TRAGACANTH AS A NOVEL EXCIPIENT IN ORAL INSULIN DELIVERY

A thesis submitted to Victoria University for the degree of Doctor of Philosophy

by

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I dedicated this thesis to Allah,

My Beloved Parents,

Wife, Son and Daughter,

for abundant love and support

ABSTRACT

Diabetes mellitus is one of the most grave and lethal non-communicable diseases. Insulin is normally used to medicate diabetes. Due to bioavailability issues, the most regular route of administration is through injection, which may pose compliance problems to treatment. The oral administration thus appears as a suitable alternative, but with several important problems. Low stability of insulin in the gastrointestinal tract and low intestinal permeation are some of the issues. Encapsulation of insulin into polymer-based particles emerges as a plausible strategy. Different encapsulation approaches and polymers have been used in this regard. Polymers with different characteristics from natural or synthetic origin have been assessed to attain this goal, with natural polymers being preferable. Natural polymer such as tragacanth, an anionic polysaccharide gum, can be alternative polymeric carrier for physiologically important peptides and proteins like insulin.

Characterisation of tragacanth was explored in the first stage of the study, for providing a foundation for possible applications. Rheological studies colloidal solution of tragacanth at pH 3, 5 or 7 were carried out by means of steady shear and small amplitude oscillatory measurements. From preliminary study, 0.5% tragacanth was selected as optimum colloidal solution and 0.2 mg/ml insulin was chosen as concentration for a model protein. Tragacanth mucoadhesivity was also analysed using an applicable rheological method and compared to chitosan, alginate and PVP. The particle size and zeta potential were measured by a zetasizer. Thermal properties of solutions were obtained using a differential scanning calorimetry. The solution exhibited shear-thinning characteristics. The value of the storage modulus (G′) and the loss modulus (G″) increased with an increase in angular frequency (Ω). In all cases, loss modulus values were higher than storage values (G″ > G′) and viscous character was, therefore, dominant. Tragacanth and

alginate showed a good mucoadhesion. Tragacanth upon dispersion created particles of a submicron size with z-average diameters (mean) ranging between roughly 431 and 581 nm, with a negative zeta potential (-7.98 to -11.92 mV). These properties were pH dependant resulting in acid gel formation at pH 3.5. Tragacanth has thus a potential to be used as an excipient for peptide/protein delivery.

Since tragacanth has a promising result to be used as a carrier in protein/peptide delivery and needs a further application, in the second study, insulin microparticles were prepared by the inclusion of insulin into a tragacanth hydrogel followed by freeze drying. The effect of the pH and concentration relationship involving polyelectrolytes offering individual particle size and zeta potential was assessed by zetasizer and scanning electron microscopy (SEM). Insulin–tragacanth interactions were prepared at varying pH (3.7, 4.3, 4.6, or 6), and concentration (0.1, 0.5, or 1% w/w) to optimize the conditions for optimal delivery of insulin. The pI of insulin can vary from 5.5 to 6.4, based on its origin. The pH 4.3; 4.6 and 6 was selected because these pH is below pI of insulin. At a pH lower than its pI value, insulin will be mainly positively charged. This insulin characteristic could be utilised to facilitate insulin–biopolymer complexes through electrostatic attraction with tragacanth (negatively charged). Individual and smaller particles with z-average diameters approximately 601 ± 19 nm (mean ± S.D.), were acquired at pH 4.6 with 0.5% of tragacanth. The acid gelation test indicated that insulin could be entrapped in the physical hydrogel of tragacanth. DSC thermograms of insulin–tragacanth showed shifts on the same unloaded tragacanth peaks and proposed polyelectrolyte–protein interactions at a pH close to 4.3–4.6. FTIR spectra of tragacanth–insulin complexes exhibited amide absorption bands featuring in the protein spectra and revealed the creation of a new chemical substance.

In the previous stage, tragacanth microparticles seem to have potential functional characteristics for oral insulin delivery by creating a complex with insulin under defined conditions followed by freeze drying. Since freeze-drying is up to 30–50 times more expensive than spray-drying and to

make the overall process more industrially applicable, spray drying method has been explored in the third research. A spray-drying process was utilized to create microparticles from insulin/tragacanth GDL acidified solutions. The complexation process was performed at two tragacanth concentrations (0.5; 1%w/w) and several pH values (3.7; 4.3; 4.6; or 6). The SEM analysis indicated that almost spherical or sub-spherical microparticles were created with a diameter of less than 10 μm. The *in vitro* insulin release of microparticles prepared at a pH 4.3 and 4.6 was substantially minimized in comparison to other pH indicating improved retention of insulin. The selection of complexation pH appears to have an impact on insulin release profile and be an important parameter in protecting against peptic digestion. This finding stem from a possible creation of an insulin/tragacanth complex and hydrogel system. The evaluation of the interaction between insulin and tragacanth at different pH values by ATR-Fourier transform infrared and differential scanning calorimetry analysis verified this hypothesis. This finding suggests that these microparticles may act as a potentially promising device for oral insulin delivery.

Keywords: Insulin release; gum tragacanth; natural polymer; encapsulation; polyelectrolyte complexes; drug delivery; hydrogels; flow behavior; acid gelation; carrier; oral administration; protein/peptides drug delivery; mucoadhesive; insulin carrier; rheology; microparticles

CERTIFICATE

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Professor

College of Health and Biomedicine

Victoria University

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This is to certify that the thesis entitled "TRAGACANTH AS A NOVEL EXCIPIENT IN ORAL INSULIN DELIVERY" submitted by Mokhamad Nur in partial fulfillment of the requirement for the award of the Doctor of Philosophy with specialization in Food Sciences and Technology at Victoria University is a record of bonafide research work carried out by him under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Werribee, Australia

Prof. Todor Vasiljevic

Thesis supervisor

Date: 03.07.2018

DECLARATION

"I, Mokhamad Nur, declare that the PhD thesis entitled "TRAGACANTH AS A NOVEL EXCIPIENT IN ORAL INSULIN DELIVERY" is no more than 100,000 words in length including quote and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature: Date: 01/07/2018

Mokhamad Nur

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Bismillaahirrahmaairrahiim.
Alhamdu lillaahi rabbil ‘aalamiin.
Allohumma sholli ‘alaa Sayyidinaa Muhammad wa ‘alaa aali Sayyidinaa Muhammad.
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Mokhamad Nur

Date: 01/07/2018

List of Publications

Peer reviewed publications

1. Nur, M., Ramchandran, L., & Vasiljevic, T. (2016). Tragacanth as an oral peptide and protein delivery carrier: Characterization and mucoadhesion. *Carbohydrate Polymers*, 143, 223-230.
2. Nur, M., & Vasiljevic, T. (2017). Can natural polymers assist in delivering insulin orally? *International Journal of Biological Macromolecules*, 103, 889-901.
3. Nur, M., & Vasiljevic, T. (2018). Insulin Inclusion into a Tragacanth Hydrogel: An Oral Delivery System for Insulin. *Materials*, 11(1), 79.

PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
2	Can natural polymers assist in delivering insulin orally?	Published	International Journal of Biological Macromolecules (Volume 103, October 2017, Pages 889-901; Impact Factor: 3.671; Web of Science Quartile: Q1)
3	Tragacanth as an oral peptide and protein delivery carrier: Characterization and mucoadhesion	Published	Carbohydrate Polymers (Volume 143, 5 June 2016, Pages 223-230; Impact Factor: 4.811; SCImago Quartile: Q1)
4	Insulin Inclusion into a Tragacanth Hydrogel: An Oral Delivery System for Insulin	Published	Materials (2018, 11(1), 79; Impact Factor: 2.654; SCImago Quartile: Q1)
5	Insulin-loaded tragacanth microparticles produced by spray drying: Towards a novel oral insulin delivery system	Under review	International Journal of Biological Macromolecules (Impact Factor: 3.671; Web of Science Quartile: Q1)

Declaration by [candidate name]: Mokhamad Nur Signature: [redacted] Date: 01/07/2018

Chaired Oral Presentations

1. Nur, M., Ramchandran, L., Dass, C. R., & Vasiljevic, T. (2014). Characterization of tragacanth for excipient in oral insulin delivery. Postgraduate Research Conference. 2 October 2014, St Albans Campus, Victoria University, Melbourne, Australia.
2. Nur, M., Ramchandran, L., Dass, C. R., & Vasiljevic, T. (2015). Characterization of tragacanth for peptide/protein delivery. Australian Institute of Food Science and Technology (AIFST) Food Science Summer School. 28 - 30 January 2015. RMIT University, Melbourne, Australia.

Awards and Achievements

1. Indonesian Directorate General of Higher Education (DIKTI) Scholarship for full-time study in a Doctor of Philosophy program.
2. Selected as an invited reviewer of an article in Nature Publishing Group (NPG) Asia Material (IF: 9.157) and an article in International Journal of Nanomedicine (IF: 4.300).

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List of Abbreviations and Acronyms

AGF	Artificial gastric fluid
AIF	Artificial intestinal fluid
ANOVA	Analysis of variance
ATR-FTIR	Attenuated total reflection-fourier transform infrared
DSC	Differential scanning calorimetry
G′	Storage modulus
G″	Loss modulus
GDL	Glucono-δ-lactone
GIT	Gastrointestinal tract
HCl	Hydrogen chloride
INS	Insulin
kDa	Kilo Dalton
LE	Loading efficiency
MW	Molecular weight
NaCl	Sodium chloride
PEC	Polyelectrolyte complex
pH	Hydrogen ion concentration
pI	Isoelectric point
pKa	Acid dissociation constant
PVP	Polyvinyl pyrrolidone
rpm	Revolution per minute
RT	Room temperature
s	Second
SAS	Statistical analysis system
SEM	Scanning electron microscope
SQ	Subcutaneous
TG	Tragacanth
w/w	Weight per weight
× g	Times gravitational force
Ω	Angular frequency
ζ	Zeta potential

CHAPTER 1

Chapter 1. Introduction

1.1 Background

Diabetes mellitus is the sixth most common cause of death in the world. There are two major types of diabetes; type 1 and 2. Insulin (Fig 1.1), a polypeptide hormone secreted from pancreas, is used to treat these patients. Type 1 diabetic patients cannot get insulin directly from their body; therefore, they will depend on external sources of insulin. On the other hand, type 2 diabetic patients can produce that hormone but during their life they may experience difficulties to produce insulin internally, therefore they still need insulin externally to maintain their blood glucose level (Zaykov et al. 2016; Sonia & Sharma 2015; Gedawy et al. 2018).

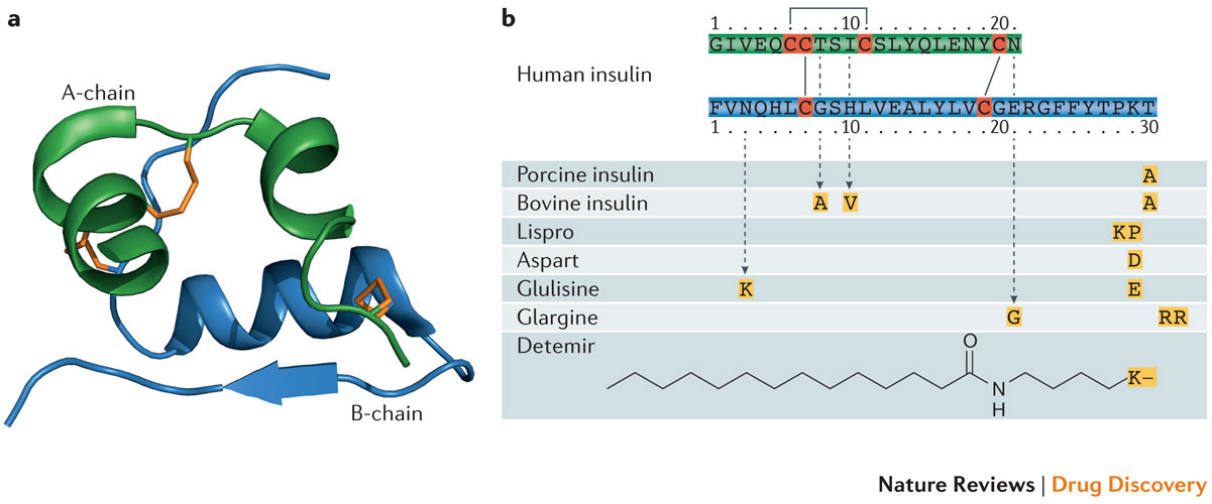

Figure 1.1 a) Structure and b) sequences of insulin (Zaykov et al. 2016)

Since the invention of insulin, insulin is administered to diabetic patients exclusively by the subcutaneous route. Because of relatively short duration of insulin action (i.e. four to eight hours) and hence the patients are required to inject insulin twice to four times every day to appropriate control of severe diabetic situation. Even though the parenteral route is acceptable in terms of efficacy, the stress and discomfort of multiple subcutaneous daily injections,

therefore researchers have sought to develop alternative route including buccal, nasal, transdermal, pulmonary and oral insulin delivery systems. Among insulin delivery systems patients and industry prefer oral route of administration because it is non-invasive, avoids injections, and reduce the risk of infections. It is also physiologically desirable since the exogenous protein imitates the physiological pathway undergoing first hepatic bypass (Arbit 2004; Sonia & Sharma 2015).

However, replicating normal physiological patterns of insulin secretion through oral insulin administration is still at its infancy. The peroral bioavailability of insulin is relatively low mainly due to acidic gastric pH, high proteolytic activity in the gut and low permeability of the intestinal epithelium. Several strategies to overcome these problems for perorally administered insulin include the addition of enzyme inhibitors and/or permeation enhancers, chemical modification, cell penetration peptides, vitamin B12, or cyclodextrin conjugation, polymeric carriers, liposomes or a colon targeting of the drug delivery system where the enzymatic activity is relatively low (Card & Magnuson 2011). Most of these strategies have produced promising results, such as retention to pH sensitivity, but the bioavailability still remains low. Bioavailability is related to the capacity of insulin to remain stable when absorbed from the human small intestine into the systemic circulation. Polymeric biomaterials are nowadays an attractive option for increasing the bioavailability of insulin. Although various natural polymeric carriers have been developed for insulin release, such systems have not shown sufficient bioavailability when administered orally (Card & Magnuson 2011; Sarmento et al. 2006b; Sonia & Sharma 2015).

To overcome this problem which is how to improve the bioavailability of insulin during oral administration, mucoadhesive polymers can be used as an alternative. These materials maintain contact with intestinal epithelium for extended periods, promoting penetration of active drug through and between cells due to the concentration gradient between nanoparticles and

intestinal membrane. Consequently, bioavailability of the drug is increased leading to improved patient compliance. In fact, insulin was observed to be directly internalized by enterocytes in contact with intestine, and retention of drugs at their absorptive sites by mucoadhesive carriers is a synergic factor (Sonia & Sharma 2012). Furthermore, uptake of nanoparticles by the M cells of the Peyers patches was demonstrated, being absorbed transcellularly, serving as a major gateway for nanoparticle absorption as well as absorption through the much more numerous gut enterocytes. Endocytosis occurs through clathrin coated pits and vesicles, fluid phase endocytosis and phagocytosis (Card & Magnuson 2011). It is well accepted that hydrophobic, negatively charged, protein loaded nanoparticles smaller than 1 mm potentially show the best absorption rate, although other factors may govern nanoparticle absorption (Sarmento et al. 2007c; Sonia & Sharma 2015).

Recent reviews indicate that, the most effective nanoparticle-based formulations in terms of pharmacological availability have been based on mucoadhesive, biodegradable, biocompatible, and acid-protected materials (Card & Magnuson 2011; Tan et al. 2010). Polymers such as tragacanth, chitosan and alginate have been described as biocompatible, biodegradable and mucoadhesive, enabling numerous pharmaceutical and biomedical applications including the design of controlled release devices. Numerous studies have been conducted to use chitosan and alginate as insulin carrier (Sarmento et al. 2007d; Tan et al. 2010; Reis et al. 2008b; Sonia & Sharma 2012; Sonia & Sharma 2015; Gedawy et al. 2018).

However, among these biopolymers, tragacanth can potentially be used as novel coating material during encapsulation process (Fig. 1.2) because it has higher mucoadhesive properties (Nur et al. 2016).

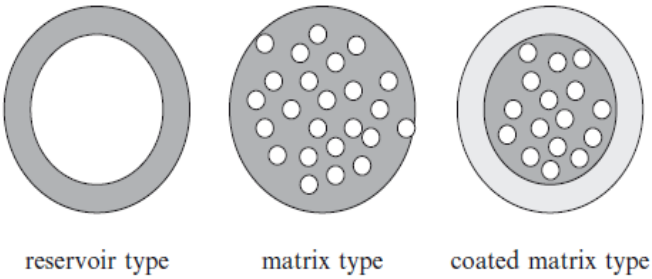

Figure 1.2 Reservoir type (left), matrix type (middle), and coated matrix type (right) encapsulates (Zuidam & Nedovic 2010).

Tragacanth (TG), is an anionic polysaccharide gum obtained from the stems and branches of different species of Astragalus. This polysaccharide shows high mucoadhesive properties (Jackson & Perkins 2001). This gum consists of two macromolecules having a total molecular weight of 850kD. Tragacanth is a highly acid-resistant gum which consists of two major fractions: 1) (water-soluble fraction) composed of tragacantic acid (ethanol-insoluble) and arabinogalactan as an ethanol-soluble minor fraction and 2) bassorin (water-insoluble but swellable fraction) (Hasandokht Firooz et al. 2012; Kaffashi et al. 2006; Mohammadifar et al. 2006).

From its zeta potential and mucoadhesive properties, tragacanth may create a complex with insulin through two types of mechanisms: polyelectrolyte complexes (PECs) and entrapment through hydrogel. PECs are made up of oppositely charged (cationic and anionic) biopolymers formulated under mild conditions to carry peptides or proteins and colloidal carriers. This complexation shows potential as a vehicle to encapsulate proteins and provide protection and sustained release (Sarmento et al. 2006b). Hydrogel is an insoluble semi permeable matrix that can be applied to entrap insulin. It can be created from polymers from animal- or plant-based derivatives (Lim et al. 2014). Factors that affect the preparation process are biopolymer concentrations, pH, emulsion size, ionic strength, and temperature. Complexes might be dried

by spray-drying or freeze-drying. Each polymer combination operates at unique conditions in terms of pH, temperature, ionic strength, polymer levels, molecular weight, charge density and cooling rate (Zuidam & Nedovic 2010; Hartig et al. 2007).

1.2. Research Objectives

This project proposes that creating complex between insulin and biopolymer, especially tragacanth can improve the bioavailability of insulin during oral insulin administration. The objective of this research is thus to develop an efficient delivery system for oral insulin administration.

With the above factors taken into consideration, the specific aims of this thesis are as follows:

- Characterize the tragacanth in oral protein/peptides delivery application; assess mucoadhesivity of tragacanth and compare it with the existing polymers, i.e. chitosan, alginate, PVP (Chapter 3)
- Create microencapsulation of insulin from tragacanth hydrogel followed by lyophilization and assess percent loading efficiency of insulin (Chapter 4)
- Study the feasibility of the spray-drying to create microparticles of tragacanth for the oral delivery of insulin and assess *in vitro* insulin release rate of the microparticles (Chapter 5).

1.3. Thesis Outline

The thesis is divided into 7 chapters. In chapter 1, background, research objectives and outline of the thesis is presented. Chapter 2 of this thesis presents a literature review of the current scientific knowledge on the proposed topic. Diabetes, insulin, the routes of insulin administration, and main approaches used for oral insulin delivery with the emphasis on

the polymeric delivery system were discussed. The scientific information about the interaction between protein and polysaccharides was also described. Chapter 3 focuses on the characterization of tragacanth to provide a foundation for possible applications in peptide/protein delivery. Tragacanth mucoadhesivity was also studied using an applicable rheological method and compared to existing polymers. Chapter 4 reports on the inclusion of insulin into a tragacanth hydrogel followed by freeze drying. The effect of the pH and concentration relationship involving polyelectrolytes offering individual particle size and zeta potential was assessed. Percent loading efficiency of microparticles was also analyzed. Chapter 5 presents a study on the feasibility of the spray-drying to produce tragacanth microparticles for oral delivery of insulin. *In vitro* insulin release rate of the microparticles was also assessed. Finally, the conclusion from the study and future research directions is provided in chapter 6. All references are listed in chapter 7.

CHAPTER 2

A. LITERATURE REVIEW

B. REVIEW ARTICLE

Chapter 2A. Literature Review

2.1. Diabetes

Diabetes mellitus is a damaging non-communicable disease. The number of diabetes patients are projected to rise to 438 million globally by the year 2030 as outlined by the World Health Organization (WHO) (Alai et al. 2015). Diabetes mellitus is an endocrine disease whereby the pancreas cannot generate adequate insulin or the body is not able to effectively use the insulin it generates. There are two primary types of diabetes: type I and type II. In type I diabetes, diabetics generate very little or no insulin. In type II diabetes, diabetics are unable to utilize insulin effectively (Alai et al. 2015). The main objective for the therapy of type I and type II diabetic is to treat the problems associated with hyperglycemia. Insulin has an essential function in diabetes medication. Diabetics with type I diabetes demand regular administration of insulin to survive. Patients with type II diabetes may or may not need exogenous insulin as a treatment method (Alai et al. 2015). Type 1 diabetes is an autoimmune disease whereby inappropriately activated T-lymphocytes destroy the insulin-producing β-cells of the pancreas. Consequently, individuals with type 1 diabetes have an absolute or almost absolute insulin insufficiency (Wong et al. 2016; Sonia & Sharma 2015).

Since insulin is the only hormone in the body which is capable of reducing blood glucose levels, type I diabetes results in elevated blood glucose levels otherwise known as hyperglycemia. If blood glucose levels remain elevated and uncontrolled, plasma proteins become glycosylated.

This causes the plasma proteins to increase in size thus resulting in the obstruction of small blood vessels in the heart, kidneys, nerves, and eyes. As a result, damage and/or destruction of these systems may occur if blood glucose levels remain uncontrolled. It is for this reason that the blood glucose levels of diabetic individuals must be maintained within the normal range of approximately 80-110 mg/dl (4.4 to 6.1 mmol/l). The standard method of calculating glucose levels in blood is in terms of a molar concentration, calculated in mmol/l (mM) (millimoles per litre or millimolar) or in mg/dl (milligrams per decilitre). The difference between the two is a factor of 18: 1 mmol/l of glucose corresponds to 18 mg/dl (the MW of glucose is around 180 g/mol) (Sonia & Sharma 2015).

Type 2 diabetes (non-insulin dependent diabetes) is more common than type 1 diabetes. While individuals with type 1 diabetes require exogenous subcutaneous (SQ) insulin, those with type 2 diabetes can initially control their blood glucose levels through diet and exercise. This is due to the fact that the B-cells of the pancreas still produce insulin although the amount may be insufficient. Eventually, however, many type 2 diabetic individuals require exogenous insulin as there is increased resistance to their own insulin (Wong et al. 2016; Sonia & Sharma 2015).

2.2. Insulin

Insulin was initially isolated in the 1920's by Frederick Banting and Charles Best to the alleviation of numerous diabetics who, prior to that period, would have been susceptible to rigorous starvation diets and a very poor quality of life (Sonia & Sharma 2015). The crude formulation produced by Banting and Best was originally isolated from a dog pancreas and

ultimately standardized with assistance from the American pharmaceutical company, Eli Lilly. After that, insulin has become one of the most extensively used peptide/protein therapies worldwide and therefore, perfectly characterized and researched. Insulin was also the first drug to be effectively mass-produced by recombinant DNA technology (Sonia & Sharma 2015).

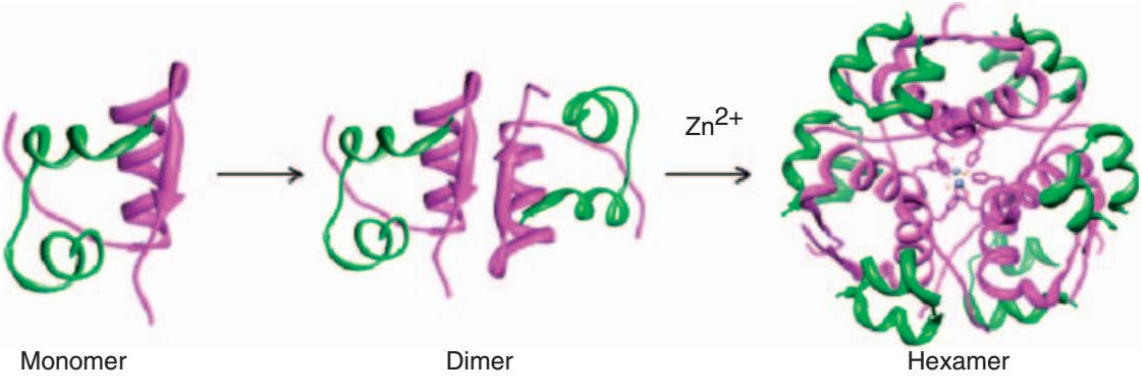

Figure 2.1 The pattern of assembly of insulin monomer, dimer, and hexamer (Vanea et al. 2014)

Insulin (Fig. 2.1) is a 5.8 kDa peptide hormone composed of 51 amino acids. The insulin monomer consists of two polypeptide chains, an A-chain of 21 amino acids and B-chain of 30 amino acids, which are linked by two disulphide bonds. The structure of insulin differs between monomers, dimers, tetramers, and hexamers in solution under the influence of ions and solvent media. Moreover, insulin is vunerable to fibril formation in organic chemicals, increased temperatures, acidic pH and vibration (Vanea et al. 2014). Insulin is a polypeptide with ampholyte behaviour. Above its isoelectric point (pH 5.3) it is negatively charged, able to interact with several types of positively charged molecules (low MW compounds or macromolecules) (Grigoras 2017).

2.3. Insulin Administration Routes

2.3.1. Insulin Pump

For diabetics who find multiple daily SQ injections of insulin prohibitive, there is a choice of the external insulin pump. The insulin pump is an equipment that delivers very small doses of insulin continuously via plastic tubing that is located subcutaneously in the skin therefore it is recognized as continuous subcutaneous insulin infusion. Insulin pumps are as small as some cell phones and can be attached to a belt or placed in a pocket. They consist of an insulin reservoir, a pump and a computer chip, which allows the user to program the pump to provide either continuous basal insulin supplementation throughout the day, a bolus of insulin prior to meals or any combination of the preceding dependent upon the user's eating habits. The advantages of utilizing an insulin pump over SQ insulin injections include more consistent insulin absorption, decreased formation of advanced glycation end products and a decreased risk of low blood sugar, or hypoglycemia (Sonia & Sharma 2015).

2.3.2. Buccal Insulin Delivery

Buccal insulin delivery is an administration of a liquid insulin formulation via a metered inhaler into the oral cavity. This kind of system provide many benefits such as a relatively large surface area for insulin absorption (Sonia & Sharma 2015). However, the inconsistency in saliva circulation in the mouth and the multilayered structure of the buccal epithelium presented significant hurdles to insulin absorption (Sonia & Sharma 2015). Generex Biotechnology has

developed an insulin delivery system, Oralin™. Oralin™ is a buccal insulin delivery apparatus in which insulin is encapsulated into mixed micelles produced from a mixture of absorption enhancers. Oral-Lyn™, is delivered by the RapidMist™ device to the oral cavity and penetrates the buccal epithelium and enters the rich network of blood vessels (Park et al. 2011).

2.3.3. Nasal Insulin Delivery

Another technique by which insulin can be administered is directly into the nasal cavity. Intranasal delivery is an attractive way for delivery of insulin because of the large surface area of the nasal epithelium (about 1500 cm^2) and easy accessibility to the systemic circulation. However, there are some barriers to intranasal delivery such as the mucociliary clearance system, increased enzymatic activity and a reduced epithelial permeability (Sonia & Sharma 2015). Moreover, when compared to SQ injections, intranasal delivery of insulin is quicker although the bioavailability is not more than 20% (Sonia & Sharma 2015). Additionally, some of the excipients such as absorption enhancers have been recognized to trigger irritation of the nasal mucosa (Sonia & Sharma 2015).

Inhalation administration route has a quicker onset of action because of the existence of alveoli in the lungs for systemic insulin absorption (Wong et al. 2016). Insulin is also less vulnerable to proteolytic destruction in the GIT. In 2006, an inhaled insulin powder product (Exubera®) was marketed by Pfizer (Wong et al. 2016; Sonia & Sharma 2015). This item was approved by FDA for adults and kids more than 6 years old. However, Exubera® was removed from the

market 1 year later. The absorption of inhaled insulin in the alveoli diverse substantially particularly for obese diabetics, smokers and diabetics with asthma and chronic obstructive pulmonary disease (Wong et al. 2016).

2.4 Oral Insulin Delivery

Among possible systemic absorption routes, oral administration of insulin is particularly interesting because it provides many benefits such as ease of administration and higher diabetics compliance (Wong et al. 2016). Moreover, the oral administration provides the distinctive feature of mimicking the physiological route for particular drugs like insulin that could get into the hepatic portal vein from the intestine and travel directly to the liver (Wong et al. 2016; Sonia & Sharma 2015). As a result, insulin delivered straight to the liver could reduce complications, like atherosclerosis, which are related to high concentrations and build-ups of insulin in the blood. On the other hand, insulin injected subcutaneously have to circulate via the body prior to reaching the liver. Despite these benefits, there are few oral peptide/protein and vaccine-based delivery systems and no insulin delivery systems on the market due mainly to low bioavailability in the GIT (Sonia & Sharma 2015). There are two main factors that are related to low bioavailability of insulin: enzymatic or acid degradation and poor absorption across the intestinal mucosa (Wong et al. 2016). Insulin, in particular, is too large and hydrophilic to cross the epithelial lining of the GIT (Wong et al. 2016).

2.5 Main Approaches Used for Oral Insulin Delivery

Numerous approaches have been applied in order to enhance the bioavailability of oral insulin designed both at preserving the insulin towards enzymatic destruction in the GIT and improving their permeability throughout the intestinal epithelial layer. These strategies can be divided into five major groups: chemical modifications to insulin, mucoadhesive system, protease inhibitors, absorption enhancers and particulate delivery systems (Sonia & Sharma 2015; Gedawy et al. 2018; Wong et al. 2016).

2.5.1 Chemical Modifications

A number of research have been conducted to change or add particular molecules to the structure of insulin to improve its bioavailability and solubility in the GIT and offer prevention of proteolytic enzymes. Insulin-transferrin conjugates undergo receptor-mediated endocytosis throughout intestinal epithelial cells, that contributes to a substantial hypoglycemic response in comparison to insulin control (Xia et al. 2000; Sonia & Sharma 2015; Park et al. 2011).

Emisphere (Eligen™ technology) established a technique, which involves a non-covalent complexation with non-acyl amino acids that leads to unfolding of the insulin structure to expose hydrophobic side chains that stimulates excellent translocation across the lipid bilayer (Park et al. 2011; Al-Hilal et al. 2013). When insulin passes across the membrane layer, the complex dissociates, and the insulin comes back to its native conformation. Even though Eligen™ technological innovation has demonstrated promising outcomes for oral delivery of

several therapeutic macromolecules, the formulation is known to trigger nausea in individuals, and the quantity of the delivery agent is orders of magnitude higher than the peptide/protein drugs, making it clinically ineffective (Park et al. 2011).

