Effect of drying techniques on microstructure and functional properties of tragacanth-insulin microparticles

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Abstract. Numerous antimicrobials, hormones and enzymes as bioactive peptides/proteins could take advantage of oral delivery. Microparticles produced by complexation of two polyelectrolytes may have prospective use as a carrier for oral administration of proteins/peptides. Food polysaccharides like alginate, carrageenan, pectin, tragacanth and dextran can be potential excipients for this purpose. In this research, microparticles were created by the inclusion of bioactive protein/peptides into a tragacanth hydrogel accompanied by drying process. Insulin was utilized as a bioactive proteins model. Bioactive protein and tragacanth microparticles created at various pH and drying methods (freeze drying and spray drying) were assessed by SEM analysis and ATR Fourier transform infrared (ATR FTIR). The SEM study revealed that sub spherical microparticle was produced using spray drying process while the porous structure was produced using the freeze-drying method. In FTIR study, the complexes between tragacanth and insulin displayed amide absorption bands appearing in the protein spectra and exhibited the formation of new chemical substances. This finding indicates that both spray and freeze dried microparticles may act as a potentially promising device for oral delivery of bioactive proteins and peptides.

1. Introduction
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) [1]. Since the invention of insulin (INS), insulin is administered to diabetic patients exclusively by the injection. 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. Because of this inconvenience, researchers have been exploring alternative delivery of insulin.

Amongst potential alternative delivery routes, oral administration of insulin is preferable since this route provides several benefits such as convenience administration of INS as well as higher diabetics conformity [2]. 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 [3-6].

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 [7]. Spray dried microparticles of INS and β-cyclodextrin can protect orally delivered INS from chemical and enzymatic degradation in the SGF [8].

In recent years, the active molecules can be encapsulated by spray drying and/or freeze drying. In spray drying, a pre-hydrolyzed sol-gel solution is atomized into a heater reactor, where the fluid is evaporated to yield microparticles. Spray drying is a very efficient one-step process, particularly advantageous because it is fast and can be scaled to an industrial level. On the other hand, therapeutics/drugs and excipient dispersed in aqueous solution could be freeze-dried to create a porous, non-shrunked product. Initially, the materials are frozen (from ~40 to ~90°C), after that dried by immediate sublimation at lowered pressure and decreased temperature (from -20 to -90°C) [9].

A gelation and mucoadhesion study indicated that tragacanth has a potential to become an excipient for the oral administration of protein/peptides, for instance, INS [10]. The use of tragacanth (TG) as an alternate choice and enhanced carrier for the oral administration of insulin as a model of bioactive peptide/protein is reported in this article. By using these series of analysis, it was designed to monitor the complexation of polyelectrolyte to become insulin excipient. Systems producing from the complexation of polyelectrolytes at different pH were freeze-dried or spray dried or and then directly analysed to verify interactions of tragacanth and insulin.

2. Materials and Method

2.1. Materials

TG powder, glucono-δ-lactone (GDL) powder and Bradford reagent were purchased from Sigma-Aldrich. The insulin sample containing 100 U/mL of INS was obtained from Novo Nordisk A/S. The water used was of Millipore level of quality. All of the reagents were of analytical grade and were used as received.

2.2. Characterisation

2.2.1. Microparticle preparation

To prepare TG 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 change is obtained more slowly with no change in the bulk volume [11]. The process was allowed to continue overnight by a mild stirring of the mixture with a magnetic rode at RT. The solution was then pumped into a Buchi-B290 mini spray drier (In vitro Technologies). 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.

Tragacanth and insulin microparticles were prepared via mixing insulin and TG colloidal solutions containing a concentration of tragacanth (0.5 or 1%w/w) at predetermined pH. The complexation was allowed to proceed overnight by gently stirring the solution with a magnetic rode at room temperature. The mixture was then centrifuged at 20,000g for 60 min at a room temperature by a high-performance centrifuge (Beckman Coulter Inc., Brea, CA). The sedimented PECs were then frozen at -20 °C and freeze-dried at 0 °C for, at least, 48 h using a freeze dryer (model FD-300, Airvac Engineering Pty. Ltd).
2.2.2. **ATR-Fourier transform infrared (FTIR) analysis**

Infrared spectra of TG (control), INS powder (control) and microparticles at various pH (4.3; 4.6), at 1% (w/w) concentration of TG were acquired 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) [10, 12] and then exported to MS Excel for analysis and graphs creation.

2.2.3. **Scanning electron microscope (SEM) analysis**

The morphological structure of microparticles was analysed by using a scanning electron microscope (SEM). Samples of 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.

