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Solid state stabilization of proteins by sugars

Why size and flexibility matter

Maarten A. Mensink

The research presented in this thesis was carried out at the Department of Pharmaceutical Technology and Biopharmacy of the University of Groningen.

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Why size and flexibility matter

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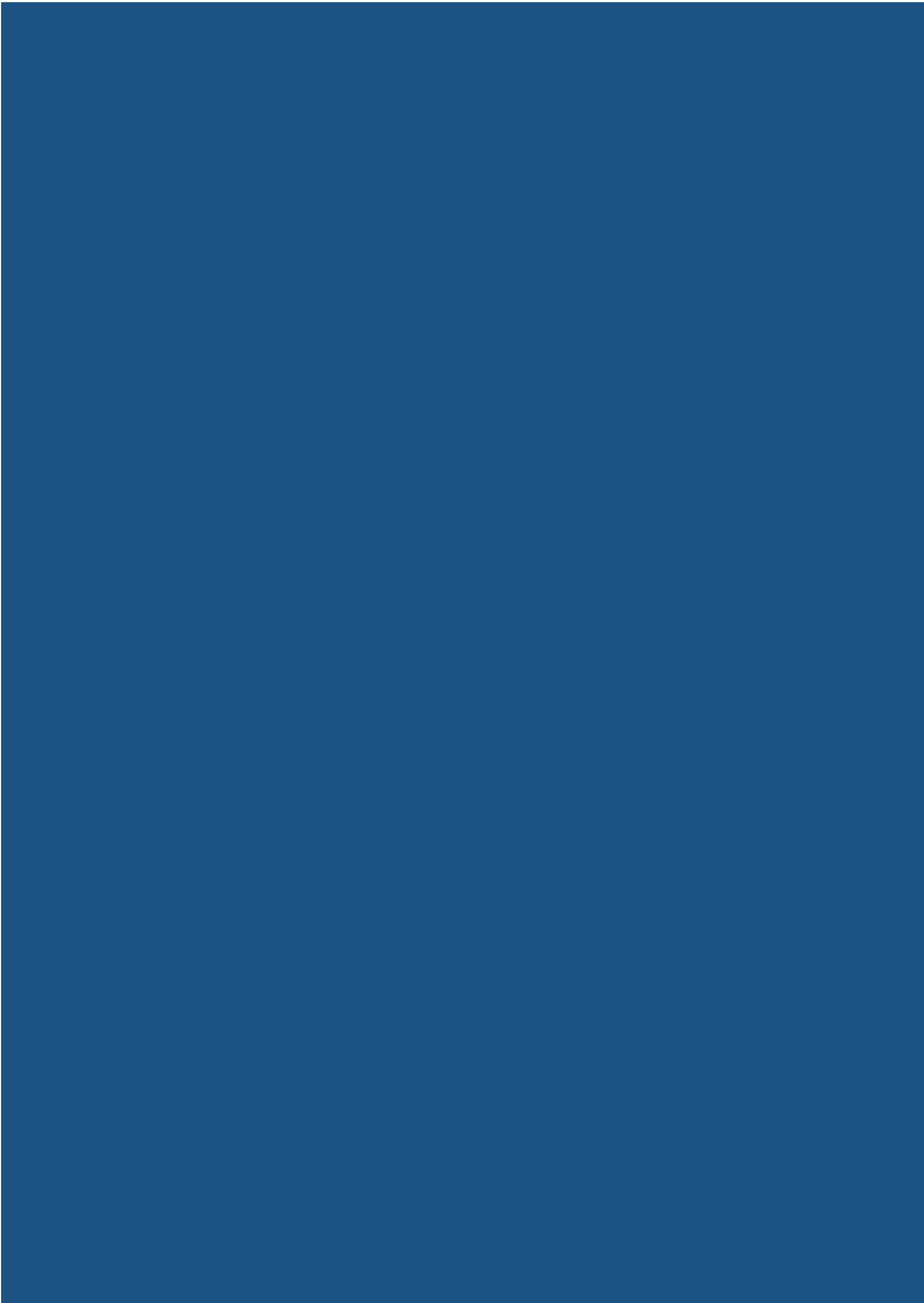
Voor mijn trotse ouders

In memoriam
Marcel Hendrik Bernhard Mensink
4 juli 1957 8 februari 2013



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1 Introduction & Thesis outline

INTRODUCTION & THESIS OUTLINE

Over the past decades protein drugs have gone from being of little therapeutic relevance to being a cornerstone of modern day medicine. Since the introduction of the first recombinant protein drug (insulin) in 1982, the number of protein drugs on the market has increased drastically to well over 100 in 2016.[1–3] Protein drugs have provided immense improvements for various therapeutic areas like metabolic disorders, oncology, and inflammatory diseases (e.g. rheumatoid arthritis). This is mainly because of their unprecedented specificity, allowing for high effectivity with limited side-effects.[1]
A large part of the protein drugs which are currently on the market are liquid formulations. A major disadvantage of liquid protein formulations is their limited shelf life.[4] Proteins in solution often deteriorate rapidly if they are not refrigerated continuously, causing them to lose functionality and potentially become immunogenic.[4] To achieve an acceptable shelf life, protein solutions are therefore dependent on refrigerated handling and storage, the so-called cold chain. Maintaining this cold chain is expensive and basically not feasible in developing, often tropical regions.[5] Circumventing this cold chain would thus reduce costs of storage and transport, and improve availability of protein drugs in those areas. Moreover, it improves safety.
Drying proteins in the presence of sugars can provide stabilization and increase protein shelf life, even at elevated temperatures, eliminating the need for the cold chain.[6] Much research has focused on how sugars stabilize proteins in the solid state in a general sense.[7,8] How sugars protect against drying and other stress conditions in a general sense is discussed in chapter 8. However, less attention has gone into why some sugars are more suitable than others and therewith which sugar characteristics are desirable for protein stabilization. In this dissertation, this latter topic will be addressed in more depth.
First it is shown that size and molecular flexibility of a sugar influence their ability to stabilize proteins (chapter 2). This was investigated by freeze-drying (lyophilizing) several sugars of different size and molecular flexibility with 4 different model proteins and comparing the ability of the sugars to maintain protein functionality during storage at elevated temperature. To enable investigation of the role of molecular flexibility of the sugars, a polysaccharide (sugar polymer) with an exceptionally flexible backbone, inulin, was used. Because inulin is unique in its molecular flexibility and plays a key role in this thesis, the physicochemical characteristics of this sugar are reviewed (chapter 3) and related to its pharmaceutical applications, including stabilization of proteins (chapter 4).
In the second part of this dissertation, it is mechanistically discussed why size and molecular flexibility of sugars are important for protein stabilization. Using in-line near infrared spectroscopy during freeze-drying it is shown that smaller and molecularly more flexible sugars form more hydrogen bonds with a model protein during the last phase of freeze-

drying because they are less affected by steric hindrance, resulting in improved storage stability (chapter 5). Similar results are found in the solid state using terahertz time domain spectroscopy, where smaller sugars show better interactions with another model protein (chapter 6). Furthermore, it is shown by solid-state nuclear magnetic resonance spectroscopy that larger sugars have a higher tendency to phase-separate from the protein, resulting in reduced protein stabilization (chapter 7). Lastly, an overview is provided of the general theories regarding stabilization of proteins by sugars in combination with the new knowledge presented here (chapter 8). There, common degradation routes and stress factors are reviewed and related to how sugars protect against them.