Other researchers have evaluated site-specific oligomeric modifications, which are believed to improve half-lives *in vivo* and offer higher enzymatic resistance when compared with native insulin (Clement et al. 2004). Cell-penetrating peptides (CPP) have also been researched to improve cellular translocation (Fukuoka et al. 2018). It was discovered that CPP-insulin conjugates improved transport by 6-8 times throughout a Caco-2 cell line (Liang & Yang 2005).

2.5.2 Protease Inhibitors

Because trypsin and chymotrypsin are the proteolytic enzymes mainly accountable for the breakdown of insulin in the GIT, researchers have focused on neutralizing these enzymes particularly (Gedawy et al. 2018). Co-administration of insulin with enzyme inhibitors provides a viable means to avoid the enzymatic barrier in obtaining the delivery of insulin and to increase bioavailability in the GIT (Park et al. 2011; Khafagy et al. 2007). The selection of enzyme inhibitors will rely on the structure of insulin. Information on the specificity of proteolytic enzyme is important to assure the stability of the insulin in the GIT (Sonia & Sharma 2015).

Some permeation enhancers like taurochenodeoxycholate, dimethyl-a-cyclodextrin or glycocholic acid enhanced the bioavailability of insulin, and capric acid, a fatty acid, was

most effective against a-chymotrypsin (Wong et al. 2016; Radwan & Aboul-Enein 2001). A number of researchers have indicated that while the use of protease inhibitors improves oral insulin bioavailability, there is still the problem of low permeability, and therefore these strategies must frequently be coupled with other methods (Liang & Yang 2005). For instance, fatty acids and bile salts have been explored as a method to retard enzymatic degradation and affect translocation across the intestinal epithelium. This is considered to happen by modifying the nature of the cellular membrane or facilitating paracellular uptake by opening tight junctions (Wong et al. 2016).

A new category of enzyme inhibitor, which is known as duck ovomucoid demonstrated 100% protective effect against trypsin and α-loading ef destruction of insulin *in vitro* for one hour at 1 : 2 ratio of enzyme inhibitor (Agarwal et al. 2000). Moreover, polymer-inhibitor conjugates for example CMC Bowman-Birk inhibitor and CMC-elastinal (CMC-Ela) have provided *in vitro* protection against elastase, *α*-chymotrypsin and trypsin. Particularly, CMC-Ela exhibited higher inhibitory activity towards elastase, such that almost 33% of insulin remained stable against proteolytic attack even after 4 hour of incubation (Marschütz & Bernkop-Schnürch 2000).

2.5.3 Absorption/permeation Enhancers

Mucosal absorption enhancers have also been explored and co-administered with insulin to enhance paracellular permeability (Gedawy et al. 2018; Park et al. 2011). Cyclodextrins, bile salts , trisodium citrates, chelating agents like EDTA, surfactants, fatty acids and terpenes have all been demonstrated to improve translocation across the intestinal mucosa (Sonia & Sharma 2015; Wong et al. 2016).

Many of these permeation enhancers have been used to insulin delivery systems (Sonia & Sharma 2015). Surfactants assist in improving transcellular transport by increasing the fluidity of the cell membrane and calcium chelators assist to enhance paracellular transport mediated by modulating the TJ of the cells by complex formation with calcium ions (Pepić et al. 2013; Delie & Blanco-Príeto 2005).

2.5.4 Mucoadhesive Systems and Mucus Penetration

The mucoadhesive properties of certain polymers have been applied to extend the residence time of the insulin at its absorption site by increasing the contact with mucosa which in fact enhance the concentration gradient of the insulin (Park et al. 2011; Gedawy et al. 2018). In polymeric mucoadhesives (Fig. 2.2), after initial intimate contact between polymer and mucus, diffusion appears to play an important role in the establishment of adhesive interactions; polymers diffuse and entangle with mucin fibers, while bonding is concurrently established (Sosnik et al. 2014). Interaction could be either covalent (*e.g.*, disulfide bridging with cysteine

residues of mucin) or non-covalent (*e.g.*, hydrogen bonding, electrostatic forces, hydrophobic interactions, van der Waals bonding). The dynamic balance between diffusion, physical entanglement and repulsive/adhesive interactions contributes to the consolidation of adhesion (Plapied et al. 2011).

The surface properties of the micro/nanoparticles will affect its transport through the mucus. The micro/nanoparticles mobility also appears to be highly influenced by surface charges. Transport rates were inversely associated with micro/nanoparticles surface potentials, with negatively charged micro/nanoparticles showing substantially greater transport rates than near neutral, or positively charged micro/nanoparticles whose transport was severely restricted, probably by particle aggregation and electrostatic adhesive interactions with mucin fibres (Plapied et al. 2011).

A balance between mucoadhesion and mucus penetration is essential for successful oral delivery. Since particles immobilized by mucus are cleared from the mucosal tissue, the elaboration of mucus-penetrating systems is a primary concern to enhance mucosal drug delivery. Particles have to be small enough to avoid substantial steric inhibition by the fibre mesh and should avoid adhesion to mucin fibres. Concurrently, they should have mucoadhesive properties to prolong retention time and contact with intestinal mucosa (Plapied et al. 2011).

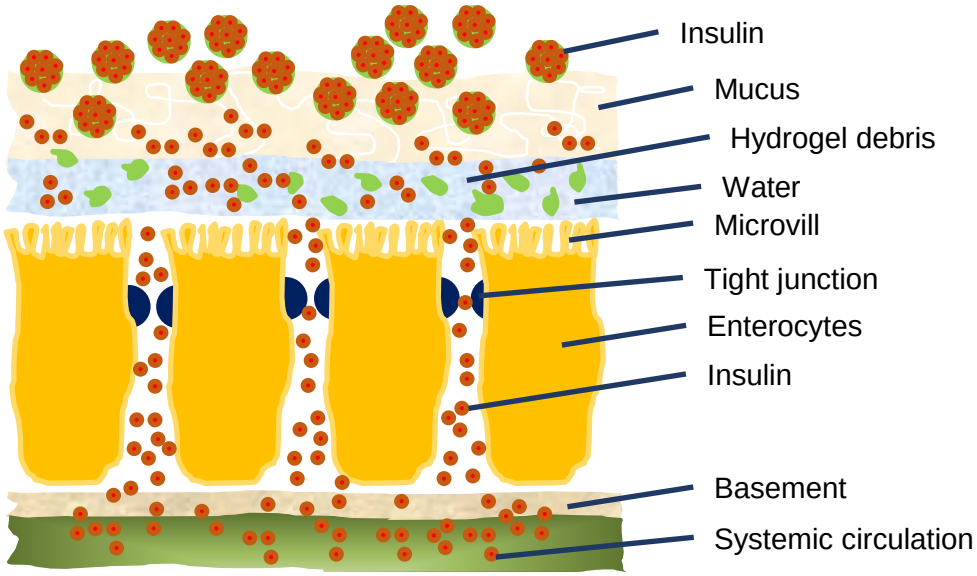

Figure 2.2 Mucoadhesive system (Chaturvedi et al. 2013; Sosnik et al. 2014)

2.5.5 Particulate Carrier Delivery Systems

Encapsulation is the phrase used to indicate the inclusion of an active agent into a particle of various wall material, like a phospholipid or polymer (Zuidam & Nedovic 2010). Two of the main reasons to encapsulate an active agent in drug delivery are for protection and to control release. The active agent can be preserved from pH extremes or hydrolytic conditions, or the individual may be protected from the active agent, which could possibly present toxicity and health problems or possess an unpleasant taste or odour (Zuidam & Nedovic 2010; Park et al. 2011). The target location for release and the release profile can be determined by excipient selection, formulation optimization and particle engineering. For instance, in mucosal drug delivery system, materials are usually chosen to enhance epithelial

permeability by reversibly opening tight junctions between cells or by improving contact time with the mucosa via bioadhesive forces (Gedawy et al. 2018). Chitosan is one of the biopolymers that can both reversibly open tight junctions and possess high mucoadhesion (Sonia & Sharma 2015).

Micro- or nanoencapsulation is the term for describing the incorporation of the active material into micro- and nano-sized particles respectively. This method is often used in the pharmaceutical industry to influence the pharmacokinetics of a therapeutic, enhance stability and minimize toxicity (Tewa-Tagne et al. 2006). “Microparticles” and “nanoparticles” are typical terms including both micro- or nanospheres and micro- or nanocapsules. Micro- or nanospheres have a matrix structure in which the peptides/protein can be absorbed either at the surface or throughout the internal of the particle. Micro- or nanocapsules, in contrast, possess a shell-type structure in which the therapeutics is coated in the center of the particle and surrounded by a protein or polymeric coating (Wong et al. 2018).

When selecting formulation techniques for encapsulated drugs, factors like shear stress, temperature and pH have to be considered to prevent denaturation or destruction of the active materials (Coppi et al. 2002). In addition, an effective method must be scalable to produce industrial levels while reducing cost. Techniques that are usually discovered in the literature using preformed polymers include ionotropic pregelation, emulsion dispersion and spray/freeze drying (Kusonwiriyawong et al. 2009; Sarmento et al. 2007b; Reis et al. 2008b).

2.6 Polymeric Delivery System

2.6.1 Ionotropic Pregelation

Ionotropic pregelation requires polyelectrolyte complexes (PECs) to produce the nano- or microparticle matrix (Sarmento et al. 2006a; Sarmento et al. 2006b) Particularly, PECs are formed when polycationic and polyanionic polyelectrolytes are put together in dilute solution. A good example of a PEC includes alginate, a polyanion, and chitosan, a polycation. The nano- or microparticles are produced by mixing alginate with calcium carbonate and continuously stirring the mixture to obtain a pre-gel state. After that, the therapeutics is put into the solution and finally, chitosan is added to the mixture which creates and stabilizes the expected nano- or microparticles. Ionotropic gelation is favourable because it a uncomplicated method based on simple methods. However, like emulsion techniques, it is challenging to be scaled up and needs a further drying step to make sure long-term stability (Sarmento et al. 2006a; Sarmento et al. 2006b).

2.6.2 Emulsion Dispersion

The emulsion dispersion technique to create polymeric nano- or microparticles is generally carried out in two major steps. A polymer and active material in solution are initially emulsified with an aqueous phase, and the solvent is then evaporated, leaving nano- or microspheres (Reis et al. 2006). This method allows control over nano- or microparticle size by altering the temperature, viscosity of aqueous and organic solvents and stirring rates. In

addition, emulsion dispersion has been demonstrated to reproducibly create particle in the nano- or micro- size range. Issues with emulsion dispersion techniques include the capability to be scaled up to an industrial level because of the energy requirements for dispersion and the number of steps needed. An extra drying step, like freeze- or spray- drying, is typically also needed to ensure stable shelf life (Bowey & Neufeld 2010).

2.6.3 Freeze- and Spray-Drying

Two most common drying methods for encapsulating insulin/peptide are freeze drying and spray drying. Therapeutics/drugs and excipient material dissolved in water could be freeze-dried to create a porous, non-shrunken structure. Initially, the material is frozen at temperatures between −90 and −40°C and after that dried by direct sublimation under low pressure and decreased temperature (between −90 and −20°C). After drying, the brittle cake acquired could be broken into smaller parts by, e.g., grinding, if required. The usage of substantial quantities of cryoprotectants (for example, 30% maltodextrin, 10% disaccharides or 10 % milk proteins) could assist to stabilize sensitive encapsulates such as liposomes or sensitive active agent such as probiotics (Zuidam & Nedovic 2010). The major drawbacks of freeze-drying are the extended operating time, the high energy use, and the open porous structure acquired, which is generally not a very good barrier between the active materials and its surroundings (Zuidam & Nedovic 2010). When compared with spray-drying, freeze-drying is approximately 30-50 times more costly (Gharsallaoui et al. 2007).

On the other hand, spray drying is the transformation of an emulsion, dispersion or suspension to a dry state by atomizing the solution and dispersing it through a hot air (Anandharamakrishnan & Padma Ishwarya 2015). Spray drying is a well-established technology presently applied in a number of industrial sectors to produce numerous food and cosmetic products (Zuidam & Nedovic 2010). It is also used in the pharmaceutical industry to create drug powders and other dry therapeutics. Both aqueous and organic solvent soluble ingredients can be dried via spray drying, with the latter performed in a closed loop operation. Spray-drying is a method based on the transformation of a liquid solution into a dry powder by atomization in a hot drying air. The spray-drying procedure (Fig. 2.3) includes four basic steps: (i) atomization of the fluid feed, (ii) drying of spray into drying gas, (iii) creation of dry particles and (iv) separation and collection of the dry product from the drying gas into drying chamber (Sosnik & Seremeta 2015). Initially, the liquid is pumped into the drying chamber by a peristaltic pump via an atomizer or nozzle that can be a pressure nozzle, a two-fluid nozzle or a rotary atomizer and the atomization happens by centrifugal, pressure or kinetic energy, respectively. The atomized droplets produced (nano to micrometer scale) are exposed to quick solvent evaporation resulting in the creation of dry particles that are separated from the drying gas by means of a cyclone that deposes them in a product collection vessel located in the bottom of the machine (Sosnik & Seremeta 2015) .

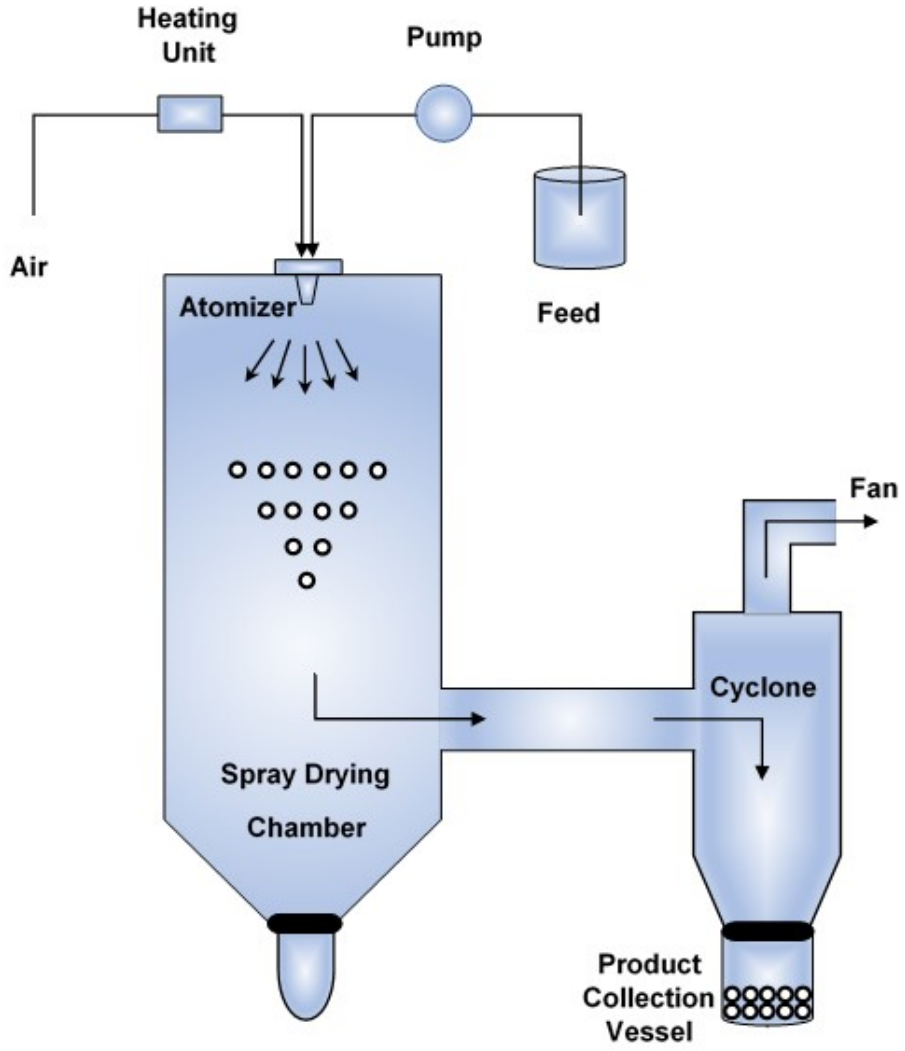

Figure 2.3 Schematic illustration of a spray dryer

To optimize spray drying process parameters, there have been numerous studies explaining the effects on the particle size, humidity and morphology of the final microparticulate products (Anandharamakrishnan & Padma Ishwarya 2015; Kusonwiriyawong et al. 2009). For instance, it has been established that particle size is directly related to the concentration and composition of the initial formulation, while the morphology of the particulate is influenced by the initial composition and drying rate (Kusonwiriyawong et al. 2009).

Another critical factor is the product yield of spray drying, which could be governed by the process parameters. The mass yield depends on the quantity of solids pumped into the spray drying unit and the mass of particles obtained. For laboratory scale operations, the yield is usually low, producing a maximum of 60%, subject to polymer used and equipment operating parameters (Ameri & Maa 2006).

One of the disadvantages of spray drying is the need to frequently operate at inlet temperatures more than of 100°C, potentially denaturing heat-sensitive protein/peptides (Ameri & Maa 2006). Numerous reports have demonstrated that optimized conditions or the addition of sugars or amino acids could stabilize peptides/proteins (Bowey & Neufeld 2010; Kusonwiriyawong et al. 2009). BSA spray dried in chitosan matrix at an air inlet temperature of 120°C maintained its integrity and secondary conformational structure (Kusonwiriyawong et al. 2009). While in the atomization phase, dispersed droplets are moisture saturated and the relative humidity level approaches 100%. The protein/peptide remains at the wet-bulb temperature, which is lower than the temperature of the air inside the drying chamber (Ameri & Maa 2006). For this reason, the therapeutic is not exposed to the real inlet temperature. In addition, as the droplet loses moisture, the temperature in the drying chamber decreases because of the latent effects of evaporative cooling (Anandharamakrishnan & Padma Ishwarya 2015). If droplets are exposed to high temperatures, there are numerous methods to prevent surface denaturation such as the addition of a surfactant (Ameri & Maa 2006).

Even though spray drying is usually regarded as a drying technology, it has also been applied as a technique to encapsulate therapeutics into the polymeric system (Reis et al. 2007). Spray drying has many advantages since it can be scaled up to a commercial level, is fast, and can be operated as either a continuous or batch basis (Anandharamakrishnan & Padma Ishwarya 2015; Bowey et al. 2013). Alternative methods like ionotropic pregelation, emulsion-solvent evaporation or spray freeze drying have been applied to create therapeutics loaded micro- and nanoparticles (Bowey & Neufeld 2010). However, these approaches have a number of drawbacks which affects final product quality and downstream processing. For example, spray freeze drying needs the selection of appropriate cryoprotectants, and can have an effect on the bioavailability of therapeutics (Bowey & Neufeld 2010). In addition, organic solvents and several processing steps, which include an additional drying stage, are often needed when ionotropic pregelation techniques and emulsion dispersion are used. A drying stage is frequently important to ensure stable shelf life for wet particulate products which can lose bioactivity caused by sedimentation and aggregation within the therapeutic formulation (Tewa-Tagne et al. 2006). On the other hand, spray drying provides the benefit of encapsulation and drying in one continuous operation. Even though organic solvents can be employed in closed loop spray drying techniques, formulations can usually be designed to an aqueous system and thus prevent both the environmental and health risks related to organic solvents. In other circumstances, when using a poorly water-soluble active material or polymer, spray drying leaves low residual solvent levels in the final product in comparison to other encapsulation

methods using the same ingredients. Because of the nature of the spray drying process, the drug is only subjected to the solvent for short durations (Anandharamakrishnan & Padma Ishwarya 2015).

When compared with the other techniques, spray drying is less dependent on the solubility or hydrophobicity of the drugs and matrix polymer and is a good substitute for hydrophilic therapeutics that cannot be coated using solvent evaporation techniques (Anandharamakrishnan & Padma Ishwarya 2015; Wang & Wang 2002). These therapeutics usually leach out during processing, and consequently cannot be successfully entrapped. Lastly, spray drying can be designed to operate under sterile conditions, which is essential in pharmaceutical applications. Spray drying also yields a high therapeutics encapsulation efficiency (EE) compared to other microencapsulation techniques (Anandharamakrishnan & Padma Ishwarya 2015).

2.7 Interaction between Peptide/protein and Polysaccharides

Polymer molecules interact with each other and with other molecules through a range of chemical and physical interactions (Israelachvili 2011). The magnitude, sign, direction and strength of these interactions can frequently be controlled by modifications in environmental conditions or solution formulation, for example, temperature, ionic strength, pH, and solvent type (McClements 2018a; McClements 2018b). These interactions are in charge of holding polymer-based delivery systems together and identifying the way that they react to adjustments

in environmental and solution conditions, for instance, whether the structures remain intact, shrink, swell, disintegrate or erode. Knowing the characteristics of these interactions and the aspects that affect them is thus important for developing colloidal delivery systems with certain functional properties.

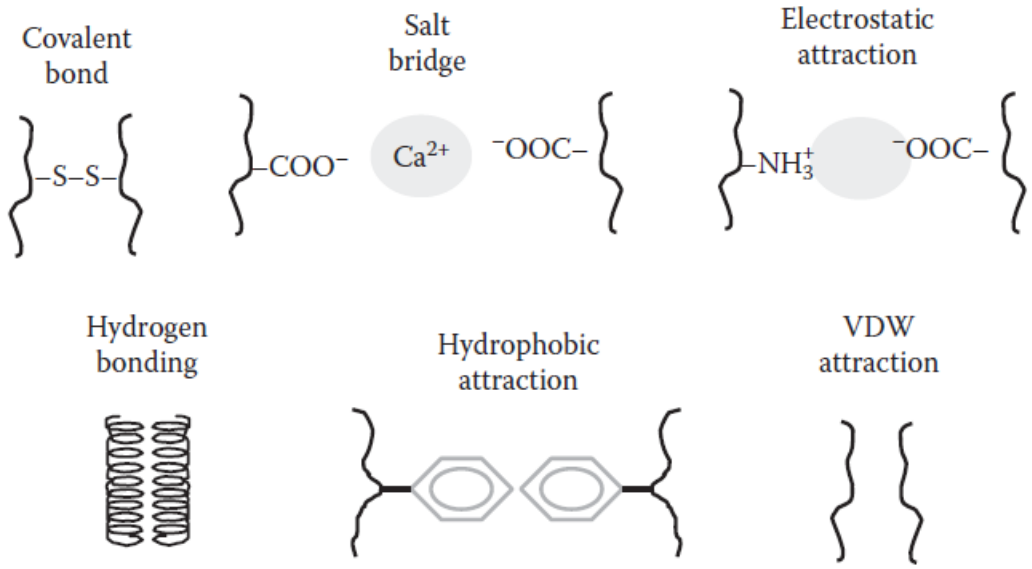

Figure 2.4 Biopolymers may interact with each other through a variety of different kinds of molecular interactions (McClements 2014).

Polysaccharides and proteins vary broadly in their electrical attributes and could be polar, nonpolar, cationic, anionic, or amphoteric (McClements 2014). Numerous polymers have functional groups that are able of becoming ionized under the suitable solution conditions. The net electrical charge on cationic (e.g., chitosan) or anionic (e.g., pectin, alginate, and carrageenan) polysaccharides is determined by solution pH in accordance with the pKa values of their ionizable groups (Fig. 2.4). The electrical charge on peptides/proteins differs from

negative, to neutral, to positive as the pH is decreased from above to below their isoelectric points (pI). However, it should be regarded that the electrical charge distribution on peptide/protein surfaces is not homogenous, with some areas being positive and other areas being negative. Therefore, peptides/proteins that possess a net positive charge could possibly still have significant patches of negative charge on their surfaces (and vice versa), that has significant effects for their capability to interact with other charged molecules (McClements 2014; McClements 2018b). The relative distribution of charges on a polymer molecule could also be modified by adjustments in its 3D conformation; for instance, the charge groups are frequently further apart in a random coil conformation than in a globular conformation. The charge status of functional groups could also rely on their particular local conditions (e.g., dielectric constant) and may be modified by solvent modifications (e.g., inclusion of an organic solvent to an aqueous polymer solution), location modifications (e.g., the adsorption of a polymer to an interface so certain groups can move from the water to oil phase), or polymer conformational modifications (e.g., the movement of a group from inside the hydrophobic core of a globular protein to its hydrophilic surface upon denaturation) (McClements 2014; McClements 2018b). Adjustments in solvent composition, polymer conformation, ionic composition, and pH could be utilized to manage electrostatic attractions, and therefore control the assembly of polymer-based delivery systems (McClements 2018b; McClements 2018a).

Chapter 2B. Review Article

The review article entitled “Can natural polymers assist in delivering insulin orally?” by Nur, M. & Vasiljevic, T. has been published in the peer-reviewed journal “*International Journal of Biological Macromolecules*” (2017), 103, 889-901. doi.org/10.1016/j.ijbiomac.2017.05.138

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

GRADUATE RESEARCH CENTRE

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book:	Can natural polymers assist in delivering insulin orally?
Surname:	Nur
First name:	Mokhamad
College:	College of Health & Biomedicine
Candidate's Contribution (%):	85

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Mokhamad Nur Digitally signed by Mokhamad Nur Date: 2018.05.19 09:42:50 +07'00'	19/05/2018
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
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College of Health and Biomedicine, Werribee Campus, Victoria University
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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Mokhamad Nur	85	Idea and concept development; reviewing the relevant literatures; manuscript preparation	Mokhamad Nur Digitally signed by Mokhamad Nur Date: 2018.05.19 09:43:34 +07'00'	19/05/2018
Todor Vasiljevic	15	Concept development; reviewing and editing the manuscript; submission to journal	Prof Todor Vasiljevic Digitally signed by Prof Todor Vasiljevic Date: 2018.05.31 19:54:08 +10'00'	31.5.2108

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Review

Can natural polymers assist in delivering insulin orally?

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ABSTRACT

Diabetes mellitus is one of the most grave and lethal non communicable diseases. Insulin is normally used to medicate diabetes. Due to bioavailability issues, the most regular route of administration is through injection, which may pose compliance problems to treatment. The oral administration thus appears as a suitable alternative, but with several important problems. Low stability of insulin in the gastrointestinal tract and low intestinal permeation are some of the issues. Encapsulation of insulin into polymer-based particles emerges as a plausible strategy. Different encapsulation approaches and polymers have been used in this regard. Polymers with different characteristics from natural or synthetic origin have been assessed to attain this goal, with natural polymers being preferable. Natural polymers studied so far include chitosan, alginate, carrageenan, starch, pectin, casein, tragacanth, dextran, carrageenan, gelatine and cyclodextrin. While some promising knowledge and results have been gained, a polymeric-based particle system to deliver insulin orally has not been introduced onto the market yet. In this review, effectiveness of different natural polymer materials developed so far along with fabrication techniques are evaluated.

Contents

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0141-8130/

1. Introduction

Diabetes is one of the most dangerous modern diseases due to its high occurrence and secondary effects, which caused almost 1.5 million mortality in 2012 worldwide [1]. Over 382 million people were diabetic in 2013 and projections are that by 2035 this number would increase to 592 million people [2]. On average, diabetes decreases life expectancy by more than 20 years for type 1 and by up to 10 years for type 2 diabetics [3].

Insulin (Fig. 1), a hormone, is the most used and effective drug to control type 1 diabetes. Since its discovery, insulin has been administered to diabetic patients solely through injection. Even though the parenteral route is satisfactory in terms of effectiveness, the tension and discomfort of multiple daily injections has raised serious concerns resulting in numerous attempts to develop a safe and an effective non-invasive route for insulin delivery [4]. Potential non-invasive routes for insulin administration are oral, pulmonary, buccal, rectal, transdermal, parenteral, nasal and vaginal [5]. Oral route is one of the most preferred ones, which has been frequently investigated as an alternative since it mimics normal physiological route of insulin in the blood [5]. However, orally delivered insulin has low bioavailability due to its large size, hydrophilicity, susceptibility to enzymatic degradation and poor absorption characteristics across the intestinal barrier [6].

In order to enhance the oral bioavailability of insulin as well as to provide a stable and biocompatible environment to the encapsulated drug, polymeric particles have been claimed to be the perfect candidates for the oral delivery of insulin [5]. Among the polymers as the excipient material, the use of biopolymers has been described as promising [5]. However, not all biopolymers have the same effectiveness as an excipient for insulin, as it mainly depends on the type of biopolymers and the particle design techniques that are used during encapsulation of insulin [7].

In this review, the effectiveness of different biopolymer materials developed so far will be assessed and classified. The fabrication techniques will be also compared. In addition, future recommendations for biopolymer candidates to deliver insulin orally will also be discussed.

2. Parameters of ideal oral insulin carrier

A drug carrier/excipient for insulin should [5]:

(i) have improved resistance against enzymes and change of pH in the stomach;

(ii) create a biocompatible and stable environment to guarantee that the insulin is biologically still active after encapsulation, and preserve and stabilize its activity during particle production and release of insulin;

(iii) decrease or avoid degradation by enzymes and increase permeability of insulin inside the intestinal membrane;

(iv) create interaction with cell-surface receptors indicating that the particles remain intact and particle size is below a certain threshold after being absorbed through the epithelial cell layer

(v) extend its intestinal residence time, thereby increasing the permeability of the mucosal epithelium to adsorb insulin and creating the intact insulin to the systemic circulation;

(vi) deliver precise quantity of insulin fast enough to control the glucose concentration in blood, and the function has to be reproducible each time insulin is delivered; and

(vii) not be harmful after administration orally.

In general, the carrier for successful oral insulin needs to cope with obstacles between the systemic circulation and insulin. Some adjustments and tests are needed to be conducted for oral insulin because insulin from normal pancreas is normally circulated via the liver to portal circulation, which has the function to control production of glucose. The possibility of oral insulin facing quick pre-systemic destruction and poor absorption in intestine may reduce its bioavailability. On the other hand, injecting insulin through the vein imitate the endogenous insulin and result in high bioavailability [8].

Ensuring sufficient bioavailability of oral insulin, preserving its bioactivity, and maximizing the desired effects during circulation in the body are very critical parameter for the success of oral insulin administration. To meet these requirements, the first goal is protection of insulin from low pH in the stomach. Furthermore, prolonged residence time is needed in intestinal environment for enhanced absorption that can be accomplished by improving permeability insulin carrier via the mucus of intestine layer. Several techniques have been developed to enhance absorption of insulin including the use of permeation enhancers, enzyme inhibitors, absorption enhancers, modifications of insulin chemical structure and pH-responsive polymeric carrier. The criteria of polymeric carrier must be safe, biocompatible, biodegradable, and having mucoadhesion properties in order to administer insulin orally to the systemic circulation [8].

3. Natural polymers

During past decade, natural polymers (polysaccharides and proteins) have been widely studied and developed as a carrier for oral insulin delivery. Natural polymers are normally considered to be harmless *in vivo*, and most of them are already in use as excipients in pharmaceutical industry. Polysaccharides show good biocompatibility and enzymatic degradation characteristics. In general, polysaccharides can be altered chemically and biochemically, and are nontoxic, harmless, highly stable, characterized with a gel forming characteristics ability, indicating their compatibility to be used for oral protein delivery. Besides the possibility of administration through alternative routes, polysaccharide based carriers have the ability to maintain stability of peptides/protein and to enhance effects of proteins during therapeutics treatment [5].

Table 1 shows some of the properties of natural polymers used for oral insulin delivery. Weakly charged polysaccharides for instance alginate or chitosan can create polyelectrolyte complexes (PECs) through electrostatic interactions with oppositely charged polymers without changing their integrity. Table 2 shows some of the properties of natural polymers used for oral insulin delivery.