3. **Results and Discussion**

Spray-drying and freeze-drying has been suggested as a practical production method to create microparticles [13]. To attain further protection during spray drying, complexation of insulin with biopolymers has been recommended [14]. The previous research showed that insulin could be potentially entrapped in tragacanth hydrogel using PEC complexation [11]. 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 insulin for tragacanth carboxylic groups was highest at pH 4.3 or 4.6, as indicated by measuring the loading efficiency of the complexes [11]. Therefore, to investigate the complexation between INS and TG on the entrapment capacity of the microparticles, different drying techniques were chosen as a variable.

To obtain an understanding of possible complexation of TG-INS and microparticle structure, FTIR measurements were carried out. Figure 1 illustrate the FTIR spectra of spray- and freeze-dried microparticles 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\(^{-1}\)) [15]. 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 [16-18].

The FTIR results indicate that the insulin structure was, in general, slightly affected after complexation with the biopolymer. The β-sheet peak became lower after complexation. Ionic interactions that happen between opposite charges of the INS and the biopolymers could be accountable for minor changes of the INS structure [19]. However, the bandwidth of β-sheet before and after INS complexation was similar indicating that the INS secondary structure was not changed substantially.

The observed peak at around 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 noted from FTIR analyses that the increase in TG concentration appeared to have a contribution to interaction since a higher band strength was observed [10, 20].

**Figure 1.** FTIR spectra of freeze dried (FD) and spray dried (SD) microparticle produced by mixing INS solution (0.1 mg/mL) and TG solution at 1% w/w concentration at various pH (4.3 and 4.6) altered by addition of GDL at RT, ~22°C and equilibrated with gentle magnetic stirring overnight. Legend: TG (control); INS (control); mixture at pH 4.3 FD; mixture at pH 4.3 SD; mixture at pH 4.6 SD; and, mixture at pH 4.6 FD. Arrows and the numbers show a wavenumber of a specific structural change.

Figure 2 shows morphology of the particles through the use of SEM. The graphic revealed nearly spherical, or sub-spherical particles were developed having a diameter of lower than 10 μm. Microparticle size is a principle factor in determining the uptake of insulin particles in the intestinal cells therefore allowing the entry to the bloodstream [8]. The TG-INS microparticles size and morphology were similar to other spray dried biopolymers including spray dried insulin in an alginate matrix [21], insulin in β-cyclodextrin and bovine serum albumin in a chitosan matrix [22]. Chitosan crosslinked with glutaraldehyde spheres having the diameter of 7.2 μm can be transported across the GI tract to the blood circulation [23].
Figure 2. SEM images of freeze dried (FD) and spray dried (SD) 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; 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 (magnification 500 ×, bars = 10 μm).
Surface morphology information for freeze-dried microspheres has been acquired by SEM analysis and is presented in Figure 2. The microsphere showed an irregular shape and had a slightly wrinkled surface. Obviously, the round shape of the spheres was lost after drying. This indicated that INS entrapped in TG. The morphology of the TG hydrogels after freeze-drying shows the porous structure. The pore surface and size were almost like other acidified gel polymers such as TG-milk [24] and pectin–sodium caseinate systems [25]. These types of structural properties are associated with the change of INS-loaded microspheres. The breakdown of gels is accompanied by the release of INS from gels. These structural characteristics have been associated with water exchange and swelling of hydrogels. Swelling properties of the gels are crucial for biomaterial change when it is applied to INS carriers [26].

4. Conclusions
The SEM study revealed that sub spherical microparticle was produced using spray drying process while the porous structure was produced using the freeze-drying method. In FTIR study, the complexes between tragacanth and insulin displayed amide absorption bands appearing in the protein spectra and exhibited the formation of new chemical substances.

