrier was proven by encapsulating the organic fraction into the carrier and demonstrating the anti-cancer effect *in vitro*. Hence the successful working of an oral delivery system, made entirely from food grade materials for targeting nutraceuticals to different parts of the gastro intestinal tract has been showcased as a step towards developing health benefiting food additives.

Lay Summary

The consumption of extracts from foods in concentrated form, in much greater amounts than what can be obtained directly from food, seeking health benefits, is on the rise. Some examples are the green tea extract or grape seed extract sold as pills. Such extracts have been given the name nutraceuticals. While the extract itself might be useful in alleviating some disease conditions when taken along with medicines, it is questionable whether in the current form as capsules, they are able to exert their influence to their full potential. This is because they can lose their activity in the stomach acid or be degraded by enzymes in the mouth and intestine. Also, since these pills are available over the counter, their consumption cannot be controlled and the pills usually offer very high doses of the extract itself in order to make sure, the extracts are available for use in the body, after being destroyed in the stomach or intestine. In order to overcome these problems, a bilayered capsule has been designed using food grade materials-mainly chitosan (obtained from shrimp shells) and starch, such that the coating layer can dissolve specifically either in the small or large intestine based on a trigger. The coating layer houses particles that are in size similar to that of the diameter of human hair. This small size facilitates better uptake in the body and such small size ensures that each cell in the body has many particles to interact with, thereby increasing the effectiveness of the particle cell interaction. The use of food grade materials eliminates the inherent toxicity of the capsule materials used in the pills and since, the capsule can release the extract on the desired region in the GIT, reduce the amount of extract needed to be consumed. The novelty lies in the fact that the carrier is entirely food grade and can target as well as encapsulate extract of different solubilities. In order to exemplify the working of the carrier, two different extracts from a plant source were filled into the food grade bilayered capsule. The outer layer made of starch, not only protects the capsule from the stomach acid, but also decides whether it is digested in the small intestine or the colon. This was demonstrated using the extract which had anti-diabetic effect. For this effect to be manifested, the extract has to be absorbed in the small intestine. The working of the capsule was proven by showing decreased blood glucose levels in diabetic animals treated with the extract filled capsules. The anti-cancer effect of

the extract was proven by its ability to kill cancer cells and this can be used to demonstrate the ability of the capsule to deliver the extract at the colon cancer affected areas in the colon. This study not only elaborates the successful working of a food grade capsule to preserve the activity of the extract while it reaches its site of action, but also has the ability to package a plant extract that has so far never been used in combination with such capsules.

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Abbreviations

AI	Active Ingredient
GRAS	Generally Recognized As Safe
GIT	Gastro Intestinal Tract
Chnp	Chitosan Nanoparticles
C+S	Soluble starch coated chnp
SS	Soluble starch
RS	Resistant starch
C+RS	Resistant starch coated chnp
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SCF	Simulated Colonic Fluid
EE	Encapsulation Efficiency
FTIR	Fourier Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance Spectroscopy
HPLC	High Performance Liquid Chromatography
HRTEM	High Resolution Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
ELISA	Enzyme Linked Immuno Sorbent Assay
TPP	Sodium Tripolyphosphate
STMP	Sodium Trimetaphosphate
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulphoxide
P2	Organic solvent soluble fraction extracted from <i>Withania coagulans</i>
P4	Water soluble fraction extracted from <i>Withania coagulans</i>

Chapter 1

Introduction

The chapter begins with outlining the basis and background for the conception of this thesis. The current market value of nutraceuticals is discussed and the importance of encapsulating them in food materials is enunciated. Once the problem with the existing systems are realized, a hypothetical design is established for solving the problems. This is followed by laying down the objectives for proving the hypothesis. Subsequently, an overview of the thesis is provided on a chapter by chapter basis, giving a short summary of what would be discussed in each chapter. The last part of the chapter outlines the novelty and findings of the work.

1.1. Background

Oral drug delivery system is undoubtedly the most practiced system for administering the AI and offers the advantages of ease of administration, patient compliance and requires minimum supervision. Besides the systemic delivery of drugs to treat diseases, the oral route offers the advantage of local delivery of drugs in many of the GIT disorders. For this delivery method to be successful, the AI has to be protected from the diverse environment of the GIT – pH difference, digestive enzymes and gut microbiome (Figure 1.1). Hence numerous research is being carried out in this field, trying to achieve perfection in targeted delivery to specific regions of the GIT where the drug absorption would be maximum resulting in maximum bioavailability. Oral controlled drug delivery systems enhances the advantages of oral delivery, by protecting the AI through its transit in the GIT tract and help deliver the drugs locally.

Figure 1.1 Challenges to the successful delivery of active ingredient through the oral route [1]. Reproduced with permission from Future Medicine LTD

The term Nutraceutical refers to a “type of dietary supplement that delivers a concentrated form of a bioactive agent, nutrient or non-nutrient, from food origin, in a dose that must

exceed those that could be obtained from normal foods in a balanced diet” [2]. According to a recent report by Transparency market research, the global nutraceutical market is estimated to reach US\$278.96 billion by 2021 [3] and is driven by the changing outlook of consumers on the health benefits and nutritional values of food. The nutraceuticals currently being tapped, target metabolic disorders, offering to curtail the occurrence of the disease at an early stage and in those severely progressed diseases cases, to act in synergy with the drugs in reducing their dosage. The therapeutic values of many nutraceuticals are being widely discussed in the literature [4, 5] and have also been reported in many traditional and ancient systems of medicine. Most of these nutraceuticals are marketed as dietary supplements and despite the claims from many nutraceutical companies, the efficacy of these, in treating a disease condition, still remains questionable. In addition, the positive role of these nutraceuticals in chronic diseases like cancer or other bacterial infections is still unknown.

In order to realize the true potential of nutraceuticals, it is important to protect and deliver them using a carrier system, as most of the nutraceuticals currently being used as dietary supplements are extremely labile compounds having low bioavailability [6]. It is hypothesized that the bioavailability of these compounds can be increased by encapsulating them in a suitable lipophilic/hydrophilic carrier depending on the nature of the compound and delivering them directly at the site of absorption. Since the encapsulated nutraceutical would still be used as a dietary supplement, it would be best to use food grade polymers as carrier materials. Also, to protect the carrier from the diverse GIT environment, an enteric coating is essential. But currently available commercial enteric coatings are not food grade or Generally recognized as safe (GRAS) [7]. Hence, a food grade enteric coating that overcomes all the problems discussed above, would help to substantiate the beneficial properties of nutraceuticals.

1.2. Problem Statement and hypothesis

There is a lack of commercial coatings made entirely from food grade materials, that can be used to bypass the barriers in the GIT, are completely safe and could be readily used in food application as additives or functional foods [8]. This is important to note as there is no regulation in the consumption of foods marketed under this category. Most of the polymers used as enteric coating in pharmaceutical products are not approved for use as a food additive and the processing parameters employed during the coating process, does not make it suitable for use with nutraceuticals which are mostly labile compounds [9]. Hence it is important to ensure that these are totally safe. Besides, there is no existing delivery system for the nutraceutical chosen. Coagulans, being of plant origin is labile in nature and has a very strong taste and smell that deters its usage [10]. Hence a suitable carrier system for these nutraceuticals would help overcome these problems.

The thesis postulates the hypothesis that food grade particulate systems are excellent vehicles for the delivery of nutraceuticals, as they can be modified to target specific parts of the GIT. The feasibility of the hypothesis was tested by comparing the performance of the food grade materials against commercial coating materials and the targeting ability of the food grade nanoparticles was tested through both *in vitro* and *in vivo* studies.

1.3. Objectives

1. Design and develop a delivery platform using biopolymers/food grade materials with the following attributes:
 - A carrier system, delivered through the oral route using food grade polymer that can encapsulate nutraceuticals, cross the mucosal barrier and deliver to the enterocytes of the intestine.
 - A coating layer that can selectively degrade either in the small or large intestine.
2. To identify a suitable nutraceutical to be loaded into the delivery system, such that the nutraceutical can be used to exemplify the targeting ability of the delivery system

3. Encapsulation of nutraceutical into the carrier and testing its feasibility as a small intestine targeted delivery system
4. Encapsulation of nutraceutical into the carrier and testing its feasibility as a large intestine targeted delivery system

1.4. Dissertation Overview

The thesis addresses the development of a food grade carrier system for the targeted delivery of nutraceuticals to different parts of the GIT, its ability to encapsulate both hydrophobic and hydrophilic nutraceuticals and the validation of the targeting ability of the carrier platform. The term nanoparticles has been used in this thesis refers to particles in the size range of 170 -200 nm obtained from the electrospraying process.

Chapter 1 provides a rationale for the conceptualization of the work that led to this thesis and outlines the problem statement, objectives and novelty of the work.

Chapter 2 reviews the literature concerning the existing oral delivery systems for nutraceuticals and the problems with existing enteric coatings. The chapter also discusses the existing literature on targeted delivery systems for the small and large intestine. The last part of the chapter summarizes the gaps in knowledge that the thesis is addressing.

Chapter 3 discusses the experimental methodologies involved in the work, while explaining the principle of each method and also the rationale for choosing the particular technique. The chapter is divided into three main parts, with the first part focusing on the principles underlying the fabrication and characterization techniques employed, the second part focusing on the techniques used for the extraction and characterization of the nutraceutical and the last part focusing on the *in vitro* and *in vivo* experimental methods.

Chapter 4 elaborates the results for the development of the carrier platform. The characterization of the carrier using various microscopic techniques followed by the evaluation of the performance of the carrier are discussed. The chapter ends with the cell

uptake studies on the nanoparticle carrier.

Chapter 5 elaborates the results on the extraction and characterization of the nutraceutical to confirm the compounds present in the extract. The chapter also demonstrates the feasibility of loading the extracts into a polymeric carrier and studying the release of the extracts by developing a HPLC method to quantify the release.

Chapter 6 presents the results displaying the anti-diabetic effect of the extracts both *in vitro* and *in vivo*, followed by the encapsulation of the extract in the delivery system, the release kinetics of the extract, *in vitro* validation of the bioactivity of the extract, after encapsulation and the ability of the carrier to target to the small intestine *in vivo*.

Chapter 7 presents the results displaying the anti-cancer effect of the extracts *in vitro*, followed by the encapsulation in the delivery system, the release kinetics of the extract from the carrier and the validation of the bioactivity after encapsulation and targeting ability of the carrier *in vitro*.

Chapter 8 draws together the results presented in the different chapters by explaining how each of these results fulfil the objectives as a step towards realizing the hypothesis. Future recommendations to further strengthen the studies carried out have also been laid down. Reconnaissance study on the bioactive property of the extracts have been included towards the end.

1.5. Novelty and outcomes

The novel aspects of this thesis include:

1. The development of gastric protective coatings for nanosized particles for selectively targeting of nutraceuticals to either the small or large intestine. The technique used for the coating process is extremely mild and does not use any toxic solvents. In contrast to existing synthetic enteric coating polymers, all the materials used in the carrier fabrication are food derived and approved to be used as a direct food additive.

Figure 1.2 Schematic 1- representing the novelty and outcomes

2. The encapsulation of the extracts within a polymeric carrier and studying the therapeutic effects of the extracts *in vitro*. For the first time, the compounds from *Withania coagulans* were encapsulated into the developed food grade delivery system in order to protect its bioactivity and enhance the bioavailability. There have been no reports so far, on the encapsulation of the extracts of *Withania coagulans*. Three different therapeutic activities of the extracts were demonstrated using three mammalian cell lines. There have been no reports on the *in vitro* testing of the therapeutic properties of the plant extract on cell lines, to understand its cellular targets and mechanism of action.

Figure 1.3 Schematic 2 – representing the novelty and outcomes

3. The successful demonstration of the ability of the different food grade coatings to retard gastric release and target specifically to the large or small intestine. This was showcased using different simulated GIT fluids to represent stomach, small intestine and large intestine, and the different coatings, being able to modulate the release of AI in the specific region for which it was intended.

Figure 1.4 Schematic 3 - representing the novelty and outcomes

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Chapter 2

Literature Review

The chapter summarizes the various literature published in relation to the topic of this thesis, reviewing the relevant contemporary work done, thereby identifying the gaps in designing a completely food grade oral delivery system for nutraceuticals. The first part of the chapter focuses on the existing delivery systems for different nutraceuticals, followed by a detailed review on the use of chitosan as an oral delivery system and the different methods of fabricating chnp. Subsequently, the existing commercial enteric coatings, the problems in using them in food materials, current studies on small and large intestine targeted delivery have been discussed. The last part of the chapter reviews the existing literature on the chosen nutraceutical Withania coagulans and its various therapeutic effects. The chapter ends with a short summary on the various research gaps ascertained from literature review, that the thesis addresses.

2.1. Delivery systems for nutraceuticals

The growing attention on nutraceuticals and the expanding market for such products has shifted the focus on the encapsulation of nutraceuticals in order to protect its activity and ensure maximum bioavailability, when consumed. The nutraceuticals that are of interest to be encapsulated and delivered in the past 5 years are mostly polyphenols like resveratrol, curcumin, catechins isolated from green tea, Quercetin, Coenzyme Q10, Vitamins like A, C, E and D using various food-based matrix carriers like lipids, whey protein, soy proteins and chitosan (Table 2.1). Since most of these nutraceuticals are hydrophobic, more attention is being directed at developing oil based emulsion carriers. Though most of the carriers were developed with the aim of oral delivery, they were not designed in such a way to overcome the barriers of the GIT.

The advantages of using nanoparticulates as drug carriers have already been realized in pharmaceutical sciences and is currently being looked into for use as nutraceutical carriers also. A number of reviews comprehensively list out the nanocarriers that have been studied so far for the oral delivery of nutraceuticals [1-6]. Some of the advantages of using particles below 500nm for oral delivery are outlined as follows [7, 8]:

- Better targeting and absorption due to increased surface to volume ratio
- Ability to cross epithelial tight junctions and paracellular transport
- Prolonged systemic circulation compared to micro particles
- Lesser chances of being attacked by the body's immune system

Table 2.1 List of Nutraceuticals studied for encapsulation in micro and nano carriers

Nutraceutical type	Examples	Carrier material used	Ref
Polyphenols	Epigallocatechin (EGCG)	β -lacto globulin	[9]
	Curcumin	Iota-carrageenan	[10]
	Red grape seed extract	Chitosan and hyaluronic acid	[11]
	Quercetin	Solid lipid nanoparticles, lipid nanocarriers and lipid nanoemulsions	[12]
	Naringenin	Phospholipid complex, Beta lacto globulin-whey protein	[13], [14]
	Resveratrol	Gliadin and Zein	[15]
Carotenoids	Beta carotene	Barley protein	[16]
Vitamins	Vitamin D3	Hydrophobins (components of fungal cell wall)	[17]
	Riboflavin	Soy protein	[18]
	Folic acid	Whey protein concentrate and resistant starch	[19]
Probiotics	Lactobacillus acidophilus	Zein alginate core-shell microcapsules	[20]

2.2. Chitosan as an encapsulation material

Some of the commonly used GRAS materials for encapsulation of nutraceuticals have been listed in Table 2.1. Various polysaccharides, proteins and lipids derived from natural sources have been used as encapsulation materials. While different materials such as whey protein, zein and phospholipids have been reported to show retarded release of the encapsulated nutraceutical in the gastric environment, they are not mucoadhesive in nature and possess a negative zeta potential. Besides these materials release the cargo immediately once they reach the small intestine.

The choice of the encapsulation material for this thesis, was based on developing a nutraceutical encapsulated nanoparticle that could allow for coating with different food grade materials, so that the nanoparticles could be released in the small intestine or large intestine, providing for controlled release of the nutraceutical from the nanoparticles. Hence the core nanoparticle should be conducive to be coated with the chosen coating material, in this case, soluble starch and resistant starch, which possess a negative charge. Hence a positive charged polymer would best suit this purpose. Though the materials discussed above can be fabricated into nanoparticle in the desired size range and also encapsulate a nutraceutical, most of them are negatively charged polymers.

Chitosan is a cationic polysaccharide obtained from deacetylation of chitin found in the shells of crustaceans [21] and has been used widely in the encapsulations of drugs [22], proteins [23], oligonucleotides [24] and nutraceuticals [25]. The characteristic feature of chitosan that makes it a candidate of choice as carrier material is its ability to adhere to negatively charged mucosal surface and capability to penetrate tight junctions between epithelial cells [26, 27]. The mucoadhesive ability of chitosan would help increase the residence time of the particles made from chitosan within the GIT, thereby increasing the chances of sustaining the release of AI within the body. Chitosan, has been proven safe for use in animals and humans [28], and is considered as GRAS by US FDA. It is also approved for use as food additive in Japan and Korea [29].

Figure 2.1 Structure of chitosan obtained from the deacetylation of chitin [30]

2.2.1. Methods of chitosan nanoparticle fabrication

A nanoparticulate delivery system based on chitosan, is envisioned for the nutraceutical encapsulation, as it has been shown in previous studies that the size of the particle, enhances the cellular uptake of the particles in the GIT [8]. Besides the increased surface area, increases the chances of adhesion to the gastric mucosa and the smaller size, allows for faster release compared to microparticles [31], which is a trade-off between the short residence times in the GIT and sustaining the release of AI. Chitosan nanoparticle have so far been fabricated by different methods like emulsion droplet coalescence [32], ionic gelation [33, 34], electro spraying [35], coacervation/precipitation [36], and self-assembly [37]. Of the methods listed above, electrospraying offers the advantage of reproducibility, as the process parameters are equipment controlled and chances of variability are minimal [38]. Electrospraying is the process by which the polymer solution is atomized under the influence of the electric field and the charged droplets are collected on a grounded collector

solution. The process parameters such as electric field strength, flow rate and viscosity of the solution, determine the size of the particles [39]. Previous reports on electrospraying chnp have yielded particles in the size range of 200 nm [40]. The parameters can be used as a guide to optimize the fabrication parameters for the current study. Electrospraying is a mild technique that does not use any toxic organic solvents and the nanoparticles can be sprayed directly into the cross linker from which it can be collected. This actuates the suitability of the method to be used in food based applications.