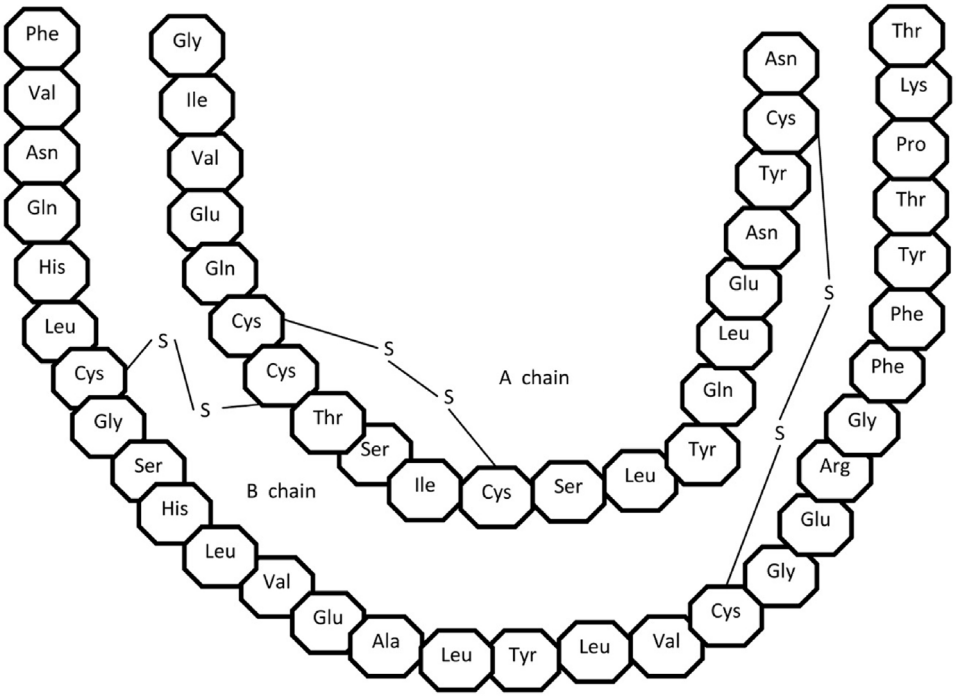

Fig. 1. Insulin structure.

Table 1
Properties of natural polymer.

Applicability	Name	Gelation mechanism	Major Structure Type	Major Monomer	Sources
Already applied for oral insulin	Chitosan	Polyphosphate cross-linking	Linear structure	2-Amino-2-deoxy-β-D-glucose	Crustaceans
	Alginate	Ca2+ cross-linking	Linear structure	β-D-Mannuronic Acid	Algal
	Pectin	Heat/sugar/(HM); Ca2+ (LM)	Highly branched coil	Glucuronate backbone	Plant cell walls
	Tragacanth	Acid gelation	Branched	D-galacturonic Acid	Exudate
	Carrageenan	Cold set gel (K+ or Ca2+)	Helical/linear structure	Sulfated galactan	Algal
Possible to be applied for oral insulin	Methyl cellulose	Reversible heat set gel	Linear structure	Methylated glucose	Wood pulp
	Xanthan gum	High concentration needed for gelling	Linear/helical (high MW)	β-D-Glucose(backbone)	*Xanthomonas campestris* exudate
	Gum arabic	Forms gels at high concentration	Branched coil domains on protein scaffold	Galactose	Acacia sap

Table 2
Natural polymer-based carriers for insulin and their pharmacological activity or bioavailability studies developed so far.

Polymer	Mean diameter (nm)	Dose (IU/kg)	Glucose level reduction (%)	Pharmacological bioavailability (%)	References
Chitosan	250–400	21	58	14.9	[61]
	200–550	50	29	NA	[79]
		100	33	NA	
	269	50	44.9	4.4	[80]
		100	51.4	3.2	
	339	100	40	3.5	
Chitosan–dextran sulfate	500	50	35	5.6	[81]
		100		3.4	
Chitosan–alginate	750	50	40	6.8	[27]
		100		3.4	
Alginate–dextran + poloxamer + chitosan + albumin	396	50	40	13.2	[82]
Chitosan–HPMCP	255	12.5	35	8.5	[66]
Dextran + Vitamin B12	192	20	70	29.4	[31]
Hyaluronic acid	182	50	40	NA	[83]

4. Natural polymers used for oral insulin delivery

4.1. Chitosan

Chitosan (CS), a copolymer of β (1–4) linked glucosamine and *N*-acetyl glucosamine (Fig. 2A), has attracted interest of many researchers in pharmaceutical area because of its excellent biodegradability, biocompatibility, and simple modification due to its reactive functional groups [8–10]. Chitosan is processed by lysozyme and decomposed gradually to amino sugars that can be metabolized completely by humans. Additionally, it has appropriate mucoadhesive properties thus it can attach to the mucosal surface and quickly open the tight junctions between epithelial cells. Chitosan (when solubilized in acid) can attain a linear structure of a high positive charge density, and behave as a cationic polymer. Chitosan solution can increase trans and paracellular permeability in a reversible, dose-dependent manner [11]. Its mode of action comprises of redistribution of cellular F-actin, interactions with the tight junction proteins occludin and ZO-1, and some plasma membrane destabilization. This action is mainly expressed due to presence of positive charge in its structure [12].

Insulin-loaded chitosan nanoparticles can also be obtained by ionotropic gelation of chitosan with tripolyphosphate (TPP) anions [23]. These nanoparticles, orally administered (21 IU/kg) to diabetic rats, resulted in a hypoglycemic effect prolonged over 15 h and a pharmacological bioavailability of approximately 15% compared with subcutaneous insulin. Chitosan has also been used as hydrophilic polymeric coating, increasing insulin transport through the intestinal membrane. Nanoparticles made by ionic cross linking of chitosan with hydroxypropyl methylcellulose phthalate (HPMCP) showed greater stability under simulated acidic conditions. Furthermore, the oral administration of chitosan/HPMCP nanoparticles in rats increased the hypoglycemic effect of insulin by more than 9.8 compared with an oral insulin solution, and such effect was even 2.8-fold higher compared with insulin-loaded chitosan/TPP nanoparticles [23].

Despite its favourable biological properties, CS is rarely used in oral administration of drugs due to high solubility at low pH and limited capacity for controlled release of drugs. To solve this problem, numerous chemical modifications of CS have been conducted via conjugation, quaternization, thiolation, substitution and grafting.

Overall, the goal of chemical modification techniques is to modify structure of chitosan or change its pKa, therefore it can overcome the absorption and enzymatic hurdles of GIT. Structural changes of polymers used in the formulation influence this modification. Positive charge of chitosan has more contribution to mucoadhesion of mucus layer than negative charge. Furthermore, at neutral environment of the intestinal pH (>6.5), chitosan cannot interact and bind well with the mucus. Consequently, the mucoadhesion of chitosan becomes weaker [13]. Hence, expanding the positive charge beyond its normal pKa (pH 6.5) of chitosan is very essential. This would lead double advantages: firstly, the stability of the insulin–chemically modified chitosan complex would be maintained, and secondly, the mucoadhesion of chitosan would remain intact, even at alkaline and neutral pH environment. In addition to these benefits, by chemical modification, the capability of chitosan to open-up the tight junctions at mucus is even improved [13].

In the conjugation approach, pKa chitosan is extended or a chelating moiety is added to the formulation. For example, CS conjugated with EGTA can make the calcium being chelated in the vicinity of insulin in the GIT [13]. Ca^{++} is widely known to have a vital function for enzymes in the GIT and can also form an apical junction in the mucus. Furthermore, thermodynamic stability of enzyme (chymotrypsin and trypsin) can be maintained by Ca^{++} [13]. Consequently, by chelating Ca^{++} ions, the effects of proteolytic enzyme can be minimized and insulin absorption in the mucus can be enhanced synergistically. A similar effect is achieved by conjugating CS with glutamine, which can extend its pKa from 6.5 to 9.13, and consequently ensuring that a positive charge can be maintained at the intestinal pH [14].

In quaternization approach, pKa of chitosan is extended by converting its primary amino group into a quaternary ammonium group or by attaching quaternary ammonium side chains to CS. Since the aim of quaternization is to enhance the solubility of CS at gastric pH environment, the addition of enteric coating is applied or the quaternized polymer is attached with hydrophobic moeity [15,16].

Thiolation method can be achieved by attaching CS to thiol moeity in order to improve mucoadhesion. Mucin-glycoprotein of the GIT can react with the thiol functional group of CS, affecting chitosan binding to the mucus via covalent bonds [18]. Consequently, the insulin can remain adhered to the mucus and decrease insulin diffusion and delay absorption. This mechanism may be the cause behind low bioavailability of insulin when thiolated CS are used and/or applied [17,18].

Substitution method is carried out by attaching functional groups to the oxygen of the hydroxyl group or primary amino nitrogen of the CS structure the hydrogen of the amino groups is substituted with a long-chain acyl functional group. This can increase the hydrophobicity of CS and enhance its resistance to the enzymatic action and permeability of formulation in the mucus layer [19]. Incompatibility of the hydrophobic formulation and hydrophilic enzymes can create enzymatic resistance. Improvement of mucosal permeability can be achieved by producing disarray in the phospholipids through acyl groups. By substituting appropriate functional group, pH responsiveness of the formulation can be modified. For instance, when changes of carboxymethyl moiety are applied to chitosan, the pH-responsive swelling and contraction of formula is reached to protect the insulin in the stomach and prevent its release at intestinal pH. Carboxymethyl substitutions in chitosan and alginate can have similar effect simultaneously. The repulsion between the COO^- of carboxymethyl CS and the COO^- of alginate at pH 7 can cause the pH responsive swelling effect [20].

In the grafting approach, CS is copolymerized with another polymer with minimum change of original properties. Grafting is applied to avoid problems associated with inability of a CS complex via electrostatic interactions with non-ionic or other cationic polymers for example polymethacrylic acid providing greater mucoadhesion. For instance, grafting chitosan with polymethacrylic acid (*N*-vinyl pyrrolidone incorporated) can reduce mucin binding of the formulation and enhance hydrophilicity [21]. It has been argued that a decrease of efficacy of mucoadhesive delivery systems caused excessive adsorption of mucin, which finally would be cleared because of the dynamic environment of mucus layer [22]. Even though mucoadhesive properties are believed to be required for oral delivery of insulin, excessive mucin binding may be counterproductive. Consequently, grafting the CS to reduce mucin binding appears essential [21].

N-Trimethyl chitosan (a partially quaternized chitosan derivative), *N*-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride, and chitosan-graft methyl methacrylate (MMA) monomers have replaced chitosan in the encapsulation of insulin since they provide greater stability through enhanced electrostatic interactions while maintaining the mucoadhesive and permeation-enhancing properties. Insulin-loaded chitosan, *N*-triethyl chitosan and *N*,*N*-dimethyl *N*-ethyl chitosan nanoparticles used in *ex vivo* studies on excised rat colon have indicated that quaternized derivatives of chitosan had greatly enhanced permeation in comparison to chitosan. Nanoparticles made of lauryl succinyl chitosan may also enhance stability of insulin at

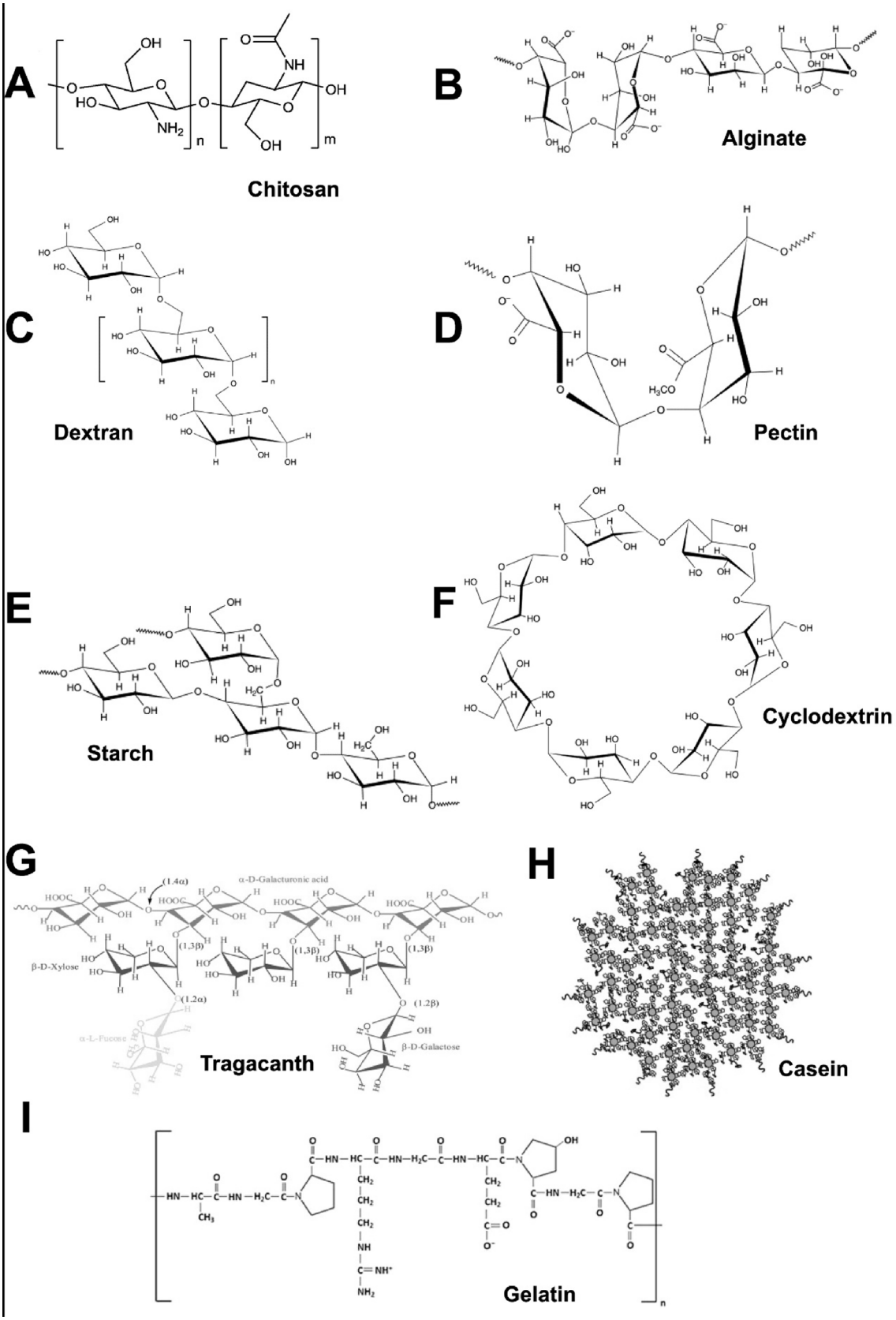

Fig. 2. Structure of some natural polymer used as vehicle in insulin delivery.

low pH. For example, the presence of succinyl carboxyl groups had an inhibitory effect on an *in vitro* release of insulin at pH 1.2. Such nanoparticles, when administered to diabetic rats, were also able to reduce blood glucose levels for approximately 6 h [23].

4.2. Alginate

Alginates (Fig. 2B) are popularly applied in biomedical applications because of their biocompatible, biodegradable and mucoadhesive properties [24,25]. Alginate are linear copolymers of L-guluronic acids (G) and D-manuronic acid (M), which are spread as blocks of G, blocks of M, or blocks of alternating G and M residues. Conformations of M-blocks are flexible while G-blocks are inflexible and MG-blocks are intermediate flexibility. The alginate derived from brown seaweed is usually neutralized with bases to create ammonium, potassium, sodium and calcium alginate salts that can be utilized for marketable materials. Otherwise, propylene oxide can react with alginic acid to create propylene glycol alginate. The monovalent salts of alginate are water soluble, while multivalent salts of alginate and alginic acid are relatively poorly water soluble and create paste-like biopolymer [26]. When added with calcium (multivalent cation), a monovalent salt of alginate can produce a gel creating ion bridges among G-block regions on various alginates structures. Gelation properties of alginate are mostly described as cold-set and thermo-irreversible. Hence, various gel characteristics can be created by either using various salts or proper selection of length and number of alginate G-blocks [26].

Low drug encapsulation efficiency can be improved by interactions with other polymers, such as CS, dextran sulfate, pectin and methylcellulose. For example, formation of alginate–chitosan capsules has been studied for the development of oral insulin formulations [27]. However, the encapsulation efficiency of insulin in alginate–chitosan capsules remained low. Ramadas, Paul, Dileep, Anitha and Sharma [28] developed an oral formulation based on liposome encapsulated alginate-CS gel capsules for insulin delivery, which consequently assisted in increasing the encapsulation efficiency of insulin. The lipid exterior appears to have helped improved absorption across biological barriers, whereas the aqueous interior of the liposome preserved the structure and conformation of insulin. Oral administration of lipoinsulin-loaded alginate–chitosan capsules reduced blood glucose level in diabetic rats [28]. Owing to the bioadhesive property of alginate, alginate coated lipoinsulin may anchor the lipoinsulin to the intestinal tract or increase the transit time of the formulation. Thus, the intimate contact with mucosa could assist in efficient absorption with increased bioavailability of insulin.

Nanoparticles, formed by alginate and dextran sulfate that nucleated around calcium and bonded to poloxamer, can be stabilized by chitosan and subsequently coated with albumin [29]. Albumin is applied to nanoparticles as the outermost layer in order to protect insulin through shielding from proteolytic degradation. The effect of this albumin layering on insulin permeation was compared with albumin-free nanoparticles that mimic the action of albumin being enzymatically removed during gastric and intestinal transport [29]. The results showed that albumin layering was important toward improving insulin uptake across the intestinal membrane, possibly by stabilizing insulin under the intestinal conditions. Moreover, insulin permeation through different intestinal *in vitro* and *ex vivo* models has also been studied. For the gold-standard Caco-2 cell monolayer, the permeation of insulin, loaded into the nanoparticles, was enhanced 2.1-fold compared to insulin in solution, 3.7-fold for the mucus-secreting Caco-2:HT29 co-culture and 3.9-fold for excised intestinal mucosa of Wistar rats. Regarding the *in vivo* studies, insulin-loaded nanoparticles proved to reduce plasma glucose levels to 40% of the basal values, with a sustained hypoglycemic effect over 24 h. Moreover, in an administrated dose of 50 IU/kg, nanoencapsulated insulin had a bioavailability of 13%, which presented a 3.0-fold increase in comparison to that of an insulin solution. Confocal microscopy studies showed internalization of nanoencapsulated insulin in the small intestinal mucosa. The same group also studied the histopathological effects of nanoparticle administration by analyzing organs and tissues of diabetic rats dosed daily for 15 days with insulin nanoparticles. No morphological or pathological alterations were observed in rat liver, spleen, pancreas, kidney or intestinal sections [29].

4.3. Dextran

Dextran, a water-soluble biopolymer, is extracted from an exocellular bacterial polysaccharide mainly containing of 1,6-linked glucopyranose units (Fig. 2C), with some 1,3-branching. The pharmacokinetic and pharmacodynamics properties application of dextran to coat insulin [30]. In order to increase bioavailability Chalasani, Russell-Jones, Jain, Diwan and Jain [31] used different levels of vitamin B12 (VB12)-coating. VB12–NPs conjugates (size: 150–300 nm) exhibited an increase in 70–75% blood glucose reductions (70–75%) and prolonged up to 54 h hypoglycemic effects *in vivo*. Low degree of cross-linking nanoparticles with VB12 derivatives of carbamate linkage appeared to be better carriers [32]. The bioavailability of carbamate linked VB12 derivatives (29.4%) was higher than that with NP conjugate of ester linked VB12 and relatively higher crosslinked particles. These NP carriers showed that a similar oral insulin efficacy can be achieved *in vivo* [31,32].

Dextran sulfate has also been complexed with chitosan in aqueous media to obtain insulin-loaded nanoparticles. This system retained insulin in a simulated gastric medium, and sustained the release of insulin up to 24 h under these conditions. Moreover, these nanoparticles reduced the basal serum glucose levels by approximately 35% in diabetic rats for more than 24 h. For doses of 50 and 100 IU/kg the pharmacological availability of insulin was 5.6 and 3.4%, respectively, which was a significant increase compared to the administration of oral insulin solutions (1.6%) [29].

4.4. Pectin

Pectin is an anionic, water soluble heterogeneous polysaccharide containing linear chains of α-(1 → 4)-D-galacturonic acid residues and 1,2 D-rhamnose with D-galactose and D-arabinose side chains (Fig. 2D) [26]. 'Pectin' usually refers to isomer group of molecules, which have some distinct characteristics. Specifically, pectin structure consist of some linear regions of α(1–4)-linked D-galacturonic acids separated by branched molecules containing numerous sugars. Galacturonic acid is probably esterified with methyl groups. Therefore, the charge on pectin fraction is influenced by the proportion of nonesterified to esterified galacturonic fractions and also the pH relative to the pKa value of the acid fractions. Pectins (pKa ≈ 3.5) have a tendency to be neutral at low pH be but negatively charged at high pH. Functional properties of pectin is majority influenced by esterified galacturonic group [26].

Pectin may be categorized as high-metoxyl (HM) or low-metoxyl (LM) pectin subject to the degree of methylation (more or less than 50%, respectively). HM pectin can create gels in high sugar level and low pH. At high sugar level, the osmotic attraction is increased; at low pH, an electrostatic repulsion between the chains is decreased. HM pectin can form a gel, which is held together by hydrophobic cross-links between helical regions in the smooth regions of the pectin backbone and hydrogen bonds [26]. Gel formed by LM pectin with the addition of calcium is caused by the capability of Ca^{2+} to create electrostatic bridges along anionic smooth regions of the pectin backbone. LM pectin can create cold-set thermo-reversible gels, while HM pectin forms cold-set thermo-irreversible gels. Some pectin can be produced to create a

gel using alternative approach. For instance, laccase enzymes can made a covalent cross-link with phenolic side groups of sugar beet pectin [26].

Gelation properties of certain pectin materials are governed by their biopolymer properties (e.g., branching, MW, side groups, and linear charge density), and also environmental conditions (e.g., temperature, sugar content, pH, and ionic strength). Pectins have a good solubility in water, but to dissolve completely, they need to be dispersed firstly in water at around 40 °C [26].

Tablets produced with calcium pectinate have good potential to be applied in colon-targeted drug delivery systems. The disadvantage of these beads lies in their microstructure. The macro porous network may create low entrapment efficiency and fast release of coated insulin, particularly for low MW substance. Hydrogel bead produced from amidated pectin and loaded with insulin resulted some sustained release of insulin, and had some anti-diabetic effect after applied *in vivo* [33].

4.5. Starch

Starch, containing amylose and amylopectin (Fig. 2E), is a widely used natural polymer applied in food and pharmaceutical industry [34]. Starch can be generated from various sources, e.g. tapioca, rice, potato, corn, and wheat. Amylopectin (branched fraction) consist of a main backbone of α-D-(1–4)-linked glucose units and a some number of α-D-(1–6)-linked branches, whereas amylose (linear factions) consist of α-D-(1–4)-linked glucose units. The ratio of amylose to amylopectin is influenced by natural sources of starches. This different of origin can also affect MW of starch as well as responsible for the variation in functional properties of starch [26].

In general, amylopectin and amylose are linked together in starch granules consisting of crystalline regions and then disjointed by amorphous regions. These crystalline regions can be interrupted by incorporation of water during heating aqueous dispersion of starch granules. Furthermore, if the concentration of starch granule is high, it can swell and increase its viscosity leading to gelatinization. When temperature further being increased, starch granules will disintegrate and molecules of amylopectin and amylose separated from granules and viscosity will be reduced [26].

In oral administration of insulin, modified starch has been commonly used. For oral insulin carrier, pH-responsive hydrogels with pendant starch poly(CMS-co-MAA-co-MEG or PBD) have been produced by free-radical crosslinked copolymerization of carboxymethyl starch (CMS), poly(ethyleneglycol monomethyl ether methacrylate) (PEGMA), and methacrylic acid. Optimization of hydrolysis rate of pH-sensitive hydrogels can be achieved by increasing the methacrylic acid content in the copolymer [35].

Zhang et al. [36] formulated a pH-responsive copolymer made of starch nanoparticles as backbone and poly(l-glutamic acid) (PGA) as graft chains. The result of *in vitro* insulin release experiment showed that the grafted copolymer had excellent pH-responsive property due to the introduction of pH-responsive PGA chain. Insulin was released more gradually from the copolymer in an artificial gastric juice (pH = 1.2) in comparison to that in artificial intestinal liquid (pH = 6.8). Hydrophobic starch acetate has also been conjugated with polyethylene glycol (PEG) forming an amphiphilic polymeric derivative. Due to pH sensitivity, the PEGylated starch acetate nanoparticles are capable of opening the tight junctions. Moreover, adhesion for PEGylated starch acetate nanoparticles was also significantly greater, at 595.7 mN mm, in comparison to that of starch acetate (179.1 mN mm) or chitosan (144.4 mN mm), which indicated a high mucoadhesiveness of PEGylated starch acetate nanoparticles and their potential use for oral insulin delivery [36].

4.6. Cyclodextrin

Cyclodextrins (CDs) are polysaccharides containing of 6–8 glucose units linked by β-1,4-glucosidic bonds (Fig. 2F). CDs are widely used during research for excipient in oral peptide drug. Its complex with insulin could protect insulin from acid degradation, denaturation and aggregation. Inulin-CD complex can contribute to the increase in insulin absorption. Nevertheless, original CDs show low solubility (in water) and cytotoxicity [37].

In an alginate and/or CS nanoparticle system, insulin is protected by complexing with cationic-β-cyclodextrin polymers (CPCDs). Owing to the electrostatic attraction between insulin and CPCDs, as well as the assistance of its polymeric chains, CPCDs could effectively protect insulin under simulated gastrointestinal (GI) conditions [38]. The cumulative insulin release in the simulated gastric fluid was much higher (40%) than that without CPCDs (18%) as insulin was mainly retained in the core of the nanoparticles and was well protected against degradation. The aggregation of the insulin molecules appears to be reduced by the complexation with β CD. CD complexes may assist in enhancing drug stability and/or absorption, whereas the particulate delivery system may serve as a platform for the encapsulation of the complexed drugs [39].

4.7. Tragacanth

Tragacanth is an anionic Iranian gum gained from the stems and branches of different species of Astragalus (Fig. 2G). It is highly acid-resistant and has high mucoadhesive properties [40]. This polymer is able to form an acid induced gel, and hence has a potential to complex with insulin, particularly at acid environment (pH below pI of insulin) [40]. In another study, tragacanth was used to encapsulate quercetin by self-assembled micelles structured of PCL core and tragacanth shell. The *in vitro* release behavior of quercetin from these micelles exhibited pH-dependence as pH appeared to control rate of quercetin release. This rate can be enhanced significantly at pH 2.2 [41].

4.8. Casein

Casein is a major milk protein fraction (almost 80% of the total milk protein) (Fig. 2H). There are four main protein types in casein with various functional and molecular attributes: αS1 (~44%), αS2 (~11%), κ (~11%), and β (~32%) [26]. Regardless of their variances, all caseins have some tertiary and secondary structure. In aqueous solutions, caseins structure is flexible and relatively disordered. Functional properties of caseins mainly depend on either highly charged or non-polar regions alongside their backbones. “Casein micelles” are clusters of caseins found in milk normally. These micelles (diameter: 50–250 nm) are linked together by mineral ion (i.e. calcium phosphate). These structures can be categorized as natural polymer that can be used to carrier of protein/peptides due to their capability solubilize hydrophobic components [26].

Caseinates, salts of the casein micelle, have isoelectric point about pH 4.6. Around this pH, electrostatic repulsion between particles will decrease causing aggregation of casein molecules. Compared to whey protein, caseins are less sensitive to change of temperature because of flexible and disordered structures. This properties is essential consideration when choosing casein for a certain application [26]. Morçöl, Nagappan, Nerenbaum, Mitchell and Bell [42] established calcium phosphate–poly(ethylene glycol)–insulin casein (CAPIC) particles to deliver insulin orally. Having characteristics of a muchoadhesive polymer and required stability at low pH, this modified casein can preserve insulin during passage in stomach and small intestine, and allow it to remain stable at the absorption site.

4.9. Gelatin

Gelatin, a biopolymer extracted from collagen (Fig. 2I) is biodegradable and widely used in biomedical and pharmaceutical industry. The charge of gelatin can be change to either negative or positive by changing the isoelectric point of gelatin at physiological pH. This mechanism can induce electrostatic interactions between gelatin and a charged biomolecule of the opposite charge creating PEC. Several method and conditions has been attempted to utilize gelatin as a carrier for controlled-release experiments. It shows that gelatin has ability to become carrier for protein/peptides via polyion complexation [43].

4.10. Carrageenan

Carrageenan (CG) is hydrocolloid polymer (MW 100–1000 kDa), composed of galactose linked by glycosidic unions units and anhydrogalactose, extracted and sourced from ocean red seaweeds [44]. It is classified based on a free hydroxyl group [26]. The pKa value of the anionic sulphate groups on CGs is around 2, which determines the degree of ionization in different media [26]. When temperature of τ- and κ- CG is increased, it will cause a disordered-ordered transition and both chains exhibit random coils. When temperature is decreased, the chain return to the ordered conformation and decrease the entropy consisting either aggregated helical dimers, aggregated mono-helices, or a double helix [44]. Different types of CG (κ, τ, λ) will indicate psychochemical properties. For instance, with the help of di- and mono- valent cations, κ- and τ- CG can create a gel. On the other hand, with either di- or mono- valent cation, λ-CG can only exhibit viscous characteristics and does not produce a gel. When oriented externally sulfate groups crated from "crosslinking" of the chains adjacent, τ-CG can make 3D networks containing of dual helices. On contrary, the presence of sulfate groups of λ-CG at the 2-postion preventing "crosslinking" and creation of more ordered 3D network [44].

κ- CG with carboxymethylated structure has been develop create microparticle (MP) that potentially preserve from low pH and enzyme [45]. Ionic gelation approach was used to create insulin- κ-CG complexes. It has a good insulin-loading capacity ($13.5 \pm 0.4\%$) and encapsulation efficiency ($94.2 \pm 2.6\%$). The MP was then functionalized with surface-lectin to enhance mucoadhesion. In vivo studies after oral administration with the rat showed that MP can extend duration of antidiabetic effect (12–24 h). This MP also exhibit low toxicity and therefore it has a potential to create more stable carrier for oral insulin delivery [45].

5. Fabrication methods

In order to deliver insulin efficiently, different approaches have been extensively introduced and applied. The following fabrication methods have been commonly trialled using natural polymer as a carrier for oral insulin delivery.

5.1. Polyelectrolyte complexes

Polyelectrolyte complexes (PECs) can be produced by mixing and solidifying two oppositely charged natural polymer colloidal solutions upon pH adjustment. Chitosan-dextran sulfate and chitosan-alginate complexes were produced by a simple drop-wise addition of the positively charge solution (chitosan) into the negatively charge solution (dextran sulphate, tragacanth or alginate) at a pre-determined pH. The insulin is located inside the polymer networks (entrapped by PECs) (Fig. 3A) [46].

The goal of PEC is to maximise the poly-cationic potential of chitosan (anionic at pH > 5.3) to create a complex with insulin, and therefore can protect insulin in acid environment of GIT. Yet, due to the fact that the insulin changes to positively charge in a pH of 1.2–5.3, CS- insulin complex is unstable. Hence, a secondary polymer is needed to get a better protection. The secondary polymers such as alginate and dextran were used to complement the ionic status of CS [13].