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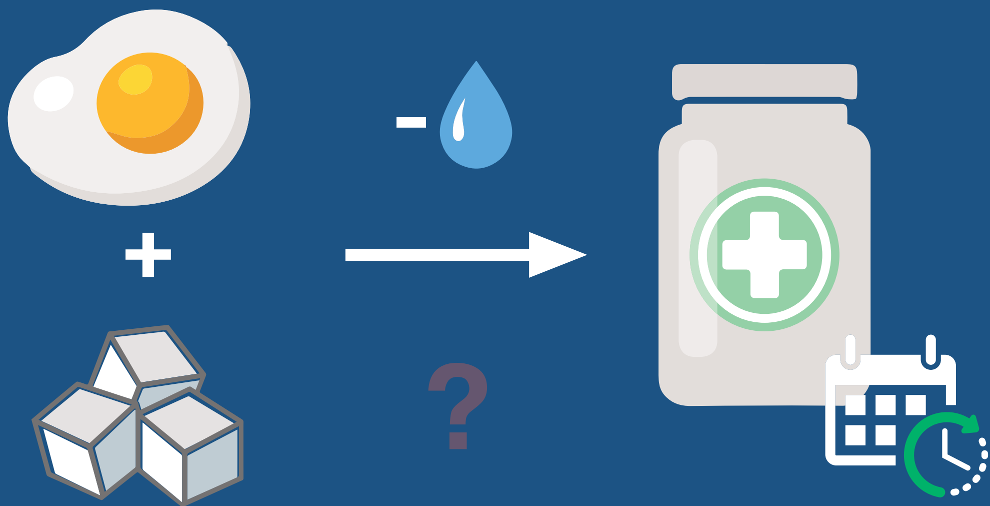

2

Size and molecular flexibility of sugars determine the storage stability of freeze-dried proteins

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Arthur de Jager, Kees van der Voort Maarschalk, Henderik W. Frijlink, Wouter L.J. Hinrichs

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ABSTRACT

Protein-based biopharmaceuticals are generally produced as aqueous solutions and stored refrigerated to obtain sufficient shelf life. Alternatively, proteins may be freeze-dried in the presence of sugars to allow storage stability at ambient conditions for prolonged periods. However, to act as a stabilizer, these sugars should remain in the glassy state during storage. This requires a sufficiently high glass transition temperature (Tg). Furthermore, the sugars should be able to replace the hydrogen bonds between the protein and water during drying. Frequently used disaccharides are characterized by a relatively low Tg, rendering them sensitive to plasticizing effects of residual water, which strongly reduces the Tg values of the formulation. Larger sugars generally have higher Tgs, but it is assumed that these sugars are limited in their ability to interact with the protein due to steric hindrance.
In this chapter, the size and molecular flexibility of sugars was related to their ability to stabilize proteins. Four diverse proteins varying in size from 6 kDa to 540 kDa were freeze-dried in the presence of different sugars varying in size and molecular flexibility. Subsequently, the different samples were subjected to an accelerated stability test. Using protein specific assays and intrinsic fluorescence, stability of the proteins was monitored. It was found that the smallest sugar (disaccharide trehalose) best preserved the proteins, but also that the Tg of the formulations was only just high enough to maintain sufficient vitrification. When trehalose-based formulations are exposed to high relative humidities, water uptake by the product reduces the Tgs too much. In that respect, sugars with higher Tgs are desired. Addition of polysaccharide dextran 70 kDa to trehalose greatly increased the Tg of the formulation. Moreover, this combination also improved the stability of the proteins compared to dextran only formulations. The molecularly flexible oligosaccharide inulin 4 kDa provided better stabilization than the similar sized but molecularly rigid oligosaccharide dextran 6 kDa.
In conclusion, the results of this study indicate that size and molecular flexibility of sugars affect their ability to stabilize proteins. As long as they maintain vitrified, smaller and molecularly more flexible sugars are less affected by steric hindrance and thus better capable at stabilizing proteins.

INTRODUCTION

Protein-based biopharmaceuticals such as recombinant monoclonal antibodies, subunit vaccines, cytokines, and hormones are generally produced as aqueous solutions. Storage of these often expensive solutions under ambient conditions may lead to fast degradation of the protein, which results in the formation of products that are inactive or even elicit unwanted immune responses. To obtain sufficient shelf life, these products are usually stored and transported refrigerated. The dependence on this so-called "cold-chain" makes these products even more expensive and transport to rural areas in tropical developing countries is often impossible. One of the potentially most effective strategies to improve the stability of proteins is to bring them in the dry state. Most degradation pathways require molecular mobility of the protein. In the dry state this molecular mobility is strongly reduced, resulting in increased stability. Freeze-drying is one of the techniques used to dry protein solutions.[1] However, during freeze-drying, proteins are subjected to freezing and drying stresses. It is well known that sugars can be used as stabilizing excipients to prevent degradation resulting from these stresses.[1]

A concomitant advantage of using sugars is that they can also contribute to an improved storage stability of the dried proteins. Two main mechanisms have been described to explain the stabilization of proteins by sugars: water replacement[2], and vitrification.[3,4] Proteins (partially) lose their hydration shells during freezing and drying, which can lead to the formation of intramolecular hydrogen bonds within the proteins, changing their three-dimensional structures. When sugars are added prior to drying, the hydrogen bonds between the protein and water (the hydration shell) are gradually replaced by hydrogen bonds between the protein and hydroxyl groups of the sugar during freeze-drying, thereby conserving the protein's three-dimensional structure.[5–7] According to the vitrification theory, the molecular mobility of the protein is strongly reduced when it is incorporated in a sugar matrix in the glassy state, resulting in a reduced degradation rate of the protein. Both water replacement and vitrification require close contact between the sugar and the protein at a molecular level to stabilize the protein.

It is well established that the water replacement and vitrification theories alone can not explain the stabilization achieved by sugars.[4,8] More recent work has refined these theories and related local vitrification in the form of fast β-relaxation in the solid state to protein stability.[8] It was found that the disaccharide trehalose stabilized model proteins by reduction of their molecular motion both during freeze-drying and in the freeze-dried solid.[9,10] Sugar molecules are significantly larger than water molecules and are therefore not able to fully replace water-protein hydrogen bonds.[10] This is in line with the water entrapment hypothesis, a refinement of the water replacement hypothesis that states that some residual water remains present around the protein, rather than that the sugars

directly interact with the protein.[11] It is now possible to relate these refined hypotheses to each other using the slaving model, which states that mobility of the protein is governed by the bulk matrix and its hydration.[12] The reader should be aware of these refinements, but for clarity purposes the long established terms vitrification and water replacement will be used throughout this chapter.