2.3. Enteric coatings

The important requirement for a carrier system to be used in oral delivery is that it should primarily protect the AI from the adverse environment of the gastrointestinal tract (GIT)—low pH in the stomach, digestive enzymes in the small intestine, and gut microbiome in the large intestine. To achieve this, polymeric coatings capable of protecting and degrading selectively in different organs of the GIT are used. Though there are a variety of such coating materials, most of these materials are made of either polymethylacrylates, poly vinyl acetate phthalate or cellulose acetate derivatives. These polymeric coatings cater to both aqueous and organic solution dispersion and require the use of plasticizers to aid in film formation. One example is the Eudragit polymers based on polymethacrylates or polyvinylacetate phthalates that are marketed by Evonik. Some recent reviews outline the detailed applications of these polymers as enteric/sustained release/colon-targeted coatings [41, 42]. Despite the research focusing on nutraceutical encapsulation, there are not many commercially available enteric coating systems targeting to small and large intestine intended for food application. Extensive literature search resulted in identifying one commercial enteric coating for nutraceuticals, developed by Colorcon®. The product is a combination of some of the synthetic polymers mentioned above, along with shellac, alginate and an appropriate plasticizer intending to protect the nutraceuticals in the stomach and delivering to the small intestine. Most of the synthetic polymers used in these commercial products are mainly intended for pharmaceutical applications and are only approved as an indirect food additive [43]. The consumption of such products when used in functional foods, as a direct food additive, is not regulated. In addition, due to the

sensitive nature of nutraceuticals, the harsh processing parameters employed during coating of these commercial polymers also make it unsuitable for these labile compounds. There is still no commercialized food grade, controlled release coating materials for targeted delivery and this opens avenues for new research in this field.

2.4. Targeted oral delivery systems for nutraceuticals

Targeted delivery systems help in increasing the bio-availability of nutraceuticals by delivering them locally. The carrier can be designed to be degraded specifically in the small intestine triggered by pH difference or intestinal enzymes or in the large intestine by the gut microbiome or intestinal pressure.

2.4.1. Small intestine targeted delivery

Research on small intestine targeted delivery systems, mainly exploit the pH difference between the stomach and intestine to trigger release. Shellac, alginate, Zein and whey protein are some of the common materials that swell in the pH of the small intestine, to release the AI, and have been used for targeting to small intestine. Chitosan alginate systems have been studied extensively for oral delivery of drugs and nutraceuticals [44, 45]. The problem with this delivery system is that it does not ensure the complete absorption of the AI as the cargo is released almost instantaneously with pH change. In addition, the AI may also undergo loss of bioactivity due to the enzymes present in the intestine [46].

2.4.2. Colon targeted delivery

The advantages of colon targeted delivery systems are that the food passing through the colon has a very long transit time, hence delivering drugs in this part of the gastro intestinal tract increases the residence time of the carrier in the body, thereby resulting in the continuous release and availability of the active ingredient for a long period. Additionally, the colon has very few proteolytic enzymes, making it a choice for delivering proteins and peptides. Some of the systems developed to target drugs and carriers to the colon are, prodrugs, pH dependent or timed release systems intending to deliver in the colon,

microbiome triggered systems and intestinal pressure controlled systems [47]. While the use of pH difference and residence times as a trigger, to tailor the release of the AI from the delivery systems has been studied widely and has been the motivation for the development of commercial products such as Eudragit and Colorcon®, the variation of the pH values and residence times from person to person, depending on body conditions, undermines the use of these colon targeted delivery systems.

An alternative to overcome such variations could be the use of microbiome triggered delivery systems, wherein the enzymes released by the gut flora, trigger the release of AI. The most common examples of such systems are the prodrugs [48]. Another approach is to use polymers that are unaffected in the upper GIT and start degrading in the colon by microbial enzymes. Pectin, Inulin, chitosan, guar gum and resistant starch are some of the materials used for targeting to the large intestine.

Starch, which is a polysaccharide made of glucose is a copolymer of linear amylose and branched amylopectin. Depending on its digestibility, starch has been classified as Rapidly Digestible Starch, Slowly Digestible Starch and Resistant Starch (RS). While the first two types of starch are digested completely in the small intestine albeit at different rates, RS escapes digestion in the small intestine and is digested mainly by the enzymes secreted by the colonic microbiome [49]. This property of resistant starch can be exploited for using it as a carrier targeted to large intestine. While the existence of a form of starch that escapes digestion known as resistant starch (RS) has been known for quite some time now, the use of this material as coating layer is being explored recently [50].

Figure 2.2 Structure of (a) amylose and (b) amylopectin - the two polymers that make up starch. Adapted from [51]

One of the preliminary work on using RS as food grade enteric coating was done by Dimantov et al. where glass beads were used as a model to coat with starch. Though the coating performed well in enzyme dissolution testes, it formed a cracked layer on drying [52]. Recently Situ et al. tested the possibility of coating insulin loaded cellulose micro particles with resistant starch and proved that the coating helped prevent the proteolytic cleavage of insulin in small intestine. The protein retained its bioactivity and the carrier was tested on diabetic rat model leading to a decrease in plasma glucose concentration [53]. Another recent work using commercial resistant starch as an encapsulating material

for folic acid, exemplifies the use of the polymer for use in nutraceutical delivery systems [19]. Patten et al. developed a colon-targeted delivery system for encapsulating long chain polyunsaturated fatty acids from fish oil, using casein and a modified form of resistant starch that is degraded specifically by the colon microbiome. The *in vivo* testing of the delivery system, using ¹⁴C-trilinolenin radiolabeled fish oil loaded into the microspheres of casein and resistant starch, prevented the premature uptake of fish oil in the small intestine and almost 50% of the loaded oil was taken up in the colon [54]. Very little work has been focused in this direction but none as an enteric coating for nanoparticulate delivery system. Hence this serves a good direction to explore for making a food grade polymer based colon targeted enteric coating.

2.5. Nutraceutical candidates for encapsulation

2.5.1. *Withania coagulans*

Withania coagulans is an herb belonging to the family Solanaceae and has been used traditionally in the coagulation of milk to make Indian cheese called paneer. The plant is native to the north western parts of India, some regions in Pakistan and Afghanistan and is known by different names as Paneer doda locally. The plant was originally identified as a substitute for animal source of rennet to make cheese [55-57]. The extract of the dried fruits of this plant called Rishyagandha is reported to have been used in the traditional Indian system of medicine called Ayurveda in the treatment of diabetes. The plant extracts have been proven widely to have numerous beneficial effects – anticancer [58, 59], anti-diabetic [60], anti hyperlipidemic [61], immunosuppressive [62], wound healing [63].

2.5.2. Anti-hyperglycemic effect

Among the many beneficial health effects, the anti-diabetic property of *Withania coagulans* is reported to be the most significant and studied extensively. Early studies on the aqueous and alcoholic extracts of *Withania coagulans* on streptozotocin induced diabetic rats have proven the ability of the both aqueous and alcoholic extracts to induce

hypoglycemia in at dosages as high as 1g/kg and 750mg/kg body weight of the rats respectively [60, 64]. In a study conducted by Datta et al. the hypoglycemic and hypolipidemic effects of the hydro-alcoholic extract of the fruits of *Withania coagulans* was tested on rats in comparison to standard drugs – glipizide and atorvastatin, commonly used in the treatment of the above diseases. It was concluded from this study that the extract acts in synergy with the drug and could help lower the doses of the drugs significantly. Also the histopathological evaluation of the pancreas of the streptozotocin-induced rats, showed a significant difference in the recovery of the beta cells of the untreated and *Withania coagulans* extract treated animals [65]. Loss of peripheral insulin sensitivity and hepatic insulin sensitivity are the most commonly observed forms of insulin resistance in type II diabetes. In a study conducted by Bharti et al. on Poloxamer-induced type 2 diabetic rats, it was shown that the aqueous extract of *Withania coagulans* fruits could normalize hyperglycemia in rats, by overcoming the above mentioned resistance mechanisms, sensitizing the muscle and fat cells to absorb glucose and liver cells to stop gluconeogenesis in response to insulin [66]. Another study by Hoda et al. has shown that the combined aqueous and organic extracts of *Withania coagulans* fruits has better anti-hyperglycemic effect on diabetic rats than those treated with Metformin [67].

In an attempt to reduce the dosage of the extract significantly, Maurya et al. extracted specific withanolide called coagulanolide from the plant *Withania coagulans* and tested the anti-hyperglycemic and anti-hyperlipidemic properties of the isolated compound in doses comparable to standard drugs metformin and fenofibrate on streptozotocin-induced diabetic rats. Coagulanolide was found to be better than metformin at the same dosage [43]. Despite these various reports on the anti-diabetic property of *W.coagulans*, there has not been any *in vitro* study on the effect of these compounds on cell lines, to understand its mechanism of action.

2.5.3. Wound healing effect

A report by Prasad et.al, outlines the wound healing property tested on streptozotocin induced diabetic rat model [63]. The study tracked the rate of wound contraction for 16

days, by studying the effect of hydro alcoholic extract of *W.coagulans* on animal wounds. The study indicates that the extract was able to promote wound healing when administered in the oral form compared to the untreated control. This is the only report on the wound healing activity of the compound, which provided an avenue for testing the effect of the extracts on cells, thereby helping to understand the mechanism by which it functions.

2.5.4. Anti-cancer effect

The anti-proliferative activity of withanolides extracted from *Withania somnifera* has been reported previously [68], while the anti-proliferative or anti- cancer effect of compounds extracted from *W.coagulans* has not been investigated before on any cancer cell lines. A sole work by Haq et al, reports the cancer chemo preventive ability of the compounds extracted from *Withania coagulans*. The study showed that the compounds were able to suppress tumor necrosis factor- α (TNF- α)-induced nuclear factor-kappa B (NF- κ B) activation in murine macrophages, which is responsible for the uncontrolled proliferation of tumor cells [69]. This opens a new area to study, how these compounds affect different cancer cell lines.

2.5.5. Clinical study

Two different short term studies conducted independently on human subjects proved the efficacy and safety of the plant extract in improving the symptoms of patients with type II diabetes [70, 71]. In the study by Alam et al., 60 patients with Type II diabetes were studied with 20 patients in the control group and 40 patients in the test group. The patients received a combination of *Withania coagulans* fruits decant and *Trigonella foenum* powder for 90 days. The patients in the treatment group showed significantly decreased blood glucose levels compared to the patients in the control group [70]. Another study using 53 patients compared the effect of *Withania coagulans* fruit powder individually and in combination with a diabetes drug against the standard drug. It was observed that the fruit powder was able to elicit a response in fasting and post prandial blood glucose levels comparable to that of the standard drug and the combination seemed to have a more pronounced effect than the drug or fruit powder alone [71]. Although long term study, on

a large population taking into consideration different contributing factors is yet to be conducted. The plant itself being used as a food additive to make cheese, does not pose any harmful side effects. But it has a very strong taste and odor that discourages the consumption of the raw extract. Also very high doses of the extract itself is required to achieve an effective treatment dosage in the body. Hence encapsulation of this nutraceutical in a carrier that offers controlled sustained release of the nutrient could offer many advantages over the free extract.

Despite these reports on the therapeutic efficacy of the plant extracts, there has been no report so far on the encapsulation of the extracts of the plant into delivery systems. Gregory et al, have attempted to encapsulate the withanolides from *Withania somnifera* in Polycaprolactone nano carriers [72] in order to increase the solubility and bioavailability. Encapsulation of the extract can help reduce the dosage and also mask the taste of the extract.

2.6. Summary

This review of existing literature thus helps in designing a robust food grade delivery system that can be used to target to different parts of the GIT. Chitosan has been identified as suitable material for the nano encapsulation of the AI. Based on the methods available, the chnp would be fabricated using electrospraying which is a mild technique that does not use any toxic chemicals. Starch has been identified as the biopolymer to be used as the coating over chnp (Figure 2.4).

Gelatinization of starch by moist heat treatment leads to the breakdown of starch granules and leaching of amylose. This amylose when subjected to a temperature of 2–4 °C for 24 hours undergoes retrogradation, which alters the rate of starch digestion in the body [49]. Retrogradation, is the realignment of the polymeric chains in gelatinized starch upon cooling [73]. Starch acquires crystallinity on retrogradation which offers gastric protection [50]. But depending on its native source, it is converted to slowly digestible starch which enables it to be digested in SIF over time. Hylon VII which has a high amylose content, when modified in this manner, is converted to resistant starch that is degraded only by the

colonic microbiome and remains unaffected in the upper part of the GIT. Thus, subjecting two different types of starch to retrogradation enables it to be protected in the stomach, but selectively degraded either in the small intestine or large intestine depending on its native susceptibility.

Some preliminary work done by Dimantov et al. and Meneguín et al. exemplify the ability of retrograded RS to retard gastric release [50, 52]. Very little work, however, has since been focused on such research and none, so far, as an enteric coating for particulate delivery systems of the nano-sized range. Based on this hypothesis, retrograded soluble starch (SS) (susceptible to digestion by amylases in the small intestine) and retrograded high amylose corn starch, Hylon VII (RS) (susceptible to digestion by enzymes in the colon) would be used as stimuli-responsive, controlled releasing coatings for gastric protection and targeted delivery of nanoparticles.

Figure 2.3 Changes in native starch granules upon gelatinization and retrogradation. Adapted from [73]. Reproduced with permission from John Wiley and Sons.

Compounds extracted from *Withania coagulans* was chosen as the nutraceutical to be loaded into the oral delivery system. Two main reasons for choosing this were:

1. The compounds from *Withania coagulans* have so far never been encapsulated into any polymeric carrier and the feasibility of encapsulating it, could be tested.
2. The therapeutic properties of the compounds: anti-diabetic and anti-cancer effects, suited well to exemplify the working of the oral delivery system, as a small

intestine targeted (systemic effect – anti-diabetic property) or large intestine targeted delivery system (local effect – anti-cancer property).

Figure 2.4 Design strategy for the food grade carrier

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Chapter 3

Experimental Methodology

This chapter outlines the materials and experimental methodologies used in this study. For each experiment, the rationale behind choosing the particular method of experimentation is explained. The chapter begins with the explanation of the methodologies used for the fabrication of chnp and the techniques used for forming a gastro resistant coating on these nanoparticles. This is followed by the enumeration of the characterization techniques used to analyze the carrier platform. The second part of the chapter deals with the extraction procedure and characterization techniques used for analyzing the nutraceutical. The last part of the chapter focuses on the in vitro experiments carried out, cell lines used and the various assays performed to quantify the behavior of the extracts and understand the endpoint results, followed by the methodology for in vivo studies on diabetic mice.

3.1. Carrier fabrication

3.1.1. Electrospraying

Electrospraying is a technique which uses electric field to disperse the polymer solution into very fine droplets. A high energy positive charge is applied to a metallic tip through which the polymer solution is flowing and forms a sphere at the tip, due to surface tension. The electric field applied to the tip then draws the spherical liquid into a cone and depending on the strength of the applied field, viscosity and flow rate of the solution, the liquid is either drawn into fibers or sprayed as droplets which can be collected on a negatively charged grounded collector [1]. **Error! Reference source not found.** Figure 3.1 shows a typical electrospray setup. In this study, Chnp was fabricated using the NANON electrospinning setup because of its ability produce particles with lesser poly dispersity, higher yield in terms of the starting material used and better encapsulation efficiency compared to the ionic gelation method. A wet electrospraying technique, where the sprayed particles were collected in a liquid bath containing the cross-linker for chitosan was followed. The cross linker used was Sodium Tripolyphosphate (TPP) obtained from Sigma. Low molecular weight chitosan ($\geq 75\%$ deacetylated, viscosity 20–300 cps, molecular weight 50–190 kDa), used in this study was also obtained from Sigma. The parameters, such as voltage, working distance, and flow rate were fixed, while chitosan solution concentration (1% and 2%) (w/v), acetic acid concentration (10%, 30%, 50%, 70%, and 90%) (v/v), and TPP concentration (1%, 2.5%, and 5%) (w/v) were varied and optimized to obtain particles in the desired size range [2]. The electrosprayed particles were collected by centrifugation at 17,000 g for 5 min, washed thrice, and stored after freeze drying for further use.

Figure 3.1 Schematic depicting the electrospray process. Reproduced with permission from Elsevier. [1]**Starch coating**

Two different types of starch were used for the coating process. Readily digestible starch, obtained from potato was used for targeting to small intestine and was designated as SS. High amylose corn starch Hylon VII® (Ingredion) was used as the resistant starch designated as RS. The inherent negative charge of the starches, due to the presence of phosphate groups, was exploited for the coating process. This was utilized for coating it over the positively charged chnp by means of electrostatic interaction. SS at a concentration of 5 mg/mL was gelatinized by heating the starch solution up to 100°C. Similarly for dissolution, RS at a concentration of 5 mg/mL was subjected to three heating cycles to be dissolved completely [3]. First, it was heated at 100°C for 45 minutes and then under pressure in an autoclave at 130°C for 30 minutes followed by boiling at 100 °C for another 30 minutes. The volume of the solution was then made up to the initial volume such that the concentration of starch in the solution remains to be 5 mg/mL. The nanoparticle suspension at a concentration of 2 mg/mL was added dropwise to the starch solution stirred at 200 rpm. Chnp and starch were allowed to react for 1 hour to form the electrostatic interactions. The as-formed particles were collected by centrifugation at 8000 g for 7 minutes. The particles were cross-linked by suspending in 1% sodium trimetaphosphate (STMP, Sigma) for 1 hour. The starch-coated chnp were stored overnight at 4°C to allow for the retrogradation of starch. The particles were then collected by centrifugation and stored after freeze drying for further use.