The principle in PEC approach is to maintain electrolytic interaction among polymers and insulin in the formulation. To create a stable interaction, pH is then modified so polymers can form complexes ionically with insulin due to opposing ionic charges. For instance, at pH range of 5.3–6.5, the ionic status of insulin is negative and CS is positive. Therefore, in that pH range, the insulin and CS remain intact and will not dissociate. Self-assembled nanobeads of insulin and is one of the easiest formulations via PEC. As mentioned before, because CS-insulin complex is unstable in acidic environment, and therefore is often added a cross-linker (i.e. tripolyphosphate (TPP) or glutaraldehyde) to the formula. This chemical can stabilze CS–insulin complex by creating ionic/covalent bonds in CS chains. TPP is more preferred to be used than glutaraldehyde because TPP is safe. On contrary, glutaraldehyde is toxic and may affect the on biological and physicochemical characteristics of insulin. It may create an interaction with insulin amino groups at a pH less than 3 which can denature the insulin.

In other PEC method, to get better stability in low pH, secondary polymers were used to the formulations. The step in addition of polymer is determined by the pH and formulation. For instance, CS can be mixed and interacted first with insulin at condition $5.3 < pH < 6.5$, in order to form multiple polymers in the formulation and create strong ionic bonds between the polymers and insulin in gastric pH and dissociate to release insulin in intestinal pH. On contrary, at pH less than 5.3, before complexing with CS, it is highly recommended to create an insulin and secondary polymer complexes first because at that pH, these constituents are oppositely charged [13].

Nanoparticle (NPs) complexes of insulin and alginate/dextran sulfate (ADS) were formed and studied albumin (ALB) and CS were used as the final coating [47]. After characterisation, it showed that these NPs can prevent 70% of insulin release *in vitro* under simulated gastric conditions and maintain a sustained release during simulated intestinal conditions. The microstructure and pH of NPs was related to the insulin release and permeation.

These ADS-NPs coated with CS/ALB showed interactions with intestinal cells compared to uncoated one causing greater permeability of insulin across Raji B/HT29-MTX/Caco-2 cell monolayers. Upon cooling and after co-incubation with chlorpromazine, permeability of complexes MP was decreased. Furthermore, the interaction of the NPs-glycocalix was crucial for insulin permeation indicated by the permeability inhibition test with natrium chlorate [47].

In another study, -γ-poly (glutamic acid) were formed a complex with CS via PEC to produce NPs. It was demonstrated that these NP resulting in a significant (bioavailability of 15.1%) antidiabetic effect after testing *in vivo* [48]. In another experiment, when insulin containing CS-γ-poly (glutamic acid) PEC NPs were filled into capsules, it has 20% of bioavailability [49]. Even though, it should be noted that the cross-linkers for example TPP was used in these studies.

5.2. Polymer-insulin complexes

The complexes of insulin and a polymer can be created by considering the isoelectric point of insulin. The isoelectric point (pI) of insulin is 5.5–6.4. At a pH below the pI value, insulin becomes positively charged and vice versa. This properties of insulin has been utilized in formation of polymer-insulin complexes via electrostatic attractions with a negatively charge polymer (tragacanth) and positively charge polymer (chitosan), that can be protonated at

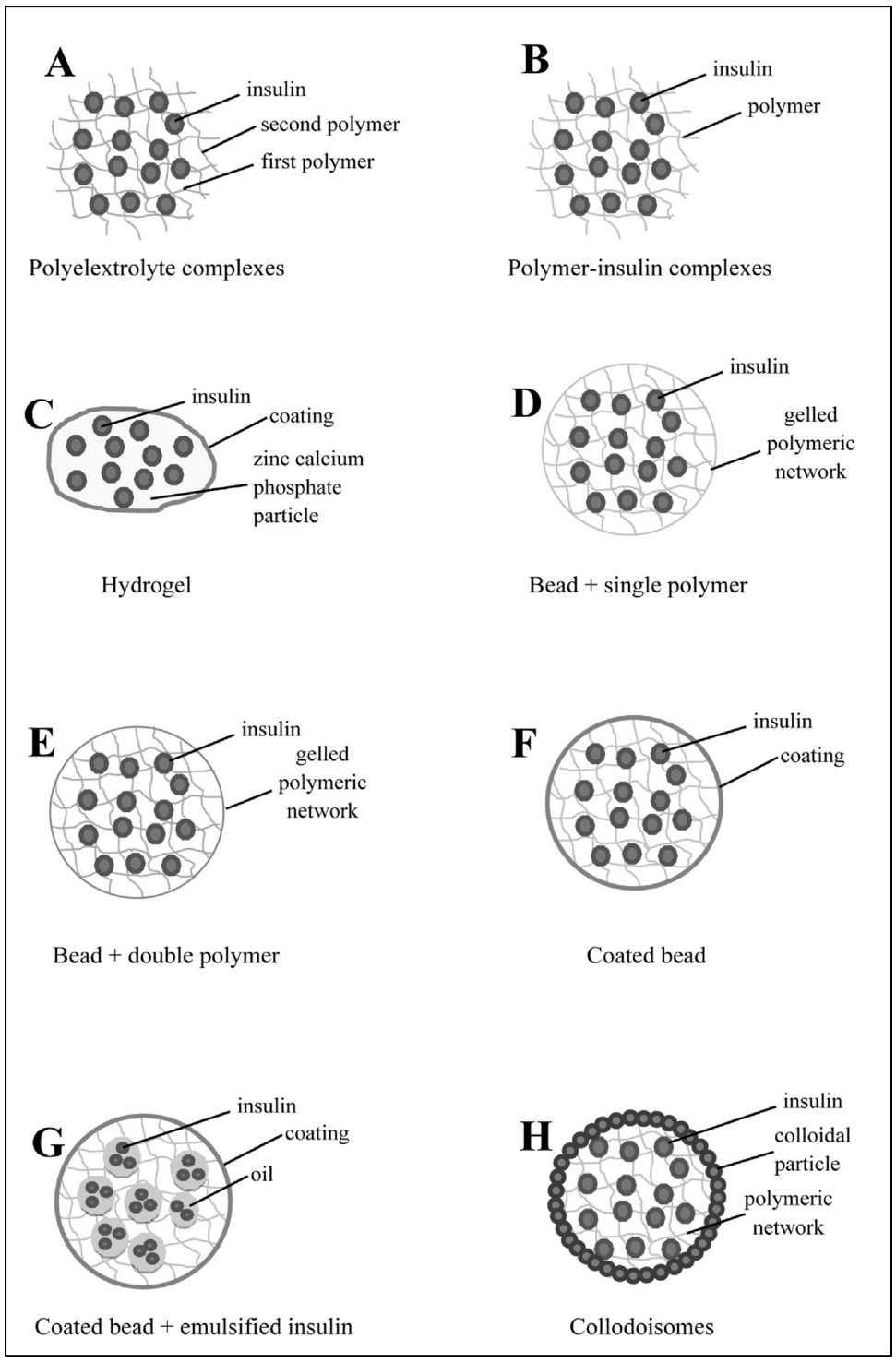

Fig. 3. Fabrication method used in insulin delivery with natural polymer as a carrier (adopted [7]).

pH values below its 6.5 (pKa). Tragacanth can has potential properties to creates a complex with insulin below pI of insulin [40]. On the other hand, chitosan can create a complex with insulin when the pH is adjusted over pI of insulin [50,51]. Both tragacanth and chitosan solutions were mixed under stirring and their complexes were formed almost instantaneously (Fig. 3B).

5.3. Hydrogel-coated particles

In addition to being used as a core structure to encapsulate insulin, dextran and alginate can also act a coating polymer for insulin. The coating mechanism of alginate polymer is caused by calcium ions from the insulin-loaded zinc modified calcium phosphate NPs immersed in alginate solution, consequently producing a coating layer on the NPs (Fig. 3C). NPs from alginate coated by

Table 3
Advantages and limitations of natural polymers used for oral antidiabetic peptide delivery.

Polymer	Advantages	Limitations
Chitosan	- Only positively charge polymer - Mucoadhesive polymer - Biodegradable with enhanced stability - Targeted drug release - Control pharmacokinetic parameters - Reduced toxicity in the peripheral healthy tissues	- Instability in acidic environment of the GI tract - Limited capacity for controlled release
Alginate	- Mucoadhesive polymer - Biodegradable with enhanced stability - Stability under acidic conditions	- Low entrapment
Dextran.	- Mucoadhesive polymer - Biodegradable with enhanced stability.	- Low entrapment
Tragacanth	- Mucoadhesive polymer - Acid gelation	- High polydispersity

zinc calcium phosphate can be used as a carrier for insulin during oral administration [52].

An *in vitro* release study of the alginate-coated NPs exhibited less than 9% release of the encapsulated insulin at low pH [52]. Shrinking of the alginate layer in acidic medium provided a diffusion barrier to the large molecular weight-hexameric insulin (MW = 32–34 kDa) used in the study. Arabic gum and CS-based NPs with the particle size from 150 to 200 nm have been produced and studied [53,54] for oral insulin carrier that demonstrated 10–38% Association Efficiency (AE) that is less than that detected in CS-TPP NPs. The maximum AE was reached if amount of arabic gum and CS used were 5 and 10 mg/mL; and the concentration of insulin added was 10 mg. To study the transport of insulin across the intestine, modified 'gut sac analysis' was used. It was showed that insulin transport with NPs was higher than free insulin. The authors stated that the observed intracellular transport is to be due to endocytosis or transcytosis, while the intercellular transport is caused by the interference of TJs after complexation of the actin filaments and the positively charged CS [53,54].

Various biopolymers can be combined to achieve better properties which are suitable for insulin oral delivery. Using new method, Woitiski et al. [55] created the complex of multipolymer layer hydrogel NPs consisting of poloxamer-188, dextran sulfate (DS), and alginate. The NPs were formed from alginic sodium salt with DS as a nucleating functional group around calcium and binding to poloxamer stabilized by CS and then coated with albumin. Psychochemical properties of the NPs depend on concentration of the polymers. The small particle size of NPs can be created by increasing the concentration of albumin and poloxamer and decreasing concentration of Ca^{2+}. However, a decrease in albumin concentration (from 1% to 0.25%) can raise the insulin EE to 90% and protect of insulin from acidic environment [56].

The best NP formulation (particle size 396 nm) [56] was made up of 0.006% insulin, 0.5% albumin, 0.20% 0.06% alginate, 0.04% poloxamer-188, calcium chloride, 0.04% dextran sulfate, and 0.07% CS, that preserve insulin from proteolytic enzyme. Alginate is disintegrated from NPs in acidic pH with very low level of insulin release (<5%) in 2 h and maximum release (~90%) at the end of 5 h at pH 6.8. The NPs indicated to be better carrier for insulin transport (3.7-fold increase via Caco-2/HT-29 co-culture; a 2.1-fold increase Caco-2 cell monolayer) than free insulin. Bioavailability and pharmacological availability of insulin is preserved by negatively charged of NPs due to protection of insulin from proteolytic enzyme and also ability to permeate through intestinal membrane. Using *in vivo* dissolution method at pH 7.4 buffer media followed by animal subcutaneous injection (1 IU/kg), the presence of insulin within the NPs was confirmed by its ability to reduce blood glucose (BGL) level up 10%. A 13% oral insulin bioavailability and 40% decrease in BGL of the basal values with a continual anti diabetic effect during 24 h was detected thus showing three times higher than free insulin at a dose of 50 IU/kg insulin coated NPs. Microstructure analysis using confocal microscope of the intestinal tissue displayed presence of insulin and NPs in small intestinal mucosa [56,57].

5.4. Beads with a single polymer

The most simple form of a particle design in biopolymeric oral insulin delivery system consists of beads or particles made of a single natural polymer (i.e. alginate or chitosan) (Fig. 3D). The sizes attained can range from 40 nm to 1.8 mm. Alginate and chitosan hydrogels are pH-sensitive. When comparing alginate and chitosan, insulin release from chitosan is higher than that from alginate, particularly at low pH, as chitosan swells under this condition. Consequently, insulin is released from chitosan bead when incubated in the acidic environment. On the other hand, in an acidic medium, alginate hydrogels shrink because of increased hydrogen bonding resulting from the protonation of the carboxyl groups in the polymeric chains, which keeps insulin trapped within the hydrogel matrix [58–61]. At neutral or slightly alkaline pH, both chitosan and alginate beads swell promoting insulin release. Gelatine and pectin have also been used to produce a bead to encapsulate insulin.

The insulin stability during acid and enzymatic degradation was analysed using a HPLC [62] and UV-based methods [63]. However, the biological activity of the insulin analysed by chemiluminescent immunoassay technique, is only 25% upon incubation at low pH [58].The performance of alginate beads to encapsulate insulin during acid environment has been assessed. Breakdown on alginate bead in consequence of low pH [64] can disrupt chemical physical stability and decrease the bioavailability of insulin [65].

Biopolymer matric can protect insulin from enzymatic degradation during encapsulation. [66]. The insulin coated in chitosan-TPP can be protected from enzymatic degradation for a minimum of 30 min whereas free insulin was completely (within 5 min) degraded by pepsin in the acidic medium. The presence of a polymeric wall creates diffusion resistance against the inward penetration of enzymes; therefore enzymatic degradation can be postponed. The reduction of insulin within chitosan-TPP nanospheres in rats blood glucose was achieved within 4 h [66].

5.5. Beads with blended polymers

Two or more natural polymers (especially charged polymers) can be blended to create wall materials with improved psychochemical properties (Fig. 3E). Dextran sulfate and alginate have

become popular natural polymers to create these blended polymers. Alginate can also be blended with whey proteins or chitosan. As an example, the functional properties of dextran sulphate-alginate can protect insulin without significant loss of insulin after 2 h incubation in the acidic environment. This performance is far better than that of blended mixtures of whey proteins–alginate or chitosan–alginate [59,67–69]. Dextran sulphate is negatively charge (anionic polymer), while insulin is positively charge under acidic medium. Therefore, the release of insulin can be protected by dextran. On the other hand, whey proteins can swell at low pH, therefore, the insulin was easily released (more than 70%) [59,67–69].

5.6. Coated beads

Beads can also be coated by a coating layer (Fig. 3F). This process is mostly caused by PECs between negatively and positively charge polymer. In alginate beads, chitosan is commonly used as coating materials. Alginate (negatively charge) and chitosan (positively charge) can create spontaneous complexation under specific pH and charge densities [70]. Under acidic environment, insulin was released about 5–45% from chitosan-alginate beads, 12–25% from albumin–chitosan-coated alginate dextran beads and less than 5% chitosan-coated alginate–dextran beads [56]. In general, 65–70% insulin was released at alkaline pH.

Bovine serum albumin (BSA), a milk protein with pI of 4.8 [71] has also been applied as a secondary layer in chitosan-coated beads and can be degraded by proteolytic enzyme [56,69]. The layer surrounding bead of polymer can act as a diffusivity barrier than can prevent the release of insulin during swollen and shrunken of the bead. It also can protect from enzymatic degradation in the stomach [72].

In general, in vivo study showed that coated beads can sustain insulin longer than uncoated one. The antidiabetic effect can over 60 h, when insulin (loaded to chitosan and coated with alginate bead) administered orally to diabetic rat [72]. Another study showed that after insulin being loaded to chitosan-coated alginate beads and then administered *in vivo*, the antidiabetic effect can be sustained for more than 18 h [27].

5.7. Coated beads with insulin emulsification technique

Fig. 3G shows the fabrication of beads using a double emulsion. In this technique, emulsified insulin which is coated with phospholipid is encapsulated with alginate. Another oppositely charges polymer (chitosan) is then used to further encapsulate the particle. This technique can improve the encapsulation efficiency [28] and enhance the insulin bioavailability [73].

5.8. Colloidosomes

Colloidosomes are microcapsules, which contain shells assembled from selective colloidal particle (Fig. 3H) [74,75]. Compatibility and elasticity of the shells can be controlled accurately by manipulating type and size of colloids and preparation environments for the creation [76,77]. Because of this controllable properties, colloidosomes are chosen to coat active material including insulin [78]. The release profiles from a reported studies [78] showed that nearly 70–90% of the encapsulated insulin was released in low pH during 2 h of incubation.

6. Conclusions and future prospects

The main issues in oral delivery of insulin are the low intestinal permeability and the enzymatic degradation, which therefore result in a low bioavailability (Table 3). Hence, to overcome these problems, different approaches have been developed and are still being investigated and developed to encapsulate insulin using various biopolymers. These polymers can be derived from synthetic or natural sources and have an ability to preserve insulin stability, bioavailability, mucoadhesion, and properly control its release. The combination of different biopolymers may accommodate all these properties together in the same carrier.

Some positive and promising results have been achieved, although obtained findings are far from the hypoglycemic effect achieved by using injected insulin. However, the insulin needed in formulation for oral delivery systems is more than that used for injected formula. This is a crucial point since the association efficiency of the carriers is important from a cost-effective point a view. The delivery of large amounts of insulin in the intestine also may result in adverse effects. Therefore, the research focus has been placed on developing oral insulin carriers using natural polymers with better performance or minimum similar to subcutaneous route profile. Studies on long term toxicity of the carriers are also needed to be conducted in order to ensure the safety of the users.

Contribution

Both authors contribute equally during preparation and writing of this article.

Conflicts of interest

No benefits have been or will be received from a commercial party related directly or indirectly to the subject matter of this article.

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CHAPTER 3

Chapter 3. Tragacanth as an oral peptide and protein delivery carrier: Characterization and mucoadhesion

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The paper entitled “Tragacanth as an oral peptide and protein delivery carrier: Characterization and mucoadhesion” by Nur, M., Ramchandran, L., & Vasiljevic, T. has been published in the peer-reviewed journal “*Carbohydrate Polymers*” (2016), 143, 223-230. doi.org/10.1016/j.carbpol.2016.01.074

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1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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In the case of the above publication, the following authors contributed to the work as follows:

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Tragacanth as an oral peptide and protein delivery carrier: Characterization and mucoadhesion

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ABSTRACT

Biopolymers such as tragacanth, an anionic polysaccharide gum, can be alternative polymeric carrier for physiologically important peptides and proteins. Characterization of tragacanth is thus essential for providing a foundation for possible applications. Rheological studies colloidal solution of tragacanth at pH 3, 5 or 7 were carried out by means of steady shear and small amplitude oscillatory measurements. Tragacanth mucoadhesivity was also analyzed using an applicable rheological method and compared to chitosan, alginate and PVP. The particle size and zeta potential were measured by a zetasizer. Thermal properties of solutions were obtained using a differential scanning calorimetry. The solution exhibited shear-thinning characteristics. The value of the storage modulus (G') and the loss modulus (G'') increased with an increase in angular frequency (Ω). In all cases, loss modulus values were higher than storage values ($G'' > G'$) and viscous character was, therefore, dominant. Tragacanth and alginate showed a good mucoadhesion. Tragacanth upon dispersion created particles of a submicron size with a negative zeta potential (−7.98 to −11.92 mV). These properties were pH dependant resulting in acid gel formation at pH 3.5. Tragacanth has thus a potential to be used as an excipient for peptide/protein delivery.

1. Introduction

An alternative approach of parenteral delivery to administer proteins and peptides orally has encouraged various efforts at delivery development. Development of high bioavailability of oral protein and peptides delivery systems can be achieved through three practical ways: (1) modification of delivery carrier; (2) physicochemical properties change of macromolecules or (3) addition of new function to macromolecules. Obviously, it is important that these methods can retain the biological activity of the protein and peptides (Mathiowitz et al., 1997; Morishita & Peppas, 2006).

The first peptide to be used as a drug was insulin (for treatment of diabetes) and since then numerous proteins and peptides drug have been reported in almost every field of medicine. There are more than 130 currently used protein therapeutics and over 1000 proteins/peptides are being tested in human clinical trials (Yadav, Kumari, & Yadav, 2011). Oral administration of drug is the most widely used route of administration, even though it is generally not practicable for protein and peptide based drugs. Due to enzymatic degradation and poor penetration of the intestinal membrane, oral bioavailability of biologicals is usually very low. Much study has been done in recent years about macromolecular drug absorption from the gastrointestinal (GI) tract, such as the barriers that limit GI absorption. Several approaches have been proposed to overcome such barriers and to create effective oral delivery systems for proteins and peptides (Morishita & Peppas, 2006).

To improve the efficiency of oral delivery of peptides and protein and overcome the gastrointestinal barriers, various carriers have been assessed and developed. Much research in recent years has developed hydrogels and carriers based on biodegradable polymers, such as polypeptides and natural biopolymers. Polysaccharides, such as chitosan, alginate, cellulose and starch, are widely applied in biomedical and pharmaceutical fields because of biocompatibility, low toxicity, and economic benefits (Gao et al., 2014).

Mucoadhesive polymers can be used to improve bioavailability of peptides and protein during oral administration. These materials can preserve contact with intestinal epithelium for extended periods, promoting penetration of active drug through and between cells due to the concentration gradient between nanoparticles and intestinal membrane. Consequently, bioavailability of the peptides and protein is increased leading to improved patient compliance (George & Abraham, 2006). Chitosan and alginate are most popular

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among mucoadhesive polymers used for protein/peptides delivery (Sonia & Sharma, 2012). Tragacanth, a polysaccharide, can potentially become an excipient during delivery system as it has demonstrated high mucoadhesive properties previously (Jackson & Perkins, 2001).

Tragacanth is an anionic polysaccharide gum (molecular weight of 850 kD) obtained from the stems and branches of different species of Astragalus. It is highly acid-resistant and comprises of two major elements: (1) tragacantic acid and arabinogalactan and (2) bassorin (Firooz, Mohammadifar, & Haratian, 2012; Kaffashi, Zandieh, & Khadiv-Parsi, 2006; Mohammadifar, Musavi, Kiumarsi, & Williams, 2006). The peptide/protein delivery mechanism of tragacanth maybe achieved through two types of mechanisms: polyelectrolyte complexes (PECs) and entrapment through hydrogel. Polyelectrolyte complexes are made up of oppositely charged (cationic and anionic) biopolymers formulated under mild conditions to transport peptides/proteins and colloidal carriers. This complexation shows potential as a vehicle to encapsulate proteins and provide protection and sustained release of protein/peptides (McClements, 2014; Sarmento et al., 2006b). Hydrogel is an insoluble semi permeable matrix that can be used to entrap protein/peptide. It can be produced from biopolymers from animal- or plant-based derivatives (Lim, Tey, & Chan, 2014).

In order to select appropriate biopolymer for protein/peptides delivery, comparing the physical and mucoadhesive properties of polymers is essential. This measurement can determine their strength to potentially improve the bioavailability of protein/peptides. The relative mucoadhesion efficiencies of materials are normally reported as ranking orders that are specific to the method of evaluation (Ivarsson & Wahlgren, 2012; Tsibouklis, Middleton, Patel, & Pratten, 2013). For this reason, tragacanth as a new material need to be tested potential candidate of mucoadhesive polymer.

This study, therefore, was aimed to (1) establish physical properties of tragacanth under a range of conditions that would allow for creation of a hydrogel and/or a polyelectrolyte complex with other biopolymer or direct complex with peptide/protein, (2) assess and compare its mucoadhesivity to others biopolymers.

2. Materials and methods

2.1. Materials

The powder form of polymers, i.e. tragacanth, low molecular weight (MW) chitosan, low viscosity alginate and polyvinyl pyrrolidone (PVP), were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Dried mucin from porcine stomach, type III and glucono-δ-lactone powder (GDL) (Sigma-Aldrich) were also used without further purification. The insulin sample containing 100 U/ml of insulin was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The water used was of Millipore quality.

2.2. Characterization of tragacanth

2.2.1. Sample preparation

Tragacanth stock solution (2% w/w) was prepared by dissolving appropriate amount of the powder in 0.1 M citric acid phosphate buffer at different pH (3, 5 or 7). Sodium azide was added during the preparation of samples (0.2 g/l) to preserve them. The resulting solution was gently stirred at room temperature and stored overnight at 4 °C. The stock solution was then diluted with the buffer to give concentrations of 0.5% (w/w) which was used in all experiments. To characterize tragacanth after loading with insulin, mixture of tragacanth in milli-Q water and insulin were prepared by mixing insulin (0.2 mg/ml) and 0.5% tragacanth at pH 3.7 by adding GDL and gently stirring overnight to create a colloidal dispersion.

2.2.2. Rheological analysis

The ability of biopolymer to form a gel and its strength under certain condition (ex. different pH level) is crucial in predicting the behaviour of polymeric carrier. These properties can be analyzed rheologically. The flow behaviour measurements of 0.5% (w/w) tragacanth solution at different pH 3, 5, or 7 were performed with a stress-controlled rheometer (MCR 301, Anton Paar GmbH, Ostfildern, Germany) using a double gap geometry (Anton Paar). The temperature was set to 20 °C (Qomarudin et al., 2015). In order to assure that the experiments were carried out inside the linear viscoelasticity region, the samples were previously submitted to stress sweep tests. The applied stress was varied from 0 to 50%, to keep sensibility in the measurements in the frequency range and to avoid noise. Apparent viscosity and dynamic oscillation measurements were performed using the following protocol: Samples were pre-sheared 500/s at 20 °C for 0.5 min and then rested for 30 s, continued frequency sweeps were carried out at angular frequency from 0.1 to 10 rad/s at a constant strain value of 5%, then rested for 30 s. Following this, apparent viscosity was measured by applying shear rate from 0.1 to 1000/s.

To assess the effect of temperature and shearing, tragacanth was also analyzed various temperatures (60, 80 or 120 °C) and shear rates (500, 1000 or 1500/s). Briefly, tragacanth mixtures were sheared and heat treated in a pressure cell (CC 25/Pr 150/A1/SS, Anton Paar, GmbH, Ostfildern, Germany) mounted on the same rheometer using a bob and cup geometry (CC 25/PR-SN, Anton Paar) under a constant shear (500, 1000 or 1500/s) and pressure (~1 bar). All treatments were conducted at a heating rate of 5 °C/min, kept at a final temperature for 60 s, and then cooled at the rate of 5 °C/min down to 20 °C. Tragacanth flow behaviour was documented using Rheoplus 32 v2.81 (Anton Paar) software. The magnetic coupling of the pressure cell was expected to reduce the sensitivity of the rheometer, hence flow data were expressed in a log scale to minimize any influence as a consequence of coupling and log-scale variations (Liyanaarachchi, Ramchandran, & Vasiljevic, 2015).

2.2.3. Particle size and zeta potential measurements

The particle size and zeta potential of the tragacanth solution at different pH (3, 5 or 7) were measured using a zetasizer (ZEN3600, Malvern Instrument Ltd., Worchestershire, UK). Tragacanth solutions were diluted 1:100 with a citric phosphate buffer and stored at room temperature for 24 h prior to particle size analysis (Qomarudin et al., 2015). All particle-size measurements were performed in a He–Ne laser beam at 658 nm. The particle size and zeta potential after loading with the insulin was analyzed at pH 3.7.

2.2.4. Determination of loading efficiency

Loading efficiency was measured indirectly after centrifugation of tragacanth and insulin dispersion after mixing insulin (0.2 mg/ml) and 0.5% tragacanth colloidal solutions at pH 3.7. The mixture was centrifuged at 20,000 × g for 60 min at room temperature by a high-performance centrifuge (Beckman Coulter Inc., Brea, CA). The amount of insulin in supernatant was measured by the Bradford method at 595 nm. The loading efficiency was calculated as:

$$\text{Loading efficiency}\,(\%) = \frac{\text{Total amuunt of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100 \quad (1)$$

2.2.5. Differential scanning calorimetry (DSC) analysis

Thermal properties of tragacanth was analyzed by DSC as described previously (Sarmento, Ferreira, Veiga, & Ribeiro, 2006a) with some modifications. Thermograms of tragacanth solutions were obtained using a DSC (Mettler Toledo, Schwerzenbach, Switzerland) equipped with an intracooler system and under an inert nitrogen gas atmosphere. Tragacanth solution (4–7 mg) at different pH (3, 5 or 7) were placed in a 40 μl aluminium pan, hermetically sealed before placing in the instrument, and heated from 20 to 200 °C at a constant rate of 10 °C/min under constant purging of nitrogen at 20 ml/min. An empty pan of equal weight served as the reference. The ΔH values and onset, endset and peak temperatures of the thermograms were recorded.

2.2.6. ATR-FTIR analysis

ATR-FTIR spectra of 0.5% tragacanth solution at different pH was obtained using a Perkin Elmer ATR-FTIR spectrometer equipped with Diamond™ ZnSe single reflection ATR plate (Perkin-Elmer, Norwalk, CT). The spectrum of each sample was obtained from 16 scans between 600 and 4000 cm^{-1} with a resolution of 4 cm^{-1} and strong apodization. This was corrected against the background spectrum of the solvent. Data acquisition and baseline manipulation was facilitated by Shimadzu IR solution software v1.40 (Qomarudin et al., 2015).

2.3. Mucoadhesion

The change of viscosity due to synergism in a mucin–polymer system was analyzed using a modified version of the viscosity tests described previously (Hassan & Gallo, 1990; Ivarsson & Wahlgren, 2012). The polymer tested were tragacanth, alginate, chitosan and PVP. All polymers except chitosan were dissolved in a simulated intestinal fluid without enzymes containing: 0.15 M NaCl and 0.01 M sodium phosphate buffer (pH 6.6) prior to the experiments. Chitosan was dissolved in 0.1 M HCl due to its poor solubility in pH 6.6 buffers.

Stock solutions, 2% (w/w) polymer, of the slowly dissolving polymers (tragacanth, chitosan, alginate) were prepared and stored at room temperature for 2 days to ensure complete dissolution and swelling of the polymers. Mucin stock solution, 6% (w/w), was prepared on the same day as the measurements to ensure that it was fresh. The stock solutions were mixed with one part buffer to provide reference samples of 1% (w/w) polymer or 3% (w/w) mucin (Ivarsson & Wahlgren, 2012). Thereafter, one part mucin stock solution and one part polymer solution were mixed together and vortexed vigorously for 15 min prior to analysis.

Apparent viscosity and dynamic oscillation measurements were performed using a stress-controlled rheometer (Anton Paar) with the following protocol—samples were presheared 500/s at 37 °C for 30 s and then rested for 30 s, continued with a frequency sweep from 0.1 to 10 Hz at a constant stress of 1 mPa, then rested for 30 s. Following this, apparent viscosity was measured by applying shear rate from 0.1 to 0.25/s A.