Various types of sugars can be used to stabilize proteins during freeze-drying and subsequent storage. To act as an appropriate stabilizer, the sugar should meet at least two important requirements. Firstly, the sugar should remain in the glassy state during storage, favoring sugars with a high glass transition temperature (Tg). At temperatures above the Tg, the sugar is in the rubbery state, displaying high molecular mobility by which vitrification is compromised. In addition, crystallization of the sugar in the rubbery state occurs easily. Crystallization can damage the protein through mechanical stresses but also results in a loss of the close contact between the sugar and proteins and therefore a loss of stability. Secondly, the sugar should contain no or only a very limited number of reducing groups. Reducing groups can react with amine groups of proteins to form a N-substituted glycosylamine, which is the first step of a cascade of reactions also referred to as the Maillard reaction or browning.[13] This reaction can proceed fast in the liquid state, but was also reported to occur in the solid state.[14]

Sugars can be categorized into mono-, di-, oligo- and polysaccharides. Monosaccharides like glucose, fructose, and galactose are not suitable as protein stabilizers during storage since these sugars have a low Tg (<40 °C) and contain reducing groups. The disaccharides sucrose and trehalose possess a much higher Tg (77 °C for sucrose and 121 °C for trehalose[15]) and do not contain reducing groups and are therefore often used as protein stabilizers.[16] Water, be it residual moisture after freeze-drying or water taken up during storage, acts as a plasticizer, which lowers the Tg strongly. This could lower the Tg to below the storage temperature, resulting in a poor stabilization or even destabilization as described above. An advantage of oligosaccharides like inulin and dextran is their high Tg values (the Tg values of moisture free inulin with an average molecular weight of 4 kDa and dextran 5 kDa are 157 °C and 176 °C, respectively)[15]. These temperatures lie far above any usual storage condition. Even if some residual moisture is present after freeze-drying, the storage temperature will still be significantly lower than the Tg. Polysaccharides exhibit even higher Tgs. As described by the Fox-Flory equation, the Tg increases with the molecular weight of the polymer.[17]

A downside of many high molecular weight sugars is the combination of a large size with the limited flexibility of the molecular chains. Due to this combination, hydrogen bond interactions with proteins are sterically hindered and efficient vitrification at the surface of the protein will become difficult to achieve.[6] This is schematically shown in figure 2.1, which shows the interaction of the adsorbing part of the sugar with the protein. It should be noted

that this illustration oversimplifies the stabilization of sugars on proteins, in order to illustrate the steric effects to the reader and that as explained in the introduction. Rigid polysaccharides are able to interact with the protein surface, but leave open gaps (figure 2.1D). For sugars with high molecular weights and a limited flexibility these gaps are large (figure 2.1D) compared to the smaller sugars (figure 2.1A). Addition of a polysaccharide with a high Tg to the disaccharide might provide benefit by combining a proper coating with a high Tg (figure 2.1C).[18] Such combinations have been previously described; i.e. disaccharides in combination with hydroxyethyl starch or dextran, but the mechanisms of stabilization were not investigated.[18,19] The obvious downside of such an approach is that the small disaccharide acts as a plasticizer for the larger polymer. For that reason the Tg of the polysaccharide has to be high enough to compensate for this Tg reduction. Since oligosaccharides are smaller than polysaccharides, oligosaccharides might achieve a more compact coating (compare figure 2.1D and E) and with that a more stable formulation. We hypothesize that compactness of the coating of proteins by oligosaccharides is also dependent on the molecular flexibility

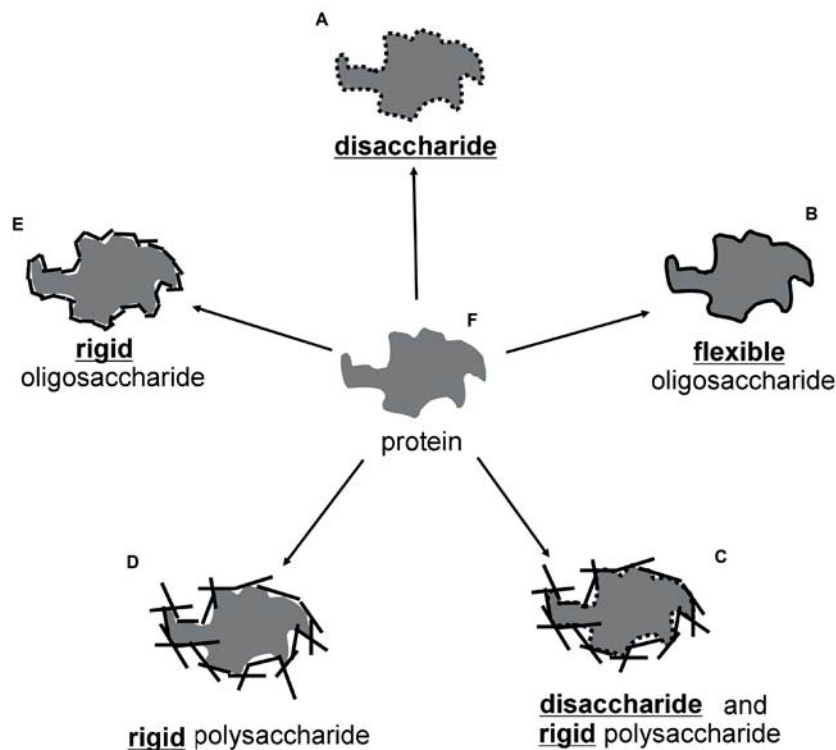

Figure 2.1 Schematic overview of the steric hindrance of different types of sugars and their compactness of coating of proteins.

of the oligosaccharide (compare figure 2.1B and E). Continuing this train of thought, rigid oligosaccharides would not be able to accommodate to the irregular surface of the protein, whereas oligosaccharides with a flexible molecular structure would be better able to do so, resulting in a more efficient stabilization by the latter oligosaccharides.
To test this hypothesis, oligosaccharides dextran and inulin of approximately the same molecular weight (6 kDa and 4 kDa, respectively, and 70 kDa for the large oligomers) were used in this study. These molecules have different flexibilities, however. Molecular dynamics simulations have shown that inulin, which has previously been applied successfully as a stabilizer of various proteins[4,20,21], is highly flexible when dissolved in water.[22] Hinrichs *et al.*[15] showed that polyethylenglycol (PEG) and inulin are compatible, while PEG and dextran are not. As a rule of thumb polymers that have similar flexibilities are compatible.[23] As PEG is a highly flexible molecule, the results of Hinrichs *et al.*[15] are in line with the simulation data and strongly indicate that inulin is more flexible than dextran. An explanation for the difference in molecular flexibility between inulin and dextran is how their backbones are constructed. Inulin is thought to have a flexible molecular structure because the backbone does not include the ring structure of the fructose units (figure 2.2).[24] The backbone of dextran runs through three atoms of the ring structure of each glucose unit, resulting in a more rigid backbone compared to inulin. Additionally, inulin is mostly comprised of furanose rings, which are smaller and more flexible than glucose units.[22,25]
Using four different-sized model proteins, we tested how size and molecular flexibility of sugars affect their ability to stabilize proteins. All proteins were freeze-dried in the presence of various sugars representing the situations in figure 2.1 and subsequently subjected to an accelerated stability test.