3.2. Characterization of the carrier platform

3.2.1. Electron microscopy

Electron microscopy was used as a tool to visualize the particles, the surface morphology, shape and size of the particles. As the particles were in the size range of 150-200 nm, field emission scanning electron microscope (FESEM) was used in order to achieve high resolution images at the size range desired. For the starch coated chnp, since the size was above 1 μm , scanning electron microscope (SEM) was sufficient to view the particles. The scanning electron microscope uses a focused beam of electrons that interact with the sample surface, producing signals, from which the information on topography or composition of the sample is obtained. In a typical SEM used in this study, a thermionic tungsten filament is used to generate an electron beam thermionically, which are then focused onto the sample by means of condenser lenses. Depending on the energy of the electron beam, the interaction volume varies, which in turn decides the type of signal produced: secondary electrons (topographical information), back scattered electrons (information on phase differences), or characteristic x-rays (information on atomic composition). A variation of the thermionic gun is the field emission gun which produces highly focused electron beams, that offer better signal to noise ratio enabling greater resolutions at higher magnifications.

FESEM (JSM 6340F) was used to determine the surface morphology and size of the particles. In order to make the samples conductive for imaging, the samples mounted onto carbon tape, were sputter coated with platinum using platinum fine coater for 20 seconds and 35 seconds at 30mA for drop cast liquid samples and powder samples respectively. For imaging powder samples, a small amount of the sample was dispersed uniformly onto the carbon tape and coated with platinum at the above-mentioned specifications. For imaging individual chnp, the freeze-dried particles were suspended in ethanol and sonicated using a probe sonicator. This suspension was drop cast onto silicon wafer, air dried and then

coated with platinum. The electron micrographs were processed using ImageJ software to calculate the size of the particles. The imaging was done at 5kV and at a working distance of 8mm. The starch coated samples were imaged using SEM (JEOL 6360), as they were of a bigger size. The powder sample was dispersed on carbon tape, coated with gold at imaged at 5kV with working distance of 20mm. Electron microscopy experiments were carried out at the Facility for Analysis Characterization Testing and Simulation (FACTS) at NTU.

3.2.2. Dynamic Light Scattering

Dynamic light scattering (DLS) is an optical technique in which the scattering of light by the particles in the suspension is used to obtain information on the hydrodynamic radius or the size distribution of the particles in suspension. It is based on the principle that particles in solution undergo Brownian motion and when a monochromatic beam of light such as Laser is shone on the particles, the light interacts with the particles either constructively or destructively, depending on their size, state of motion and distance from one another. This interference leads to a change in the wave pattern which can then be analyzed using the photon correlation function to determine the decay time, which in turn can be used in the Stokes-Einstein equation (Equation 3.1) to determine the particle diameter. The instrument does the same for multiple particles at different instances and the software computes the size distribution.

Equation 3.1 Stokes-Einstein relation

$$D_h = k_B T / 3\pi\eta D_t$$

Figure 3.2 Working principle of Dynamic Light Scattering technique [4]

The size distribution of the chnp were characterized with Malvern Zetasizer (Zetasizer Nano ZS) using Dynamic Light Scattering technique, as the technique offers a quick measure of the overall size distribution of the nanoparticle suspension based on the principle above. The samples were checked for size distribution and polydispersity index. A disposable cuvette was used to measure size distribution. Each Sample was measured in three runs with every run measured twenty times each. The data reported is the average of three samples measured as above.

3.2.3. Laser Doppler Electrophoresis

This technique is used for measuring the surface charge or zeta potential of a nanoparticle in suspension, in combination with a convergent beam of light as in dynamic light scattering. When charged nanoparticles in suspension are subjected to an applied electric field, the particles move due to the interaction between two charged particles and also in response to the applied field. The direction and velocity of the particle is dependent on the

particle charge and the electric field. This movement causes a doppler shift in the light that is being scattered by the particles in motion, which can be used to determine the velocity of the particles and in turn the surface zeta potential.

Zeta potential measurements were performed using the Malvern Zetasizer Nano ZS. Samples were placed in a disposable folded capillary cell to measure for zeta potential. Each Sample was measured in three runs with every run measured twenty times each. The data reported is the average of three samples measured as above. The instrument was also used to check the surface charge of the particles after coating them with starch to confirm the formation of a coating layer.

Figure 3.3 Principle of zeta potential measurement [5]

3.2.4. Confocal microscopy

Confocal Laser Scanning Microscope (CLSM) is an optical microscopy technique that offers better resolution compared to a fluorescent microscope. In principle, sample that is capable of producing a fluorescent signal is illuminated using a coherent source of light

such as Laser. The light passes through a pinhole aperture and is focused on the sample. The fluorescence from the sample also reached the detector through a pinhole aperture placed in from of it. This eliminates all rays that are out of the plane and collects only the confocal rays, thereby eliminating secondary fluorescence usually observed in wide field microscope. This results in increased resolution of the images obtained. CLSM was used in this study, as a means of confirming the coating of starch over chnp. For this, fluorescently tagged chitosan and starch were used to fabricate the starch coated chitosan particles. The particles were then observed under confocal microscope to visualize the two layers. The improved resolution of the microscopic technique greatly helped to confirm the presence of a coating layer. Confocal microscopy was done at the Advanced Biofilm Imaging Facility, SCELSE).

Fluorescently tagged polymers were used so that they could help visualize the presence of core shell morphology under the confocal laser scanning microscope (CLSM). Chitosan was labeled with rhodamine B, starch and RS were tagged with fluorescein isothiocyanate (FITC) following the reported protocols [6, 7] . Starch was stained with FITC by incubating the particles with a solution of FITC followed by washing to remove excess dye. The florescent tagged polymers were used to prepare the particles with same procedure outlined above for nanoparticle fabrication and enteric coating. For imaging, about 10 μ L of the particle suspension was dropped on to a glass slide covered with a cover slip and imaged using Zeiss LSM confocal laser scanning microscope (Carl Zeiss, Germany) with a 20X objective. For FITC labelled polymers, the excitation peak was centered at 488 nm and emission peak wavelength of 519 nm. For rhodamine tagged polymers, the excitation and emission peaks were at 553 and 627 nm respectively. Images were processed and analyzed using the Zen lite software. Since the resolution of the optical microscope was limited to 200 nm, the chitosan nanoparticle and a layer of thin coating on the particle could not be visualized distinctively using the confocal microscope. Hence chitosan micro particles were fabricated using the same protocol as above for chnp, but using a higher concentration of chitosan. The as formed micro particles were coated with starch and imaged by confocal microscopy incorporating fluorescent polymers in the fabrication process.

CLSM was also used to cellular uptake of chnp. For this, Caco2 cells were cultured on cover glass coated with gelatin in 6-well plates at a cell density of 10^5 cells/well and incubated for 2 h. After the incubation period, the nanoparticles were removed, the cells were washed with PBS and fixed using 3.7 % formaldehyde for 30 min. Cell nuclei was stained using DAPI and imaged using confocal microscope [8].

3.3. Extraction and characterization of withanolides from plant source

Withanolides were extracted from the plant *Withania coagulans* sourced from an herb supplier in India. The extraction process was performed according to previously reported procedure with slight modifications [9]. A known weight of the berries from the plant were crushed mechanically using pestle and mortar and the coarse powder obtained was soaked in a 1:1 mixture of water and ethanol for 24 hours. The extract was collected by filtering out the coarse powder using Whatman filter paper. The hydro alcoholic extract was concentrated using a rotary evaporator. An aliquot of the concentrated extract was mixed with a 1:1 mixture of water and ethyl acetate and extracted to obtain a water soluble fraction and ethyl acetate soluble fraction. The ethyl acetate soluble fraction was subjected to column chromatography over silica gel (230-400 mesh size) using a mixture of chloroform and methanol as the mobile phase. The fraction collected from this column was assigned as P2. The water soluble fraction was concentrated using rotary evaporator and subjected to column chromatography over silica gel (230-400 mesh size) using a mixture of water and methanol as the mobile phase. The fraction collected from this column was assigned as P4. When P2 and P4 are referred to collectively, it is called as coagulans

3.3.1. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier-Transform Infrared (FTIR) spectroscopy is an absorption spectroscopy technique which uses the interaction between infrared (IR) radiation interaction and the chemical bonds in molecules, to understand the structure of the molecule (Figure 3.4). When IR radiation interacts with a molecule, the molecule absorbs energy and the chemical bonds

in the material undergo different kinds of vibration characteristic of the bond type. The detector in the FTIR instrument recognizes this absorption corresponding to different wavelength or wave numbers and records it as a spectrum with transmittance in the y-axis and wave number in the x-axis. This spectrum can be used to identify characteristic chemical groups present in the materials and identify them qualitatively.

Figure 3.4 Basic construction of a FTIR spectrometer [10]. Reproduced with permission from Springer Nature.

Perkin Elmer® FTIR- Frontier [Perkin Elmer Inc., Shelton, USA] was used to characterize the extracts. Sample preparation involved mixing the extract with potassium bromide (KBr) in the ratio of 1:100 and pelletizing using a hydraulic press, the samples were analyzed in the range of 600-4000 cm^{-1} at 32 scans per sample.

3.3.2. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is an analytical technique used to determine the structure of molecules. When the spinning nucleus of an atom is subjected to a magnetic field, the nuclei undergo an energy transfer and undergoes precision motion. When a resonant radio frequency is used to excited such a spinning nucleus, it generates radio waves that are recorded by the detector. The resonant frequency, energy of the radiation absorbed, and the intensity of the signal are proportional to the strength of the magnetic field. Hydrogen atom is the most

common nucleus used for excitation. The resonant frequency of the nuclei is dependent on the functional groups surrounding the atom. Based on the chemical shifts reported, information on the structure of the molecule can be obtained.

The samples were characterized using ^1H NMR. NMR spectra were recorded on a (Bruker) AV400 MHz spectrometer, using DMSO- d_6 & D $_2$ O as solvents with TMS as internal reference.

Figure 3.5 Basic arrangement of a NMR spectrophotometer [11]

3.3.3. MALDI-TOF

Mass spectrometry is an important characterization tool to evaluate the molecular weight of any unknown sample or to characterize a sample with a mixture of compounds having different molecular weights. MALDI (Matrix Assisted Laser Desorption Ionization) is a soft ionization technique, wherein the sample is mixed with a matrix compound that can absorb UV light (nitrogen LASER light at 334 nm) and ionize the analyte molecule as a whole. The analyte molecule is detected based on the Time of flight (TOF) i.e. the time the analyte molecules take to reach the detector, depending on the mass to charge ratio. The sample to be analyzed is mixed with the analyte molecule and allowed to crystallize. Upon evaporation of the liquid, the sample consists of dry crystals of the sample mixed with the

matrix. When this mixture is irradiated with laser, the matrix assists in volatilization and ionization of the samples. These charged ions are then accelerated towards the detector due to the potential difference applied between the sample slide and the ground. Lighter ions with a smaller m/z value and more highly charged ions move faster through the tube until they reach the detector. As such, depending upon the mass-to-charge ratio (m/z) value of ions, the time of flight differs accordingly from ions to ions. Thus, the time-of-flight (TOF) is directly proportional to the mass of the molecule and has mass resolution of approximately 0.03%. Based on the TOF, the software computes the m/z value and chromatogram is generated by presenting intensity (y-axis) versus m/z (x-axis).

The rationale behind using this characterization technique is to identify the different compounds, by means of identifying their molecular weights, in the two fractions P2 and P4. P2 dissolved in methanol and P4 dissolved in water were added to the analyte plate and mixed with the matrix molecule. The samples once dry were analyzed using AXIMA performance MALDI-TOF.

Figure 3.6 Working principle of a mass spectrometer [12]

3.3.4. High Performance Liquid Chromatography (HPLC)

HPLC is an analytical chromatographic technique that involves the separation, identification and quantification of molecules in a mixture. The basic principle of the technique involves eluting a liquid solvent (mobile phase) of desired polarity, through a tightly packed adsorbent column, into which the sample is injected. Depending on the competitive affinity of the sample to the column or to the mobile phase, it is eluted out of the column into the detector which measures the absorbance of the molecule reaching it at that particular time. The output is displayed as a peak with intensities depending on the concentration of the analyte molecule at a retention time specific for each molecule in the sample. The concentration of the analyte can be quantified by computing the peak area and comparing with a calibration curve. HPLC was used in this study to quantify the amount of P2 and P4 encapsulated into the delivery system and also to evaluate the release kinetics of these fractions from the carrier.

Concentration of P2 in the supernatant was evaluated using HPLC (Shimadzu Prominence UFLC with a photo diode array detector SPD-M20A, Ascentis C18 column (10cm, 2.1cm I.D and 5 μ m) with methanol-water (3:2) as the mobile phase at a flow rate of 0.4 mL/min, maintained at 30⁰C. Suitable standards were prepared in methanol to obtain a calibration curve. Similarly, for P4, the mobile phase used methanol-water (1:1), at a flow rate of 0.2 mL/min. HPLC was performed at the Analytical Instrument Facility at SCELSE.

3.3.5. Encapsulation efficiency and Release kinetics studies

The encapsulation efficiency (EE) of both P2 and P4 were calculated using an indirect method by measuring the amount of extract in the supernatant from which the particles were collected. Concentration of coagulans in the supernatant was evaluated using HPLC as described above. The EE was then calculated based on Equation 3.2

Equation 3.2 Formula for calculating encapsulation efficiency

$$EE = \frac{\text{Initial amount of drug} - \text{Amount of drug in supernatant}}{\text{Initial amount of drug}} * 100 \%$$

Release kinetics of P2 was studied from bare chnp and RS-coated chnp (C+RS) and for P4 from bare chnp and soluble starch coated chnp (C+S), sequentially under three different conditions – SGF, SIF and SCF in order to mimic *in vivo* conditions. The SGF and SIF were prepared according to the US Pharmacopeia guidelines [13] and SCF was prepared by adding 2 mg mL⁻¹ lysozyme and 20 µL/mL α-amylase from *Bacillus licheniformis* to phosphate buffered saline (PBS) [14, 15]. P2 release studies were done in SGF, SIF and SCF with 0.1% tween 20 added to it. pH 4.4 medium was prepared by adjusting the pH of PBS to 4.4 using 0.1N HCl.

All release studies were performed by suspending the particles in the release media at 37°C in an incubator fitted with a rotating wheel at a speed of 20 rpm over a period of 48 h. The time frames in each of the simulated fluids were chosen to mimic the residence times of food in each region of the GIT – stomach (2 h), small intestine (3 h) and large intestine (43 h). The release was carried out in SGF for the first 2 h, followed by replacing the release medium to SIF for 3 h and then to SCF until the completion of the study. For the C+S particles, as the enteric coating is intended to break down in the small intestine and chnp taken up by enterocytes, the particles were transferred to a pH 4.4 buffer (PBS adjusted to pH 4.4) after SIF, to mimic the conditions after cell uptake. Aliquots were drawn from the release medium at fixed time points and for each time point, the amount of release medium withdrawn was replaced with fresh medium to maintain sink conditions. For P2, the nutraceutical in the aliquots had to be extracted from the simulated GIT fluids into methanol in order to be evaluated by HPLC. This was done by extracting it into Dichloromethane (DCM), which was then evaporated and re-suspended in methanol. The samples thus prepared were analyzed using HPLC as mentioned in section 3.3.4. For P4, the release media collected at each time was freeze dried and suspended in the mobile phase and analyzed using HPLC.

3.4. Cell Culture

Four different cell types were used in this study to understand the function of the coagulans extracts *in vitro*. The media used for each cell line is denoted in **Error! Reference source not found.** All cells were cultured in a 37°C incubator maintained at 5% CO₂ condition.

Table 3.1 Cell lines used and their culture conditions

Cell line	Cell type	Media
Caco2 (colorectal adenocarcinoma cells)	Human colon epithelial cells	DMEM supplemented with 10% FBS, 1% penicillin streptomycin solution, and 1% NEAA
CRL 1831 (normal colon cells)	Human colon epithelial cells	DMEM/F-12 supplemented with 10% FBS and 1% penicillin streptomycin
HDF (normal skin cells)	Human dermal fibroblast cells	DMEM supplemented with 10% FBS and 1% penicillin streptomycin solution,
MIN6 (Pancreatic beta cells)	Mouse epithelial cells from the pancreas	DMEM supplemented with 10% FBS and 0.001% βmercaptoethanol

3.4.1. Cell viability assay

In order to evaluate the toxicity of the extracts and also its ability to kill cancer cells, the percentage of cells that are actively viable, needs to be estimated. Commercially available cell viability assays use tetrazolium salt, which is reduced by metabolically active cells to generate a colored formazan dye. These dyes are non-toxic to the cells. CCK-8 is one such assay that uses WST-8 to generate a colorimetric reaction. The mechanism of action of the

assay is illustrated in Figure 3.7. The end result is absorbance measured at 450 nm. % cell viability is calculated based on Equation 3.3 after subtracting the absorbance value of the blank media.