This increase in viscosity due to adhesion (η_a) can be calculated from Eq. (2), where η_t is the total viscosity of the system, η_m is the viscosity of a pure mucin sample, η_p is the viscosity of a pure polymer sample and η_a is the viscosity resulting from adhesion (Hassan & Gallo, 1990).

$$\eta_a = \eta_t - \eta_m - \eta_p \quad (2)$$

2.4. Acid induced gelation

GDL-induced acidification was conducted to assess the ability of tragacanth to entrap peptide/protein via acid induced gelation (Dissanayake, Kelly, & Vasiljevic, 2010). By using rheometer

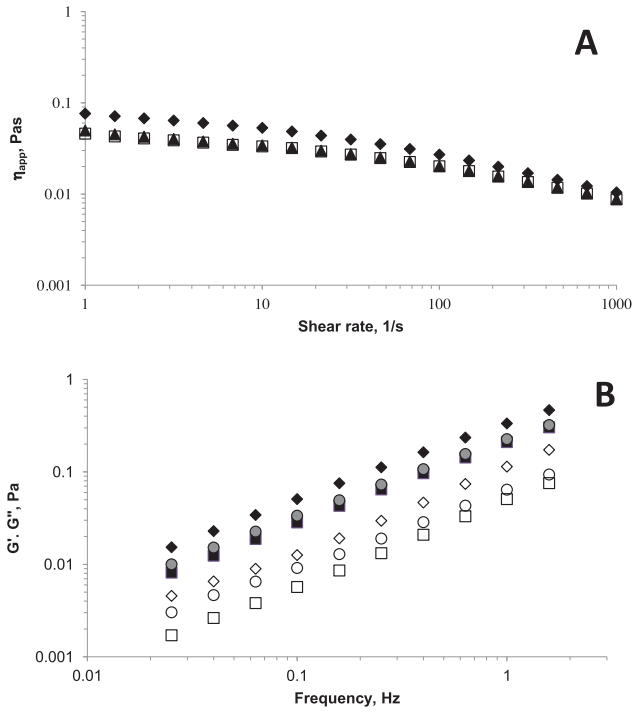

Fig. 1. (A) Apparent viscosity of 0.5% (w/w) tragacanth during shear rate ramp at different pH at 20 °C (◆ pH 7; □ pH5; ▲ pH3); (B) Small amplitude oscillatory measurements data at different pH for 0.5% tragacanth solution (◊ storage modulus pH 7; ◆ loss modulus pH7; □ storage modulus pH 5; ■ loss modulus pH 5; ○ storage modulus pH 3; ● loss modulus pH 3).

described in Section 2.2.1, dynamic small amplitude oscillatory measurements was conducted to identify an acid gel point following the modified procedure described by Dissanayake et al. (2010). Glucono-δ-lactone powder (GDL) was used to promote the acid gelation of tragacanth. The GDL powder (0.5% w/w) was added to the 0.5% aqueous tragacanth solution at 20 °C. A portion of the sample (3.9 ml) was introduced immediately into the measuring system (double gap) at 20 °C for 100 min. The pH change was simultaneously recorded every 2.5 min in another portion of the sample using a pH metre (Model 8417; Hanna Instruments, Singapore) until pH reached 3.2. This method was also applied to measure rheological characteristics of tragacanth after loaded with the insulin.

2.5. Statistics

The data obtained from particle size, zeta potential and DSC measurements were organized in a randomized block design with pH as the main factor. These tests were replicated at least once with subsequent sub-sampling giving a number of independent observations of at least ($n \geq 4$). Results were analyzed using one way ANOVA, SAS (1996). Tukey's Studentized Range (HSD) test was used for multiple comparisons of means. The level of significance was preset at $P = 0.05$.

3. Result and discussion

3.1. Physicochemical properties

The steady state and dynamic rheological properties of tragacanth as a protein/peptide carrier was investigated experimentally. Fig. 1A illustrates the flow curves of 0.5% tragacanth solution at different pH at 20 °C. It was apparent that in all the cases tragacanth exhibited shear-thinning behaviour as the viscosity decreased with increase in shear rate. This behaviour is in agreement with data

obtained for tragacanth at slightly different pH (Firooz et al., 2012) and concentrations (Chenlo, Moreira, & Silva, 2010; Keshtkaran, Mohammadifar, Asadi, Nejad, & Balaghi, 2013). The apparent viscosity values were in the same range (from 0.001 to 0.05 Pa s) but with some differences. The viscosity of tragacanth at pH 7 was slightly higher than that at lower pH (3 and 5). This could be attributed to the marginal shrinkage of tragacanth molecules during acidification as a result of a reduction in electrostatic repulsion among the uronic acid residues of the polysaccharide. The pH-induced evolution of viscosity of the mixed dispersion was rather complicated compared to each individual component (Firooz et al., 2012).

Dynamic frequency sweeps were obtained in the 0.1–10 rad/s of angular frequency range. Dynamic oscillation measurements data at different frequencies and different pH for tragacanth is displayed in Fig. 1B. Storage modulus (G') and loss modulus (G'') increased with an increase in angular frequency (ω). In general, the values of loss modulus were higher than storage modulus values ($G'' > G'$) indicating that viscous character was dominant. This confirms that in the tested range, the systems behaved like a typical dilute solution. The G' and G'' at pH 7 appeared slightly higher than at other pH, indicating more viscous behaviour at a higher pH. The differences between G' and G'' values, at the same frequency, decreased with an increase in gum concentration. This behaviour was in agreement with the characteristics of entangled semi dilute polymer solutions (Chenlo et al., 2010; Mohammadifar et al., 2006).

From Fig. 2, viscosity of all tragacanth dispersion appeared to be independent of shearing. The only effect of shearing occured at 60 °C and 500/s (Fig. 2A), under which conditions tragacanth was slightly more viscous than at other temperature and shearing rate. This suggests that the use of low shear rates and moderate temperatures may create more viscous, gell like state required from tragacanth in order to enhance complexing with active materials. Furthermore, temperature sweep did not affect the viscosity of tragacanth dispersions. The viscosity during heating period remained fairly similar to that during the cooling period regardless of shear rate, suggesting that tragacanth would not be affected by heating and other environmental adjustments i.e. pH, ionic strength would be needed to induce gelation. Therefore, it was necessary to further examine the ability of tragacanth to form hydrogels by other gelation methods such as acid gelation.

It can be seen from the Fig. 3 that tragacanth formed an acid induced gel at pH 3.5 as storage modulus became greater than the loss modulus. If this is linked to zeta potential (secondary y-axis), the possible entrapment of peptide or protein by tragacanth could be accomplished below isoelectric point (pI) of polypeptide. For example, the pI of insulin varies from 5.5 to 6.4, depending on its source. At a pH greater than its pI value, insulin is predominantly negatively charged and vice versa (Lim et al., 2014). This property of insulin can be used to form polymer-insulin complexes via electrostatic interactions with an anionic polymer such as tragacanth. At pH around 3–4, tragacanth may coacervate and at the same time may also form a hydrogel that can entrap insulin, since at this pH insulin is positively charged which is also around the determined gelling point of tragacanth.

Measuring particle size and zeta potential of a biopolymer is crucial in understanding its potential to form polyelectrolyte complexes such as coacervates since they have a potential to deliver protein/peptides. Coacervates can be created by interaction of two oppositely charged biopolymers through electrostatic attraction, supported by other forces, e.g. hydrogen bonds and hydrophobic interactions. The aggregation of the polysaccharide solutions has a direct relationship with the charge of the polysaccharide, where different charges can make different polysaccharide behaviours. The zeta potential (ζ) of the solution can be related with the charge of

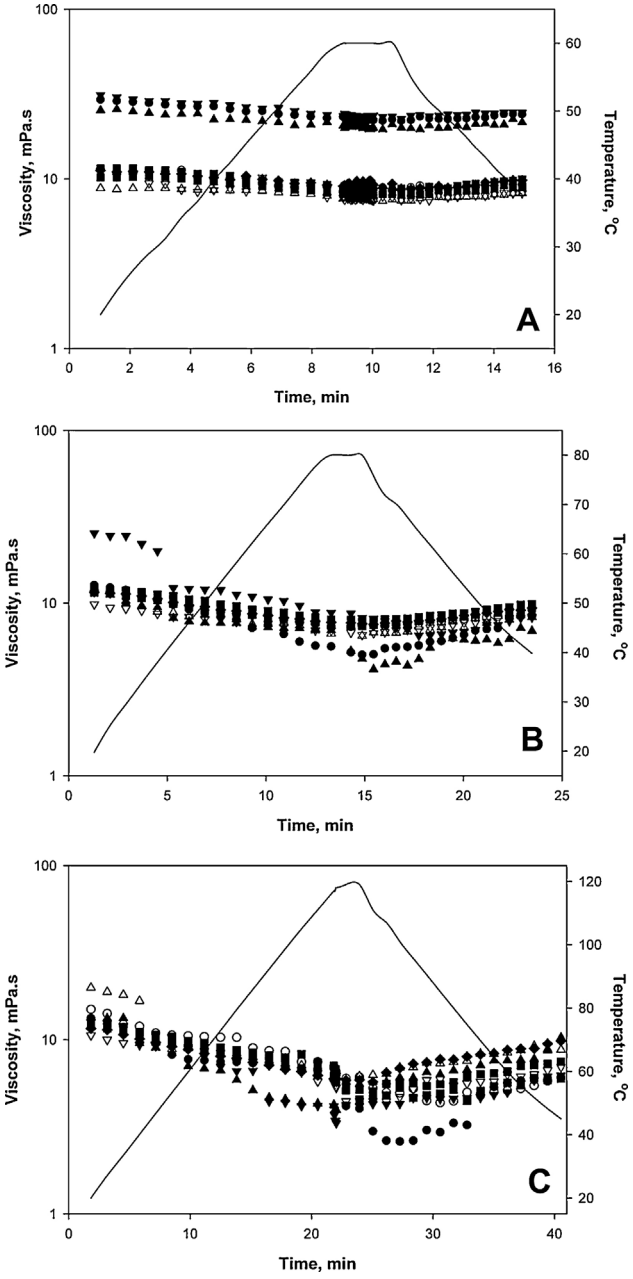

Fig. 2. Viscosity of tragacanth dispersion as a function of a constant shear rate (500, 1000 or 1500/s) and pH (3, 5 or 7), heated to a different temperature: (A) 60 °C; (B) 80 °C; and (C) 120 °C. Legend: ● pH 7 sheared at 500/s (pH7/500); ▲ pH5/500; ▼ pH3/500; ○ pH7/1000; ▽ pH5/1000; ■ pH3/1000; ■ pH7/1500; ◆ pH5/1500; ■ pH3/1500; and —temperature.

the polysaccharide (Carneiro-da-Cunha, Cerqueira, Souza, Teixeira, & Vicente, 2011).

Zeta potential analysis (Fig. 3A) confirmed that tragacanth was negatively charged (from −7.98 to −11.92), which was clearly pH dependant. This is in agreement with values reported by Yokoyama, Srinivasan, and Fogler (1988). This indicated that the solution was not physically stable and may result in sedimentation in the solution (Yokoyama et al., 1988). The pH dependence of the electrical charge on ionic polysaccharides depends on the pKa values of their ionizable side groups (McClements, 2014). Tragacanth primarily contains of arabinose, glucose, xylose, galactose, rhamnose, fucose and galacturonic acid residues in the gum structure (Balaghi, Mohammadifar, Zargaraan, Gavlighi, & Mohammadi, 2011). Almost, similar to pectin and alginate, tragacanth has a

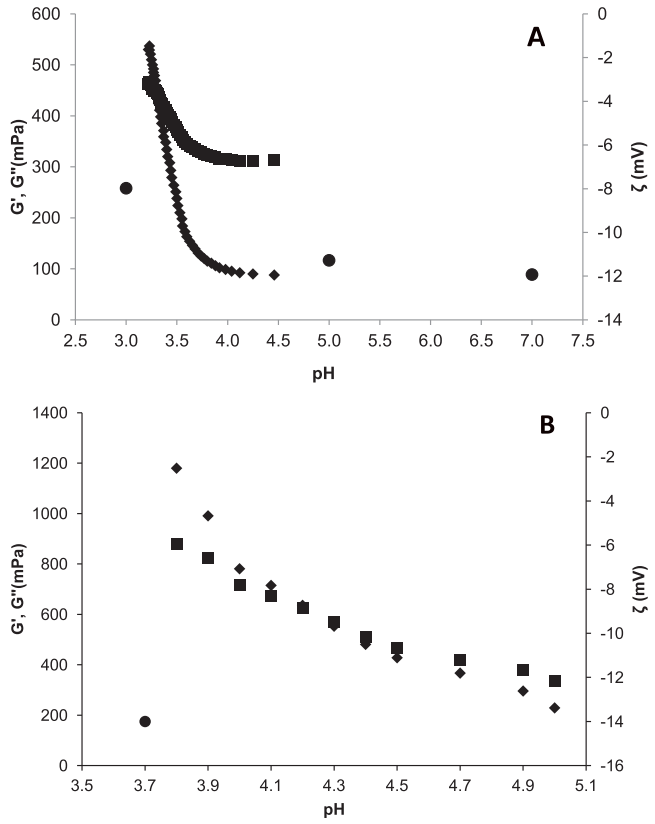

Fig. 3. Changes of storage modulus (G') and loss modulus (G'') during acid induced gelation of 0.5% (wt/wt) tragacanth only (A) and a dispersion containing insulin (0.2 mg/ml) and 0.5% tragacanth (B) by addition of 0.5% glucono-δ-lactone. Measurements were performed at 20 °C at constant strain (1%) and frequency (1 Hz). Secondary y-axis: zeta potential of tragacanth at pH 3; 5 and 7. Legend: ◆ storage modulus; ■ loss modulus;● zeta potential.

carboxyl group which is galacturonic acid. Therefore, the charge differences in tragacanth are triggered by this carboxyl group. The most common anionic side groups on polysaccharides are carboxylate groups and sulphate groups. The negative charge of alginates and pectins is derived from carboxylate groups but agars and carrageenans derive their negative charges from sulphate groups (McClements, 2014). The p*Ka* for tragacanth was previously reported to be 3 (Yokoyama et al., 1988).

Particle of tragacanth (Table 1) were submicron in size which may be really relevant for developing peptide/protein carrier. For example, insulin can be administered through different approaches in the GIT. The smaller the particle size (less than 2 μm), the better the insulin delivery because it can directly diffuse through the intercellular spaces of the intestinal epithelial cells or are absorbed through Peyer's patches (Lim et al., 2014). When the pH declined from 7 to 3, the particle size decreased significantly ($P < 0.05$). The particles in colloidal delivery systems often depend on forces that cause them to collide subjecting to the magnitude and range of the attractive and repulsive interactions between them (Israelachvili, 2011). The main attractive forces between particles in colloidal dispersions are hydrophobic attraction, van der Waals, depletion and, as well as bridging effects (McClements, 2014). From this characteristic, it is apparent that hydrogel from tragacanth could be formed by promoting their self-association under appropriate solution conditions. The charge and size of the biopolymer particles that have been created can also be controlled by modification of initial biopolymer concentration, holding time, holding temperature, ionic strength, and pH (McClements, 2014). From the result, the appropriate condition for peptide and protein delivery/carrier to make a complex with tragacanth is at pH 3–5 (pH under isoelectric pH of protein/peptides).

Preliminary testing (rheology, particle size, zeta potential and loading efficiency) of the suitability of tragacanth as a carrier of insulin was also carried out (Fig. 3B). Insulin (0.2 mg/ml) was selected as a model peptide to be loaded and mixed with 0.5% (w/w) tragacanth. Rheological GDL-induced acidification test indicated that tragacanth and insulin had a gelling point around pH 4.1. After tragacanth solution was loaded with the insulin, the average particle size of the complex at pH 3.7 was 566 nm with the zeta potential value of −14. Under these conditions, the loading efficiency of insulin was 78%. This result indicated that insulin was able to form a complex with tragacanth at pH 3.7. However, further investigations into the impact of variations of pH and concentrations of insulin and tragacanth need to be carried out to optimize the conditions for optimal delivery of insulin.

Differential scanning calorimetry (DSC) can be utilized to describe the thermal behaviour of biopolymers and polyelectrolytes which is related to their structure, association state and hydrophilic properties. Shifts of endothermic and exothermic peaks are usually related to the interactions between polymers and peptides (Sarmento et al., 2006a). DSC can be used to follow a large number of gelation processes such as the conformational changes of biopolymers (Djabourov, Nishinari, & Ross-Murphy, 2013). Experimental data from DSC can also confirm information concerning the source of the topology in polymeric composites (Kasapis, Norton, & Johan, 2009). Overall, change of pH had some effect on the thermal properties of tragacanth (Table 1). Most of the DSC thermograms exhibited endotherms peaking in the temperature between 110 and 134 °C. The transitions associated with loss of water were related to the hydrophilic nature of functional groups of biopolymers. Furthermore, the particle size of the samples to some extent may affect this transition (Sarmento et al., 2006a).

As demonstrated in Table 1, changing the pH (3, 5, or 7) influenced the enthalpy and onset value, but not peak and endset value of the tragacanth solution. This could be due to the higher stability of complexes at lower pH, thus more energy was needed to remove remaining water adsorbed to nanoparticles (showed by endothermic peak move to greater value), and less energy was released by breaking ionic interactions and during nanoparticle thermal decomposition (showed by exothermic peak move to greater value) (Sarmento et al., 2006a).

Detection of anionic side of polysaccharide is essential in polyelectrolyte complexes (PECs) to facilitate peptide/protein delivery. If anionic side is determined, appropriate condition of peptide/protein can be formulated. The most common anionic side of polysaccharides are sulphate groups and carboxylate groups. Pectins and alginates derive their negative charge from carboxylate groups whereas carrageenans and agars derive their negative

Table 1
Particle size and thermal properties of 0.5% tragacanth solution at pH 3, 5 or 7.

pH	Particle size (nm)	ΔH (J/g)	Onset (°C)	Peak (°C)	Endset (°C)
3	581.15^{a}	−1909.5 ± 23.43^{a}	143.7 ± 1.37^{ab}	144.77 ± 1.55^{a}	155.12 ± 1.25^{a}
5	465.55^{bb}	−1909.8 ± 28.33^{a}	142.87 ± 0.91^{b}	144.03 ± 1.13^{a}	154.10 ± 2.49^{a}
7	431.60^{b}	−1848.2 ± 15.35^{b}	145.57 ± 1.67^{a}	146.84 ± 1.66^{a}	155.76 ± 1.99^{a}

a–c means with different letters with in the same column differed significantly ($P < 0.05$).

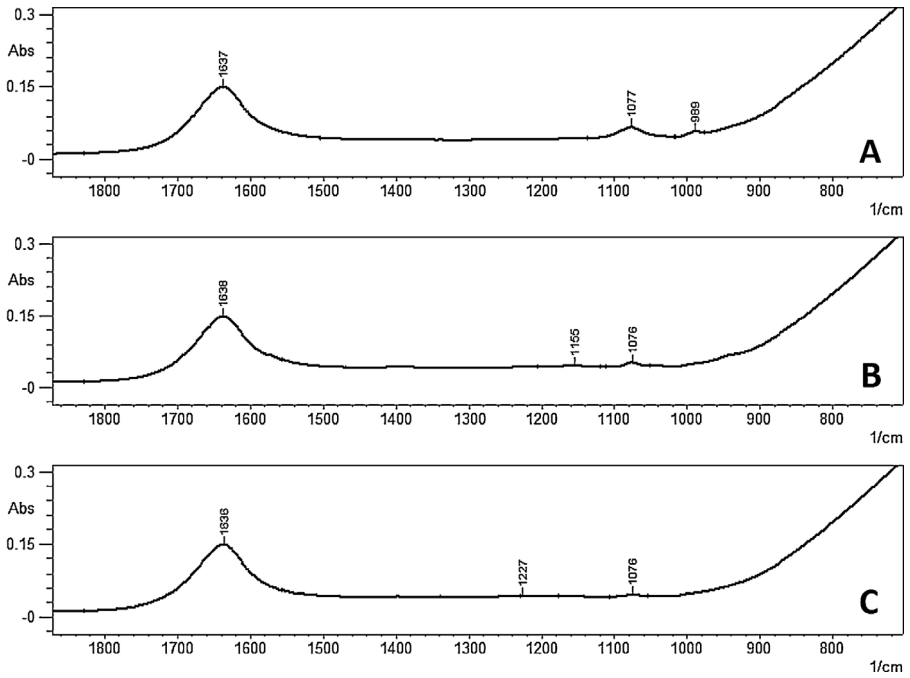

Fig. 4. FTIR spectra of 0.5% tragacanth solution at; (A) pH 7; (B) pH 5; (C) pH 3.

charges from sulphate groups (McClements, 2014). Almost, similar to pectin and alginate, tragacanth contains galacturonic acid. For example, the carboxyl group (—COO^-) of the anionic polymer may interact with the amino group (—NH_3^+) of cationic polymer (chitosan or a protein) form an ionic complex between the two compounds via polyelectrolytes interactions and peptide entrapment (Sarmento et al., 2006a). For that reason, during FTIR analysis, the focus was on monitoring the changes in carboxyl group of tragacanth.

The typical FTIR spectra of tragacanth solution under different pH can be seen in Fig. 4. The bands related to symmetric stretching of carboxylate groups and methyl groups in methyl esters of galacturonic acid (1417 and 1368 cm^{-1}, respectively), the vibrational modes of COOH in galacturonic acid and its salts and esters include asymmetric stretching (1740–1600 cm^{-1}). Polygalacturonic acids have maximum absorption band in this area, with very strong absorptions at 1017 and 1100 cm^{-1}, maximum absorption bands at 1043 and 1070 cm^{-1} representing the presence polysaccharides that contain galactose for instance galactans and arabinogalactans (Fattahi et al., 2013)

At different pH level (3, 5 and 7) asymmetric stretching of carboxylate group shifted from 1637 cm^{-1} (pH 7) to 1638 cm^{-1} (pH 5) and 1636 cm^{-1} (pH 3). This shift may be due to differences in zeta potential of tragacanth at different pH (Fig. 3A) that can influence the carboxylate group (McClements, 2014). Such changes in charge may affect the carboxylate group (McClements, 2014), which can cause a shift in carboxylate group peak observed in FTIR spectra. However, the absorbance value at that peak is not different. These structural characteristics can help optimize complexation of tragacanth by changing the pH. For example, at pH close to 3 the complexation of tragacanth may occur better than other pH.

3.2. Mucoadhesive properties

Several strategies can be performed to analyze mucoadhesion when using a rheometer. In this experiment, we used the method to measure the viscosity at different shear rates (Hassan & Gallo, 1990; Ivarsson & Wahlgren, 2012) and also determine the viscoelastic properties (Callens, Ceulemans, Ludwig, Foreman, & Remon, 2003). The results of the rheology measurements are presented in Fig. 5, showing the polymer viscosity (η_p) and mucin viscosity (η_m) as references, and the total viscosity (η_t) of the mucin–polymer mixtures. Mucin, chitosan and polyvinyl pyrrolidone (PVP) exhibited a very low viscosity, and it can be observed that after mixing with mucin, the viscosity of PVP did not change, showing an absence of interactions between them. Tragacanth and alginate showed shear-thinning behaviour with an increase in viscosity after mixing with mucin (Fig. 5). According to these measurements, at lower shear rates, the order of polymer mucoadhesion was tragacanth > alginate > PVP > chitosan. Thus, we found that tragacanth had the greatest potential for oral delivery of protein/peptides.

Frequency sweep experiment (Fig. 6) was achieved in the viscoelastic region of each sample, maintaining the structure of the system intact during the test. By conducting small stress amplitude oscillations measurement at an entire range of frequencies, the type of network structure can be determined. The major

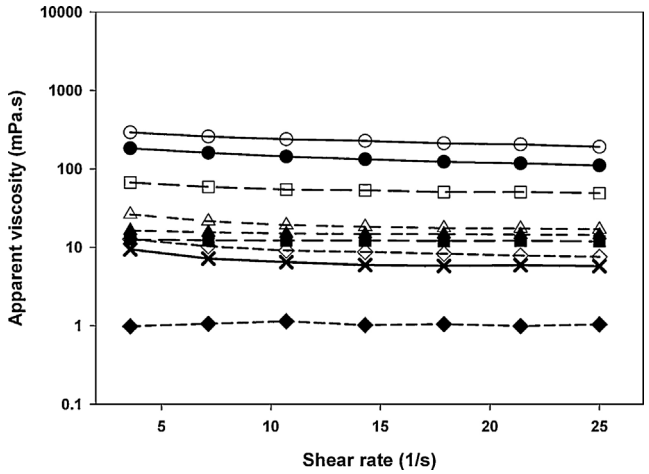

Fig. 5. Flow behaviour of mucin and polymers. Legend: ● tragacanth; ■ alginate; ◆ PVP; ▲ chitosan; ✕ mucin; ○ tragacanth/mucin; □ alginate/mucin; ◊ PVP/mucin; ▽ chitosan/mucin.

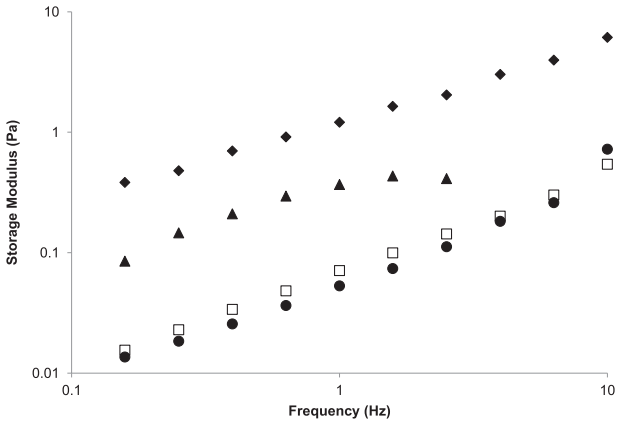

Fig. 6. Frequency sweep curves of mucin mixed with tragacanth, alginate and PVP at pH 6.6, 37 °C. Legend: ◆ alginate and mucin; □ mucin; ● PVP and mucin; ◆ tragacanth and mucin.

difference between physical entanglements and a network of secondary bonds is found in the low frequency range: in a network with secondary bonds the bonds are fixed irrespective of the time scale while in an entangled network, the polymers can disentangle if the time is long enough (low frequency) (Callens et al., 2003).

From Fig. 6, it can be seen tragacanth exhibited stronger viscoelastic properties than alginate when mixed with mucin. This indicated that tragacanth had greater mucoadhesion than alginate. This mucoadhesive property could mean that tragacanth may be suitable for site specific protein/peptide carrier due to prolong interaction with mucin and thereby control the release of protein/peptide. Mucoadhesive drug delivery systems work by increasing the drug residence time at the site of activity or resorption. For instance, polyanion carrageenan, a mucoadhesive polymer, helped to increase the area available between the cells, permitting the movement of molecules across the layers. These systems were effective in oral insulin therapy (Chaturvedi, Ganguly, Nadagouda, & Aminabhavi, 2013). Microparticles from lectin and *k*-carrageenan reported improved adhesion and absorption of insulin due to ionic interactions between the amino groups of insulin and negatively charged sulphate groups in carboxymethylated *k*-carrageenan imparting sustained release of insulin (Leong et al., 2011).

4. Conclusion

The conducted work found that different temperatures and shearing had no major effect on the viscosity of tragacanth solution. Behaviour of tragacanth in solution was pH dependant with zeta potential nearing neutrality towards pH 3 which resulted in gel formation. The tragacanth particles had a suitable size (submicron) that could assist in drug deliver. This biopolymer also showed higher mucoadhesion than alginate, PVP or chitosan. Based on these findings, tragacanth has a potential to be used as an excipient for oral peptide/protein delivery, due to its ability to form polyelectrolyte complexes and a hydrogel at an appropriate pH. Tragacanth formed a complex with insulin at pH 3.7 indicating its suitability as a carrier of insulin. However further investigations are required to optimize the conditions for maximum complexing and delivery of insulin.

Acknowledgments

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CHAPTER 4

Chapter 4. Insulin Inclusion into a Tragacanth Hydrogel: An Oral Delivery System for Insulin

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The paper entitled “Insulin Inclusion into a Tragacanth Hydrogel: An Oral Delivery System for Insulin” by Nur, M., & Vasiljevic, T. has been published in the peer-reviewed journal “*Materials*” (2018), 11(1), 79. doi.org/10.3390/ma11010079

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GRADUATE RESEARCH CENTRE

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Title of Paper/Journal/Book:	Insulin Inclusion into a Tragacanth Hydrogel: An Oral Delivery System for Insulin
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I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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Mokhamad Nur	85	Concept development; experimental design and execution; data analysis and interpretation; manuscript preparation	Mokhamad Nur Digitally signed by Mokhamad Nur Date: 2018.05.19 09:43:34 +07'00'	19/05/2018
Todor Vasiljevic	15	Experimental design; data analysis and interpretation; reviewing the manuscript; submission to journal	Prof Todor Vasiljevic Digitally signed by Prof Todor Vasiljevic Date: 2018.05.31 19:52:19 +10'00'	31.5.2018

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Article

Insulin Inclusion into a Tragacanth Hydrogel: An Oral Delivery System for Insulin

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Abstract: Nanoparticles or microparticles created by physical complexation between two polyelectrolytes may have a prospective use as an excipient for oral insulin administration. Natural polymers such as tragacanth, alginate, dextran, pullulan, hyaluronic acid, gelatin and chitosan can be potential candidates for this purpose. In this research, insulin particles were prepared by the inclusion of insulin into a tragacanth hydrogel. The effect of the pH and concentration relationship involving polyelectrolytes offering individual particle size and zeta potential was assessed by zetasizer and scanning electron microscopy (SEM). Insulin–tragacanth interactions at varying pH (3.7, 4.3, 4.6, or 6), and concentration (0.1%, 0.5%, or 1% w/w) were evaluated by differential scanning calorimetry (DSC) and ATR Fourier transform infrared (ATR-FTIR) analysis. Individual and smaller particles, approximately 800 nm, were acquired at pH 4.6 with 0.5% of tragacanth. The acid gelation test indicated that insulin could be entrapped in the physical hydrogel of tragacanth. DSC thermograms of insulin–tragacanth showed shifts on the same unloaded tragacanth peaks and suggested polyelectrolyte–protein interactions at a pH close to 4.3–4.6. FTIR spectra of tragacanth–insulin complexes exhibited amide absorption bands featuring in the protein spectra and revealed the creation of a new chemical substance.

Keywords: insulin; protein; peptides; PEC; hydrogels; gum tragacanth; insulin carrier; rheology; drug delivery; biopolymers

1. Introduction

Development of an appropriate carrier system for the oral delivery of insulin is still the main related problem due to compromised bioavailability hindered by the epithelial barriers of the stomach and gastrointestinal destruction by proteolytic enzymes [1–3]. Thus, a suitable insulin carrier really should provide biocompatibility as well as stabilisation under conditions in the gut in order to assure that the primary fraction of the insulin would be biologically active when delivered on site [1–3].

Biopolymers, for example, chitosan, dextran sulphate, and alginates, have been extensively studied due to their suitability for encapsulating proteins/peptides [4,5]. However, after encapsulation with these biopolymers, the bioavailability of insulin after oral administration remained low. These biopolymers can be complexed with insulin using various strategies, which include polyelectrolyte complexation (PEC), emulsification, ionotropic pregelation, and spray drying. Particles formed through polyelectrolyte complexation have demonstrated potential for use as an oral insulin carrier. PEC is generally created as soon as negatively and positively charged polyelectrolytes are mixed together through electrostatic attractions [5]. To be able to form a complex, the two polymers need to be ionised as well as carrying opposing charges. Therefore, the reaction could directly take place

within pH values in the area of (typically) the pKa interval of the two polymers. At the time of complexation, electrolytes may sometimes form a hydrogel [6]. However, in the case that the ionic attraction is very strong, precipitation could occur and prevent hydrogel creation. Precipitation could be prevented when electrostatic interaction is destabilised through enhancement of the ionic strength, for example, by adding salts (NaCl). Salts can diminish the interactions between positively and negatively charged electrolytes just by adding to the counter-ion environment. Therefore, basically no phase separation occurs, and a viscous and macroscopically homogeneous mix that could create a gel at a low temperature is also acquired [6].