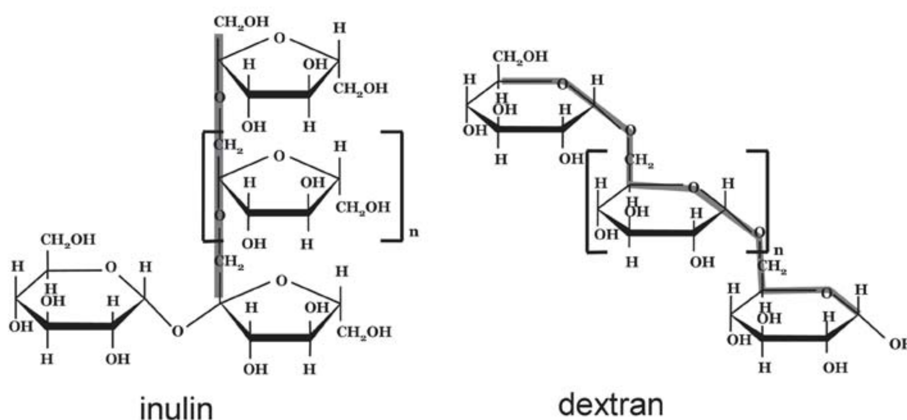

Figure 2.2 Molecular structure of flexible inulin and rigid dextran. The backbone is highlighted in grey.

MATERIALS AND METHODS

MATERIALS

Hepatitis B surface antigen (HBsAg) was provided by the Serum Institute of India Ltd. (Pune, India). Insulin was provided by MSD (Oss, The Netherlands). A suspension of LDH from rabbit muscle in 3.2 M ammonium sulfate, dextran 6 kDa, dextran 70 kDa, bovine serum albumin (BSA), magnesium chloride, *ortho*-nitrophenyl-β-galactoside, reduced β-nicotinamide adenine dinucleotide disodium salt hydrate (NADH), sodium pyruvate, potassium sodium tartrate tetrahydrate, 3,5-dinitrosalicylic acid and phenol were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). β-galactosidase was obtained from Sorachim (Lausanne, Switzerland). Trehalose was obtained from Cargill (Amsterdam, The Netherlands). Lactose monohydrate was obtained from DMV-Fonterra excipients (Goch, Germany). Inulin 4 kDa was a generous gift from Sensus (Roosendaal, The Netherlands). Sodium sulfite was obtained from Spruyt Hillen BV (IJsselstein, The Netherlands). Acetonitril was obtained from Biosolve (Valkenswaard, The Netherlands). Sodium hydroxide and glucose were obtained from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) consisted of 10 mM phosphate, 137 mM sodium chloride and 2.7 mM potassium chloride (pH 7.4). Phosphate buffer for the β-galactosidase assay consisted of 75 mM disodium hydrogen phosphate and 25 mM potassium dihydrogen phosphate (pH 7.3). The buffer used for the LDH assay solutions was a 0.1 M solution of monopotassium phosphate adjusted to pH 7.5 with sodium hydroxide. Hepes buffer (HB) consisted of 2 mM Hepes at a pH of 7.5.

PREPARATION OF POWDER FORMULATIONS

In this study, four different proteins were used: insulin (6 kDa), hepatitis B surface antigen (HBsAg) (a virus like particle consisting of around 100 subunits with a molecular mass of 25 kDa each[26]), lactate dehydrogenase (LDH) (140 kDa), and β-galactosidase (540 kDa). For each protein, six powder formulations were prepared by freeze-drying in the presence of either one or two sugars or no sugar at all (control). The sugars used in this study were trehalose, dextran 6 kDa, dextran 70 kDa, inulin 4 kDa or a mixture of dextran 70 kDa and trehalose (1:1 weight ratio).

First, protein solutions of 1 mg/mL in HB were prepared. The LDH suspension was dialyzed overnight at 4 °C against HB using a ThermoScientific Slide-a-lyzer cassette with a molecular weight cut-off of 7 kDa prior to dilution. Next, the sugar was dissolved upon heating at a concentration of 62.25 mg/mL in HB. After cooling the sugar solution to room temperature, the protein and sugar solution were mixed in a 1:4 v/v ratio resulting in a final protein concentration of 0.2 mg/mL and sugar concentration of 49.8 mg/mL (protein:sugar ratio = 1:249 (w/w)). Next, 200 µL of this solution was pipetted into a 4 mL HPLC glass vial, which was then immersed into liquid nitrogen until the protein-sugar solution was frozen.

Subsequently, the vials with the frozen solutions were placed on a precooled shelf (-35 °C) of a Christ Epsilon 2-4 freeze-dryer (Salm & Kipp, Breukelen, The Netherlands). The frozen solutions were then freeze-dried at a pressure of 0.220 mBar and a condenser temperature of -85 °C for 24 hours. Thereafter, the pressure was decreased to 0.050 mBar while the shelf temperature was increased to -15 °C at a rate of 10 °C/min. This temperature was maintained for 2 hours, after which the temperature was further increased to 0 °C at a rate of 5 °C/min and lastly to 20 °C at a rate of 1 °C/min. The last part of the secondary drying was maintained for a minimum of 8 hours.

DIFFERENTIAL SCANNING CALORIMETRY

Differential Scanning Calorimetry (DSC) was used to determine the Tg of the powder formulations after freeze-drying and after storage. The samples (about 2 to 3 mg) were analyzed in an open aluminum pan and placed in a Q2000 DSC (TA Instruments, Ghent, Belgium) and were preheated for 3 minutes at 80 °C to remove the residual water. Next, the sample was cooled to 20 °C and subsequently raised to 240 °C at 20 °C/min. The inflection point of the step transition in the thermograph was taken as the Tg. Additionally, placebo sugar samples, containing sugar and buffer, were stored at 60 °C in open DSC pans. After 1 week, pans were hermetically sealed and measured to determine the effect of water uptake during storage on the Tg. DSC settings were identical, except for the preheat step which was left out. The Tg of the maximum freeze concentrated fraction, the Tg', was determined by rapid cooling of approximately 40 mg of sugar solution (49.8 mg/mL in HB) to -60 °C and subsequently heating the sample to 30 °C at 20 °C/min.