For all toxicity and cell viability assays, cells were seeded onto 96 well plate at a cell density of 10^4 cells per well, with 5 replicates. The cells reached confluency after 24 hours. To begin with, the media is removed after 24 hours and fresh media was added to all wells. The extracts whose toxicity is desired to be tested, was added as per the required concentrations intended to be tested. After the stipulated time points (2, 24 or 48 hours), the media containing the coagulans extracts was removed and replaced with fresh media. To this, 10 μ l of the CCK8 assay reagent (Dojindo Molecular Technologies, Inc) was added and incubated at 37°C for 2 hours. The absorbance was measured at 450nm and the cell viability was calculated.

Figure 3.7 Principle of cell viability detection using CCK-8 assay [16]

Equation 3.3 Formula for % cell viability calculation

$$\% \text{ cell viability} = \left(\frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \right) * 100$$

3.4.2. Glucose stimulated insulin secretion assay (GSIS)

For the insulin release study, cells were seeded on 6 well plates at the density of 1×10^5 cells per well and studied using the Glucose stimulated Insulin Secretion assay (GSIS). Briefly, once the cells reach confluency after 48 hours, DMEM was removed, the cells were washed and starved by incubating in Ca-10 buffer with 0.5mM glucose. After 2 hours starvation, the buffer was removed and the cells were incubated for another 30 minutes in Ca-10 buffer with 3.3 mM glucose and the samples were collected to be analyzed by ELISA for the insulin secreted under low glucose condition. After this 30 minute starvation, the cells were stimulated by treating with Ca-10 buffer containing 16.7 mM glucose and the P4 treatments were added. The samples from the wells were collected to be analyzed by ELISA for insulin secretion under the stimulated condition in the presence or absence of P4. Insulin index was calculated using the Equation 3.4. For studying the effect of P4 encapsulated particles, on MIN6 cells, the particles were subjected to different simulated fluids and the release media collected at each time point, was added to the cells after 100 times dilution and GSIS assay similar to that of free P4 was carried out.

Equation 3.4 Formula used to calculate insulin index

$$\text{Insulin index} = \frac{\text{Amount of insulin secreted in high glucose condition}}{\text{Amount of glucose secreted in low glucose condition}}$$

3.4.3. ELISA

Enzyme-linked immunosorbent assay abbreviated as (ELISA) is a diagnostic tool used to detect a particular substance using antibodies specific to that substance and an enzyme that produces a colored reaction, in order to quantify the substance. The insulin ELISA kit from Merckodia, used in this study for insulin detection, works on the principle of sandwich ELISA. The schematic of the process is depicted in Figure 3.8. Briefly, the primary monoclonal antibody specific for one of the antigenic determinants on the insulin molecule, is coated onto the micro titration wells. Insulin in the sample is then bound to this antibody.

Another specific antibody for the second antigenic determinant on the insulin molecule is added, thereby sandwiching the molecule between the antibodies. A secondary antibody conjugated to an enzyme is then added. The enzyme then reacts with a substrate to give a colored product, which is read as absorbance signal and used to quantify the insulin in the sample based on the calibration curve.

Figure 3.8 Schematic showing the steps involved in sandwich ELISA [17]

3.4.4. Flow cytometry

The basic working of a flow cytometer, involves a flow cell which allows for stream lining of the cells using a sheath fluid and exposing each cell to the Laser source. The resulting fluorescence from the cells are recorded on a detector and data are represented based on fluorescence intensity with respect to the forward scatter or side scatter. The equipment allows for multiparametric analysis of thousands of cells per second. Flow cytometry was used in this study to quantify the uptake of fluorescent chnp by Caco2 cells.

For cytotoxicity studies, cells were seeded on a 96-well plate at a density of 10^4 cells/well. Cells reached confluence after 48 h. The cells were then incubated with chnp for 2, 4, 6 and 24 h after which the nanoparticles were removed and cell viability assessed using CCK-8 kit. For cell uptake studies, cells were seeded in a 6-well plate at a cell density of 10^5 cells/well. The cells reached confluence after three days. Fluorescent chnp prepared by loading coumarin 343 was used for the cell uptake studies. Coumarin 343 was loaded into chnp using cyclodextrin to load a hydrophobic molecule in chnp [18]. Briefly 10 mg/mL cyclodextrin was dissolved into chitosan solution and then 0.2 mg/mL of coumarin 343 was added to this solution. The dye was solubilized due to the presence of cyclodextrin.

This chitosan solution was then used to fabricate chnp as mentioned in section 3.1.1. Fluorescent chnp at a concentration of 0.5 mg/mL was added to the wells and incubated for 2, 4 and 6 h. After the incubation period, the nanoparticles were removed, the cells washed thrice with PBS and then collected by trypsinization for analysis by flow cytometry (BD accuri C6 flow cytometer, SCELSE).

Figure 3.9 Basic working of a flow cytometer [19]

3.5. *In vivo* studies

The effect of the two fractions, P2 and P4 were tested on ICR mice induced with diabetes using alloxan. Male ICR mice, aged 5 weeks were obtained from Nomura Siam International, Thailand. On arrival, the animals were housed in stainless steel cages (2 mice

per cage) at the Laboratory Animal Facility, Suranaree University of Technology. After acclimatization for a week, the mice were induced with diabetes by peritoneal injection of alloxan (120 mg/kg), based on the protocol adapted from Alam et.al. [20]. Food and water were given *ad libitum*. Fasting blood glucose (FBG) levels were monitored after 3 days of alloxan injection. The animals with FBG ≥ 250 mg/dL were used for the study. In order to keep the initial FBG levels same across the groups, the animals were grouped such that the average of the FBG in each group was not statistically different across the groups. For the initial study, animals were divided into different treatment groups and treated with P2 or P4, with deionized water and glibenclamide being used as negative and positive controls respectively. The extracts P2 and P4 were dissolved in its respective solvents, DMSO and water and was fed by oral gavage every day for the entire experiment period. The body weight and fasting blood glucose was monitored on day 0 at the start of the experiment and at day 5 at the end of the experiment. A set of control animals that were not induced with diabetes also received similar treatments over 15 days, to assess for any toxicity of the fractions.

For studying the effect of encapsulating the extracts into the carrier system, chnp, C+S and C+RS particles loaded with P4 were prepared and fed at the concentration to match the 50mg/kg every day, calculated based on encapsulation efficiency. The particles were suspended in DI water and fed to the animals by oral gavage. The animals were grouped as in Table 3.2. The *in vivo* experiments were carried out at Suranaree University of Technology, Thailand. All procedures were approved and conducted following the guidelines of the Institutional Animal Care and Use Committee, Suranaree University of Technology (B 2559/00020.001 (SUT Laboratory Animal Facility), U1-01433-2558 (Investigator)).

The major limitations of the alloxan induced diabetes model is high mortality if severe diabetes is induced or auto-reversion to normoglycemic state, due to regeneration of β cells if the diabetes is mild [21, 22]. To overcome this limitation, the animals were grouped such that each group had animals with a gradient of blood glucose from mildly diabetic to severely diabetic. In addition, each group consisted of 5 animals to begin with, to allow for

deaths of any severely diabetic animals and data from three animals were alone chosen for the analysis. The study was limited to only 10 days, as chances of auto-reversal to normoglycemic state was high beyond this time.

Table 3.2 Grouping of animals for in vivo study

Normal +DI	Non-diabetic mice fed deionized water
Normal+DMSO	Non-diabetic mice fed DMSO
Normal+P2 50mg/Kg	Non-diabetic mice fed P2
Normal +P4 50mg/Kg	Non-diabetic mice fed P4
DM+DI	Diabetic mice fed deionized water
DM+DMSO	Diabetic mice fed DMSO
DM+P2 50mg/Kg	Diabetic mice fed P2
DM+P4 50mg/Kg	Diabetic mice fed P4
DM+glibenclemide 20 mg/Kg	Diabetic mice fed Glibenclemide
DM+chnp	Diabetic mice fed chnp
DM+ C+S	Diabetic mice fed C+S particles
DM+ C+RS	Diabetic mice fed C+RS particles

3.6. Statistical analysis

All experiments were carried out in triplicates and data were expressed as mean $\pm$ standard deviation. Comparisons between test groups were made with one-way ANOVA, followed by Tukey's HSD post hoc test, on Origin Pro 9.0 software.

All experiments were carried out at the School of Materials Science and Engineering, NTU, except where mentioned otherwise.

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Chapter 4

Carrier fabrication and characterization

The chapter focuses on the fabrication of an oral delivery system using food grade polymers and its characterization. The first part of the chapter discusses the results on the fabrication of chnp using electrospraying. The particles were characterized using DLS and FESEM and was found to have a diameter of around 175 nm with a positive zeta potential of +20mV. The coating of starch on chnp are described subsequently and the coated particles were characterized using confocal microscopy to confirm the presence of a coating layer. The second part of the chapter discusses the results on the evaluation of the functionality of starch as a gastro protective coating by comparing it with other commercial polymers. The chapter ends with the results on the cellular uptake study of chnp.

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4.1. Fabrication and characterization of chnp

Chnp, in this study, were fabricated using electrospraying as outlined in section 3.1. The basic working parameters were adapted from the work by Arya et.al, with the voltage set at 25 kV, flow rate at 0.2 mL/h, the working distance at 7 cm, and needle gauge size as 25G [1]. Optimization was done for the acetic acid concentration, chitosan concentration and collector solution. Since viscosity plays an important role in electrospraying, two different concentrations of chitosan solutions at varying acetic acid concentrations, were used for optimization studies. Above 2% (w/v) concentration, the solution was too viscous to obtain nanoparticles, and below 1% (w/v) concentration, the formation of a stable jet was impaired. As 2% (w/v) chitosan solution, led to the formation of particles larger than 500 nm, the chitosan concentration was subsequently fixed at 1% (w/v). At lower acetic acid concentrations, the conductivity of the solution was very poor and the formation of a stable jet was impaired. It was possible to electrospray only for concentrations above 10% (v/v) acetic acid concentrations. At 10 and 30% (v/v) acetic acid concentration, the droplets formed were not distinct, due to the lower surface charge, resulting in conjoined or aggregated particles. At concentrations greater than 50% (v/v), i.e. at 70 and 90% (v/v), the increased surface charge resulted in the formation of beaded fibers, leading to the formation of polymer patches rather than nanoparticles on the collector. At a concentration of 50% (v/v), the droplets were well defined and could be collected as individual beads on the collector. The final acetic acid concentration was fixed at 50% (v/v).

The composition of the collector solution is an important determinant to get a powdered sample, in the wet electrospraying method used here. The collector also acts as the cross-linker for chitosan. The cross-linker used for chitosan was TPP. The zeta potential of the particles was influenced by pH and concentration of the collector. Any concentration of TPP above 1% (w/v), resulted in negative zeta potential of chnp due to complete coverage of the amine groups on chitosan. Also electrospraying using this TPP concentration, led to film formation due to its inherent surface tension. In order to overcome the surface tension and disperse the particles while they were being sprayed, 0.5% (w/v) Tween 80 was added to TPP. This resulted in well dispersed particles with a zeta potential of +20.8 mV and a

hydrodynamic size of 175.8 ± 37.4 nm (Table 4.1) with a polydispersity index of 0.205. Figure 4.2 (a-d) shows the FESEM images of the chnp at different acetic acid concentrations. Figure 4.2 (e & f) shows the FESEM images of particles obtained using the optimized parameters as reflected in Figure 4.1.

Figure 4.1 Graphic showing the optimized parameters used for electrospaying of chnp. The optimized parameters are indicated in bold fonts. Reproduced with permission from John Wiley and Sons.

Figure 4.2 Field-emission scanning electron microscopic images of chnp. Electrosprayed chnp at different acetic acid concentrations: a) 30%, b) 50%, c) 70%, and d) 90%. Electrosprayed chnp with optimized parameters collected on e) aluminum foil, f) 1% TPP solution with 0.5% Tween 80. Particles were collected from solution by centrifugation at 15,000 rpm, freeze dried, and imaged by dispersing in ethanol and drop casting on silicon wafer. Reproduced with permission from John Wiley and Sons.

Table 4.1 Zeta potential and size of Chnp, C+S and C+RS measured using DLS

	Zeta potential [mV]	Hydrodynamic size - diameter [nm]
Chnp	+20.8±3.01	175.8±37.4
C+S	-16.66±1.65	6534±1760
C+RS	-7.15±1.87	5862±910

4.2. Starch coating on chnp and characterization

The electrostatic interaction between chnp and starch was used as basis to achieve a polysaccharide coating on chnp. The starch granules in SS and RS has a negative charge due to the presence of native phosphate groups [2] This was exploited to form a starch coating on chnp. The formation of a coating layer was confirmed by the inversion of positive zeta potential of chnp to a negative charge on the starch coated particles as shown in Table 4.1 and also by an increase in particle size (Figure 4.3). The particles, as they are made from hydrogels, tend to aggregate, and need to be sonicated to achieve individual particles. The hydrodynamic size of the particles measured from DLS was 6534±1760 nm for C+S and 5862±910 nm for C+RS. There was a variation in the size of the particles when coated with starchFSS or RS, as clusters of chnp were coated with starch rather than individual particle being coated separately. This was also evident in the size distribution of the particles, with the polydispersity index ranging from 0.5 to 0.8.

The formation of a coating on chnp was confirmed by confocal microscopy using fluorescently tagged polymers. However, the resolution of the optical microscope was limited in visualizing the distinctive core shell morphology of the C+S or C+RS. Hence to confirm the coating of starch on chitosan, chitosan microparticles coated with starch were used. For this, chitosan microparticles were fabricated using the technique similar to that

of chnp but varying the concentration of the chitosan solution alone and subsequently coated with starch. Our rationale is that if the layers could be visualized on the microparticles, then they should be similarly present on the nanoparticles that were also fabricated by the same method. Figure 4.4 shows the distinctive presence of fluorescent Rhodamine B tagged chitosan microparticles and a layer of FITC tagged starch hydrogel surrounding the particle. Based on the collective data from DLS, FESEM and fluorescence microscope, the starch coating on chitosan is conclusively evident.

Figure 4.3 Scanning electron microscopic images of (a) starch coated chnp (b) RS coated chnp. Reproduced with permission from John Wiley and Sons.

Figure 4.4 CLSM images of starch coated chnp showing the chitosan core and starch shell. (a) C+S (b) C+RS. The left panel shows the rhodamine tagged chitosan, middle panel shows the FITC tagged starch and the right panel shows the merge of the two. Reproduced with permission from John Wiley and Sons.

4.3. Evaluation of starch as a protective coating

In order to evaluate the efficacy of starch as an enteric coating, films made of SS and RS were prepared by casting them in a petri dish after dissolution and retrogradation. The films were dried overnight at 37°C. The starch films, were suspended in Simulated Gastric Fluid (SGF), Simulated Intestinal Fluid (SIF) and Simulated Colonic Fluid (SCF) for 2, 4 and 6 h each and the % weight loss was calculated using the formula below:

$$\% \text{ weight loss} = \frac{\text{Initial dry wt of the film} - \text{final dry wt of the film}}{\text{Initial dry wt of the film}} * 100\%$$

The study was done to assess the ability of the starch films to withstand the conditions in the stomach and to test its susceptibility to the conditions in the small and large intestines over different time intervals, in comparison to the commercial enteric polymers. Films of Eudragit L100 (EL100) and Eudragit S100 (ES100) were prepared by solvent casting and was subject to the same treatment as that of the starch films in SGF, SIF and SCF for different time intervals. The EL100 dissolves above pH 6.0 and was compared to SS. ES100 dissolves above pH 7 and was compared to RS.

Based on the graph in Figure 4.5, it was observed that retrograded SS films, showed almost no loss in weight in SGF for 2h and 4 h, and only less than 5% weight loss after 6 h. The SS films, as expected, showed drastic weight loss with increasing time in SIF and 100% weight loss was observed at 6 h. For the commercially available, non-polysaccharide-based EL100 films, a similar trend was observed to that of SS (Figure 4.5(b)). It was observed that there was no significant difference in the % weight loss observed in EL100 and SS films in SGF even after six hours, while both dissolved in SIF. The RS films, resisted degradation in both SGF and SIF with almost no weight loss in SGF, and with a less than 5% weight loss in SIF at 6 h. The RS films started showing signs of degradation only when subjected to SCF which contained bacterial enzymes similar to that in the colon with an increasing loss in weight with increasing time and a maximum of 60% weight loss observed at 6 h as shown in Figure 4.5(c). When compared with ES100 films (Figure 4.5(d)), there was no significant difference in the % weight loss observed in ES100 and RS films in SGF and SIF even after six hours, while both dissolved in SCF. Thus, it was evidenced from this study that both SS and RS possess the gastro protective ability and would release the AI only at its target site, comparable to that of commercial synthetic enteric polymers.

Figure 4.5 Comparison of degradation of commercial enteric coating polymers and starch in simulated GIT fluids at various time points. (a&b) soluble starch and EL100 represent coatings for small intestine targeted delivery (c&d) resistant starch and ES100 represent coatings for large intestine targeted delivery *significantly different at $P < 0.001$ using One-way ANOVA, post-hoc tukey test, $n=3$.