Numerous studies have assessed the feasibility of various PECs for the delivery of insulin including alginate–chitosan [7,8], chitosan–poly(γ-glutamic acid) [9], and chitosan–arabic gum systems [10]. The benefit of these hydrogel systems is that the drug can be encapsulated easily by creating a water-based ionotropic gel, which protects the bioactive structure of insulin [11]. One of the main factors affecting functional properties of insulin–biopolymer complexes is pH. In alginate–insulin systems, if the environment pH is reduced from 6 to 4, the insulin encapsulation efficiency of the complexes increases [12]. This is also in accord with other research which showed that the encapsulation efficiency of insulin was around 93% [13] and 97% [14]. A possible explanation for the observed difference may be attributed to the environmental pH since it was under the isoelectric point of insulin (pI = 5.3), which gave preference to alginate–protein electrostatic attraction [15]. In another study, involving the dextran sulphate–insulin complexation, the protection has been attributed to the ionic interaction between the amino acid residues in the insulin molecules and the sulphate groups in the dextran sulphate [11]. This mechanism has also been studied when the complexation of polyion and protein decreased the release of the protein [16]. The glycemic effect was prolonged with the promotion of sustained insulin availability in vivo when dextran sulphate was included as a physical mixture in the delivery systems composed of chitosan and/or polyethylenimine [11,17].

To promote insulin absorption in the intestinal area, the PEC needs to adhere to the gastrointestinal lining at the site of delivery. Therefore, polymers with enhanced mucoadhesive properties are selected [18,19]. These types of mucoadhesive particles have the ability to extend the residence time around the site of release, trigger contact with the intestinal barrier, and produce a drug concentration gradient, which stimulates the transmission of the insulin via the intestinal membrane layer [18,20]. Recently, we reported that tragacanth gum (TG) could be used as a new polymer to deliver proteins and peptides. It is highly acid-resistant with high mucoadhesive properties [18]. Tragacanth offers special functional properties since it is safe, nontoxic, biocompatible, biodegradable, and stable over a broad range of pH [21]. Moreover, it is the most effective natural emulsifier intended for low pH O/W emulsions [21]. TG offers distinct surface activity attributes and decreases water surface tension efficiently even at very low quantities—lower than 0.25%—as well as encouraging emulsification. The zeta potential of tragacanth is about −21 mV. This can be related to the carboxylic groups of galacturonic acid (negatively charged) which is the primary chain of tragacanthin (water-soluble fraction of TG). TG is a viscous, odour-free biopolymer primarily containing two components: tragacanthin (water-soluble) and bassorin (swellable). The ratio between the soluble and insoluble fractions of TG gum in water differs considerably and depends on various Astragalus species [21]. Interaction with other material via hydrogen bonding and crosslinking can be initiated by using these TG functional groups (i.e., carboxylic acid and hydroxyl) [21].

The TG polymer has the capacity to create a gel via the carboxylic groups of tragacanth. Therefore, tragacanth provides a possibility to create an interaction with insulin, particularly in an acidic environment (under the pI of insulin) [18]. In other research, TG was applied in quercetin encapsulation through structuring of the TG shell and polycaprolactone (PCL) core self-assembled micelles. The quercetin release from these micelles showed a pH dependence. The rate of release was increased considerably at pH 2.2 [21].

A gelation and mucoadhesion study indicated that tragacanth has the potential to become an excipient for the oral administration of protein/peptides, for instance, insulin [18]. It is conceivable

that tragacanth properties may enhance the drug loading capacity, encapsulation efficiency, and the stability of the insulin encapsulated in the tragacanth particles through ionic attraction between tragacanth and the amino groups of the amino acid residues in insulin. Herein, we report our result on the use of tragacanth as an alternative choice and enhanced carrier for the oral administration of insulin. The approach was designed to monitor the complexation of the polyelectrolyte in becoming an insulin excipient. Systems produced from the complexation of polyelectrolytes at different pH and stoichiometric relationships involving polyelectrolytes were freeze-dried and/or directly analysed to verify interactions between tragacanth and insulin.

2. Materials and Methods

2.1. Materials

Tragacanth was obtained from Sigma–Aldrich (Castle Hill, Australia). GDL (Glucono-δ-lactone) powder from Sigma–Aldrich was also applied with no additional purification. The insulin sample that contains 100 U/mL of insulin was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). The water utilised was of a Millipore level of quality.

2.2. Characterisation

2.2.1. Microparticle Preparation

TG stock solution (2% w/w) was well prepared by dissolving a proper amount of the powder in MilliQ water at different pH levels (3.7, 4.3, 4.6, or 6), which was adjusted by adding an appropriate quantity of glucono δ lactone (GDL) powder. GDL dissociates in water, releasing gluconic acid and lowering the pH of the solution. The advantage of this type of pH adjustment is that the change is achieved at a slower rate without any change in the bulk volume. Sodium azide was included throughout the preparation of samples (0.2 g/L) to prevent microbial growth. The resulting solution was gently stirred with a magnetic rod at room temperature and kept overnight at 4 °C.

Tragacanth and insulin microparticles were prepared via mixing insulin (0.2 mg/mL) and TG colloidal solutions containing a different concentration of tragacanth (0.1%, 0.5%, or 1% w/w) at a predetermined pH. The complexation was allowed to proceed overnight by gently stirring the solution with a magnetic rod at room temperature. The mixture was then centrifuged at 20,000× *g* for 60 min at a room temperature using a high-performance centrifuge (Beckman Coulter Inc., Brea, CA, USA). The sedimented PECs were then frozen at −20 °C and freeze-dried at 0 °C for at least 48 h using a freeze-drier (model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia).

2.2.2. Acid-Induced Gelation

GDL-induced acidification was carried out to evaluate the capability of TG to entrap insulin through acid-induced gelation [18]. Dynamic small amplitude oscillatory analysis was carried out using a stress-controlled rheometer (MCR 301, Anton Paar GmbH, Ostfildern, Germany) with a double gap geometry (DG 26.7-SN. 24845, Anton Paar) to determine an acid gel point following a previously described method [18]. The required amount of the GDL powder was added to a TG solution at 20 °C. An exact volume of the sample (3.9 mL) was added directly into the testing system (double gap) at the same temperature and allowed to stabilize for 100 min (time sweep) during which time changes in the viscoelastic behaviour of the colloidal solution were recorded. The change of pH was concurrently noted every 2.5 min in the remaining part of the sample by using a pH meter (Model 8417; Hanna Instruments, Singapore) during the same period.

2.2.3. Particle Size and Zeta Potential Analysis

The particle size and zeta potential of the polymeric complexes created by mixing the insulin solution (0.2 mg/mL) and the TG solution (0.1%, 0.5%, or 1% w/w tragacanth) at different pH (3.7, 4.3,

4.6, or 6) were analysed using a zetasizer (ZEN3600, Malvern Instrument Ltd., Worcestershire, UK) with a He–Ne laser beam at 658 nm. An appropriate aliquot of the insulin–tragacanth mixture was diluted 1:100 with MilliQ and stored overnight before the measurement [18].

2.2.4. Measurement of Loading Efficiency

Loading efficiency was determined indirectly following centrifugation of the insulin and TG dispersion upon mixing insulin (0.1 mg/mL) and TG colloidal solutions at different pH and tragacanth concentrations. The PEC was centrifuged at 20,000× g for 60 min at room temperature using a high-performance centrifuge (Beckman Coulter, Brea, CA, USA). The quantity of insulin in the supernatant was analysed using the Bradford procedure at 595 nm [18]. The loading efficiency was measured as

$$\text{Loading efficiency } (\%) = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100. \quad (1)$$

2.2.5. DSC (Differential Scanning Calorimetry) Analysis

Thermal characteristics of particles were analysed using DSC, as explained earlier [15], with some adjustments. Thermograms of TG solutions were gained by using a DSC (Mettler Toledo, Schwerzenbach, Switzerland) fitted with an intracooler system and under an inert nitrogen gas atmosphere. A sample (4–7 mg) obtained under described experimental conditions (variable pH and tragacanth concentration) was put in a 40 µL aluminium lightweight pan, hermetically enclosed just before insertion into the DSC, and then heated from 20 to 350 °C at a constant rate of 10 °C/min within constant purging of nitrogen at 20 mL/min. An empty pan with the same weight functioned as the reference. The ΔH values, onset, endset, and peak temperatures of the thermograms were documented.

2.2.6. FTIR Analysis

FTIR spectra of the particles at different pH (3.7, 4.3, 4.6, 6) and concentrations of tragacanth (0.1%, 0.5%, and 1%) were acquired by using a Perkin Elmer ATR-FTIR spectrometer equipped with a Diamond TM ZnSe single reflection ATR plate (Perkin-Elmer, Norwalk, CT, USA). The actual spectrum of every sample was acquired from 16 scans from 600 to 4000 cm^{-1} having a resolution of 4 cm^{-1} as well as strong apodisation. This particular measurement was adjusted towards the background spectrum of the solvent. Baseline manipulation and data acquisition were gained using Shimadzu IR solution software v1.40 [18,22].

2.2.7. Scanning Electron Microscope (SEM)

Particle morphology was studied using scanning electron microscopy (SEM). For SEM analysis, samples of microparticulate complexes were installed on metal stubs, gold covered under vacuums and then analysed in a JEOL NeoScope JCM-5000 A SEM (10 kV, Tokyo, Japan).

2.3. Statistics

The information acquired from particle size analysis was arranged in a randomised block design using pH as the primary factor. These assessments were duplicated at least once, with subsequent subsampling providing a number of independent observations of at least $n \geq 4$. Final results were analysed using one-way ANOVA, SAS (1996). Tukey's Studentized Range (HSD) analysis was applied for multiple comparisons of means. The degree of significance was predetermined at $p = 0.05$.

3. Results and Discussion

The application of a biopolymer as a multiparticulate excipient for protein/peptide delivery has long been extensively recorded in the scientific literature [2,23]. This kind of matrix currently has great potential to be applied for the controlled release of drugs because of its relatively small molecular size.

Furthermore, bioavailability and drug absorption could be improved as a result of a large surface area to volume level ratio, which leads to significantly more intimate contact along with the mucus layer [11,23].

In this study, initially, microparticulate polyelectrolyte complexes between insulin and tragacanth were created, including the mild mixing of two aqueous solutions of tragacanth and insulin. To test the ability to create a gel, GDL was added to the mixture, and an acid gelation test was conducted.

It can be seen from Figure 1 that during the acid gelation test, as the pH was decreasing, at pH around 4.3, the storage modulus was greater than that at other pH levels. Almost all of the systems with viscoelastic properties possess both viscous (liquid) and elastic (solid) elements, in which the shear stress is between 0 and 90 degrees. In these systems, the stress element that is in-phase with the shear strain is in charge of the elastic element and is identified as the storage modulus (G′), which depicts the material elasticity. The value of the storage modulus is proportional to the quantity of permanent interactions and the strength of the interactions existing in the biopolymers. Therefore, G′ is a measure of the structure of the biopolymers [24]. A time sweep offers the viscoelastic properties of biopolymers as a function of time, in which the strain, frequency, and temperature are kept constant. The gel networks of biopolymers continue to develop throughout a time sweep. This can be noticed from the increase in the value of the storage modulus as a function of time [24]. In our system, during the time sweep, a change of pH was measured (as described in Section 2.2.2). Therefore, a storage modulus vs pH graph was created. The increase in the storage modulus (maximum at pH 4.3), which is close to the gelling point of tragacanth and insulin [18], was an indication that insulin was likely entrapped in the tragacanth gel (hydrogel creation). Carboxylic groups from tragacanth may be involved in this process. Most of the pH-sensitive biopolymers consist of pendent basic or acidic groups, which either take or give protons in reaction to the solvent pH [25]. Polyacid biopolymers are unswollen in an acidic environment since their acid groups are unionised and protonated [25]. Upon increasing the pH, a negatively charged polymer would swell. The opposing patterns are noticed in polybasic biopolymers, considering that the ionisation of the basic groups increases the following decline in pH [26]. Some instances of pH-sensitive biopolymers having anionic groups are polycarboxylic acids (PAA) or poly-methacrylic acid (PMA) and poly-sulfonamides (derivatives of p-aminobenzene sulfonamide). In an acidic environment, hydrophobic interactions dominate and carboxyl groups are protonated, resulting in volume withdrawal involving the biopolymer consisting of carboxyl groups. In a basic environment, carboxyl groups dissociate into carboxylate ions, causing higher charge density in the biopolymer, resulting in swelling. The chain configuration of a weak polyacid is a function of the pKa of the biopolymer [27].

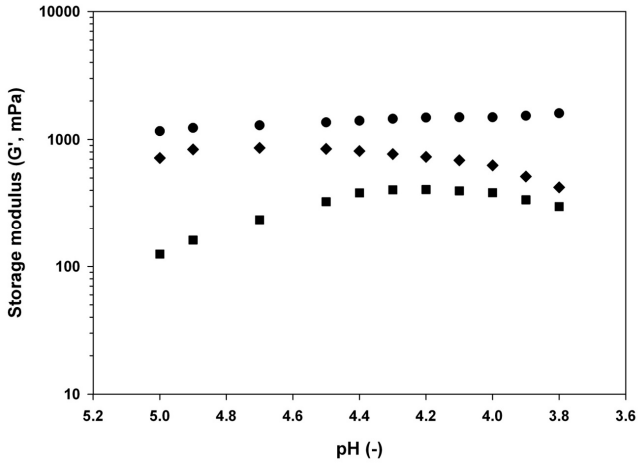

Figure 1. Evolution of storage modulus (G′) during acid-induced gelation of tragacanth dispersions at ♦ 0.1% w/w, ■ 0.5% w/w, and ● 1% w/w. Measurements were performed at 20 °C at constant strain (1%) and frequency (1 Hz).

This type of gel is called a physical gel due to the fact that the networks tend to be retained by molecular entanglements and/or weak forces including hydrophobic, H-bonding, or ionic interactions [28]. The network porosity of such hydrogels changes along with electrostatic repulsion. An ionic gel consisting of carboxylic and/or sulfonic acid groups demonstrates either immediate or slow changes in their particular dynamic or equilibrium swelling behaviour due to the change in the environmental pH. The ionisation degree of those hydrogels is determined by a number of pendant acidic groups within the gel, causing enhanced electrostatic repulsions involving negatively charged carboxyl groups at various chains. This, consequently, leads to increasing hydrophilicity of the network as well as a higher swelling ratio within a higher pH [28]. On the other hand, a hydrogel consisting of basic pendant groups, including amines, will ionise as well as demonstrate electrostatic repulsion in an acidic environment [28].

It can also be noticed from Figure 1 that tragacanth at a higher concentration (1%) exhibited stronger viscoelastic properties than at the lower concentrations (0.5% and 0.1%). If this is linked to pI, the achievable entrapment of a protein and/or a peptide by TG is most likely achieved under the isoelectric point (pI) of insulin [18]. For instance, the pI of insulin can vary from 5.5 to 6.4, based on its origin. At a pH higher than its pI value, insulin will be mainly negatively charged [29]. This insulin characteristic could be utilised to create insulin–biopolymer complexes through electrostatic attraction with tragacanth. At pH around 4.3 and 4.6, TG may undergo coacervation with insulin as well as simultaneously creating a hydrogel which is able to entrap insulin since, at this pH, insulin can be positively charged close to the determined gelling point of TG [18,30].

After a mixture of a gel-like formation was created, it was then freeze-dried, and a loading efficiency (LE) was examined at different levels of pH and concentrations. The pH variety was selected to obtain opposite charges of electrolytes as well as suitable complex creation. Within this particular pH range, electrostatic attraction involving proteins and biopolymers occurs. It can be observed from Figure 2 that the reduction of pH from 6 to 3.7 resulted in an increase of LE, especially at pH 4.6. However, if the pH of the aqueous solution was set to 3.7 or 4.2, the particles become much larger (>800 nm). In this pH range, some parts of tragacanth begin to precipitate [31], which may play a role in the increased mean particle size (Table 1). Therefore, insulin was partly bound ionically to the insoluble uronic acid of tragacanth. The interaction involving biopolymers and insulin is mostly ionic. However, one should also consider hydrogen bonding as well as van der Waals forces [32,33]. It can be seen from Table 2 that negative zeta potential (ZP) values are acquired because of the carboxylic groups [34] of tragacanth [18]. Moreover, the ZP values depend on the dispersion pH [34]. In general, if the ZP values are less than −10 mV (in most cases, from −25 to −30 mV, Table 2), it can predict an excellent colloidal stability because of the high energy barrier between particles [34]. Positive amino radicals of insulin are highly and electrostatically interacted with by carboxylic/sulphate groups. At pH 4.3 or 4.6, insulin is primarily positively charged (pI of insulin: 5.3) [35] and is therefore attracted to the partly negative tragacanth, while at pH 6, positive charges are minimised on the amino acid, which could prevent attractive interactions with the negative charges on the tragacanth. As a result, the insulin LE is less at pH 6 than at other, lower pH. For that reason, pH 4.6—at which microparticles were produced, and a high insulin LE was acquired—was selected as the most appropriate pH. The outcomes acquired suggested that the affinity of insulin for tragacanth carboxylic groups is greatest at pH 4.3 or 4.6, as pointed out by examining the LEs of the created complexes. A tragacanth concentration of 0.5% (w/w) tends to be the optimum concentration for complexation. Particularly, at pH 4.3, the LE increased from 65% (0.1%, w/w of TG) to 89% for TG concentration 0.5% (w/w).

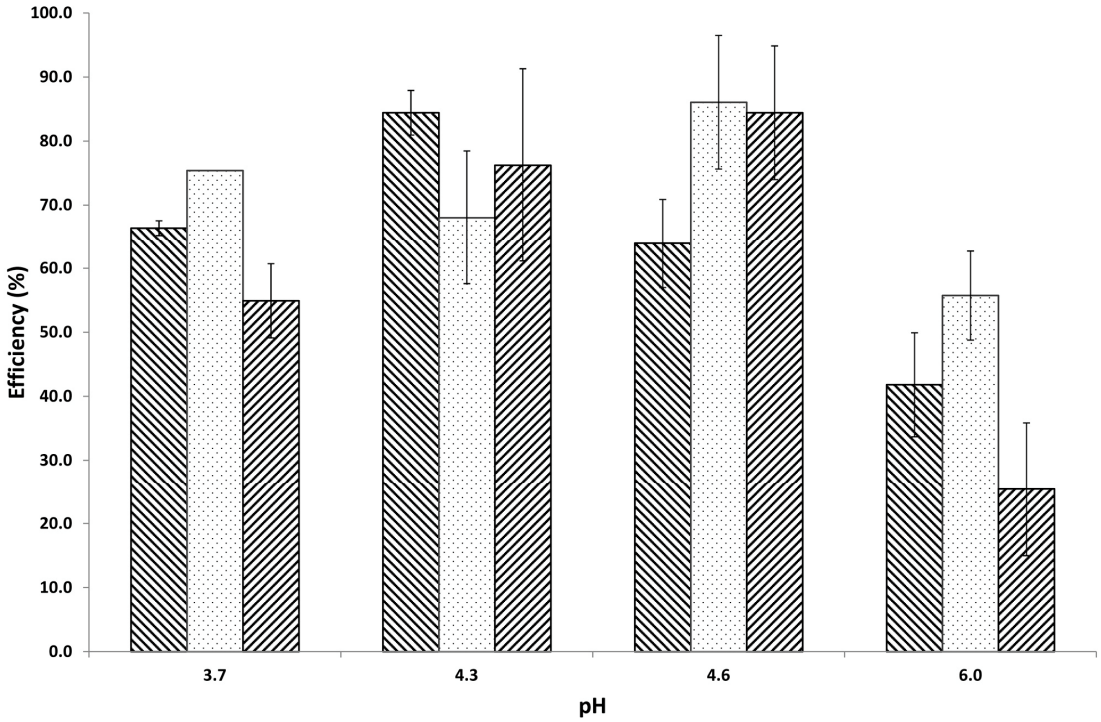

Figure 2. Loading Efficiency (LE) of insulin complexing with tragacanth at different concentrations (▧ −0.1% w/w, ▨ −0.5% w/w, and ▩ −1% w/w) at different pH (3.7, 4.3, 4.6, or 6) adjusted by addition of GDL at room temperature and equilibration under very low magnetic stirring overnight.

Table 1. Particle size of polymeric complexes created by mixing insulin solution (0.2 mg/mL) and tragacanth solution at 0.1%, 0.5%, or 1% w/w concentration at different pH (3.7, 4.3, 4.6, or 6) adjusted by addition of glucono δ lactone (GDL) at room temperature and equilibration under very low magnetic stirring overnight. The results are presented as means of at least five independent observations ($n \geq 5$) with ±SE.

pH	Particle Size, nm		
	Tragacanth Concentration, % w/w		
	0.1	0.5	1
3.7	1105 ± 48	1382 ± 141	839 ± 22
4.3	667 ± 37	811 ± 20	957 ± 56
4.6	651 ± 09	601 ± 19	649 ± 25
6	566 ± 23	373 ± 08	719 ± 05

Table 2. Zeta potential of polymeric complexes created by mixing insulin solution (0.2 mg/mL) and tragacanth solution at 0.1%, 0.5%, or 1% w/w concentration at different pH (3.7, 4.3, 4.6, or 6) adjusted by addition of GDL at room temperature and equilibration under very low magnetic stirring overnight. The results are presented as means of at least five independent observations ($n \geq 5$) with ±SE.

pH	Zeta Potential, mV		
	Tragacanth Concentration, % w/w		
	0.1	0.5	1
3.7	−22.3 ± 0.6	−26.0 ± 0.6	−29.1 ± 0.5
4.3	−38.9 ± 1.2	−19.2 ± 2.5	−30.9 ± 1.9
4.6	−36.3 ± 1.7	−7.5 ± 0.4	−18.1 ± 0.6
6	−38.1 ± 1.3	−39.5 ± 4.9	−40.8 ± 8.4

Insulin and biopolymer complexes could be produced at the isoelectric point (pI) of insulin. The pI of insulin is around 5.5–6.4. At a pH under the pI value, insulin is positively charged; the converse is also true [2]. These attributes of insulin have been used in the creation of insulin–biopolymer complexes through electrostatic interaction with a negatively charged biopolymer (alginate) as well as positively charged biopolymer (chitosan), which are protonated at pH values under its pKa (6.5) [2]. TG has the propensity to form a complex with insulin at a pH under the pI of insulin [18]. Conversely, a positively charged biopolymer such as chitosan can form a complex with insulin if the pH is altered over the pI of insulin [36,37].

DSC thermograms in Figure 3 show variations involving individual biopolymers and, after complexation, suggest ionic interactions indicated by the change of endothermic peaks as well as by the shift in exothermic peaks associated with decomposition temperature. The DSC curves exhibit a wide endothermic peak between 100 and 200 °C for isolated polyelectrolyte and its physical mixture. All samples exhibited exothermic peaks between 220 and 285 °C.

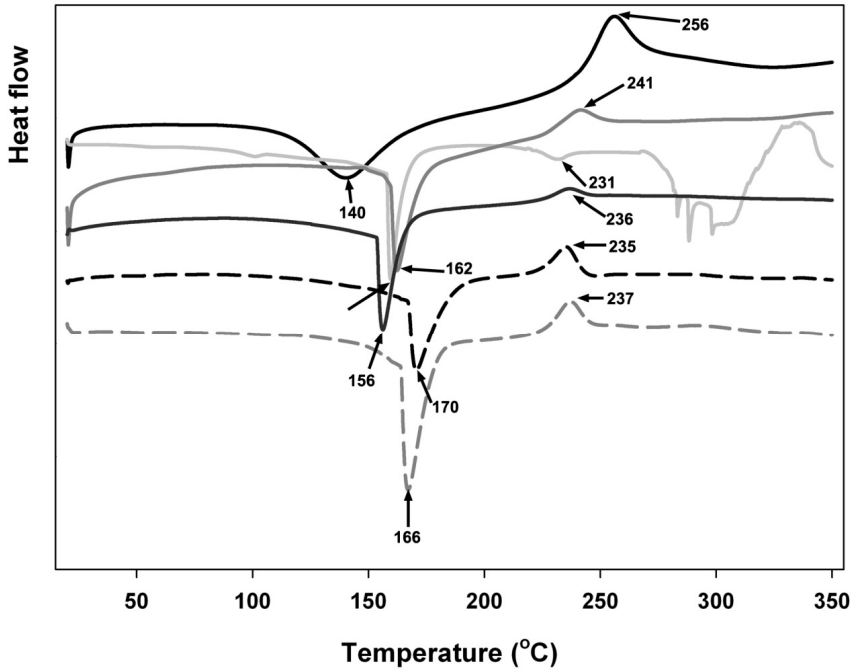

Figure 3. DSC thermograms of polymeric complexes created by mixing insulin solution (0.1 mg/mL) and tragacanth solution at 0.1%, 0.5%, or 1% w/w concentration at different pH (3.7, 4.3, 4.6, or 6) adjusted by addition of GDL at room temperature and equilibration under very low magnetic stirring overnight. Legend: ▬ tragacanth (control); ▬ insulin (control); ▬ mixture at pH 3.7; ▬ mixture at pH 4.3; ▬ ▬ mixture at pH 4.6; and, ▬ ▬ mixture at pH 6.0. Arrows and numbers indicate the temperature of phase transition.

Individual biopolymers were recognised by the occurrence of first endothermic peaks at 140 °C for tragacanth only and 156 °C, 162 °C, 166 °C, 170 °C for the mixtures at different pH, respectively, and also by the occurrence of greater exothermic peaks at 256 °C for tragacanth only and 235 °C, 336 °C, 237 °C, 241 °C for the mixtures at different pH, respectively. Exothermic peaks are correlated with the destruction of polyelectrolytes as a result of dehydration as well as depolymerisation reactions, most likely due to the incomplete decarboxylation of the protonated carboxylic groups as well as oxidation reactions of the polyelectrolytes, while endothermic peaks are related to the reduction of water connected to the hydrophilic groups of the biopolymers [38–40].

Exothermic and endothermic peaks shifted to greater temperatures when the pH of the microparticles was reduced from 6 to 4.3. It was noticed that lowering the pH resulted in greater

stability of the microparticle; therefore, more energy was required in order to eliminate residual water adsorbed onto the mixture (endothermic peak changed to increased value), and a lesser amount of energy was discharged by breaking ionic attractions and through microparticle thermal decomposition (exothermic peak change to increased value) [41].

Like alginate and pectin, TG possesses a carboxyl group (galacturonic acid). Consequently, the charge variations in TG are caused by this particular carboxyl group. The most typical negatively charged side groups on polysaccharides are usually sulphate groups or carboxylate groups. The negative charge of agars and carrageenans comes from sulphate groups, but pectins and alginates obtain their negative charges from carboxylate groups [42]. TG has a previously documented pKa value of 3 [18].

The optimum ionic attraction between tragacanth and insulin was achieved at lower pH. Almost similar to alginate, tragacanth gel shrunk at lower pH levels due to a decrease in the pore size of the tragacanth matrix [18,43]. For that reason, at pH 4.3 or 4.6 it is feasible that microparticles introduced a more powerful robustness than at pH 6. Interactions between tragacanth and insulin have been identified to become pH-dependent together with more powerful complexes that were previously acquired at pH close to 4.2–4.7 [18].

The inclusion of insulin within microparticulate complexes can certainly be seen by the postponing of its endothermic peak. The two endothermic peaks in connection with insulin, which are related to water loss and the denaturation process [42], started to be indistinct and changed themselves into a single peak following entrapment in the microparticulate complexes. Insulin-loaded models achieved this particular endothermic peak at a lower temperature in comparison to insulin-free models, apparently showing an attraction involving the protein and the tragacanth. Furthermore, looking at the exothermic conditions of insulin-loaded and unloaded particles, the onset point began at a lower temperature for insulin-loaded microcomplexes; this probably suggests that entrapped insulin initiated decomposition at higher temperatures (235–241 °C) than when analysed in isolation from the particles (231 °C).

The obtained FTIR spectra are represented in Figure 4a–c, and show two shoulders on the complex absorption bands in Amide I (~1644 cm^{-1}) as well as in Amide II (~1531 cm^{-1}) that are properties of the protein spectra. The monomer of insulin has numerous ionizable groups because of six amino acid residues able to attach a positive charge and ten amino acid residues able to attain a negative charge [44,45]. These kinds of characteristics are, therefore, probably responsible for the entrapment of insulin into tragacanth microparticles.

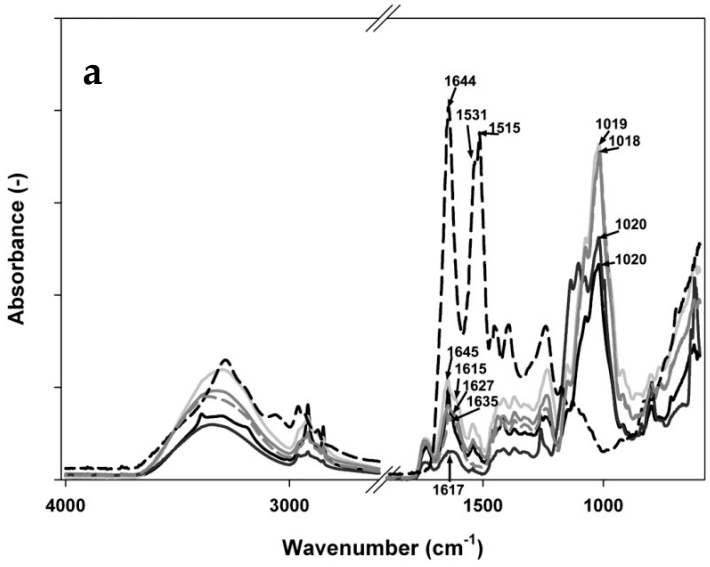

Figure 4. *Cont.*

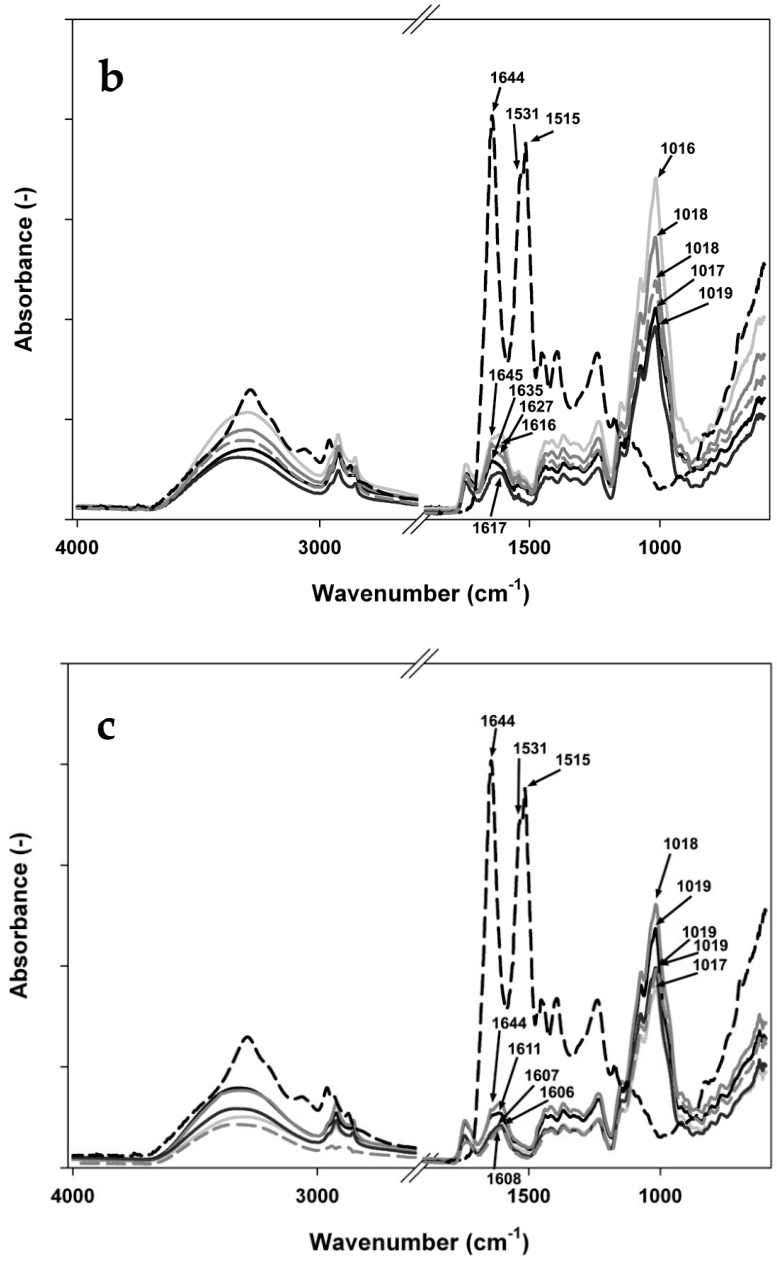

Figure 4. FTIR spectra of polymeric complexes created by mixing insulin solution (0.1 mg/mL) and tragacanth solution at (**a**) 0.1%, (**b**) 0.5%, or (**c**) 1% w/w concentration at different pH (3.7, 4.3, 4.6, or 6) adjusted by addition of GDL at room temperature and equilibration under very low magnetic stirring overnight. Legend: ▬ tragacanth (control); ▬ insulin (control); ▬ mixture at pH 3.7; ▬ mixture at pH 4.3; ▬ ▬ mixture at pH 4.6; and, ▬ ▬ mixture at pH 6.0. Arrows and numbers indicate a wavenumber of a particular structural change.