SUMNER ASSAY

The amount of reducing groups in each sugar was measured by means of the Sumner assay according to the procedure described by Franssen *et al*.[27]. To a glass tube, 1.0 mL of an aqueous sugar solution was added. Next, 1.5 mL of aqueous Sumner assay solution was added, containing 200 mg/mL NaK-tartrate, 10 mg/mL dinitrosalicylic acid, 10 mg/mL NaOH and 2 mg/mL phenol. Finally, 100 µL of a freshly prepared 0.24 M of Na_2SO_3 was added. The glass tube was vortexed and then placed in a waterbath at 95 °C for 15 minutes. After cooling to room temperature the absorbance was measured at 630 nm using a Unicam UV 500 spectrophotometer (ThemoSpectronic, Cambridge, UK) and compared to a calibration curve of 0.10 – 1.00 mg/mL glucose solutions. The amount of reducing groups of a sample was related to that of glucose, which was thus by definition 100% reducing.

WATER CONTENT DETERMINATION

The amount of residual moisture after freeze-drying was determined by Karl Fischer coulometric water titration using an 831 KF Coulometer (Metrohm Applikon, Schiedam, The

Netherlands). Prior to analysis, the powder was dissolved in Hydranal®-Coulomat AG (Karl Fischer reagent).

STORAGE STABILITY TESTING

In order to evaluate the storage stability after freeze-drying, the formulations were stored at 60 ºC at a relative humidity <10%. Immediately after freeze-drying and after 1, 2, and 4 weeks of storage, samples were analyzed by an assay specific for each protein: reversed phase high-performance liquid chromatography (RP-HPLC; for insulin), ELISA (for HBsAg) or by enzymatic activity assay (for β-galactosidase and LDH) as well as by intrinsic fluorescence spectroscopy (all proteins). The results of all analysis were compared to the result immediately after freeze-drying and expressed as a percentage of this result (*i.e.* the results immediately after freeze-drying were taken as 100%). Since changes in activity during processing were found to be small and similar for all formulations per protein (data not shown), this aspect was not further considered in this work.

RP-HPLC

A modified gradient RP-HPLC method, based on the United States Pharmacopeia assay for insulin[28], was used to determine the amount of undeamidated insulin after freeze-drying and after storage at 60 °C. Prior to analysis, the powder formulations were reconstituted in 0.01 N hydrogen chloride to a concentration of 80 µg/mL. An Ultimate 3000 HPLC (Dionex) with a ChromSpher C18 column (3 mm x 250 mm) was used. A gradient of two mobile phases was applied. Mobile phase A consisted of 10% acetonitrile, 70% Milli-Q water and 20% Na_2SO_4 buffer (2 M, pH 2.3) while mobile phase B consisted of 40% acetonitrile, 40% Milli-Q water and 20% Na_2SO_4 buffer (2 M, pH 2.3). The gradient scheme was: 0 – 2 minutes, 65% mobile phase B; 2 – 16 minutes, mobile phase B gradually increased to 100%; 16 – 17 minutes, mobile phase B decreased to 65%; 17 – 27 minutes, mobile phase B kept at 65%. The flow rate was 1.0 mL/min and the column was kept at a temperature of 40 °C. For the detection of insulin a UV detector was used, set at a wavelength of 214 nm. The peak area was determined and compared to the peak areas of freshly prepared reference samples.

HBSAG ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The ability to induce an immune response is depending on the antigenicity of the vaccine, which is depending on the epitopes of the vaccine. The integrity of the epitopes on the ‘a’ determinant of HBsAg was investigated using a Murex HBsAg version 3 ELISA kit (Murex Biotech Limited, Dartford, UK). The powder formulations were reconstituted and diluted in PBS to a concentration of 1 ng/mL and pre-incubated for 1 hour at 37 °C in microwells coated with a mixture of mouse monoclonal antibodies specific for different epitopes on the ‘a’ determinant of HBsAg. Next, affinity purified goat antibodies to HBsAg, conjugated to

horseradish peroxidase, were added to the wells and these wells were incubated at 37 °C for 30 minutes. After washing, a substrate solution containing 3,3', 5,5'-tetramethybenzidine (TMB) and hydrogen peroxidase was added. The conversion of TMB by peroxidase was stopped after 30 minutes with sulfuric acid and measured spectrophotometrically at 415 nm with a Benchmark Microplate reader (BioRad, Herculas, CA, USA). The absorbance was compared to unprocessed HBsAg, which was stored in a refrigerator during the stability study.

LDH ACTIVITY ASSAY

Functionality of LDH was determined by measuring its ability to convert pyruvate into lactate. At pH 7.5, LDH converts pyruvate and NADH to lactate and NAD+. NADH absorbs at 340 nm, while NAD+ does not, thus allowing this reaction to be monitored spectrophotometrically. A unit of activity is defined as the conversion of 1.0 µmole of pyruvate per minute at pH 7.5 at 37 ºC. LDH containing samples were diluted to a concentration of approximately 0.25-0.025 unit/ml with a solution of 0.01% BSA in 0.1 M potassium phosphate (pH 7.5) buffer. The analysis was carried out in a flat-bottom 96-wells plate (Greiner Microlon®600 F-bottom). 50 µL 8 mM sodium pyruvate in the aforementioned phosphate buffer was added to 100 µL of the diluted LDH solution. The plate was then incubated at 37 ºC for 10 minutes. Lastly, 50 µL of a 1.2 mM freshly prepared NADH solution in the same buffer was added to start the reaction. The absorption at 340 nm was measured every minute for up to 1 hour using a Biotek Synergy HT multi-detection microplate reader. The reaction rate was determined from the slope of the linear part of the absorption-time curves. A correction for the slope of references without LDH was made. Reaction rates were shown to be linear to the concentration of LDH for a range from 0.5 to 0.001 unit/mL (data not shown). Because a reference solution of LDH was not stable over 4 weeks (even when stored refrigerated), activities of the samples were related to their activities directly after freeze-drying.

β-GALACTOSIDASE ACTIVITY ASSAY

The structural integrity of β-galactosidase was evaluated using an enzymatic activity assay, based on the rate of conversion of a colorless substrate, ortho-nitrophenyl-β-galactoside, into the yellow product, ortho-nitrophenol, by β-galactosidase. First, the powder formulations were reconstituted in 0.1 M phosphate buffer (pH 7.3) and diluted to a concentration of 10 µg/mL. Next, the wells of a 96-wells plate (Greiner Microlon®600 F-bottom) were pre-incubated with 20 µL β-galactosidase solution (samples at 10 µg/mL, calibtration curve: 4 – 20 µg/mL), 230 µL of 1.0 mM $MgCl_2$ and 0.008% bovine serum albumin (BSA) in phosphate buffer (0.1 M, pH 7.3) for 10 minutes at 37 °C. Afterwards, 20 µL of a 34 mM *ortho*-nitrophenyl-β-galactoside solution in phosphate buffer (pH 7.3) was pipetted into the wells and the absorption at 415 nm was measured 10 times with an interval of 30 seconds using a

Benchmark microplate reader (BioRad, Herculas, CA, USA). The absorption was plotted as a function of time and the slope of this straight line was taken as a measure of the enzymatic activity of β-galactosidase and was compared to unprocessed β-galactosidase, which was stored in a refrigerator during the stability study.