4.4. Chnp toxicity and cell uptake studies

For studying the ability of chnp to be taken up by the cells in the GIT, the Caco2 cells have been used as the *in vitro* model [3]. Cellular uptake was studied for 2, 4 and 6 h using chnp loaded with coumarin 343, to enable detection by flow cytometry. Since chnp were non-toxic to the cells within 6 h, and any cell uptake observed should be due to the active endocytosis of the particles by live cells. Based on the analysis of flow cytometry data, as seen in Figure 4.6(a), cellular uptake of chnp, peaked after 2 h incubation, which decreased progressively with time. This could be due to the aggregation of the nanoparticles with

time, which led to an increase in size thus retarding cell uptake. The cell uptake was also visualized using confocal microscopy (Figure 4.6(b)) which clearly showed that chnp was taken up by the Caco2 cells, indicated by the localization of fluorescent particles around the nucleus inside the cells. The above results provide an indication that these chnp would be able to enter the enterocytes through endosomal uptake, leading to the eventual release of the AI into the systemic circulation owing to the pH encountered by chnp in the lysosome as simulated in the release kinetic studies.

Figure 4.6 Cell uptake of chnp using flow cytometry and confocal microscopy. (a) shows the quantification of cellular uptake using flow cytometry. * Significantly different from the rest of the time points. $P < 0.05$ using One-way ANOVA and post-hoc tukey test, $n=3$ (b) the uptake fluorescent chnp into the caco2 cells and localized around the nucleus.

4.5. Discussion

Chnp in this work, have been produced by electrospraying, which is a well-known technique for making nanofibrous scaffolds with remarkable reproducibility [4], but less prevalent for making micro/nanoparticles for delivery. The technique works on the principle that a polymer solution, under the influence of an electric field is either drawn into fibers or sprayed into particles, depending on the process parameters, which are then attracted to the oppositely charged collector. It is highly versatile in the sense that parameters such as voltage, flow rate, needle gauge size, concentration of the polymer solution and the tip to target distance can be varied to achieve different sized particles. The advantage of using this technique is that it is easily reproducible and scale up is easy. In addition, this green synthesis technique does not utilize any organic solvents. The concentration of chitosan and acetic acid were the major determinants of the size of the electrosprayed particles. Consistent with previous reports, at low acetic acid concentrations, the particles were large and aggregated [1] Hence an optimal acetic acid concentration that resulted in discrete particles for 1% (W/V) chitosan concentration was chosen. Chnp fabricated using these optimized chitosan and acetic acid concentration, was found to be within the 200 nm range and shown to be taken up by enterocytes as evident from Figure 4.6. These chnp thus form the core of the delivery system that will house the AI.

Retrograded SS and RS have been investigated as a gastric protectant as well as for targeted release functionality. Gelatinization of starch by moist-heat treatment led to the breakdown of starch granules and leaching of amylose. This amylose when subjected to a temperature of 2-4⁰C for 24 h, undergoes retrogradation, which alters the rate of starch digestion in the body [5] Starch, acquires crystallinity on retrogradation which offers gastric protection [6] But depending on its native source, it is converted to slowly digestible starch [5] which enables it to be digested in SIF over time. Hylon VII which has a high amylose content, when modified in this manner is converted to resistant starch that is degraded only by the colonic microbiome and remains unaffected in the upper part of the GIT. Thus, subjecting two different types

of starch to retrogradation, enables it to be protected in SGF, but selectively degraded either by SIF or SCF depending on its native susceptibility. This hypothesis was substantiated by the results shown in Figure 4.5, where retrograded SS and RS was degraded selectively by SIF and SCF respectively. The performance of the starch films was also found to be comparable to that of commercial enteric coating films.

The electrostatic complexation of biopolymers such as chitosan, alginate and polyglutamic acid for use as a drug carrier has been studied previously [7, 8]. The starch coating procedure adopted, capitalizes on the electrostatic interaction between chnp and starch followed by cross linking the hydrogel. Hence, it eliminates the use of any organic solvents and does not require any special equipment as compared to the protective coatings on tablets formed using spray drying. This would significantly reduce cost and time from an industrial application perspective. Previous work on RS as food grade enteric coating was done by Dimantov et al. where glass beads were used as a model to coat with starch. Though the coating performed well in enzyme dissolution testes, it formed a cracked layer on drying [9]. Recently, Situ et al. tested the possibility of coating insulin-loaded cellulose microparticles with resistant starch and proved that the coating helped prevent the proteolytic cleavage of insulin in the small intestine [10]. These works exemplify the feasibility of coating RS on microparticles using a specialized equipment such as fluidized bed reactor or spray coater. The current study focuses on achieving such coatings on nano particulate carriers without the use of any specialized equipment. Besides, the RS (Hylon VII) can not only act as the protective coating but also serves the purpose of a dietary fiber/prebiotic and help promote the general health of the gut [11]. Thus, using a polysaccharide coating holds potential for a dual functional carrier system, acting as a prebiotic while targeting the delivery of the AI of interest.

4.6. Summary

In this work, a nano carrier with the capability of encapsulating both hydrophobic and hydrophilic AI was fabricated solely from food grade materials using a sustainable approach. Since this delivery system uses only biopolymeric materials obtained from food sources, it can be readily employed for the delivery of nutraceuticals as a food additive. The performance of the carrier was evaluated under simulated GIT conditions and the polysaccharide enteric coating was found to protect the nano carrier and retard any release in the SGF. By using either SS or RS as coating onto chnp, it was found that specific release of the encapsulated drug or nutraceutical can be achieved in the small intestine or colon respectively. Chnp was also shown to be taken up by the Caco2 cells. This study holds promise for a nano-carrier that can be exploited for targeted delivery of drugs or nutraceuticals to the GIT.

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Chapter 5

Coagulans as a nutraceutical candidate

The Indian medicinal plant Withania coagulans was chosen as a source to extract the nutraceutical coagulans, because of its widely reported medicinal properties as well its role in food processing. Two different fractions were extracted from the plant source. The chapter outlines the results of characterization of the extracts using FTIR, NMR and MALDI. The characterization results, verify that the extract obtained, have a similar structural backbone, to that of the ones reported previously. Following this, the feasibility of loading the extracts (P2 and P4) into polymeric carrier was investigated using PLGA microparticles system as a model. HPLC was used as a means to quantify the extracts. Hence, the chapter also discusses the method developed to quantify the extracts and this HPLC method was used to study the release kinetics of the extracts from the PLGA microparticles. The bioactivity of P2 and P4 was tested on three different cell lines to find out its beneficial properties. Of these, the wound healing effect of the extracts is explicated in this chapter, while the anti-diabetic and anti-cancer effects are discussed in the subsequent chapters. The in vitro studies on HDF cells, indicate promising results on the wound healing effect of the aqueous extract, P4.

5.1. Extraction and characterization of coagulans

Coagulans was extracted from *Withania coagulans* plant as outlined in section 3.3. The crude extract (P1), the organic and aqueous fractions (P2 and P4) were characterized using FTIR, NMR and MALDI to confirm for the presence of the steroidal lactones as outlined in previous reports. The extraction efficiency, was calculated based on the yield of P2 and P4 using Equation 5.1 and the values are listed in Table 5.1. It is evident from the extraction efficiencies that proportion of water soluble components are higher in the fruits compared to the organic soluble ones.

Equation 5.1 Formula for calculation of extraction efficiency

$$\text{Extraction efficiency} = \frac{\text{Weight of fruits used for extraction}}{\text{Weight of extract obtained}} * 100$$

Table 5.1 Extraction efficiencies of P2 and P4

Extraction efficiency	
	(%)
P2	0.132
P4	3.13

5.1.5. FTIR

From the FTIR spectra of the crude extract, P2 and P4, represented in Figure 5.1, the presence of characteristic cyclic ether of α - β unsaturated ketone peak at 1080 cm^{-1} and the peak for δ lactone at 1660 cm^{-1} , confirmed for the presence of withanolide like structure. Other notable peaks confirming the withanolide structure, were observed at 3380 cm^{-1} attributed to the $-\text{OH}$ stretch and at 1600 cm^{-1} and 2940 cm^{-1} corresponding to the aromatic ring stretch, represented in Table 5.2. The presence of these characteristic peaks, provides

a preliminary confirmation on the compounds present in the fractions, based on comparison with previous reports [1].

Figure 5.1 FTIR spectra of extracts from *Withania coagulans*: crude extract (P1), fractions P2 and P4

Table 5.2 Characteristic groups of the extract observed from FTIR and their respective wavenumbers

Functional group	Wave number obtained (cm ⁻¹)	Wave number reported [2, 3] (cm ⁻¹)
-OH stretch	3380	3200-3600
Aromatic ring stretch	1600 and 2940	1580-1615 2950-3100
Cyclic ether	1080	1070-1140
α - β unsaturated ketone and δ lactone	1657-1661	1663 and 1715

5.1.6. NMR

Further conformation on the similarity of the extracted fraction to that of the reported ones [1, 3] was done using H-NMR. Based on the NMR peaks obtained, those characteristics for the steroidal lactone ring, can be attributed to the peaks at 1.75 and 1.9 for P2 (Figure 5.2) and at 1.87 and 2.15 for P4 (Figure 5.3). The structural backbone of the steroidal rings is depicted as an insert within the Figure 5.2 and Figure 5.3. The α - and β olefinic hydrogens of the conjugated enone was clearly evident at 5.39 and 6.45 for P4, while only the β olefinic hydrogen at 6.65 was evident for P2. The C-18, C-19 and C-21 methyl groups were also prominently evident as listed in Table 5.3.

Figure 5.2 NMR spectrum of the organic fraction (P2). The inset shows the structural backbone of steroidal lactones.

Figure 5.3 NMR spectra of aqueous fraction (P4). The inset shows the structural backbone of steroidal lactones

Table 5.3 Characteristic peaks observed in the NMR spectra of the extract fractions P2 and P4

	Reported	Fraction 1 (P2)	Fraction 2 (P4)
C-18, C-19 and C-21 methyl groups	1.20, 1.17 and 1.24 singlet	1.24, 1.20	1.12, 1.14 and 1.09
Chemical shift of the deshielded C-21 methyl singlet and the appearance of the C-22 methine	double doublet at 4.67	5.1	
Methyl groups attached to the conjugated lactone group	1.74 and 1.87	1.9 and 1.75	1.87 and 2.15
The α - and β olefinic hydrogens of the conjugated enone	5.62 and 6.55 respectively	6.65	6.45 and 5.39

5.1.7. MALDI

Mass spectrometric analysis using MALDI-TOF equipment was used to identify the molecular weight of the dominant compound. The fractions P2 or P4 in their respective solvents was mixed with the matrix and analyzed. A dominant peak for P2 was obtained at 493.67 m/z, while other smaller peaks were also observed. The structure of the molecule for the dominant peak in P2 was hypothesized to be similar to that of the insert in Figure 5.4, based on the similarity in molecular weights from previous reports [1, 2]. For P4, three dominant peaks at, 491.06, 530.53 and 567.9 m/z were observed. The molecule at 567.9 was fragmented and lead to peaks at 265 and 303 m/z. The mixture of compounds, are hypothesized to have the structures represented in the insert in Figure 5.5 [4].

HPLC was used for quantitative determination of the extracts, after being loaded into polymeric carriers. As the fraction was not a pure compound, HPLC gave rise to more than one peak. In order to decipher which peak matches closely with the starting material, MALDI-TOF was used to analyze the samples. The sample collected at each retention time which gives rise to a peak in HPLC, was analyzed using MALDI, for a strong single peak 493.6 m/z, which also gave an idea of the structure of the abundant compound reported previously as shown in Figure 5.4. The same was repeated with samples at each peak observed at specific time points. The retention time that could give rise to a strong single peak at 493.67, was chosen as the retention time for P2. Similarly, for P4, the peak at 491m/z was the most abundant and the different peaks in HPLC was analyzed using MALDI, to identify, which retention time peak would provide the strong signal at 491 m/z.

Figure 5.4 Characterization of P2 using mass spectrometry

Figure 5.5 Characterization of P4 using mass spectrometry

5.2. Encapsulation of the extracts in a model polymeric carrier system

In order to test the feasibility of loading the fractions P2 and P4 into polymeric carriers, the fractions were loaded into PLGA microparticles as a model carrier system. This would help understand if the extracts can be encapsulated which has never been done before and also to have an idea of the release kinetics of the extracts from a well-established polymer like PLGA which can later be translated into other polymeric carrier systems. For this, PLGA microparticles were prepared as using the solvent evaporation technique [5] and loaded with either P2 or P4. Both P2 and P4 loaded microspheres had a smooth spherical morphology with an average size of 81.28 μ m and 67.13 μ m respectively as in Figure 5.6. While the cross section of P2 microparticles, revealed a solid core, the P4 microparticles had a hollow core. This hollow core can be attributed to the double emulsion technique that was used to fabricate these particles. This difference in core morphology, besides their solubility, influenced the release profile of the fractions from the microparticles. It is inferred from the results that the extracts, both hydrophobic and hydrophilic can be encapsulated into a polymeric carrier system.

Figure 5.6 Coagulans extract encapsulated in PLGA microparticles (a&b) P2 loaded PLGA microparticles and its cross section (c&d) P4 loaded microparticles and its cross section.

5.3. HPLC method development for studying release profiles of P2 and P4

The HPLC method, developed for the quantification of the extracts was validated using the PLGA microparticle model carrier with P2 and P4 loaded into it and their release studied using PBS as the release medium. The HPLC was also used to determine the encapsulation efficiency of the extracts which was later used to calculate the cumulative release percentage. The release profiles of P2 and P4 from PLGA microparticles is shown in Figure 5.7. The release pattern indicates that the kinetics of release depends on the core morphology and the solubility of the drug in the polymer, with a substantial burst release of the hydrophilic P4 and a cumulative release of up to 90 % in two weeks, being observed from the hollow core P4 particles. The release of P2 from the solid core particles lags behind, with a cumulative release of only up to 65% in the time frame studied. This

experiment sets the platform for further studies where HPLC is being used as an important tool to quantify the release of extracts from the carrier system.

Figure 5.7 Release profiles of coagulans extract from PLGA microparticles measured using HPLC. The release study was carried out at 37°C, n=3.

5.4. Discussion

Withania coagulans, with its numerous touted beneficial properties, and proven previously in a few studies, was chosen as the nutraceutical candidate to be encapsulated into the carrier system developed in this work, thereby adding a new dimension of novelty to the work. The encapsulation of this nutraceutical, has many advantages such as, protecting the extract and releasing it only at the target site, in turn helping to improve the bioavailability, taste masking and dosage reduction. The extraction was successfully carried out, keeping the previous protocols as a basis, but refraining from complete purification to obtain single compound extracts, as it was beyond the scope of this thesis. The extracts P2 and P4 are theorized to be a mixture of compounds containing the steroidal lactone backbone, confirmed by FTIR and NMR studies. The dominance of a particular molecular weight

over other similarly structured, but different molecular weight compounds was confirmed from MALDI-TOF studies. On comparison of the molecular weight obtained from MALDI, to that of reported structures, the structure of the dominant compound has been predicted and depicted in Figure 5.4 and Figure 5.5. HPLC, used as a tool for quantification of the extracts, also revealed that the extracts are not pure compounds but a mixture of compounds that are quite close in molecular weight. The MALDI peak was matched to that of the retention times of the peaks in HPLC in order to do quantification studies. Though the beneficial properties of the extracts are widely touted, an attempt to study the mechanisms of action, systemically both *in vitro* and *in vivo* is lacking. This work aims to bridge the gap by studying the effect of the extracts *in vitro*, in their respective cell lines. The beneficial properties of coagulans extracts is addressed in the subsequent chapters.

As the extract from coagulans has never been encapsulated in a delivery system, as a preliminary experiment, the extracts were loaded into the well-known PLGA microparticle system, as this system is known for its ability to encapsulate both hydrophilic and hydrophobic components before moving into the food based delivery system. It was indeed possible to encapsulate the extracts into PLGA carrier system and its release profile from the system was studied by optimizing HPLC methods for both P2 and P4, which forms the platform for studying their release from chitosan and starch based carriers subsequently. PLGA was solely chosen for its ease of handling and well established protocols for fabrication and characterization.

5.5. Summary

The chosen nutraceutical, was extracted from the plant *Withania coagulans* as two different fractions with different solubilities, essentially giving rise to a hydrophilic and hydrophobic fraction to base further tests on. The extracts were characterized using standard techniques such as FTIR, NMR and MALDI in order to deduce the structure of the compounds present in the extract and their similarities to the previous reports. The extracts were loaded into a model carrier system to evaluate their feasibility to be encapsulated and the release of the extracts was studied using HPLC.

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Chapter 6

Coagulans loading in nano carrier-small intestine targeted delivery

The functioning of the chnp coated with starch, as a small intestine targeted delivery system is demonstrated in this chapter. For this, the anti-diabetic effect of coagulans was chosen as an effect to exemplify the absorption of the nutraceutical in the small intestine. The anti-diabetic effect of the extracts was tested in vitro and in vivo, in order to determine the fraction that is non-toxic and effective and also to understand the mechanism of action. The in vivo results revealed P4 to be better than P2. This was also confirmed in vitro by the ability of P4 to promote insulin secretion from beta cells. Following this, P4 was loaded into the carrier system (C+S) and its release from the carrier was studied in different simulated GIT fluids to confirm the working of the starch coating and also to estimate the amount of P4 released in each segment of the GIT. P4 loaded particles were also tested on their ability to promote insulin secretion from beta cells, in order to access the bioactivity of P4 encapsulated into the particles. The working of the carrier was further confirmed from in vivo studies of P4 loaded particles, proving that starch coated particles are indeed able to lower the blood glucose of diabetic mice better than uncoated chnp, substantiating that starch coating is able to target to the small intestine for efficient systemic absorption.

The chnp coated with starch, was developed as a carrier for nutraceutical molecules, to achieve a targeted delivery of the active ingredient (AI) to the small intestine, with minimal loss in the stomach. Of the widely reported therapeutic properties of coagulans, the anti-diabetic effect was chosen as an effect that can be used to prove the small intestine targeting ability of the carrier, as the AI needs to be absorbed in the small intestine to elicit a systemic response i.e. lowering of blood glucose.