The bands at 1416 and 1369 cm^{-1} are associated with the symmetric stretching of carboxylate groups as well as the methyl groups in methyl esters of galacturonic acid, respectively, while the actual vibrational modes of COOH in galacturonic acid and its salts and esters contain asymmetric stretching (1740–1600 cm^{-1}). Polygalacturonic acids possess the highest possible absorption band in this region, having quite strong absorptions at 1017 and 1020 cm^{-1}, and optimum absorption bands at 1018 and 1019 cm^{-1} representing the occurrence polysaccharides which have galactose, for example, arabinogalactans and galactans [18,46].

It can be seen that tragacanth carboxyl peaks close to 1627 cm^{-1} (symmetric COO– stretching vibration) and 1416 cm^{-1} (asymmetric COO– stretching vibration) broadened and shifted a little from 1627 to 1616 cm^{-1} and 1416 to 1415 cm^{-1} following complexation with insulin. Moreover, the FTIR spectrum of tragacanth exhibits a peak associated with an amide bond at 1645 cm^{-1}. Noticed shifts in the absorption bands of the carboxyl groups, amide bonds, and amino groups could be assigned to an ionic attraction between the carboxyl group of tragacanth and insulin [47]. Also, the peak absorbance of amino groups of tragacanth at 1149 cm^{-1} existed right after complexation. Similar observations were noted previously [48–50]. These findings indicate an effective interaction between biopolymers corresponding to the stoichiometric ratios between them, which indicate the occurrence of TG at the end of the mixture [15,51].

Surface morphology information for freeze-dried microspheres has been acquired by SEM analysis and is presented in Figure 5a. The carrier exhibited an irregular shape and had a somewhat wrinkled surface. Apparently, the spherical shape of the microspheres was lost after drying. We speculate that insulin entrapped in tragacanth, as in Figure 5b, explains the results of SEM observation. It could be seen that, at the beginning, the tragacanth creates a homogenous network from the core to the edge. Insulin is then entrapped inside the network. The negatively charged tragacanth creates a complex with the positively charged insulin [52,53]. The microstructure (SEM) of the tragacanth hydrogels after freeze-drying exhibits the porous morphology, with pore size greater than the submicron range. The pore structure and size were similar to other acidified gel biopolymers including tragacanth-milk [54] and pectin-sodium caseinate systems [55]. These morphological characteristics are related to the exchange of insulin-loaded microparticles. The destruction of hydrogels is followed by the discharge of insulin from hydrogels. These morphological properties have been connected with water exchange and swelling of hydrogels. Swelling characteristics of hydrogels are essential for material transfer when applied to insulin carriers [56].

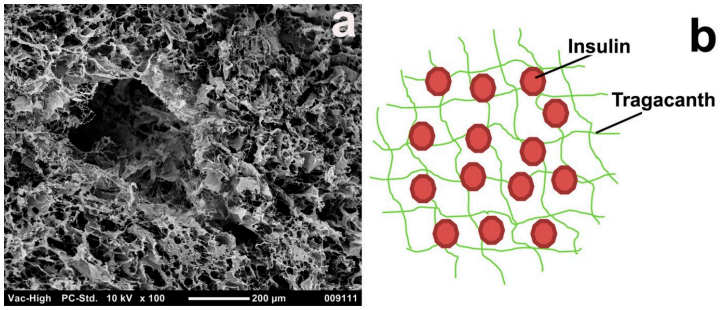

Figure 5. SEM image of (**a**) a complex between insulin (0.1 mg/mL) and 0.5% of tragacanth at pH 4.6 with the best exhibited efficiency and (**b**) proposed polyelectrolyte complexation (PEC) model of insulin entrapment in a tragacanth network.

4. Conclusions

Insulin was entrapped in physical hydrogel and polyelectrolyte complexes (PECs) created using biodegradable biopolymer—tragacanth. Microparticulate complexation between tragacanth and insulin was revealed by FTIR and DSC measurement. These microparticles appear to have potential functional properties for oral insulin delivery, especially those that contain tragacanth polyelectrolytes at pH 4.3 and 4.6, although additional in vivo research should be carried out to ensure the presence of these properties.

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analysed and interpreted the data; Mokhamad Nur wrote the paper with critical contributions from Todor Vasiljevic.

Conflicts of Interest: No rewards have already been and/or will be gained from a commercial party associated with the topic of this research.

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CHAPTER 5

Chapter 5. Insulin-loaded tragacanth microparticles produced by spray drying: Towards a novel oral insulin delivery system

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The paper entitled “Insulin-loaded tragacanth microparticles produced by spray drying: Towards a novel oral insulin delivery system” by Nur, M., & Vasiljevic, T. has been submitted to peer-reviewed journal “*International Journal of Biological Macromolecules*”. Manuscript number: IJBIOMAC_2018_2892

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5.1. Introduction

Type 1 diabetes is a generalized disorder of the glucose metabolism, in which the pancreas produces little or no insulin (INS). The unique medication still remains regular INS injection. However, a poor estimation of normal physiological INS secretion, complications, as well as poor patient compliance may lead to inadequate outcomes, which thus have triggered an exploration of a substitute for subcutaneous injection, for example using the oral route (Lim et al. 2017; Sonia & Sharma 2015; Zaykov et al. 2016). While a number of attempts have been made, numerous barriers for successful delivery still exist, for example, activity of proteolytic digestive enzymes at various locations in the gastrointestinal epithelia could limit the absorption of insulin by hydrolytic breakdown of the insulin structure (Nur & Vasiljevic 2017; Wong et al. 2018; Sonia & Sharma 2015). To overcome these issues, numerous strategies have been developed, for example, the formulation of carrier systems, the inhibition of the enzymatic degradation, and the chemical modification of the insulin (Nur & Vasiljevic 2017; Sonia & Sharma 2015; Peppas & Kavimandan 2006).

To overcome the issues created by the gastric pH and proteolytic destruction in the gastrointestinal tract (GIT), the formulation of oral insulin delivery systems such as microparticles and microspheres has demonstrated promising outcomes. For example, the alginate microspheres produced by ionotropic gelation appeared to decrease INS release in simulated gastric fluid (SGF) after the addition of calcium ions (Martins et al. 2007). Spray dried microparticles of insulin and β-cyclodextrin can protect orally delivered INS from chemical and enzymatic degradation in the SGF (D'Souza et al. 2015). Chitosan crosslinked with glutaraldehyde microspheres appeared to be transferred across the GI tract to the blood circulation (Wei et al. 2008). Chitosan and dextran were coated into complexes to propose further electrostatic interaction, protect encapsulated INS from the gastric pH environment,

facilitate mucoadhesiveness, and enhance gastrointestinal permeability (Balabushevich et al. 2013; Wong et al. 2018). Moreover, it was revealed that microparticles could be taken up by Peyer's patches; the percentage of exposure uptake from microparticles not more than 10 μm was much higher than that of the bigger microparticles (Coppi et al. 2001; Wong et al. 2018). To reduce insulin inactivation and the damage of its biological functionalities, a gentle microencapsulation technique, preventing direct exposure to the increase of temperature and organic solvents, need to be applied. Among several other microencapsulation methods, the spray-drying process appears to be regarded as suitable for insulin encapsulation (Coppi et al. 2001).

Spray-drying is a rapid, economic single step process which has a potential for the large-scale encapsulation of protein/peptide drugs, which can be designed as a continuous drying process (Faheem & Haggag 2015). Moreover, it generally produces particles with a homogeneous size distribution characterized by high encapsulation efficiency (Anandharamakrishnan & Padma Ishwarya 2015; Quaglia et al. 2003). The spray drying process includes atomization of a liquid feed or suspension into tiny droplets that are sprayed together with hot gas usually air into a drying chamber. In contrast to other techniques used in microparticle formulations, for example, spray freeze drying, emulsion dispersion or ionotropic pre-gelation, spray drying is less time consuming and less dependent on the solubility of the therapeutic substance and matrix biopolymer (Coppi et al. 2001; Anandharamakrishnan & Padma Ishwarya 2015). One of the disadvantages of spray drying is potentially denaturation of proteins due to the use of inlet temperatures more than 100°C. Numerous reports, however, have suggested that the incorporation of biopolymers, amino acids, or sugars in addition to careful control of feed rate to maintain drying at a constant wet bulb temperature appear to protect proteins throughout spray drying and preserve their biological activity (Bowey et al. 2013; Bowey & Neufeld 2010). For instance, spray dried insulin in an alginate matrix retained its bioactivity (Bowey et al.

2013) and bovine serum albumin in a chitosan matrix maintained its secondary structure and integrity in addition to bioactivity (Kusonwiriyawong et al. 2009).

To select for appropriate insulin matrix/excipient in a spray drying process, natural polymers are preferred candidates as insulin release carrier since they are safe, highly inert to peptide drugs, without a requirement for organic solvents. Chitosan, alginate, dextran, starch and pectin, as some examples of biopolymers, have been applied extensively in particle designs of insulin excipient due to their non-toxic attributes (Sonia & Sharma 2015). Protein biopolymers, such as gelatine and caseins, have also been explored to create insulin-loaded particles for oral administration with promising outcomes and good safety profile (Sonia & Sharma 2015). In this regard, the use of tragacanth gum (TG) has been proposed to prepare microparticles in order to control the release of insulin (Nur et al. 2016; Nur & Vasiljevic 2018). TG is an acid-resistant, heterogeneous, highly branched and negatively charge carbohydrate polymer comprising of two main fractions - water-soluble fraction (tragacanthin) and water-swellable fraction (bassorin), which represent 60-70% of the total gum. TG has shown potential to be used as a stabilizer, emulsifier, thickener and suspending agent (Nur & Vasiljevic 2017; Nur et al. 2016). It is also recognized to be safe when administered orally. Moreover, TG has been characterized by a high mucoadhesion, a property that could be potentially used to increase insulin residence time at the site of resorption, therefore enhancing overall insulin bioavailability and effectiveness (Nur & Vasiljevic 2017; Nur et al. 2016; Jabri et al. 2018).

Natural weakly charged polymer and insulin complex could be created by exploiting their interactions at the isoelectric point (pI) of INS. The pI of INS is approximately 5.5 - 6.4 (McClements 2018b). With changes in pH, insulin overall electrostatic potential changes and it becomes either negative or positive at pH values above or below its pI, respectively (Nur & Vasiljevic 2017). This characteristics of INS have been applied in creation of INS-natural

polymer complexes via electrostatic attraction with an anionic (alginate) and cationic polymer (chitosan) (Nur & Vasiljevic 2017).

In our previous work, we reported that tragacanth microparticles seem to have potential functional characteristics for oral INS delivery by creating a complex with insulin under defined conditions followed by freeze drying (Nur & Vasiljevic 2018). Thus, to fully utilise on benefits described above and make the overall process more industrially applicable, spray drying method has been explored in this research. Therefore, this research aimed to study the feasibility of the spray-drying to create microparticles of tragacanth for the oral delivery of insulin. In particular, conformational properties assessed by FTIR, DSC, morphology of created particles and their sorption properties in addition to insulin release were evaluated.

5.2. Materials and Methods

5.2.1. Materials

Tragacanth (TG) powder, glucono-δ-lactone (GDL) powder and Bradford reagent were acquired from Sigma-Aldrich (Castle Hill, NSW, Aus). The insulin (INS) sample containing 100 U/ml of INS was acquired from Novo Nordisk A/S (Bagsvaerd, Denmark). The water used was of Millipore level of quality. All of the reagents were of analytical grade and were utilized as received.

5.2.2 Characterization

5.2.2.1 Microparticle preparation

To prepare tragacanth stock solution (2% w/w), an appropriate quantity of the TG powder was dissolved in MilliQ water. Sodium azide was added during the preparation of solution (0.2 g/L) to protect against microbial growth. The TG solution was then mildly stirred by a magnetic rode at room temperature (RT), ~22°C and stored overnight at 4°C.

TG and INS complexes were created by mixing INS and TG solution to achieve a final concentration of INS (0.1 mg/mL) and TG (0.5 and 1% w/w). The solution was then adjusted to achieve various pH levels (3.7; 4.3; 4.6; or 6) by adding a proper amount of GDL powder. GDL dissociates in MilliQ water releasing gluconic acid decreasing pH of the solution. The benefit of this particular type of pH adjustment is that the pH alteration is obtained more slowly with no change in the bulk volume (Nur & Vasiljevic 2018). The process was allowed to continue overnight by mildly stirring the mixture with a magnetic rode at RT. The solution was then pumped into a Buchi-B290 mini spray drier (In vitro Technologies, Noble Park, VIC, Australia). The feed solution flow rate was kept at 12–14 mL/min, with an aspirator setting of 100% and 150–160 °C and 90–100 °C inlet and outlet temperatures were used, respectively. Moreover, blank (TG) microparticles were created with no insulin as a control.

5.2.2.2 ATR-Fourier Transform Infrared (FTIR) analysis

IR spectra of the spray dried TG (control), INS powder (control) and microparticles at various pH (3.7; 4.3; 4.6; 6) and concentration of TG (0.5% and 1%) were obtained by using a Perkin Elmer ATR-FTIR spectrometer fitted with Diamond TM ZnSe single reflection ATR plate (Perkin-Elmer, Norwalk, CT). The spectrum of every sample was obtained from 16 scans from 600 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and strong apodization. This analysis was adjusted

towards the background. The raw data were obtained by using a Perkin-Elmer Spectrum™ 10 software. The records were then exported to DX file extension so it can be analysed using the applicable software. Data and peak identification were then acquired by using Shimadzu IR solution software version 1.40 (Shimadzu Corporation, Kyoto, Japan) (Nur et al. 2016; Qomarudin et al. 2015) and then exported to MS Excel for analysis and graphs creation.

5.2.2.3 Differential Scanning Calorimetry (DSC) analysis

Thermal properties of TG (control), INS powder (control) and microparticles at various pH (3.7; 4.3; 4.6; 6) were analysed using a DSC (Mettler Toledo, Schwerzenbach, Switzerland) equipped with an intracooler system and under an inert nitrogen gas atmosphere. A powder sample (4–7 mg) acquired under described experimental settings were placed in a 40 µL aluminium pan, hermetically sealed prior to insertion into the machine, and after that heated from 20 to 350 °C at a constant rate of 10 °C/min accompanied with a continuous purging of nitrogen at 20 mL/min (Nur & Vasiljevic 2018). A blank pan with the same weight used as the reference. The onset, endset, peak temperatures and ΔH values of the thermograms were recorded.

5.2.2.4 Scanning electron microscope (SEM) analysis

The morphological structure of microparticles was analyzed by using a scanning electron microscope (SEM). Samples of spray dried TG (control), INS powder (control) and microparticles at various pH (3.7; 4.3; 4.6; 6) and concentration of TG (0.5% and 1%) were installed on metal stubs, gold covered under vacuum and after that assessed in a JEOL JSM 7800 F (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 5 kV and a working distance of 12 mm.

5.2.2.5 Sorption analysis

The sorption data of microparticle was acquired by using an Aqualab vapour sorption analyser (Graintec Scientific Pty Ltd, Anzac Avenue, Toowoomba QLD, Australia) having an automatic isotherm generator. DDI (Dynamic Dewpoint Isotherm) technique, which produces dynamic isotherms and DVS (Dynamic Vapour Sorption) technique, that produces static isotherms, were both carried out at a RT (Chandrapala & Vasiljevic 2017).

5.2.2.6 *In Vitro* Release of Insulin

Insulin release analysis was focused entirely on the release behaviour in the gastric and intestinal environment (Li et al. 2016; Woitiski et al. 2010). Approximately 2.0 g of NaCl and seven mL of HCl (37 %w) were mixed in 1 L of Milli-Q water to prepare artificial gastric fluid (AGF, pH 1.2). To prepare artificial intestinal fluid (AIF, pH 6.8), KH_2PO_4 (6.8 g) was added and dissolved in 500 mL of MilliQ water, and the pH was then changed to 6.8. The solution was then diluted to 1 L by the addition of MilliQ water (Li et al. 2016). Pepsin and pancreatin enzyme-free fluids were utilized to analyse the insulin retention and release to minimize the enzymatic interferences (Woitiski et al. 2010). Spray dried insulin-loaded microparticles (10 mg) were incubated in 10 mL AGF at pH 1.2, under continuous magnetic stirring (100 rpm, 2 h) at 37°C. Samples at proper intervals were taken and separated from the microparticles by centrifugation (5000*g*/5 min) to acquire the supernatant for INS content analysis. The fresh replacement solvent was added to keep a constant volume. To check the progress of microparticle transferring from the stomach into the intestine, after 2 h, the buffer was altered to pH 6.8. The microparticles (sediment) after separated from the supernatant from previous interval (at pH 1.2) were transferred into 10 ml AIF at pH 6.8, with continuous magnetic stirring (100 rpm) at 37°C for three h. Samples at proper intervals were taken and separated from the microparticles by centrifugation (5000*g*/5 min) to acquire the supernatant for INS content

analysis. The fresh replacement solvent was also added to keep a constant volume. Tests were carried out 3 times and insulin release expressed as a percentage of initial insulin loading (Zhang et al. 2011; Reis et al. 2008a; Martins et al. 2007). The quantity of insulin was measured spectrophotometrically using the Bradford method at 595 nm using controls without INS as blanks (Nur et al. 2016; Martins et al. 2007).

5.2.3 Statistics

Sample analysis was replicated at minimum once having subsequent sub-sampling presenting some independent analysis of at least ($n \geq 3$). When necessary, the results were then assessed by using one-way ANOVA, SAS (1996). Multiple comparisons of means were then analyzed by using Tukey's Studentized Range (HSD). The significance degree was fixed at $p=0.05$.

5.3. Result and Discussion

Spray-drying has been suggested as a feasible manufacturing process for producing microparticles (Ameri & Maa 2006). Tragacanth microparticles containing encapsulated insulin were created by spray drying by a continuous flow of TG/INS solution, atomized via a nozzle having pre-heated air and accumulated in a cyclone separator. The compressed air and feed were introduced co-currently into the drying chamber. In a co-current spray drier, atomised droplets come into contact with the hot air and heat is moved by convection to the droplets. In the beginning, the drying rate rises until the tiny droplets approach the wet-bulb temperature (thermal energy of hot air utilized for evaporation) (Anandharamakrishnan & Padma Ishwarya 2015) and the relative humidity approaches 100%. The drying rate then continues to be constant since the solvent is constantly evaporated at the droplet surface area, which remains saturated with the solvent. This enables the microparticle to be kept at the wet-bulb temperature, that is lower than the outlet temperature of the drying air (Bowey & Neufeld 2010). When drying is accomplished, air temperature declines immediately due to the latent effects of evaporative

cooling. The contact period of the hot air with the atomized droplets is limited to a couple of seconds, ultimately utilizing benefits of low temperature and short residence time of microparticles (Bowey & Neufeld 2010). Because the particles are rarely exposed to the high temperatures, thermal inactivation of heat sensitive materials (Anandharamakrishnan & Padma Ishwarya 2015) such as INS can be minimized (Bowey & Neufeld 2010).

To achieve greater protection during spray dring process, complexation of INS with natural polymers has been suggested (Bowey & Neufeld 2010). Our previous findings showed that INS could be potentially entrapped in TG hydrogel using PEC complexation (Nur & Vasiljevic 2018). Maximum loading efficiency (LE) can be achieved by proper selection of complexation pH and concentration of TG. The previous finding proposed that the affinity of INS for TG carboxylic groups was highest at pH 4.3 or 4.6, as indicated by measuring the loading efficiency of the complexes (Nur & Vasiljevic 2018). A TG concentration of 0.5% (*w*/*w*) appeared to be the optimum concentration for complexation (Nur & Vasiljevic 2018). Therefore, to investigate the complexation between INS and TG on the entrapment capacity of the microparticles during spray drying, four different pH values (3.7; 4.3; 4.6; 6) and TG concentration (0.5 and 1 %, w/w) were selected as a formulation variable.

To obtain an understanding of possible complexation of TG-INS and microparticle structure, FTIR measurements were carried out. Fig. 5.1A and 5.1B illustrate the FTIR spectra of spray dried microparticles at various TG concentrations (0.5 and 1% w/w) over the range of 2000-600 cm^{-1}. A comparison of the spectra of microparticles illustrates the existence of 3 strong absorption bands at 1600-1700 cm^{-1}, 1400-1500 cm^{-1} and about 1045 cm^{-1}. The region between 1600-1700 cm^{-1} is typically identified as Amide I band representative of a protein secondary structure with peaks created as a result of C-O stretching vibration. As expected, INS control presented the maximum spectral intensity height due to a greater quantity of INS and increased

free amino groups, while INS-TG microparticle showed a minimized absorption intensity due to a dilution effect. Our research group has identified that between 1600 and 1700 cm^{-1}, Amide I bands have been generally associated with β-sheet (1623-43 cm^{-1} and 1689-1698 cm^{-1}); α-helical (1654-1658 cm^{-1}); β-turn (1666-1687 cm^{-1}); random coils (1646-1650 cm^{-1}) and 3_{10}-helix (1660-1666 cm^{-1}) (Grewal et al. 2018). In addition, Amid II region located between 1400 and 1500 cm^{-1} showed twisting vibration of N-H groups and stretching vibrations of C-N groups (Grewal et al. 2017a; Grewal et al. 2017b; Piccirilli et al. 2013).

The FTIR results show that the INS structure was, overall, slightly affected after complexation with the polymer. The β-sheet peak became lower after complexation. Ionic attractions that happen between opposite charges of the INS and the biopolymers could be accountable for minor changes of the INS structure (Sarmento et al. 2007a). However, the bandwidth of β -sheet before and after INS complexation was similar indicating that the INS secondary structure was not changed substantially.

The noticed peak at approximately 1040 cm^{-1}, typical for most polysaccharides, has been related to stretching of C-O linkages. Because this particular band may indicate the occurrence of guluronic and galacturonic units, TG microparticles were about to possess increased peaks. The FTIR spectrum of the TG control verified this statement by providing a significant peak in that region. Stretching vibrations (COO-) of asymmetric and symmetric carboxylate group were indicated by the adsorption band between 1400 cm^{-1} and 1600 cm^{-1}. It can be observed from FTIR analyses that the increase in TG concentration appeared to have a contribution to interaction since a higher band strength was noticed (Fattahi et al. 2013; Nur et al. 2016).

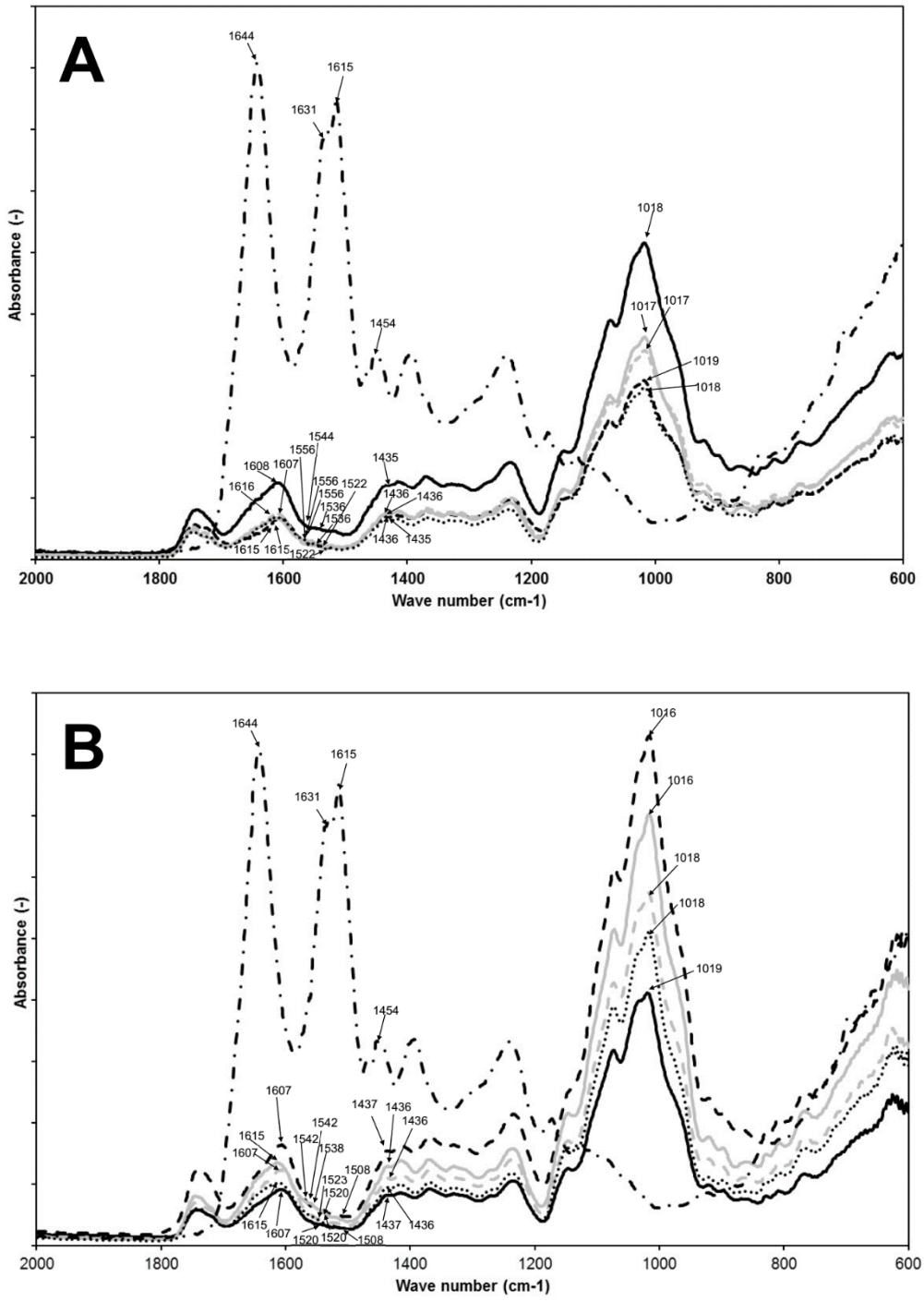

Figure 5.1 FTIR spectra of spray dried microparticle produced by mixing INS solution (0.1 mg/mL) and TG solution at (A) 0.5 or (B) 1% w/w concentration at various pH (3.7, 4.3, 4.6 or 6) altered by addition of GDL at RT, ~22°C and equilibrated with gentle magnetic stirring overnight. Legend: ····· TG (control); – · – INS (control); — — mixture at pH 3.7; —— mixture at pH 4.3; – – mixture at pH 4.6; and, —— mixture

at pH 6.0. Arrows and the numbers show a wavenumber of a specific structural change.

As seen from FTIR analyses, TG carboxyl peaks at around 1435 cm^{-1} (symmetric COO− stretching vibration) as well as 1556 cm^{-1} asymmetric COO− stretching vibration) were enhanced and changed from 1436 to 1435 and 1556 to 1524 cm^{-1} right after mixed with INS. Also, the FTIR spectrum of TG shows a peak of an amide bond at 1615 cm^{-1}. The observed change in the absorption bands of the amide bonds, carboxyl groups and amino groups, might be attributable to an ionic interaction between INS and the carboxyl group of TG (Nur & Vasiljevic 2018). Moreover, the peak absorbance of C-O stretching of TG at 1019 cm^{-1} appeared upon complexation. Similar findings had been observed previously (Wu et al. 2012). DSC thermograms of INS, TG and spray dried microparticles after complexation are presented in Fig 5.2. The thermograms show a broad endothermic peak between 140 and 172°C for isolated polyelectrolyte and its physical mixture. Microparticles showed exothermic peaks starting from 230 to 256°C. The shift in endothermic peaks and the change of exothermic peaks related to decomposition temperature may indicate ionic attraction between TG and INS (Nur & Vasiljevic 2018).

The polymers were identified by the presence of first endothermic peaks at 140°C for TG only and 147, 154, 169, 172°C for the TG-INS at various pH, respectively, and higher exothermic peaks at 256°C for TG only and 230, 233, 238, 241°C for the TG-INS at various pH, respectively. Endothermic peaks are connected to the decrease of moisture content associated with hydrophilic groups of biopolymers. On the other hand, exothermic peaks are related to the breakdown of biopolymers due to dehydration and depolymerisation reactions most likely to the incomplete decarboxylation of the protonated carboxylic groups as well as oxidation reactions of the polyelectrolytes (Zohuriaan & Shokrolahi 2004; Mimmo et al. 2005; Soares et al. 2004).