INTRINSIC FLUORESCENCE SPECTROSCOPY

Steady state fluorescence spectroscopy measurements were performed using a QuantaMaster™ 40 spectrofluorometer (PTI, Birmingham, AL, USA) in a similar manner as reported previously.[20] Samples were measured in a rectangular quartz cuvette with a path length of 10 mm. The temperature was maintained constant at 20.0 °C during all measurements. Before measuring, the lyophilized samples were reconstituted with PBS to a protein concentration of 10 μg/mL and 1.5 mL of this solution was placed in a cuvette. An excitation wavelength of 295 nm was used for LDH, β-galactosidase and HBsAg to specifically excite the tryptophan residues. Emission scans were performed from 300 to 400 nm. For insulin, which does not contain tryptophan residues, an excitation wavelength of 279 nm was used to excite the tyrosine residues. Emission scans were performed from 280 to 340 nm. For all scans excitation slits of 2.5 nm and emission slits of 2.5 nm were used and all scans were performed at a speed of 100 nm/s. For each sample 5 scans were performed and the result was averaged and corrected for background caused by PBS.

RESULTS

GLASS TRANSITION TEMPERATURE (Tg)

Table 2.1 shows the Tg values of the different sugars and of the different protein formulations, immediately after freeze-drying and evaporation of residual moisture (3 min preheat at 80 °C). The Tg values of the various formulations did not depend on the type of protein used and were similar to values found elsewhere.[15,29] The highest glass transition temperature was found for dextran 70 kDa based formulations and the lowest for formulations containing trehalose. The glass transition temperature of the formulations containing both dextran 70 kDa and trehalose was about the same as the glass transition temperature of the inulin 4 kDa based formulations. The Tg of dextran 6 kDa was between that of inulin 4 kDa and dextran 70 kDa. The glass transition temperature did not change during storage (data not shown). The influence of protein on the Tg was negligible, as was shown by the minor differences between the Tg of the protein containing powders and the Tg of the corresponding sugar. The Tg of the placebo samples measured in hermetically sealed pans (without preheat) after storage was much lower than the samples measured in open pans, as can be explained by the plasticizing effect of water. The Tg of the larger sugars was lowered more than for the smaller sugars. Water has a Tg of approximately

-109 °C[30,31], the relative difference in Tgs between water and the sugars is thus larger for the larger sugars, providing a possible explanation for the larger drop in Tg. The Tg of all the samples is still 25-30 °C above the storage temperature of 60 °C for trehalose and even higher for the other samples. The Tg' values of all the formulations are higher than the shelf temperature used during primary drying and no visual collapse was observed. The moisture content of the trehalose and inulin formulations was low (<2%) and slightly higher in the formulations containing dextran (3–4%).

Table 2.1 Tg and Tg' of pure sugars and different protein formulations (protein-sugar ratio 1:249 (w/w)) , and water content of placebos immediately after freeze-drying (n=1 for Tg determination; n=3 for water content and Tg' determinations; n.d.= not determined). Formulations contained < 1% buffer on a dry substance basis.

	Glass transition temperature (°C)						Tg' (°C)	Water content (weight % ± S.D.)
Sugar	**Insulin**	**LDH**	**β-galactosidase**	**HBsAg**	**Pure sugar**	**Pure sugar (closed pan after storage)**	**Pure sugar solution**	**Pure sugar (after freeze drying)**
Trehalose	121	122	121	121	122	88	-27.9±0.7	1.8±0.1
Dextran 70 kDa + trehalose (1:1)	159	159	158	159	159	110	-19.7±0.8	3.0±0.1
Dextran 70 kDa	223	224	224	223	224	167	-11.2±0.4	4.1±0.3
Dextran 6 kDa	192	190	192	190	193	144	-14.1±0.2	4.2±0.2
Inulin 4 kDa	154	155	154	155	156	119	-16.9±0.9	1.1±0.2
Lactose	n.d.	n.d.	n.d.	n.d.	119	92	n.d.	n.d.
Trehalose + lactose (4:1)	n.d.	n.d.	n.d.	n.d.	121	89	n.d.	n.d.

REDUCING GROUPS

The Sumner assay was performed to determine the amount of reducing groups in each sugar. Reducing groups of the sugar can react with amino acids in the protein. Table 2.2 shows that trehalose is a non-reducing sugar, oligosaccharides inulin 4 kDa and dextran 6 kDa contained some reducing groups, and polysaccharide dextran 70 kDa contained little reducing groups. Dextran is a non-reducing sugar, apart from one glucose unit at the end of the chain, which can form a reducing group by ring opening.[32] For the smaller dextran, these end groups are relatively more abundant compared to the larger dextran, explaining the difference in amount of reducing groups found. In theory, inulin should not contain any reducing groups because of the way the fructose and glucose groups are linked.[21] However,

Table 2.2 Percentage of reducing groups relative to glucose as determined by Sumner assay (n=3)

Sugar	Percentage reducing groups relative to glucose
Trehalose	0.1 (±0.0)
Dextran 70 kDa	0.6 (±0.0)
Dextran 6 kDa	10.9 (±0.8)
Inulin 4 kDa	5.0 (±0.1)
Lactose	82.1 (±3.1)

if the glucose end group of inulin is cleaved, a reducing fructose group at the end of the chain as well as a reducing glucose are created. This could explain the amount of reducing groups found by the Sumner assay, which were similar to previously reported values.[21]
Even though dextran and inulin contain some reducing groups, no Maillard browning was observed during storage at 60 °C. In fact, even for proteins freeze-dried in the presence of lactose, which contains a large number of reducing groups, as was confirmed by our test (table 2.2), no browning was detected. This may be explained by the near absence of water present in the various samples. It should however also be noted that the first stages of the Maillard reaction occur without browning.

STORAGE STABILITY

To investigate the stability of the four proteins after freeze-drying in the presence of the various sugars, all formulations were stored at 60 °C and analyzed after 1, 2, and 4 weeks of storage and compared to the result immediately after freeze-drying. Samples were analyzed using an assay specific for each protein and by fluorescence spectroscopy.

Protein specific assays

Figure 2.3 shows an example of the activity versus time profile of the formulations containing LDH and the various sugars. The other proteins show similar, but numerically different profiles, their activity versus time profiles can be found in the supporting information. Standard deviations of the assays were generally smaller than 5% and were not shown for clarity reasons. To obtain an overview of the data, a kinetic fit of the decrease of protein that remained intact during storage at 60 °C was made. As protein degradation can occur through many pathways, there was no obvious kinetic fit to be used. The function that provided the highest correlation coefficients (R) (equation 2.1), a logarithmic function, was therefore used. A square root of time based function, as used in other studies, also seemed consistent with the data, but yielded lower R-values. R-Values were generally larger than 0.95 and always larger than 0.90.