References
[1] Alai M S, Lin W J, Pingale S S 2015 Application of polymeric nanoparticles and micelles in insulin oral delivery J. Food Drug Anal. 23 351-358.
[2] Wong C Y, Martinez J, Dass C R 2016 Oral delivery of insulin for treatment of diabetes: status quo, challenges and opportunities J. Pharm. Pharmacol. 68 1093-1108.
[3] Morishita M, Peppas N A 2006 Is the oral route possible for peptide and protein drug delivery? Drug Discov. Today 11 905-910.
[4] Nur M, Vasiljevic T 2017 Can natural polymers assist in delivering insulin orally? Int. J. Biol. Macromol. 103 889-901.
[5] Sonia T A, Sharma C P 2012 An overview of natural polymers for oral insulin delivery Drug Discov. Today 17 784-792.
[6] Peppas N A, Kavimandan N J 2006 Nanoscale analysis of protein and peptide absorption: Insulin absorption using complexation and pH-sensitive hydrogels as delivery vehicles Eur. J. Pharma. Sci. 29 183-197.
[7] Martins S, Sarmento B, Souto E B, Ferreira D C 2007 Insulin-loaded alginate microspheres for oral delivery – Effect of polysaccharide reinforcement on physicochemical properties and release profile Carbohydr. Polym. 69 725-73.1
[8] D’Souza B, Bhowmik T, Uddin M N, Oettinger C, D’Souza M 2015 Development of β-cyclodextrin-based sustained release microparticles for oral insulin delivery Drug Dev. Ind. Pharm. 41 1288-1293.
[9] Zuidam N J, Nedovic V 2010 Encapsulation technologies for active food ingredients and food processing Springer New York.
[10] Nur M, Ramchandran L, Vasiljevic T 2016 Tragacanth as an oral peptide and protein delivery carrier: Characterization and mucoadhesion Carbohydr. Polym. 143 223-230.
[11] Nur M, Vasiljevic T 2018 Insulin inclusion into a tragacanth hydrogel: an oral delivery system for insulin Mater. 11 79 1-14.
[12] Qomarudin Q, Orbell J D, Ramchandran L, Gray S R, Stewart M B, Vasiljevic T 2015 Properties of beta-lactoglobulin/alginate mixtures as a function of component ratio, pH and applied shear Food Res. Int. 71 23-31.
[13] Ameri M, Maa Y-F 2006 Spray Drying of biopharmaceuticals: stability and process considerations Drying Technol. 24 763-768.
[14] Bowey K, Neufeld R J 2010 Systemic and mucosal delivery of drugs within polymeric microparticles produced by spray drying Biodrugs: Clin. Immunother. Biopharma. Gene Therapy 24 359-377.
[15] Grewal M K, Huppertz T, Vasiljevic T 2018 FTIR fingerprinting of structural changes of milk proteins induced by heat treatment, deamidation and dephosphorylation Food Hydrocoll. 80 160-167.

[16] Grewal M K, Chandrapala J, Donkor O, Apostolopoulos V, Stojanovska L, Vasiljevic T 2017 Fourier transform infrared spectroscopy analysis of physicochemical changes in UHT milk during accelerated storage Int. Dairy J. 66 99-107.

[17] Grewal M K, Chandrapala J, Donkor O, Apostolopoulos V, Vasiljevic T 2017 Predicting sediment formation in ultra high temperature-treated whole and skim milk using attenuated total reflectance-Fourier transform infrared spectroscopy Int. Dairy J. 74 39-48.

[18] Piccirilli F, Mangialardo S, Postorino P, Lupi S, Perucchi A 2013 FTIR analysis of the high pressure response of native insulin assemblies J. Mol. Struct. 1050 159-165.

[19] Sarmento B, Ferreira D C, Jorgensen L, van de Weert M 2007 Probing insulin’s secondary structure after entrapment into alginate/chitosan nanoparticles Eur. J. Pharma. Biopharma. 65 10-17.

[20] Fattahi A, Petrini P, Munarin F, Shokoohinia Y, Golozar M A, Varshosaz J, Tanzi M C 2013 Polysaccharides derived from tragacanth as biocompatible polymers and gels J. Appl. Polym. Sci. 129 2092-2102.

[21] Bowey K, Swift B E, Flynn L E, Neufeld R J 2013 Characterization of biologically active insulin-loaded alginate microparticles prepared by spray drying Drug Dev. Ind. Pharma. 39 457-465.

[22] Kusonwiriyawong C, Pichayakorn W, Lipipun V, Ritthidej G C 2009 Retained integrity of protein encapsulated in spray-dried chitosan microparticles J. Microencap. 26 111-121.

[23] Wei W, Wang L-Y, Yuan L, Yang X-D, Su Z-G, Ma G-H 2008 Bioprocess of uniform-sized crosslinked chitosan microspheres in rats following oral administration Eur. J. Pharma. Biopharma. 69 878-886.

[24] Nejatian M, Hatami M, Mohammadifar M A 2013 Effect of gum tragacanth exuded by three Iranian Astragalus on mixed milk protein system during acid gelation International J. Biol. Macromol. 53 168-176.

[25] Matia-Merino L, Lau K, Dickinson E 2004 Effects of low-methoxyl amidated pectin and ionic calcium on rheology and microstructure of acid-induced sodium caseinate gels Food Hydrocol. 18 271-281.

[26] Li L, Jiang G, Yu W, Liu D, Chen H, Liu Y, Huang Q, Tong Z, Yao J, Kong X 2016 A composite hydrogel system containing glucose-responsive nanocarriers for oral delivery of insulin Mater. Sci. Eng. C 69 37-45.