6.1. Anti-diabetic effect of coagulans

The anti-diabetic effect of coagulans was tested *in vitro*, to study its effect on beta cell function and *in vivo*, to identify the effective fraction

6.1.1. *In vitro* studies on MIN6 cells

The effects of the extracts were studied simultaneously, *in vitro*, in order to assess the mechanism by which the extracts facilitate their anti-diabetic effect. The most common effect studied primarily would be the effect on beta cell function, to promote insulin secretion [1]. Pancreatic beta cells (MIN6) was used for the *in vitro* tests. The working concentration was estimated by studying the toxicity of P2 and P4 on MIN6 cells. The cells were seeded in 96 well plates at a density of 2×10^4 cells per well. Confluency was reached after 24 hours and different concentrations of P2 and P4 as shown in Figure 6.1 were added to the cells and incubated for 2 hours. Each concentration had five replicates. After 2 hours, CCK8 assay was done and % cell viability was calculated based on Equation 3.3. For P2, all concentration chosen, except for the 0.1 μ M concentration, displayed considerable amount of toxicity on beta cells in 2 hours, discouraging further studies using the P2 fraction. P4 was relatively non-toxic to the cells even at high concentrations, within the 2 hours tested. Based on the toxicity studies, P4 was chosen to evaluate the beta cell function.

Figure 6.1 Toxicity of different concentrations of (a) P4 and (b) P2 on MIN6 cells, studied over 2 hours. *significantly different from 0.1, 0.5 and 1 μM concentrations, $P < 0.05$ using One-way ANOVA, post-hoc Tukey test, $n=5$

Beta cell function was evaluated based on the insulin secretion response, using the GSIS assay and quantified using the ELISA assay. For this study, cells were seeded on 6 well plates at a density of 1×10^5 cells per well. Confluency was reached after 48 hours. Based on the toxicity studies, five different concentrations of P4 (0.1, 0.5, 1, 2 and 5 μM) were chosen to evaluate the insulin secretion ability using the GSIS assay. The amount of insulin released is represented as fold change. The fold change in insulin was compared to that of untreated cells. As in Figure 6.2, of the five concentrations chosen, though all concentrations showed better insulin fold change than, the untreated, 1 μM showed a significant surge in insulin secretion compared to all the other concentrations. Based on this, 1 μM was chosen for subsequent *in vitro* studies.

Figure 6.2 Insulin fold change observed for the mouse pancreatic beta cells (MIN6) on treatment with different concentrations of P4. * Significantly different from all groups at $P < 0.05$ using One-Way ANOVA and post-hoc Tukey test, $n=3$.

6.1.2. *In vivo* studies of free extract

The extraction procedure for the extracts was followed based on reported protocols with some modifications. Despite this fact, in order to confirm beyond doubt that the two fractions, that were obtained, possess anti-diabetic effects and more importantly, their non-toxicity, the fractions were tested *in vivo* on alloxan induced diabetic mouse model as elaborated in section 3.5. The dosage of the fractions was fixed based on previous reports [2] at 50mg/kg. The animals in the normal control group were fed DI water, P2 or P4 at the chosen concentration, for 5 days and their glucose tolerance was measured using Oral Glucose Tolerance Test (OGTT) on the 5th day. While both extracts were not toxic to the animals, the group that received P4 every day, showed better glucose tolerance compared to the group receiving DI water, as seen in Figure 6.3 from the difference in the area under

the curves. This led to the inference that P4 was able to produce hypoglycemic condition better than P2. The glucose lowering effect of P2 and P4 was also compared on diabetic mice. The glucose lowering effect of P4 was confirmed from the positive effects of P4 seen in diabetic mice compared to that of P2. As shown in Figure 6.4, after 5 days of continuous treatment with the extracts, the severely diabetic animals, that were fed P4, showed reduced fasting blood glucose levels, compared to that of the group treated with P2, which was not any different from the diabetic control group receiving only DI water. Since P4 was able to elicit a glucose lowering effect within 5 days of treatment and the experiment aimed at comparing the efficiency of the two extracts, the results after 10 days of treatment is not shown here. After 10 days of treatment, P2 was also able to lower the blood glucose in diabetic mice. Since P4 produced the same effect within a short time, it was chosen for encapsulating into the nanoparticles.

Figure 6.3 Glucose tolerance of normal (non-diabetic) control mice fed DI water, P2 or P4. (a) Glucose levels measured for the glucose tolerance test, over 120 minutes (b) Area under the glucose tolerance curves in (a). Area under the curve (AUC) values helps to compare the glucose tolerance values statistically. The DI water treated group is significantly different from P4 treated group. * $P < 0.05$ using One-way ANOVA and post-hoc Tukey test, $n = 3$.

Figure 6.4 Fasting blood glucose levels of the normal mice fed DI water and diabetic mice, fed DI water, P2 or P4, depicted on day 0 and day 5. The treatment with the extracts was compared against untreated diabetic mice. * $P < 0.05$ using One-way ANOVA, post-hoc Tukey test, $n=3$.

6.2. Encapsulation of P4 into the nano carrier

P4 was encapsulated into chnp during the electrospraying process itself by mixing P4 into the chitosan solution to be electrosprayed. The EE was calculated from the concentration of P4 obtained after collecting the chnp based on Equation 3.2 and was found to be $39 \pm 8\%$

6.2.1. Release study of P4 from Chnp and C+S

P4 was encapsulated into the food grade carrier system as a means of targeting the delivery of P4 to the small intestine, where it can be taken up by the enterocytes to reach the systemic circulation. Since the aim of the coating is to prevent enteric release and facilitate release in the subsequent compartments of the GIT, sequential release of P4 was studied in

SGF for 2 hours, followed by SIF for 3 hours and finally the release pattern was observed in pH 4.4 medium as the final compartment. The pH 4.4 medium mimics the pH of the lysosome, which would be the compartment inside the cell into which the nanoparticles would end up after being taken up by the enterocytes of the small intestine. The uptake of these chnp in the Caco2 model system has been demonstrated previously [3]. Based on the sequential release study carried out for both the starch coated and uncoated chnp as a comparison (Figure 6.5), the uncoated chnp released up to 40% of P4 in SGF within 2 hours, while the C+S particles released less than 20% of P4, indicating that the starch layer is able to retard the release in SGF that mimics the low pH condition in the stomach. On being transferred to SIF, the uncoated chnp that were swollen and releasing P4 in SGF, continued to release P4 and up to 60% cumulative release was observed at the end of 5 hours. For the C+S particles, while the starch coating is digested in SIF, there were no enzymes or conducive pH to trigger the release of P4 from chnp. Hence only 10% release is observed in SIF for C+S particles. Once the particles were transferred to the pH 4.4 medium, the slightly acidic pH promotes the swelling of chnp and rapid release of P4 was observed within the first few hours itself. On being transferred to pH 4.4 medium, it was observed that the uncoated chnp released less than 7% of P4 in the first 2 hours, while more than 20% P4 release is observed from the C+S particles. This can be attributed to the burst release due to the swelling of chnp released from C+S particles on encountering an acidic pH, while the uncoated chnp had already exhibited its burst release in SGF and such drastic change is not observed. This vast difference in release in lysosome will give a leverage to the better performance of the C+S particles as 70% of the encapsulated P4 is still intact after passing through SIF and the burst release in pH 4.4 medium would help increase the systemic absorption of P4 which in turn can help bring down the blood glucose more gradually over a longer period of time.

Figure 6.5 Release kinetics of P4 from chnp and C+S under simulated GIT conditions. *Significantly different ($P<0.05$) from C+S at the corresponding time point, One-way ANOVA, post-hoc, Dunn-Sidak test, $n=3$.

Mathematical model to explain for drug release, indicates that the release of P4 from chnp follows first order kinetics with a regression co-efficient (R^2) value of 0.972, indicating that the release of the hydrophilic drug P4 from chnp is by diffusion and it is dependent on the concentration of P4. The modeling of release of P4 from chnp using the first order release kinetics, fits well to explain the huge burst release observed in the first 5 hours due to the initial swelling of the nanoparticles in SGF, leading to faster diffusion of P4 from the matrix. The release of P4 from C+S, was modeled in two stages, one indicative of the retarded release in the upper GIT because of the presence of the SS coating layer and another indicative of the release kinetics after the degradation of the SS coating layer. Both the stages follow zero order kinetics, indicating a controlled release phenomenon independent of the concentration of P4. Since the AI, P4 is hydrophilic, majority of the

release happens by means of diffusion. The Korsmeyer-Peppas model (Power law) was used to understand the mechanism of release, as it is a more comprehensive model, developed based on the Higuchi model, to study the drug release from polymeric matrices, mainly hydrogels [4]. The release data of P4 from C+S, showed good fit into the Korsmeyer-Peppas model with an R^2 value of 0.97. Based on the n value of 0.512 (exponent of release), obtained from the equation Figure 6.6, it follows an anomalous transport, with the mechanism being governed equally by both diffusion and swelling ($0.43 < n < 0.85$ for spheres). Based on the n value, being close to 0.5, it could be postulated that, the first stage of release in the upper GIT, is governed majorly by diffusion, as in the case of Fickian model, while the later stage of release could be a combination of swelling, mainly due to the degradation of the SS coating and diffusion in a time-dependent manner.

Figure 6.6 Mathematical modelling of the release kinetics of P4 from chnp and C+S. (a) release profile from chnp fit to first order kinetics (b) release from C+S fit to zero-order kinetics and (c) Korsmeyer-Peppas model

6.2.2. Effect of P4 release media on MIN6 cells

With the knowledge that the starch coating is able to retard the release of P4 in SGF and might facilitate the release within the enterocytes itself, it is imperative to ensure and prove that this P4 is bioactive to elicit its response. For this purpose, the release media collected from the particles that were subjected to sequential release study-SGF, SIF and pH 4.4 medium, were added to the MIN6 cells and GSIS was carried out to measure for insulin secretion on adding the release media. For this purpose, 3 different groups were selected. One is the blank release media itself, to prove that the release media does not have any inherent toxicity, leading to cell lysis and increased insulin being observed in the supernatant. The next group is free P4 added to SIF or pH 4.4 medium, collecting the sample after the respective treatment times in each compartment. The third group is the C+S particles subjected to sequential release and release media collected from SIF and pH 4.4 at respective treatment times. The concentration of P4 in the release media for the 2nd and 3rd group is chosen to match 1 μ M after dilution. Since the release of P4 in SGF, is not desired as it would not promote effective absorption in the small intestine, the effect of

SGF and that of P4 encapsulated into uncoated chnp on the bioactivity is overlooked. From the results shown in Figure 6.7, it is evident that the blank release media itself does not have any effect on the insulin secretion from MIN6 cells. The line indicated as untreated baseline is the basal insulin secretion observed with just glucose stimulation only with no treatment. And the line indicated as P4 in the graph, is the insulin fold change observed for the treatment of 1 μ M on MIN6 cells. For free P4 in release media, while the SIF release media does not promote insulin secretion, P4 in pH 4.4 media promotes insulin secretion, though it does not touch the P4 line. This could lead to conclude two things: 1. The enzymes in SIF alter P4 making it to lose its bioactivity. 2. Since P4 is free, there is partial loss of bioactivity in pH 4.4. A similar trend of bioactivity loss in SIF, was observed for the P4 released from C+S particles, while P4 released in pH 4.4 from C+S had its bioactivity completely preserved because of the encapsulation within C+S particles and P4 did not have to encounter the SIF. This proves that the bioactivity of P4 is intact in the C+S particles.

Figure 6.7 Insulin fold change observed for the mouse pancreatic beta cells (MIN6) on treatment with release media from C+S particles compared against free in the same release media and blank release medium alone

6.2.3. *In vivo* studies of the nano carrier

While the *in vitro* study proves the bioactivity of P4 is intact after encapsulation into the particles, the efficacy of these particles *in vivo* is a question. Hence uncoated chnp and chnp coated with soluble starch were tested on diabetic animals. The test groups in this study were diabetic animals that were fed by oral gavage, chnp and C+S as test formulations, Glibenclamide and distilled water as controls. A non-diabetic control group, fed Distilled water was also included. The amount of encapsulated P4 fed each day was 50mg/Kg. Based on the results, the group that received chnp treatment was similar to the negative control group that received only distilled water. It is evident that the chnp does not have any effect on the fasting blood glucose levels due to the preemptive release of P4 in the stomach and SIF leading to its loss of bioactivity, as observed in Figure 6.8. The

animals in the group, that received C+S treatment, showed marked decrease in blood glucose levels even after 5 days of treatment comparable to the positive control group that was treated with the drug Glibenclemide. This proves that the delivery system not only retards pre-mature release of the drug but also enables it to be absorbed systemically to exert an effect.

Figure 6.8 Fasting blood glucose levels of diabetic mice before and after 5 days of oral gavage with P4 encapsulated starch coated chnp and uncoated chnp. The groups were also compared against positive and negative controls and normal control mice. * $P < 0.05$, one way ANOVA post-hoc Dunn-Sidak test, $n=3$.

6.3. Discussion

The concept of using food as medicine has been practiced from ancient times and the extracts from some of these foods are also being marketed commercially as nutraceuticals such as resveratrol, curcumin and green tea extract. On the other hand, there are extracts from medicinal plants that have even led to the development of commercial drugs such as

Digitoxin, Paclitaxel and Quinine. The renewed public interest towards such compounds extracted from foods or medicinal and marketed as nutraceuticals, has led to the increased consumption of such products that can be purchased off the counter, or sometimes even added to food. In an effort to combine such alternative system of medicine with that of the burgeoning field of encapsulation technology, extracts from the berries of *Withania coagulans* have been encapsulated into a food based polymeric carrier, with two converging aims: one is to provide a suitable delivery system for the extract for which no such system exists and also to prove the small intestine targeting ability of the carrier *in vivo*.

The basic characterization of the fractions P2 and P4, extracted from *Withania coagulans*, was in agreement with previous reports [5]. Extensive characterization was not done as the aim was not to extract a pure compound, but to study the combined effects of the compounds. The basic characterization of the fractions using FTIR and NMR, ensured that the compounds reported to have anti-diabetic effect are indeed present [6]. Hence the structure of the compounds present can only be predicted to match that of the steroidal lactone backbone reported for similar withanolides extracted from the plant, but the exact structure could not be elucidated. Further, based on the mass spectrometric data of P4, it is evident that it is not a pure compound and there could be more than one compound in P4. The same was also observed in the HPLC chromatograms, which gave rise to multiple peaks. The peak corresponding to the most abundant compound was used for the purpose of quantification.

Any compound/drug that lowers blood glucose, exerts its action by three different ways: One is by promoting insulin secretion from the pancreatic beta cells, or by sensitizing the adipocytes to insulin, promoting glucose uptake or inhibiting gluconeogenesis from hepatocytes. There is a dearth when it comes to the testing of the extracts from *Withania coagulans in vitro*, which could largely help in the understanding of the mechanism of action of the extract. Since most of the plant extracts that exert anti-diabetic effect, act by promoting insulin secretion from beta cells, it was hypothesized that *Withania coagulans* promotes insulin secretion by sensitizing or stimulating the beta cells through activation of Ca²⁺ channels and in turn exocytosis of insulin from insulin containing granules [2]. This hypothesis was confirmed by testing both P2 and P4 on mouse pancreatic beta cells at

different concentrations and P4 was indeed found to promote insulin secretion at a certain concentration, in agreement with *in vivo* results. This could be attributed to the presence of saponins, flavonoids, tannins, alkaloids, terpenoids and sterols in the extract, previously shown to be present in the extracts of *Withania coagulans* [7]. Such bioactive components have been associated with hypoglycemic activity [8].

The *in vivo* testing of the free extracts showed that P4 was able to decrease fasting blood glucose in diabetic animals, which is a key indicator on the level of the intensity of the disease. While previous reports have shown that the compounds extracted from both the aqueous and organic fraction were able to decrease FBG in diabetic mice over 30 days [2], the same effect was observed within 5 days for P4 (Figure 6.4). P2 could not show any effect within this 5-day period. There have been no reports so far discussing the decrease in FBG within such short time frames. Besides, the fraction used in this study is not a pure compound, hence the effect could not be directly compared to that of previous reports and the effect observed might be a combined effect of more than one compound.

Alloxan is a glucose analog molecule that has selective toxicity towards the pancreatic beta cells of rodents. The toxicity of the molecule is mediated by selective uptake and accumulation in beta cells through the GLUT2 glucose transporter. Once inside the cells, the molecule reacts with thiols forming reactive oxygen species and free radicals, leading to cell kill [9]. The lowering of FBG in such alloxan induced diabetic models in the absence of any insulin supplementation, could only mean that the few beta cells left behind in mouse are either restored back or the remaining cells are induced to promote insulin secretion. Since the insulin secreting ability of P4 from beta cells was proven *in vitro* (Figure 3.4), it can be concluded that beta cells have been regenerated in the diabetic mice that led to the decrease in FBG. The presence of these compounds has been reported to support the regeneration of beta cells. In addition, the anti-oxidant property of coagulans [10], might also be responsible for the regeneration of beta cells, as the main mechanism by which alloxan causes diabetes is by generating ROS and free radicals. A study on the free radical scavenging activity of *Withania somnifera* on STZ injected mice, was shown to restore β cell function [11]. Since the Withanolides in both *Withania somnifera* and *Withania*

coagulans possess the same structural backbone comprising that of steroidal lactones, the study gives insight into the mechanism by which Withanolides are able restore the function of β cells.