(2.1) $F(t) = -A \ln(t) + B$

where F(t) is the amount of protein still intact, relative to the initial activity, t time in days and the fitting factor A provides information about the rate of degradation of the specific formulation. B is a fitting value that completes the equation and is not used further. Figure 2.4 depicts the degradation rate constants and the accompanying correlation coefficients. Since a logarithmic fit cannot incorporate t=0, t=0.01 was used for the fits.
For insulin, LDH, and ß-galactosidase, it was found that the rate of degradation was lowest when trehalose was used as a stabilizer. HBsAg was best stabilized by inulin 4 kDa. However, the differences between stabilizing capacities of trehalose and inulin 4 kDa were small. Furthermore, it was found that all four proteins were more stable when freeze-dried in the presence of the inulin 4 kDa than when freeze-dried in the presence of the dextran 6 kDa. Remarkably, the insulin formulation with dextran 6 kDa was even less stable than the formulation of insulin only (no sugar). Proteins freeze-dried in the presence of dextran 70 kDa were more stable than when the smaller dextran was used. The stabilizing capacity of dextran 70 kDa was substantially increased when it was mixed in a 1:1 weight ratio with trehalose.

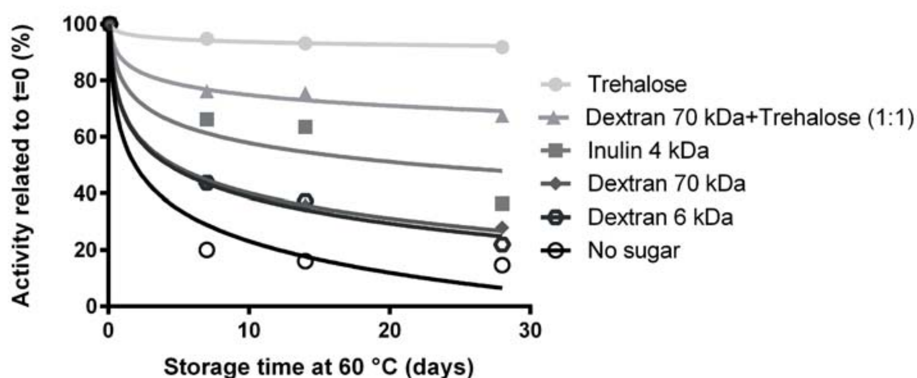

Figure 2.3 Storage stability of LDH formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. The relative standard deviation was between 0 and 3%, error bars are not shown for clarity purposes.)

Intrinsic fluorescence spectroscopy

To monitor irreversible changes in the conformation of the proteins during storage, intrinsic fluorescence spectroscopy was used. Conformational changes lead to changes in the local environment of tryptophan or tyrosine (in the case of insulin) residues, which influences the fluorescence intensity of these residues. Additionally, formation of insoluble aggregates could also influence the fluorescence intensity. Any changes in the fluorescence intensity

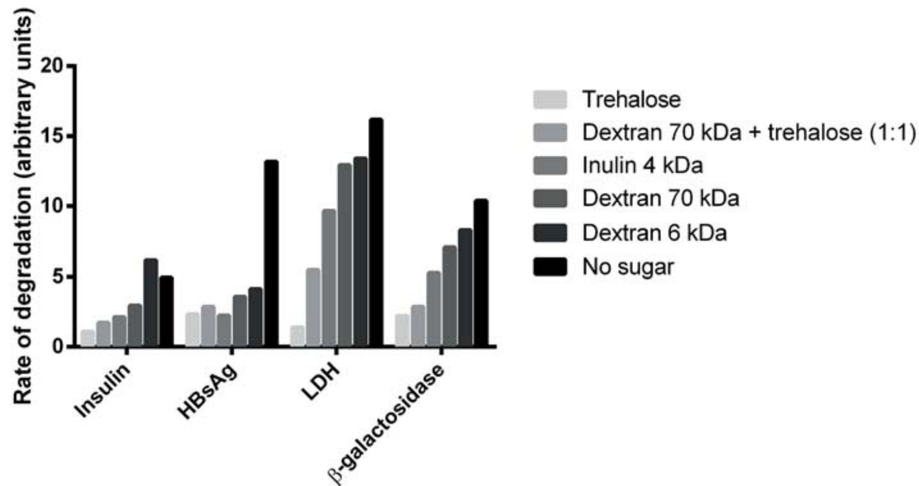

Figure 2.4 Rate of degradation of the proteins during storage for the various formulations (fitting factor A from equation 2.1).

are thus undesired. As with any protein analysis after reconstitution, fluorescence only measures irreversible changes during storage as some changes could have reverted with reconstitution.

Figure 2.5 shows the changes in maximum fluorescence intensity of the formulations containing LDH and the various sugars. The other proteins show similar, but numerically different profiles; their fluorescence intensity versus time profiles can be found in the supporting information. Similar to the protein specific assays, a logarithmic function (equation 2.1) was used to fit the rate of change in tryptophan fluorescence intensity over time. Fitting factors (R) were at least 0.90 except for the combination of insulin and trehalose (R = 0.756). Here too, the fitting parameter (A) from equation 2.1 was taken as a measure for the degradation rate for comparison of the different formulations.

Figure 2.6 shows the same trends as found with the protein specific assays. Proteins freeze-dried in the presence of trehalose showed the least change in maximum fluorescence intensity, except for β-galactosidase. For β-galactosidase, the most stable formulation was achieved when using a combination of dextran and trehalose, yet it should be noted that the differences between the various formulations were relatively small for this protein. When both oligosaccharides (molecularly rigid dextran 6 kDa and flexible inulin 4 kDa) are compared, the changes in conformation were for every protein smaller when they were freeze-dried in the presence of inulin 4 kDa. Conformational changes in the structure of insulin and HBsAg freeze-dried in the presence of dextran 6 kDa were even greater during storage than when no sugar was used. Proteins freeze-dried in the presence of dextran 70 kDa yielded products

that were more stable than proteins freeze-dried in the presence of dextran 6 kDa but not as stable as when inulin 4 kDa was used. Furthermore, with the exception of HBsAg, the decrease in maximum fluorescence intensity was small when a mixture of dextran 70 kDa and trehalose was used, compared to the formulations containing dextran 70 kDa only.