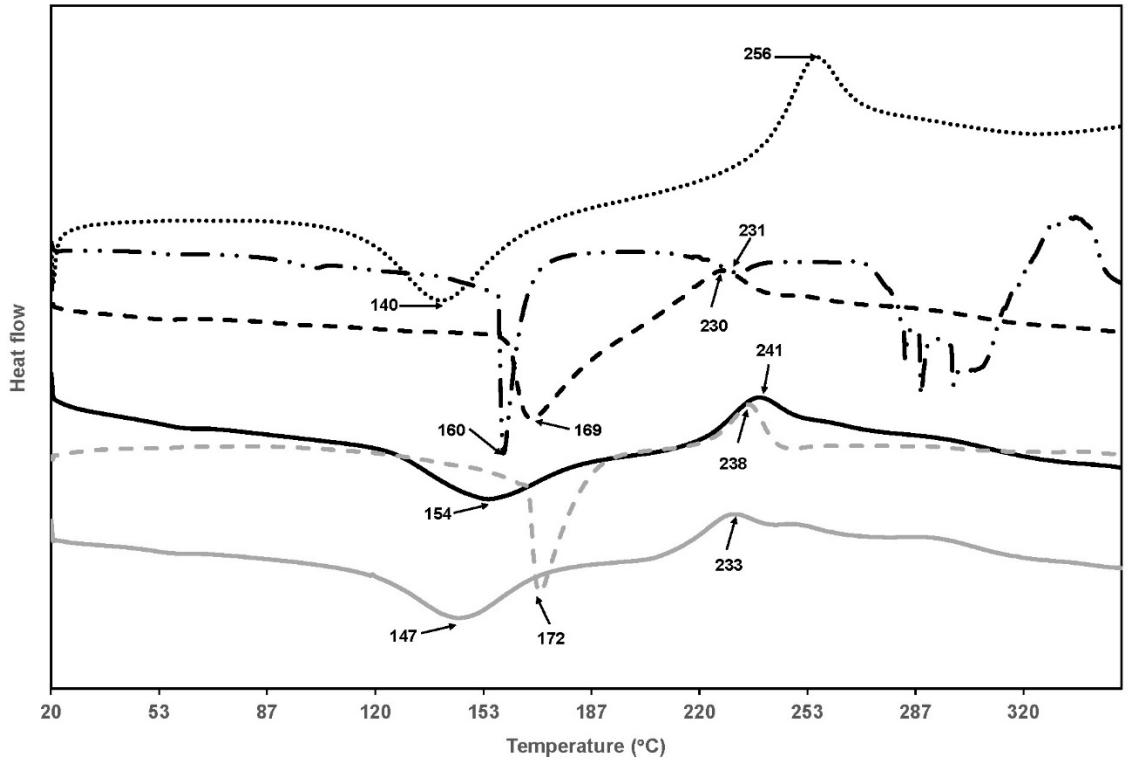

Figure 5.2 DSC thermograms of spray dried microparticle produced by mixing INS solution (0.1 mg/mL) and TG solution (1% w/w) concentration at various pH (3.7, 4.3, 4.6 or 6) altered by addition of GDL at RT, ~22°C and equilibrated with gentle magnetic stirring overnight. Legend: ····· TG (control); – · – INS (control); — — mixture at pH 3.7; ——— mixture at pH 4.3; – – mixture at pH 4.6; and, ——— mixture at pH 6.0.

Endothermic and exothermic peaks changed to higher temperatures when pH of particles was lowered from 6 to 4.3. It could be observed that by reducing the pH, it led to higher stability of particles, consequently more energy was needed to remove residual water adsorbed onto mixture (endothermic peak shifts to higher value), and a less quantity of energy was released by breaking ionic interactions and during particles thermal decomposition (exothermic peak shifts to higher value) (Nur & Vasiljevic 2018; Sarmento et al. 2006a).

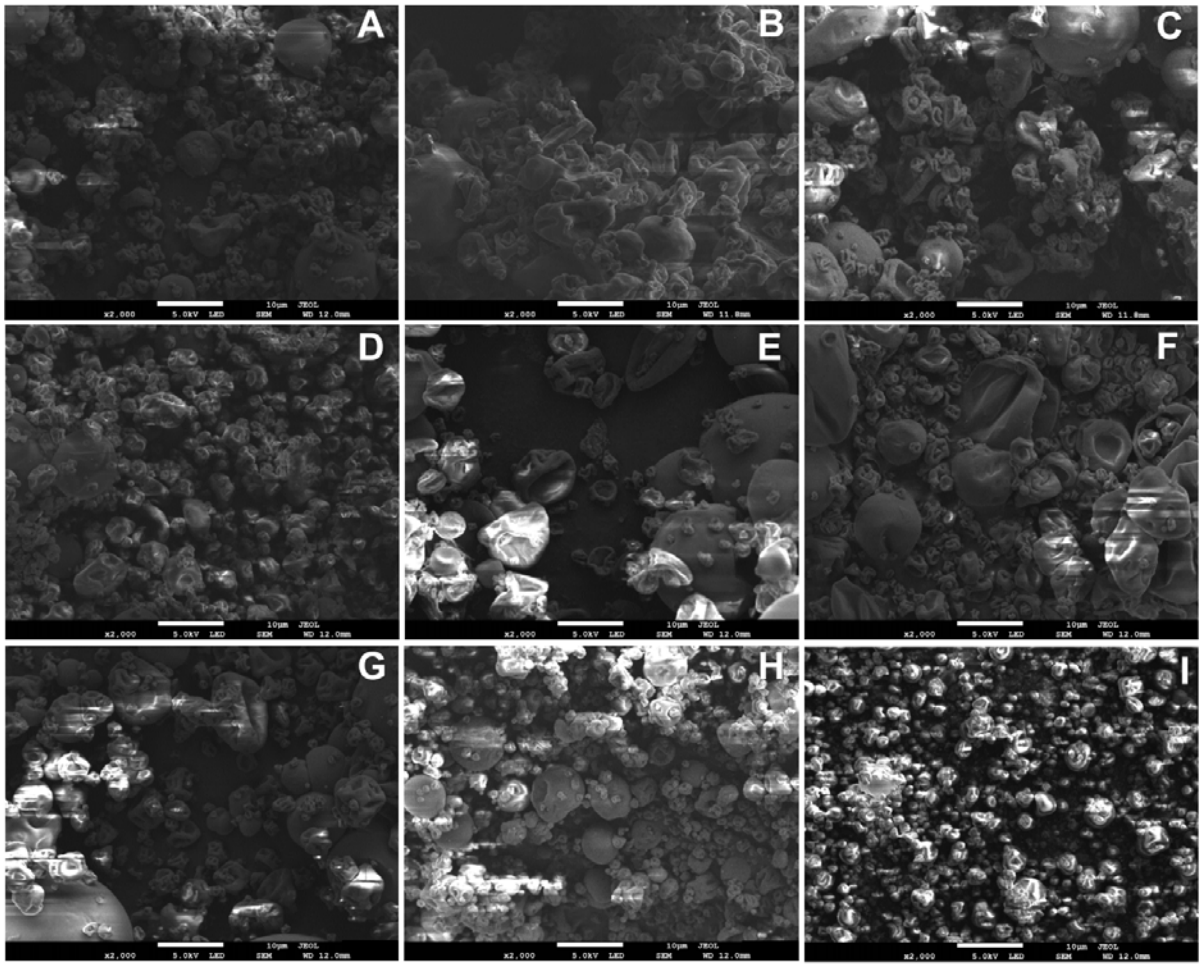

Figure 5.3 SEM images of spray dried microparticles produced by mixing INS solution (0.1 mg/mL) and TG solution at different concentration and at different pH altered by addition of GDL at RT, ~22°C and equilibrated with gentle magnetic stirring overnight. Legend: a) TG (control); b) INS+0.5%w/w TG at pH 3.7; c) INS+0.5%w/w TG at pH 4.3; d) INS+0.5 %w/w TG at pH 4.6; e) INS+0.5%w/w TG at pH 6.0; f) INS+1%w/w TG at pH 3.7; g) INS+1%w/w TG at pH 4.3; h) INS+1%w/w TG at pH 4.6; i) INS+1%w/w TG at pH 6.0 (magnification 2000 ×, bars = 10 μm).

Fig. 5.3 illustrates morphology of the created microparticles by using SEM. The data showed almost spherical, or sub-spherical microparticles were created with a diameter of less than 10 μm. Particle size is a main factor in determining the uptake of INS microparticles in the

intestinal cells and thus enabling the access to the bloodstream (D'Souza et al. 2015). For this, microparticles ranging from 1 to 10 μm are ideal (D'Souza et al. 2015), and we have optimized our formulation to obtain a size of less than 10 μm. The TG-INS microparticles size and morphology were similar to other spray dried biopolymers including spray dried insulin in an alginate matrix (Bowey et al. 2013), insulin in β-cyclodextrin and bovine serum albumin in a chitosan matrix (Kusonwiriyawong et al. 2009). Chitosan crosslinked with glutaraldehyde microspheres having the diameter of 7.2 μm can be transported across the GI tract to the blood circulation (Wei et al. 2008).

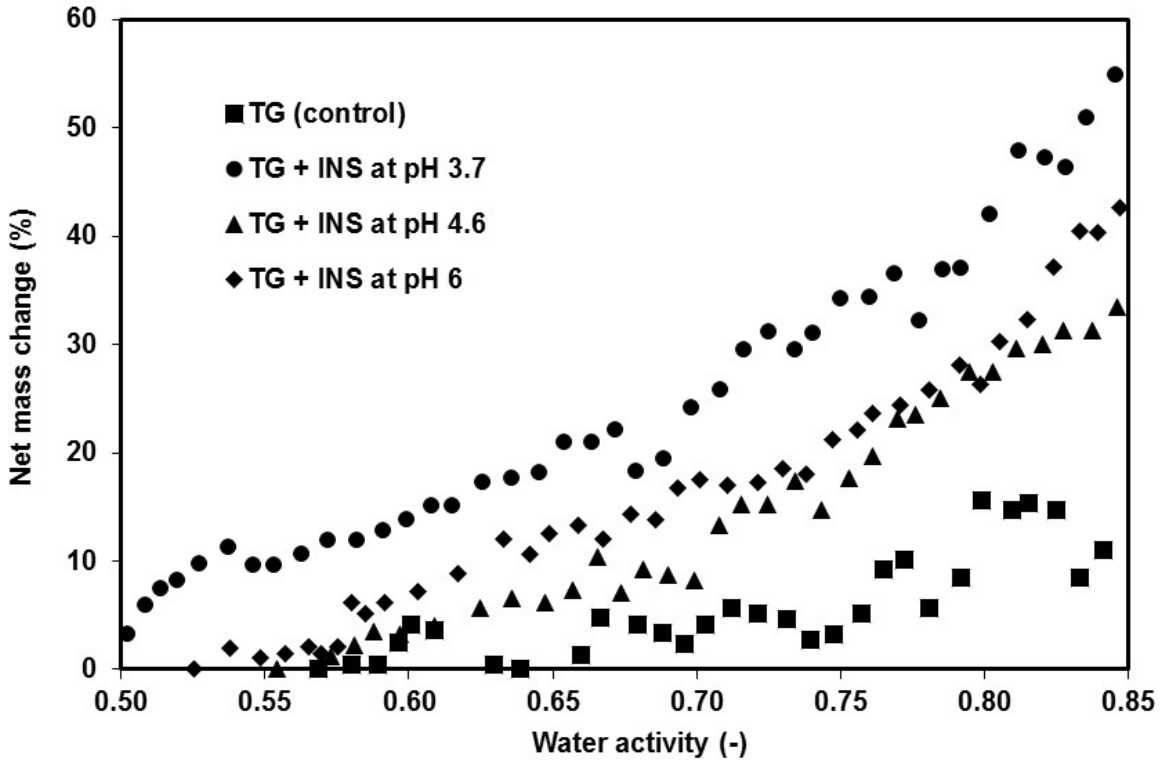

Figure 5.4 Sorption curves of the spray dried microparticles prepared by mixing tragacanth (TG) solution (1% w/w) and insulin (INS) solution (0.1 mg/mL) at different pH

altered by the addition of GDL at RT, ~22°C and equilibrated with gentle magnetic stirring overnight.

The sorption properties of TG microparticle without and with INS after drying can be seen in Fig. 5.4. Most microparticles exhibited nearly linear curves regardless the formulation having a gradual increase of net mass change when water activity was increased. Microparticles absorbed water steadily start from water activity 0.5. This kind of pattern confirmed hydrophilic formulation. Complexation between insulin and tragacanth increased the corresponding moisture because of increased water sorption capacity.

The sorption curves pointed out that microparticles displayed necessary changes in water activity as they adsorbed a substantial amount of water at higher water activities. An increasing pattern of net mass change was acquired with the change in pH of complexation medium. This information also recognized the functional properties of TG as plasticizer since plasticizers lead to an increase in elongation sorption and water sorption (Tonyali et al. 2018). Among all microparticles of TG-INS, the fitted curve of TG-INS prepared at pH 4.6 was the closest to the control (pure TG) particularly around water activity of 0.8. Heterogeneous hydrophilic properties, highly branched, and primarily water swellable part of TG (bassorin) could retain water within biopolymer network and thus enhance water interaction (Tonyali et al. 2018).

Impact of formulation on the INS release due to exposure to gastrointestinal pH as an imitative physiological trigger was performed in AGF (artificial gastric fluid, pH 1.2) and then AIF (artificial intestinal fluid, pH 6.8) at 37 °C (Sonia & Sharma 2015). Fig. 5.5 shows the release profile of INS from microparticles under artificial gastric medium and then intestinal environment. Overall, the INS release value at pH 1.2 was lower than that at pH 6.8. No more than 9-27% of loaded-INS was released in the first hour from microparticles. These values indicate that TG could prevent release of INS during gastric passage. After that, release of

insulin at pH 1.2 increased to 40-60% in 2 hours, followed by a reduction in release rate. It is possible that some insulin was associated with the particle surface and desorbed in contact with the aqueous environment because the tragacanth nuclei did not have the capacity to entrap all protein (Sarmento et al. 2007a; Sarmento et al. 2006b). After changing the pH to 6.8, approximately 79-97% of loaded-INS was released from microparticles. The lower release of INS during low pH could be related to a tight TG network. TG hydrogel at low pH collapsed creating an impermeable network structure preserving INS against the acidic environment and enzymatic degradation. However, at a higher pH (6.8), TG swells as it creates an ionic state, creating a more porous structure thus allowing more room for INS release (Nur & Vasiljevic 2018). This mechanism appears very similar to an alginate system (Sarmento et al. 2007c; Woitiski et al. 2010).

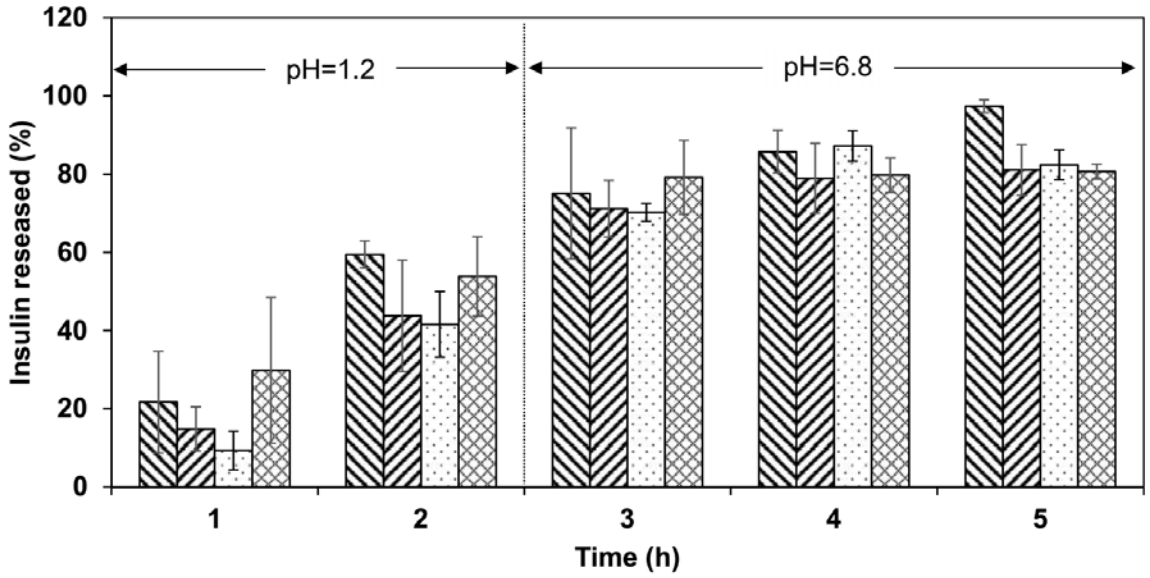

Figure 5.5 *In vitro* insulin release profiles of microparticles in AGF (pH 1.2) for 2 h followed by 3 h in AIF (pH 6.8). Spray dried microparticles of INS and 1% (w/w) TG at various pH were altered by the addition of GDL at RT, ~22°C and equilibrated with gentle magnetic stirring overnight. (n = 3, mean ± S.D.) Legend: mixture at pH 3.7; mixture at pH 4.3; mixture at pH 4.6; and, mixture at pH 6.0.

INS release behaviour among microparticles obtained at different pH (3.7, 4.3, 4.6 or 6.0) clearly indicated that prepared mixture at pH 4.3 or 4.6 has the lower release of INS than the preparation of mixture at pH 3.7 or 6, thus would be a better delivery system. The greatest ionic interactions between TG and INS were attained at low pH. Nearly identical to alginate system, TG hydrogel has shrunk at low pH, at which a reduction of the pore size of TG matrix can be attained (Nur et al. 2016). Therefore, at pH 4.3 and 4.6 it is possible that microparticles exhibited stronger complexation than at pH 6.0. Complexation between TG and INS are known to be pH-dependent (Nur et al. 2016). Consequently, complexation of INS-TG microparticles is useful to prevent insulin from the destruction of proteolytic throughout the gastrointestinal movement. This mechanism was also noticed in alginate and chitosan systems (Sonia & Sharma 2015; Li et al. 2016; Sarmento et al. 2007c).

5.4. Conclusions

Spray drying provides a one-step process for encapsulating insulin within tragacanth microparticles. Polyelectrolyte microparticle present potential as an oral delivery carrier system for insulin as well as possibly other protein/peptide drugs. The FTIR and DSC results indicated that the change on infrared peaks as well as shifts on endothermic and exothermic peaks noticed between INS and final TG-INS complexes were an indication of ionic attractions that resulted in the creation of new chemical substance. INS structure was, overall, slightly affected after association with the polymer but the secondary structure was not altered substantially. The SEM analysis indicated that almost spherical or sub-spherical microparticles were created with a diameter of less than 10 μm. The choice of appropriate pH for complexation appears to have an effect on *in vitro* insulin release profile and be an essential parameter to prevent insulin release, particularly INS-TG complex which prepared at pH 4.3 and 4.6.

Further research is required to assess the possibility of adding another biopolymer such as chitosan, alginate, carrageenan and dextran sulphate in improving protection of insulin during gastric environment and enhancing insulin sustained release in the intestinal environment.

CHAPTER 6

Chapter 6. General Conclusion and Recommendation

6.1 General Conclusion

Considering that diabetics (especially type 1) need multiple administration of insulin (INS) every day during their lifetime, the prospective insulin market is so massive that nearly all oral peptide/protein delivery technological innovation are concentrated on insulin delivery. The main problems in the oral delivery of insulin are the low intestinal permeability and the enzymatic degradation which therefore results in a low bioavailability. Hence, to overcome these problems, different approaches have been developed to encapsulate insulin by using various polymers. These polymers can be gained from synthetic or natural sources and have an ability to preserve insulin stability, bioavailability, mucoadhesion, and properly control the release. The combination of different factors and biopolymers may accommodate all these properties together in the same carrier.

It is for this reason that the intention of this thesis was to contribute towards a development of an oral insulin delivery system which takes advantage of the large absorptive surface area of the small intestine. Several factors are considered important in the development of such a system. Firstly, the nature of the polymer must be taken into consideration. For our system, we utilize the tragacanth as an excipient. Secondly, the size of the device must be taken into consideration. It has been shown that particle uptake by the enterocytes of the small intestine increases as particle size decreases.

With the above factors taken into consideration, this thesis characterized the tragacanth in an oral protein/peptides delivery application. The thesis also explored the design and assembly of microparticles via a freeze dryer and spray drying technique for the potential applications in oral

insulin delivery. Particle size, zeta potential, mucoadhesion, flow behaviour, morphology and microstructure could be directly controlled by manufacturing conditions and formulations, thus establishing a direct relationship between process parameters and the physicochemical properties and functionalities of microparticles.

In chapter 3, the main scientific outcomes from the work were the understanding of tragacanth functional properties. The conducted research found that different temperatures and shearing had no major effect on the viscosity of tragacanth solution. The viscosity of all tragacanth dispersion appeared to be independent of shearing. Furthermore, temperature sweep did not affect the viscosity of tragacanth dispersions. The viscosity during heating period remained fairly similar to that during the cooling period regardless of shear rate, suggesting that tragacanth would not be affected by heating and other environmental adjustments ie pH, ionic strength would be needed to induce gelation.

The solution exhibited shear-thinning characteristics. The value of the storage modulus (G′) and the loss modulus (G″) increased with an increase in angular frequency (Ω). In all cases, loss modulus values were higher than storage values (G″ > G′) and viscous character was, therefore, dominant. This confirms that in the tested range, the systems behaved like a typical dilute solution. The *G'* and *G″* at pH 7 appeared slightly higher than at other pH, indicating more viscous behaviour at a higher pH. The differences between *G′* and *G″* values, at the same frequency, decreased with an increase in gum concentration.

The behaviour of tragacanth in solution was pH dependant with zeta potential nearing neutrality

towards pH 3, which resulted in gel formation. Tragacanth upon dispersion created particles of a submicron size with a negative zeta potential (-7.98 to -11.92 mV). These properties were pH dependant resulting in acid gel formation at pH 3.5. The tragacanth particles had a suitable size (submicron) that could assist in drug delivery.

During DSC analysis, changing the pH (3, 5, or 7) influenced the enthalpy and the onset value, but not the peak and endset values of the tragacanth solution. This could be due to greater stability of complexes at lower pH, thus more energy was needed to remove remaining water adsorbed onto nanoparticles. From the FTIR analysis, the bands related to symmetric stretching of carboxylate groups and methyl groups in methyl esters of galacturonic acid (1417 and 1368 cm^{-1}, respectively), the vibrational modes of COOH in galacturonic acid and its salts and esters include asymmetric stretching (1740–1600 cm^{-1}). Polygalacturonic acids have a maximum absorption band in this area, with very strong absorptions at 1017 and 1100 cm^{-1}, maximum absorption bands at 1043 and 1070 cm^{-1} representing the presence polysaccharides that contain galactose, for instance, galactans and arabinogalactans. Tragacanth showed higher mucoadhesion than alginate, PVP or chitosan. Tragacanth and alginate showed shear-thinning behaviour with an increase in viscosity after mixing with mucin. According to these measurements, at lower shear rates, the order of polymer mucoadhesion was tragacanth > alginate> PVP > chitosan. Furthermore, tragacanth exhibited stronger viscoelastic properties than alginate when mixed with mucin. This indicated that tragacanth had greater mucoadhesion than alginate. This mucoadhesive property could mean that tragacanth may be suitable for site-

specific protein/peptide carrier due to prolong interaction with mucin and thereby control the release of protein/peptide. Based on these findings, tragacanth has a potential to be used as an excipient for oral peptide/protein delivery, due to its ability to form polyelectrolyte complexes and a hydrogel at an appropriate pH. From preliminary study, 0.5% tragacanth was selected as optimum colloidal solution and 0.2 mg/ml insulin was chosen as concentration for a model protein.

In chapter 4, insulin was successfully entrapped in physical hydrogel and polyelectrolyte complexes (PECs) created using biodegradable biopolymer-tragacanth. Microparticulate complexation between tragacanth and insulin was revealed by FTIR and DSC measurement.

From the FTIR study, tragacanth carboxyl peaks close to 1627 cm^{-1} (symmetric COO– stretching vibration) and 1416 cm^{-1} (asymmetric COO stretching vibration) broadened and shifted a little from 1627 to 1616 cm^{-1} and 1416 to 1415 cm^{-1} following complexation with insulin. Moreover, the FTIR spectrum of tragacanth exhibited a peak associated with an amide bond at 1645 cm^{-1}. Noticed shifts in the absorption bands of the carboxyl groups, amide bonds, and amino groups could be assigned to an ionic attraction between the carboxyl group of tragacanth and insulin.

The inclusion of insulin within microparticulate complexes can certainly be confirmed by DSC by the postponing of its endothermic peak. The two endothermic peaks in connection with insulin, which are related to water loss and the denaturation process, started to be indistinct and changed themselves into a single peak following entrapment in the microparticulate complexes.

Insulin-loaded models achieved this particular endothermic peak at a lower temperature in comparison to insulin-free models, apparently showing an attraction involving the protein and the tragacanth. Furthermore, looking at the exothermic conditions of insulin-loaded and unloaded particles, the onset point began at a lower temperature for insulin-loaded microcomplexes; this probably suggests that entrapped insulin initiated decomposition at higher temperatures (235–241 °C) than when analysed in isolation from the particles (231 °C).

During the acid gelation test, as the pH was decreasing, at pH around 4.3, the storage modulus was greater than that at other pH levels. The value of the storage modulus is proportional to the quantity of permanent interactions and the strength of the interactions existing in the biopolymers. Therefore, G′ is a measure of the structure of the biopolymers. A time sweep offers the viscoelastic properties of biopolymers as a function of time, in which the strain, frequency, and temperature are kept constant. The gel networks of biopolymers continue to develop throughout a time sweep. This can be noticed from an increase in the value of the storage modulus as a function of time. In our system, during the time sweep, a change of pH was measured. Therefore, a storage modulus vs pH graph was created. The increase in the storage modulus (maximum at pH 4.3), which is close to the gelling point of tragacanth and insulin, was an indication that insulin was likely entrapped in a tragacanth gel (hydrogel creation).

Maximum loading efficiency (LE) can be achieved by proper selection of complexation pH and concentration of tragacanth. The finding proposed that the affinity of insulin for tragacanth carboxylic groups was highest at pH 4.3 or 4.6, as indicated by measuring the loading efficiency

of the complexes. LE at pH 4.3 and 4.6 is approximately 80%, while at pH 3.7 was ~65% and the lowest was at pH 6.0 (~50%). A tragacanth concentration of 0.5% (w/w) appeared to be the optimum concentration for complexation. These microparticles appear to have potential functional properties for oral insulin delivery, especially those that contain tragacanth polyelectrolytes at pH 4.3 and 4.6. If the pH of the aqueous solution was set to 3.7 or 4.2, the particles become much larger (>800 nm). In this pH range, some parts of tragacanth begin to precipitate. Surface morphology information for freeze-dried microspheres has been acquired by SEM analysis. The carrier exhibited an irregular shape and had a somewhat wrinkled surface. At the beginning, the tragacanth creates a homogenous network from the core to the edge. Insulin is then entrapped inside the network. The negatively charged tragacanth creates a complex with the positively charged insulin. For further studies, Field Emission Scanning Electron Microscopy can give better details of the surface morphology. Moreover, Transmission Electron Microscopy studies could give details of the internal structure of the microparticles.

In chapter 5, a spray dryer was successfully used to encapsulate insulin with tragacanth. Spray drying provides a one-step process for encapsulating insulin within tragacanth microparticles. Polyelectrolyte microparticles present potential as an oral delivery carrier system for insulin as well as possibly other protein/peptide drugs. The pH 3.7 and 6.0 was still included during the research in this chapter because different drying method was used in this chapter. The FTIR and DSC results indicated that the change of infrared peaks as well as shifts in endothermic and exothermic peaks noticed between INS and final TG-INS complexes were an indication of ionic

attractions that resulted in the creation of new chemical substance. INS structure was, overall, slightly affected after association with the polymer but the secondary structure was not altered substantially. The SEM analysis indicated that almost spherical or sub-spherical microparticles were created with a diameter of less than 10 μm.

Most microparticles exhibited nearly linear curves regardless the formulation having a gradual increase of net mass change when water activity was increased. Microparticles absorbed water steadily start from water activity 0.5. This kind of pattern confirmed hydrophilic formulation. Complexation between insulin and tragacanth increased the corresponding moisture because of increased water sorption capacity. The choice of appropriate pH for complexation appears to have an effect on *in vitro* insulin release profile and be an essential parameter to prevent insulin release, particularly INS-TG complex which prepared at pH 4.3 and 4.6.

6. 2 Future Research Direction

Optimum microparticles formula for oral insulin delivery at pH 4.3 and 4.6 was 0.5% TG for freeze drying method and 1% TG for spray drying technique. The promising results from this study warrant further investigation of tragacanth micro/nanoparticles for oral insulin delivery. for Future work in research and development may need to address some issues.

To overcome the aforementioned shortcoming (some burst release in GIT) of pure tragacanth hydrogel/microparticles, several approaches could be proposed: coating of tragacanth hydrogel with polycationic chitosan, and reinforcement of tragacanth matrices with other natural

polymers like alginate, chitosan, whey protein, kappa carrageenan and dextran sulphate. Ionic crosslinking of tragacanth using crosslinking agents such as Ca^{+2} and Ba^{+2} ions could be potentially developed to create more pH-responsive hydrogel.

Reducing the size of tragacanth particle from micro to nano scale in the future is important to maximize the absorptive cellular intestinal uptake of insulin. Further development of the spray drying technique could be used as a strategy to produce uniform and smaller tragacanth particle, such as the use of nano spray drier and/or micro-fluidic jet spray-drier.

Further characterization using other techniques such as Thermogravimetric Analysis/Differential Thermogravimetry, X-Ray Diffraction and Transmission Electron Microscopy could be conducted to give better understanding of tragacanth insulin interaction. Study on insulin release from the tragacanth microparticles also could be carried out, so that kinetic release profile can be acquired. Moreover, LE study of microparticle using the spray drying method is needed to assess the efficiency of the technique. Studying physicochemical characteristics of microparticles with different ratio of insulin and tragacanth polymer using spray drying method is also important to increase LE and improve release study.

Although *in vitro* drug release test is an indispensable tool for the development of controlled release systems, an *in vivo* assessment (or simulated *in vivo*) of their behaviors should be done to provide a better prediction of the real bioavailability of these drug delivery systems. If the bioavailability of tragacanth based oral insulin delivery system remained low, absorption enhancers may be added to the formulations, such as sodium lauryl sulphate or bile salts. While

the other polymers technique taken up to now have demonstrated some results in protecting insulin from destruction and in enhancing absorption from the GIT, none of the techniques suggested or under development have been verified to be adequate for clinical applications. Therefore, tragacanth microparticle should be tested clinically after successful *in vivo* analysis.

For oral aministration of insulin, to be clinically valuable, it needs to deliver a precise quantity of insulin rapidly sufficient to maintain the blood sugar concentration, and the functionality needs to be reproducible whenever oral insulin is administered. Consequently, recently developed oral insulin delivery systems needs to be evaluated in terms of simple scale-up manufacturing process, insulin quality, degree of toxicity and bioavailability. Because the majority of therapeutic peptides/proteins need persistent administration, the side effects on intestinal absorption of age, individual variations, physiological circumstances and long-term oral administration of absorption carriers must also be properly assessed. Although oral administration of insulin has no potential side effects, it is essential to examine whether the other biological or chemical substances included to increase uptake of insulin in the system could possibly have safety issues, particularly if consumed continuously over extended periods of time.

CHAPTER 7

Chapter 7. References

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APPENDIX

Table 1. Dynamic light scattering results showing z-average diameters of 0.5% tragacanth solution at pH 3, 5 or 7. The results are presented as means of at least four independent observations ($n \geq 4$) with ± SE.

pH	Particle size (nm)
3	581 ± 15
5	466 ± 27
7	432 ± 21

Table 2. Dynamic light scattering results presenting z-average diameters of polymeric complexes created by mixing insulin solution (0.2 mg/mL) and tragacanth solution at 0.1, 0.5, or 1% *w*/*w* concentration at different pH (3.7, 4.3, 4.6, or 6) adjusted by addition of glucono δ lactone (GDL) at room temperature and equilibration under very low magnetic stirring overnight. The results are presented as means of at least five independent observations ($n \geq 5$) with ± SE.

pH	Particle Size, nm		
	Tragacanth concentration, % *w*/*w*		
	0.1	0.5	1
3.7	1105 ± 48	1382 ± 141	839 ± 22
4.3	667 ± 37	811 ± 20	957 ± 56
4.6	651 ± 09	601 ± 19	649 ± 25
6	566 ± 23	373 ± 08	719 ± 05