6.4. Summary

The ability of starch as a food grade enteric coating to retard the premature release of the AI in the stomach was proven in diabetic animal model, by the reversal of diabetes upon administering the starch coated nanoparticles containing the AI ingredient responsible for causing the hypoglycemic effect. The work set the platform to ultimately prove the above hypothesis by extracting a nutraceutical from a medicinal plant source and testing its efficacy on diabetic animals as well as testing the mechanism as well as the bioactivity of the encapsulated extract *in vitro*. Thus, the work advocates the feasibility of encapsulating the extracts from *Withania coagulans* into a polymeric carrier and also the ability of the food grade nano carrier to encapsulate a nutraceutical and deliver it at the target site.

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Chapter 7

Coagulans loading in nano carrier-large intestine targeted delivery

The functioning of the chnp coated with resistant starch, as a large intestine targeted delivery system is demonstrated in this chapter. For this, the local effect of the nutraceutical in the large intestine, needs to be demonstrated. The anti-cancer effect of coagulans on colon cancer cell line was studied for both the fractions P2 and P4. Simultaneously, the toxicity of the fractions was tested against fibroblasts as well normal colon cells to elucidate how safe the extracts were. Though both P2 and P4 were non-toxic to normal cells, compared to standard anti-cancer drugs, P2 was able to elicit 50% cell kill at a much lower concentration and lesser time with a SI of above 15. Based on this, P2 was encapsulated into the carrier system (C+RS) and its release profile was studied in different GIT fluids. The working of the coating was confirmed by further evaluating the ability of P2 loaded particles to kill the cancer cells. The in vitro studies showed that C+RS particles were able to kill the cancer cells only after being subjected to SCF where RS would be broken down to release the encapsulated P2 from chnp.

The chnp coated with resistant starch, was developed as a carrier for nutraceutical molecules, to achieve a targeted delivery of the active ingredient (AI) to the large intestine, with minimal loss in the stomach and small intestine. The chemo preventive ability of coagulans has been described previously [1]. Drawing from this the anti-cancer ability of the extract was tested against colon cancer cells to prove the large intestine targeting ability of the carrier, as the AI needs to be delivered locally in the large intestine to elicit a local response i.e. killing of the cancerous cells.

7.1. Anti-cancer effect of coagulans

7.1.1. *In vitro* toxicity studies on colon cancer cell lines

The ability of the extracts P2 and P4 to kill colon cancer cells was evaluated against Caco2 cells based on the method outlined in section 3.4.1. Different concentrations of the extracts, P2 and P4, were tested on Caco2 cells for 24 and 48 hours, to identify whether the extracts had any toxic effects on Caco2 cells. The minimum concentration at which P2 was able to kill 50% of the cells (IC₅₀) is shown in Figure 7.1. It was observed that after 24 hours of treatment, P2 exhibited two killing windows, one at a slightly lower concentration of 0.5-2 μ M and another wide range at a higher concentration between 20-100 μ M. The exact value for the IC₅₀ was obtained from the linear regression analysis performed on the viability of Caco2 cells over a range of P2 concentrations 1.89 $\pm$ 0.4 μ M was obtained as the IC₅₀ for the low concentration window and 81.96 $\pm$ 8.5 μ M was obtained as the IC₅₀ for the higher concentration window. This high concentration IC₅₀ window was neglected since it was possible to achieve cell kill at a lower concentration of P2 itself. Also, linear regression analysis was not performed for the cell viabilities obtained for 48-hour treatment, as the extract is effective within 24 hours.

In the case of treatment with P4, Caco2 cells did not exhibit any cell death up to 24 hours even at very high concentrations of P4. The extract was able to elicit its anti-cancer ability only after 48 hours of treatment. As shown in Figure 7.2, P4 does not exhibit any toxic effect against Caco2 cells up to 10 μ M and begins to show a concentration dependent toxic effect beyond 10 μ M, achieving 50% cell kill around 250 μ M. The linear regression analysis

of the cell viabilities against varying concentrations of P4 resulted in the IC₅₀ value of $259.59 \pm 38.7 \mu\text{M}$.

Figure 7.1 Effect of varying concentrations of P2 on the viability of Caco2 cells after 24 hours treatment. * $P < 0.05$ using One-Way ANOVA, post-hoc Tukey test, $n = 5$.

Figure 7.2 Effect of varying concentrations of P4 on the viability of Caco2 cells after 48 hours treatment. * $P < 0.05$ using One-Way ANOVA, post-hoc Tukey test, $n = 5$.

7.1.2. IC₅₀ and Selectivity Index (SI)

The selective ability of the extracts to kill cancer cells and remain safe on normal cells was assessed by treating the Caco2 cell line and two normal cell lines – HDFa and CRL 1831 with various concentrations of P2 and P4 over specific time points. The IC₅₀ was determined for all cell lines and the SI was calculated as the ratio of IC₅₀ of normal cells to that of the IC₅₀ of Caco2 cells [2]. Based on the results shown in Table 7.1 and Table 7.2, it is evident that both P2 and P4 are able to selectively kill only the cancer cells. Also, the SI of both P2 and P4 were much higher compared to the commercial drugs Doxorubicin (DOX) and 5-Fluorouracil (5FU). 5FU is commonly used in the treatment of colon cancer and used as the hydrophobic model drug to compare against the extract. DOX is a hydrophilic drug and is not preferred for the treatment of colon cancer due to its severe cardiotoxicity. It has been used in the study as a model hydrophilic drug to compare with the extract. While P4 did not exhibit cytotoxicity within 24 hours, P2 elicited its response during this time. Hence P2 was chosen as the active compound for further studies.

Table 7.1 Comparison of IC₅₀ and selectivity of P2 on cancerous and normal cell lines

Cell line	IC ₅₀ (μM)			SI		
	P2	DOX	5 FU	P2	DOX	5 FU
Caco2	1.89±0.4	51.3±13.4	106.94±24.27	-	-	-
HDFa	29.79±6.7	5.76±2.25	41.81±13.8	15.76	0.1	0.39
CRL1831	35.95±12.7	66.34±4.41	148.5±20.7	19.02	1.29	1.38

Table 7.2 Comparison of IC₅₀ and selectivity of P4 on cancerous and normal cell lines

Cell line	IC ₅₀ (μM)			SI		
	P4	DOX	5 FU	P4	DOX	5 FU
Caco2	259.59±38.7	16.3±7.7	53.92±5.87	-	-	-
HDFa	1218±178.3	1.69±0.64	29.87±4.032	4.69	0.1	0.55
CRL1831	5000±127.2	12.08±1.2	93.59±1.97	19.23	0.74	1.73

7.2. Encapsulation of P2 into the nano carrier

P2 was encapsulated into chnp during the fabrication process itself. Being hydrophobic, P2 loaded chnp, were prepared using β CD as the solubilizing agent [3] Briefly, β CD at a concentration of 5 mg/mL was dissolved in chitosan solution. P2, was then mixed with this β CD chitosan solution at a rate of 2 mg/mL. The presence of β CD solubilized the hydrophobic nutraceutical within its core. This chitosan solution was then electrosprayed under the same conditions as mentioned in section 3.1.1. The amount of P2 encapsulated in the particles was determined using an indirect method by measuring the amount of P2 in the supernatant from which the particles were collected. Concentration of P2 in the supernatant and the encapsulation efficiency was evaluated using HPLC as outlined in section 3.3.4. The EE was found to be $55 \pm 8\%$.

7.2.1. Release study of P2 from chnp and C+RS

The release kinetics of P2 was studied in different simulated gastric fluids as mentioned in section 3.3.5. As shown in Figure 7.3, the release kinetics of P2 from chnp was compared to that of C+RS in order to ascertain the ability of the RS coating to retard the release of the nutraceutical in the upper GIT. While uncoated chnp displayed a burst release of up to 40% within 2 hours in the SGF, the RS coating, restricted the release to less than 20% during the first 2 hours in SGF. Sequential transfer to SIF, further triggered the release of another 20% from the uncoated chnp, leading to loss of 60% of the encapsulated P2 within 5 hours in the upper part of the GIT. The same was not true for the RS coated chnp which continued to delay the premature release of P2 resulting in the cumulative release of nearly 25% P2 in 5 hours in the upper GIT, preserving 75% of P2 for release in the colon. On transfer to SCF, chnp continued to release P2 and nearly 100% cumulative release was observed in 48 hours. The release of P2 from RS coated chnp, was triggered on contact with SCF and a steady release was observed up to 48 hours, cumulating to 80% total release within this time.

Figure 7.3 Release kinetics of P2 from chnp and C+RS sequentially in different GIT fluids. *Significantly different ($P<0.05$) from C+RS at the corresponding time point, One-way ANOVA, post-hoc, Dunn-Sidak test, $n=3$.

The release of P2 from chnp was found to follow first order kinetics as observed from Figure 7.4 (a) with an R^2 value of 0.9227. This concentration dependent release pattern of P2 from uncoated chnp could be attributed to the swelling of chnp in SGF and the subsequent release of the AI. P2, being hydrophobic, the release was also controlled by the dissolution of the AI in the release media. Similar to SS coated chnp, the release of P2 from RS coated particles also exhibited zero order release kinetics in two stages. The minimal release observed in the upper part of the GIT, fitted well with the zero-order kinetic model with an R^2 value of 0.9743 and the second stage of P2 release in the colon, had an R^2 value of 0.939. Similar to P4, the release mechanism of P2 from C+RS was studied using the Korsmeyer-Peppas model, which exhibited good fit to the release data with an R^2 value of 0.9814. With the n value being 0.5268, the release of P2 from C+RS was attributed to an

anomalous transport mechanism, following a combination of diffusion and swelling. The swelling in this case happens mainly by the degradation of polymers in the colonic enzymes.

Figure 7.4 Mathematical modelling of the release kinetics of P2 from chnp and C+RS. (a) release profile from chnp fit to first order kinetics (b) release from C+RS fit to zero-order kinetics and (c) Korsmeyer-Peppas model

7.2.2. Effect of release media on Caco2 cells

Caco 2 cells were cultured in the media mentioned under table 3.1 and the once the cells reached 80% confluency, cells were seeded in 96 well plate at the rate of 1×10^4 cells per well. The cells reached confluency after 48 hours and were used for testing. P2 loaded chnp we prepared following the procedure outlined in section. The particles were then coated with RS as they were intended to target to the large intestine.

The amount of chnp were taken such that the IC 50 concentration is met, with the % of P2 released in 2hrs in SGF alone, with the % of P2 released in 3hrs in SIF alone and with the % of P2 released in 24hrs in SCF alone, after being diluted 40 times. Release samples cannot be added directly to the cells as they contain enzymes and low pH in the case of SGF. Particles cannot be added directly to the cells as they need the enzymatic conditions to be simulated.

This was matched in weight and coated with RS. The rationale behind this is to test the hypothesis that the amount of P2 released from equal amounts of chnp would be different depending on the presence or absence of coating. Hence similar amounts of chnp, both

coated with RS and uncoated are subject to SGF, SIF and SCF individually, and the ability of the coating to retard the release was ascertained *in vitro*.

The test groups were:

1. Blk – Blk SGF, SIF or SCF diluted 40 times.
2. P2 – Free P2 in SGF, SIF or SCF in 2h, 3h or 24h respectively in concentration to match IC 50 after 40 times dilution.
3. Chnp – Uncoated chnp loaded with P2
4. C+RS – Chnp coated with RS.

From the results, it is evident that the release media itself does not have an effect on the cells. Free P2 and chnp groups in SGF displays cytotoxicity whereas C+RS does not kill the cells (* C+RS significantly different from P2 and chnp at $p < 0.05$). This proves that the coating is able to effectively retard the release of P2 in SGF. A similar trend is observed in SIF for C+RS. But the free P2 and chnp groups also do not display any cytotoxicity in SIF. This could be because P2 is being degraded in SIF (an aspect observed for P4 in SIF). In SCF, due to the degradation of RS by the bacterial amylase, P2 is released which in turn is reflected as the killing of caco2 cells.

Figure 7.5 Effect of the release media from P2 encapsulated chnp and C+RS on the viability of Caco2 cells, compared against free P2 and blank release media. Free P2, P2 encapsulated chnp and P2 encapsulated C+RS were subject to each of the release medium individually, and the ability of the RS coating to retard the release of P4 was compared and evaluated based on the viability of Caco2 cells. Decrease in cell viability indicates release of P2. * $P < 0.05$, One-way ANOVA, post-hoc Tukey test, $n = 4$.

7.3. Discussion

Colon cancer is the third leading cancer type in both men and women, after prostate/breast and lung cancers, where new cases are estimated to occur as of 2018, compared to previous years [4]. With the increasing incidence of drug resistance being observed, the focus is shifting towards natural compounds extracted from plant or food sources that have cancer cell killing or mitigating properties [5]. The synergistic use of such compounds in order to overcome drug resistance or lessen the drug concentration is also being considered [6]. The

anti-cancer effect of the organic fraction, P2 extracted from the plant *Withania coagulans* has been considered in this chapter as a means to exemplify the large intestine targeting ability of the oral delivery system developed.

The Caco2 cell line, derived from the human epithelial colorectal adenocarcinoma cells, have been used as a colon cancer model to study the ability of the extract in killing colon cancer cells. The *in vitro* studies of the pure extracts revealed its ability to kill Caco2 cells. In order to access the safety of the extracts when treated on normal cells, two normal cells lines-dermal fibroblasts (HDFa) and normal colon cells (CRL 1831), were subjected to varying concentrations of the extracts. The study revealed the selective ability of the extracts, both P2 and P4 to kill only the Caco2 cells, while leaving the HDFa and CRL1831 unharmed. Based on the concentration and time required to elicit a response, P2 was chosen as the active compound with shorter response time (24 hours). The SI of two commercial drugs, 5-Fluorouracil and Doxorubicin were also compared as a control. The low selectivity indices observed for the two drugs is in agreement with previous reports. A study by Shahin et al., exemplifies the poor SI of Doxorubicin when breast cancer cell lines (MCF 7 and MDA-MB-435) were compared against non-cancerous MCF10A cells [7]. Similarly, low SI of 5FU against various colon cancer cell lines compared to that of mouse fibroblasts has been observed [8]. The high SI observed for P2 compared to that of the commercial drugs, used to treat colon cancer, strengthens the functionality of the extract as a potential anti-cancer agent, opening avenues for further research into its commercialization.

It is imperative to understand the mechanism by which P2 elicits its selective anti-cancer effect, before putting it to functional use. Though, studying the mechanism of action of P2 is beyond the scope of this thesis, the mechanism of action is hypothesized based on literature evidence, which could provide a good starting point.

The anti-cancer effect of such plant derived molecules are usually attributed to the secondary metabolites such as flavonoids and alkaloids [9]. The anticancer activity of steroidal lactones, extracted from *Withania somnifera*, with similar structural backbones to

that P2, have been reported previously [10]. These reports, attribute that the anti-cancer effect of the extracts from *Withania somnifera* could be linked any of the following mechanisms individually or in combination: (i) reinforcement of cellular detoxification system (ii) selective inhibition of tumor cell proliferation and induction of apoptosis (iii) suppression of tumor angiogenesis; (iv) blockade of metastasis (v) alteration of tumor cell metabolism or (vi) eradication of cancer stem cells [11]. A study on the cancer chemo preventive ability of the compounds extracted from *Withania coagulans*, by Haq et al, showed that the compounds were able to suppress tumor necrosis factor- α (TNF- α)-induced nuclear factor-kappa B (NF- κ B) activation in murine macrophages, which is responsible for the uncontrolled proliferation of tumor cells [12]. This could also be one of the responses happening at the cellular level to suppress tumor.

P2 was encapsulated into the delivery system in order to protect its bioactivity and deliver it locally in the large intestine. RS, with its ability to resist digestion by the intestinal amylase, protected the release of P2 in SGF and SIF as seen in Figure 7.3. The simulated condition in SCF, containing bacterial amylase, mimicking the colon, helped to degrade the RS coating and release P2 at its intended location. *In vitro* studies of P2 encapsulated chnp and C+RS, revealed the ability of the coating layer to not only retard the undesired release of AI in the upper GIT but also preserve the bioactivity of the encapsulated AI, as observed from Figure 7.5. An additional observation worth discussing is the inability of the RS coated chnp, encapsulated with P4, to lower blood glucose levels in diabetic mice, as seen in Figure 6.8. The inability of the C+RS particles, to reduce blood glucose, helps conclude that RS does not degrade in the stomach or small intestine, as the degradation of the coating, would have caused the release of P4 in the small intestine, thereby eliciting a response.

7.4. Summary

The ability of RS as a food grade enteric coating to retard the premature release of the AI in the upper GIT was investigated in this chapter. The anti-cancer property of P2 and P4 were tested on Caco2 cell model and P2 was found to be better than P4 with a higher SI

and shorter treatment time. The hypothesis that the food grade coating can help to target to large intestine was tested by encapsulating P2 into the delivery system and studying its release profile, followed by testing the ability of the P2 encapsulated particles to act only in the presence of colonic enzymes.

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Chapter 8

Conclusions and future recommendations

This chapter draws together the discussions and conclusions from different results presented in Chapters 4-7 as a means to understand how these results encompass the main hypothesis and objectives. The conclusions drawn also help to understand to what extent the aim of this thesis has been fulfilled in developing an oral delivery system targeting to different parts of the GIT using food grade materials. Following this, the protractive direction of the thesis is also discussed. The detailed investigation of the chnp uptake using a triculture model, and the biokinetics study of chnp using a GIT simulator would help understand the fate of the nanoparticles in an environment better representative of what is in vivo. This forms a bridging study before moving into animal models to test the biokinetics of chnp. Purification and isolation of the extracts P2 and P4 in order to identify single molecule compounds responsible for the anti-cancer and anti-diabetic effect is also recommended as another important step towards standardizing these nutraceuticals. The last part of the chapter discusses some reconnaissance work done on the wound healing properties of the extract, done as a part of screening the extracts for the presence of bioactive properties.

8.1. Conclusions

The work for this thesis was inducted with the primary aim to develop an oral delivery system for targeted delivery to the small or large intestine using food grade polymers, so that it can be used to encapsulate nutraceuticals, intended to be used as an additive in fortified or functional foods, thereby protecting the bioactivity and enhancing the bioavailability of the nutraceutical. The lack of a delivery system that could perform such diverse functions, formed the motivation for this thesis. Hence, it was hypothesized that a food grade polymer could be modified effectively to target to different parts of the GIT. In order to test this hypothesis, four main objectives were outlined.

The primary objective was to design and develop a delivery platform using biopolymers/food grade materials using mild techniques that do not use any toxic solvents. After extensive literature survey, chitosan and starch were chosen as the materials to be used in the fabrication of the delivery system. Chitosan was chosen for its mucoadhesive property and starch, for its selective digestibility depending on its source. The design of the delivery system was laid out in a way to maximize the interaction of the carrier with the cells lining the GIT, to allow increased residence times and better cellular uptake for systemic absorption. This was facilitated by incorporating a nano sized core into the design of the carrier. Naturally, chitosan was the core material to take advantage of its mucoadhesive and transient tight junction opening properties, while starch, with its selective digestibility, was to be used as the coating material. In addition, starch was rendered gastro protective by means of retrogradation.

With this design in place, the delivery system was developed by a two-step fabrication process. Chnp were fabricated by means of electrospraying, as a reproducible technique without the use of harsh chemicals. The prepared chnp were characterized extensively and was found to be in the desired size range to facilitate uptake by the enterocytes of the GIT. Following this, the chnp were coated with two different types of retrograded starch, depending on its intended target, the small or large intestine. The coating was by means of electrostatic interactions followed by using a food grade cross linker. The formation of a

coating layer of starch on chnp was verified successfully by means of zeta potential measurements, which indicated charge inversion and the presence of a core shell morphology was visualized using confocal microscopy. The next important step in fulfilling the first objective was to compare the selective degrading ability of the starch coating in different simulated intestinal fluids to that of commercially available non-food grade synthetic polymers. The performance of the two different retrograded starches was comparable to that of the commercial Eudragit polymers, with the soluble starch resisting degradation in the SGF and degrading in SIF and the RS resisting degradation in SGF and SIF and degrading in SCF. The ability of chnp to be taken up by the enterocytes was studied using Caco2 cell monolayer as a model and the nanoparticles were indeed able to localize within the cells after 2 hours of incubation. These results, provided substantial evidence on the successful development of a delivery platform.

The second objective was to choose a suitable nutraceutical to be loaded into the delivery system, such that the nutraceutical can be used to exemplify the targeting ability of the delivery system. Based on its numerous therapeutic properties reported, *Withania coagulans* was chosen as the nutraceutical, and the anti-diabetic and anti-cancer properties of the extracts were chosen to exemplify the small intestine and large intestine targeting ability of the delivery system. In this regard, two different fractions – aqueous (P4) and organic (P2) were extracted from the fruits of the plant. The extracted fractions were characterized to identify the structure of the compounds present and the basic structural backbone of the compounds in P2 and P4 were found to be similar to that of the compounds reported previously. It was also deduced that the fractions obtained were not pure compounds and was a mixture of more than one compound. The fractions were not further purified as the combined therapeutic effect was intended to be studied and the purification and isolation of a single compound with therapeutic effect was beyond the scope of this work. The feasibility of encapsulating the selected nutraceutical within a well-known polymeric carrier was also tested as a preliminary study before being encapsulated into the actual carrier itself.

The last two objectives, focuses on testing the targeting ability of the delivery system. The third objective was to test the small intestine targeting ability of the extract by means of the anti-diabetic effect of the AI. The anti-diabetic effect of the extracts was confirmed *in vivo* using diabetic mouse model. P4 was found to be more effective than P2 and was chosen as the fraction to be loaded into the delivery system. The *in vitro* testing of P4 on beta cells, led to the confirmation of the hypothesis that the preliminary mechanism of action could be the promotion of insulin secretion from beta cells. With these information, P4 was encapsulated into the delivery system made of chitosan and starch and its working was tested *in vitro*, by a sequential release study in simulated gastric fluids. The results were supportive of the selective degrading and gastro protective property of starch, manifested by a retarded release of P4 in SGF, slow release in SIF and the rate of release picking up subsequently. It was also observed from *in vitro* studies, that the encapsulation prevented the AI from being degraded and preserved the bioactivity of the AI to be utilized at the intended site of action. The working of the P4 loaded delivery system was tested *in vivo* and the preliminary results were indicative of the successful small intestine targeting ability of the starch coated chnp.

The last objective, to verify the hypothesis, was to prove the large intestine targeting ability of the delivery system. The reported anti-cancer effect of the extract was used to exemplify this property. The effect of the extracts P2 and P4 on colon cancer cell line was studied and based on the time required to elicit the response, concentration and SI, P2 was found to have potent, selective toxicity against colon cancer cells compared to P4. While investigating the mechanism of action against cancer cells was beyond the scope of this thesis, some of the possible routes of mediating tumor cell toxicity were hypothesized. Following this, P2 was encapsulated into the delivery system suitable for large intestine targeting and the release profile indicated the ability of RS coating to retard the release of P2 in SGF and SIF. *In vitro* studies on delivery systems encapsulated with P2, further confirmed the ability of the carrier to release the AI only in the presence of bacterial amylases and also its ability to protect the bioactivity of the encapsulated AI.

Thus, by fulfillment of the four main objectives outlined above, led to the verification of hypothesis that food grade materials could be used as excellent delivery vehicles and can be used as a means of GIT targeting. The food grade coating stands out from commercial enteric or stimuli-responsive coatings in that the currently available coatings can only be used at the macroscale for coating microparticles. Also, these commercial non-GRAS materials are used only as film coatings on pharmaceutical tablets and are required to be spray-coated onto tablets at specific inlet and outlet air temperatures, which would adversely affect the sensitive nature of the AI. Hence, the use of food grade materials such as starch and chitosan serves as a good basis in developing food grade, GIT-targeting nanoparticles for drug and nutraceutical delivery. The final nano-carrier is composed of GRAS materials that are approved to be used as a direct food additive.

8.2. Future recommendations

Some of the studies listed below, helps to add on to the scientific knowledge and better understand the nutraceutical loaded delivery system, as a step towards developing a robust system, intended for use in foods.

8.2.1. Evaluation of cellular uptake using triculture model

The triculture model developed by DeLoid et al, mimics the intestinal epithelial barrier, using three different types of cells [1]. Caco2 cells form the enterocyte monolayer, HT29-MTX cells differentiate into goblet cells and Caco2 cells in the presence of soluble factors from Raji B cells, differentiate into M cell. The three cell types are representative of the cells found in the human intestine. The cells are cultures on a Transwell membrane as shown in Figure 8.1. for 21 days to allow for the differentiation into the particular cell type. Following this, a preliminary toxicity study would be carried out to assess the safe concentration to work on. The lactate dehydrogenase and presto blue assays would be used as the endpoint analysis to quantify the cytotoxicity. Fluorescent chnp at the chosen concentration, would be added to the apical compartment and its ability to pass through the

monolayer into the basolateral compartment would be evaluated over different time points. At each time point, the media from the basolateral compartment would be checked to quantify the number of particles that can cross over. A qualitative study on the cellular uptake can be done by visualizing the cellular monolayer using confocal microscopy.

Figure 8.1 Triculture model to study nanoparticle uptake in GIT [1]. Reproduced with permission from ACS publications.

8.2.2. Biokinetics study using the GIT simulator

The fate of the starch coated delivery system under a dynamic condition, would give an idea of how the system would behave *in vivo*. The GIT simulator developed by DeLoid et al, at Harvard would serve this purpose comprehensively. The simulator, as shown in Figure 8.2, comprises of three phases to mimic the digestion in the mouth, stomach and small intestine by matching the time, composition and dynamic environments in each of these phases. The simulator allows for collection of sample at each phase for analysis. Drug or fluorescent dye loaded chnp can be used to assess the amount of encapsulated material released at each phase of the simulator. This would help assess the functioning of the starch coating. The digesta from the last small intestine phase, can be added to the triculture model, described above to simulate the ability of chnp to be up taken, after going through he GIT simulator.

Figure 8.2 Schematic of the GIT simulator [1]. Reproduced with permission from ACS publications.

8.2.3. Purification of the extracts P2 and P4

The therapeutic effects of the extracts P2 and P4 have been demonstrated *in vitro* in this work. Since the extracts are not pure compounds, the next step would be to purify the fractions, with the aim of isolating a single molecule compound that is responsible for the therapeutic properties. This would involve, rigorous purification using chromatographic columns and extensive characterization to elucidate the structure. Following this, the purified compounds could then be screened using the *in vitro* assays mentioned in sections 3.4.1, **Error! Reference source not found.** and 3.4.2 to identify the compound with the

ost potent therapeutic effect. Subsequently the mechanism of action of these compounds could be elucidated using pancreatic islet cell model or cancer cell lines.

8.2.4. *In vivo* studies

In order to further strengthen the preliminary *in vivo* data obtained in section, 6.2.3, the experiment would be repeated to increase the number of animals in each group. This increased number of animals can then be used to compute the statistics. Similarly, *in vivo* studies using cancer mouse model could be done to prove beyond doubt the large intestine targeting ability of the delivery system.

8.3. Reconnaissance studies

In an attempt to understand and hypothesize the mechanisms involved in the beneficial effects of the coagulans extracts, three different effects of the extracts: wound-healing effect, anti-diabetic effect and anti-cancer effect were evaluated *in vitro*. While the anti-diabetic and anti-cancer effects, have been discussed in chapters 6 and 7, as a means to exemplify the targeting ability of the delivery system, the observed results for the wound healing property of the extracts, does not warrant a place in these chapters. Hence these results are explained here as findings observed while investigating the various therapeutic effects of coagulans *in vitro*

8.3.1. Wound healing effect

The toxicity of the extracts, at a range of concentrations, for both P2 and P4 were tested on HDF cells, in order to determine the working concentration for the wound healing studies. The cells were seeded at the concentration of 2×10^4 cells per well in 96 well plates. After 24 hours, the cells reached confluency and different concentrations of P2 and P4 dissolved in DMSO and water respectively, were added to the cells. Each concentration had five replicates. CCK 8 was used to assess the viability of cells and % cell viability was calculated based on Equation 3.3. As in Figure 8.3, all the four concentrations of P2

evaluated, showed potent toxic effects after 48 hours treatment on the cells, while P4, even at higher concentrations, showed only minimal toxicity even after 48 hours treatment. At low concentration, P4 was essentially non-toxic to the cells and also promoted cell proliferation. Hence this concentration was chosen for the subsequent studies.

Figure 8.3 Effect of (a) P2 and (b) P4 on the viability of HDF cells

8.3.2. Scratch wound assay

The scratch wound assay is a standard technique used to study rate of cell migration in the presence or absence of a particular molecule or compound. Briefly, HDF cells were seeded onto 25 mm cell culture dishes at a density of 1×10^5 cells. Once the cells reached confluency after 24 hours, a scratch was made running through the diameter of the dish using a sterile 1000 μL pipette tip. The resulting cell debris was washed off using PBS and fresh media was added, to which the samples were added. The experiment was repeated three times to obtain replicates. Once the samples were added, the dishes were viewed under light microscope and photographed at five different points along the scratch, which were also marked to enable subsequent follow up for 48 hours. At every 12-hour interval, the dishes were photographed using the software attached to the microscope at the pre-marked points. The images were then analyzed using ImageJ to determine the open wound area and the wound closure rate was calculated based on Equation 8.1.

Equation 8.1 Formula for % wound closure calculation

$$\% \text{ wound closure} = \left(100 - \frac{\text{Area of scratch devoid of cells } T_n}{\text{Area of scratch devoid of cells at } T_0} \right) * 100$$

Where, T_n refers to the different time points at 0, 12, 24, 36 or 48.

As shown in Figure 8.4, the experiment involved four test groups. Untreated cells (group 1-untreated) were the negative control. A positive drug control was not included as the mechanism of action of P4 is not known, which would severely hamper the choice of a right control. The concentration of free P4 (group 2-P4-1 $\mu\text{g/mL}$) was chosen based on the toxicity studies as elaborated in section 8.3.1. The same concentration was matched in P4 encapsulated in PLGA (group 3-P4 PLGA) to be release over 48 hours and for the P4 added in parts group, P4 added every 12 hours (group 4-P4-1 $\mu\text{g/mL}$ added in parts) such as to match the 1 $\mu\text{g/mL}$ concentration over 48 hours. All the test groups except group 1, showed complete wound closure at 48 hours, proving that P4 is in fact able to promote wound closure and was significantly different from group 1 at all-time points. In order compare the effect of encapsulating P4 and releasing it in a controlled sustained manner, to that of adding free P4, P4 was added every 12 hours to mimic sustained release. This is seen in

the groups 3 and 4 showing statistically significant faster wound closure than group 2 after 12 and 36 hours, the time points at which fresh P4 is added.

Figure 8.4 Wound healing effect of free P4 and P4 encapsulated in PLGA microparticles compared against untreated control and free P4 added in parts at specific time intervals on HDF cells over 48 hours. * $P < 0.05$ One-Way ANOVA, post-hoc Tukey test, $n = 3$.

The wound healing effect stems from the toxicity studies of P2 and P4 on HDF. From the studies, it was inferred that P2 is not only toxic to the cells but may also not possess the compounds that promote wound healing activity. On the other hand, at low concentrations, P4 was able to promote cell proliferation and almost double the cells compared to untreated cells after 48 hours. This led to the inference that P4 would certainly contain compounds essential for wound healing as proliferation of fibroblasts is an important step in wound healing. A study of wound healing effect of coagulans on mice, also orients towards the increased proliferation of fibroblasts as the mechanism of action [2]. While the molecular processes and signaling pathways underlying proliferation and migration of the fibroblasts

needs to be evaluated to support the migration of fibroblasts at the wound site, it could be hypothesized that the anti-oxidant property of coagulans could be one of the reasons for the wound healing property. When there is a wound, hydrogen peroxide causes injury to fibroblasts and damages its migrative ability by inhibition of epidermal growth factor receptor internalization [3]. Coagulans with its known anti-oxidant property [4], could help control oxidative damage, thereby promoting fibroblast migration.

References

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Appendix

Linear regression analysis

The regression analysis was done by plotting the % cell viability observed vs log concentration (μM). IC 50 was calculated from the equation obtained by fitting the data points to a linear trendline. An R^2 value of above 0.95 was considered to be best fit. Of the samples tested, one representative graph for each condition is shown.

P2

As mentioned in section 7.1.1, cell kill was observed at a lower concentration of P2, within a narrow window also the higher concentrations. This gave rise to two trends in the cell kill as observed in the graph above. Based on the equation obtained from the linear regression graph in the lower concentration window,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}) = \text{Antilog} \left[\frac{50 - 52.576}{-0.9906} \right]$$

$$\text{IC}_{50} = 1.418 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 116.29}{-46.55} \right]$$

$$\text{IC}_{50} = 26.54 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 112.84}{-43.503} \right]$$

$$\text{IC}_{50} = 27.82 \mu\text{M}$$

DOX

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 63.052}{-8.014} \right]$$

$$\text{IC}_{50} = 42.60 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 64.403}{-22.111} \right]$$

$$\text{IC}_{50} = 4.48 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 89.656}{-21.47} \right]$$

$$\text{IC}_{50} = 70.3 \mu\text{M}$$

5 FU

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells(IC50)} = \text{Antilog} \left[\frac{50 - 125.77}{-35.68} \right]$$

$$\text{IC 50} = 132.75 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells(IC50)} = \text{Antilog} \left[\frac{50 - 98.286}{-30.98} \right]$$

$$\text{IC 50} = 36.19 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells(IC50)} = \text{Antilog} \left[\frac{50 - 125.91}{-34.698} \right]$$

$$\text{IC 50} = 154.07 \mu\text{M}$$

P4-48 hours

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 117.57}{-28.653} \right]$$

$$\text{IC}_{50} = 238.64 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 233.2}{-58.101} \right]$$

$$\text{IC}_{50} = 1422.75 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 368.81}{-85.654} \right]$$

$$\text{IC}_{50} = 5273.12 \mu\text{M}$$

DOX

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 79.877}{-25.466} \right]$$

$$\text{IC}_{50} = 14.90 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 55.409}{-16.598} \right]$$

$$\text{IC}_{50} = 2.1 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 64.923}{-13.942} \right]$$

$$\text{IC}_{50} = 11.75 \mu\text{M}$$

5 FU**Caco2**

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells(IC50)} = \text{Antilog} \left[\frac{50 - 134.57}{-49.487} \right]$$

$$\text{IC 50} = 51.88 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells(IC50)} = \text{Antilog} \left[\frac{50 - 161.57}{-73.406} \right]$$

$$\text{IC 50} = 33.10 \mu\text{M}$$

From the equation obtained from the linear regression graph,

$$\text{Concentration of P2 required to kill 50\% cells (IC}_{50}\text{)} = \text{Antilog} \left[\frac{50 - 144.7}{-48.193} \right]$$

$$\text{IC}_{50} = 92.26 \mu\text{M}$$