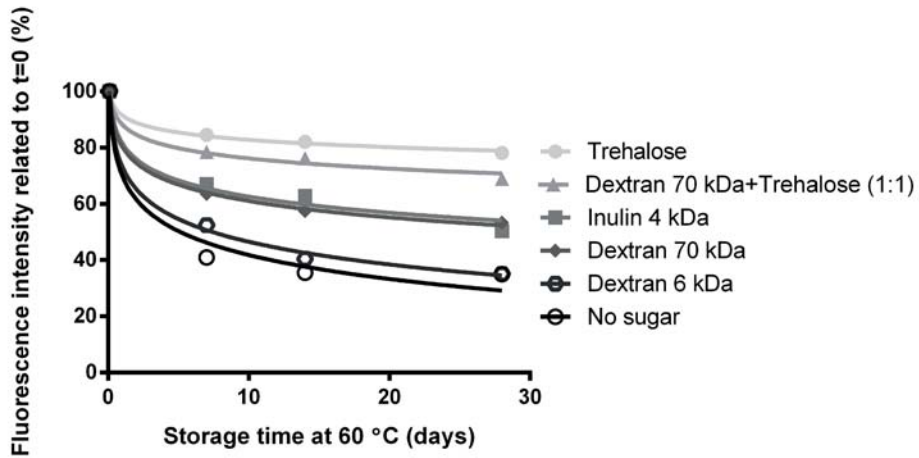

Figure 2.5 Maximum fluorescence intensity of LDH formulations after 1, 2, and 4 weeks of storage at 60°C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)

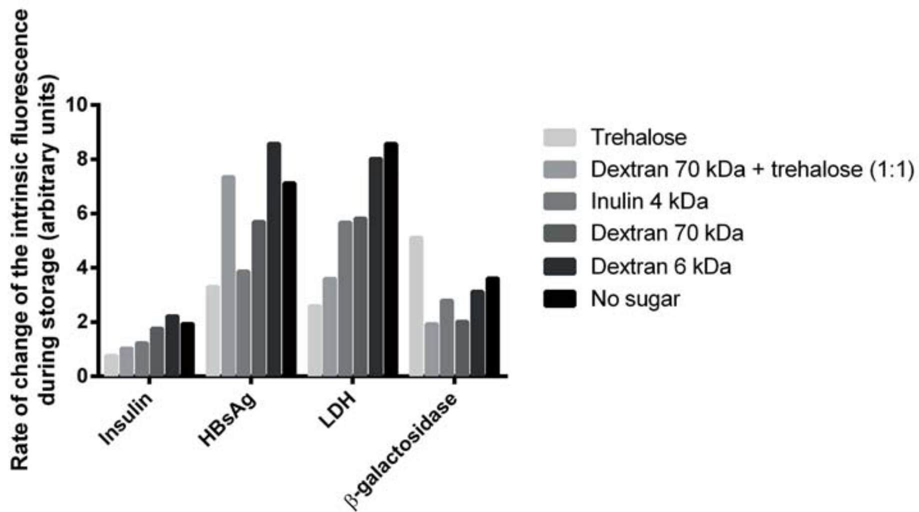

Figure 2.6 Rate of change (fitting factor A from equation 2.1) of the intrinsic tryptophan (or tyrosine for insulin) fluorescence of the proteins during storage for the various formulations.

Degradation by reducing sugars

The Sumner assay indicated that the amount of reducing groups differed for the different sugars. Even though no browning was observed upon storage, it was important to rule out the effect of reducing groups on stability, before making any statements about the influence of sugar size and molecular flexibility on their ability to stabilize proteins. Therefore stability tests with proteins LDH and β-galactosidase were carried out to assess the effect of presence of reducing sugars. These stability tests were carried out with the same protein, but at a later date. The obtained degradation rates were not the same as the initial test, but this did not influence the conclusions drawn from these experiments, as these were based on the relative differences between the reducing formulations. Table 2.3 shows the results of the stability test with reducing sugars.

Lactose has the same molecular weight and a similar glass transition temperature as trehalose, but unlike trehalose it is a reducing sugar. Trehalose, lactose and the mixture of the two provided very similar results. Because the changes in fluorescence intensity were relatively small for β-galactosidase, R values are relatively low. The relative differences between the formulations, however, remain unaffected. There is no clear relationship between the amount of reducing groups and the rates of degradation. We can thus rule out the effect of reducing groups on storage stability of the proteins when comparing formulations in these experiments.

Table 2.3 Rate of degradation and rate of change of intrinsic fluorescence intensity of the proteins (fitting factor A from equation 2.1) with various reducing sugars during storage with the corresponding correlation coefficient (R) of the fit (shown in parentheses) and amount of reducing groups of the sugar as determined by the Sumner assay.

	Activity assay		Intrinsic tryptophan fluorescence		Reducing groups relative to glucose
Sugar	**LDH (140 kDa)**	**β-galactosidase (540 kDa)**	**LDH (140 kDa)**	**β-galactosidase (540 kDa)**	
Trehalose	6.10 (0.948)	11.33 (0.851)	4.28 (0.864)	1.54 (0.355)	0.1% (±0.0)
Trehalose + lactose (4:1)	6.12 (0.947)	12.62 (0.871)	4.43 (0.888)	1.02 (0.227)	16.5% *
Lactose	6.18 (0.952)	12.39 (0.824)	4.19 (0.913)	0.96 (0.222)	82.1% (±3.1)

* is based on calculation

DISCUSSION

In this study, we tested the influence of the size and molecular flexibility of sugars on their protein stabilizing abilities. It was hypothesized that small sugars (disaccharides) were hardly sterically hindered in interacting with proteins and that for oligosaccharides the quality of the interaction between the sugar and protein depended on molecular flexibility. Proteins freeze-dried in the presence of the smallest sugar (the disaccharide trehalose) were best preserved, in regard to functionality as well as the change in conformation during storage. Furthermore, it was confirmed that the flexible oligosaccharide (inulin) indeed stabilized the proteins better than the rigid oligosaccharide (dextran).
From the degradation rates (A, from equation 2.1 for change in either activity or fluorescence) of the different formulations, shown in figures 2.4 and 2.6, it is possible to calculate the relative improvement stability of that formulation compared to the unstabilized protein, using equation 2.2.

(2.2) $$\text{Relative improvement of stability} = (1 - (A_{formulation} / A_{no\ sugar})) \times 100\%$$

The protein without any sugar is the negative control and by definition has 0% improved stability. A perfectly stabilized formulation, would have no change in activity during storage, and thus a degradation rate ($A_{formulation}$) of 0, and with that a 100% improved stability compared to the formulation without sugar.
In table 2.4 the rank order of the stabilizing capacity of the sugars for each protein group is shown, based on the relative improvement of stability from both the protein specific assays and intrinsic fluorescence spectroscopy. The formulation with the highest relative improvement of stability was ranked 1, the one with the lowest improvement was ranked lowest. In almost all cases, proteins were most stable when freeze-dried in the presence of trehalose, followed by the formulations containing a mixture of dextran 70 kDa and trehalose. This result is independent of the analytical tool used and is roughly independent of the protein and protein size used.
Furthermore, molecularly flexible inulin 4 kDa based formulations were more stable than formulations based on rigid dextran 6 kDa and 70 kDa, according to the protein specific assays as well as intrinsic fluorescence spectroscopy results. Formulations containing dextran 6 kDa or no sugar at all were least stable. Interestingly, the trends between the sugars are the same for all the proteins despite the large differences in their size (i.e. from 6 kDa for insulin to 540 kDa for β-galactosidase) and nature.
As described and illustrated in the introduction, small molecules like disaccharides should be able to interact closely with the protein's surface irrespective of its irregular nature. Larger molecules are less capable of doing so because of steric hindrance. A disadvantage of using

Table 2.4 Rank order of the stabilizing capacity of the sugars for each protein and median rank of sugar over all four proteins according to the proteins specific assay and intrinsic fluorescence spectroscopy. The rank is based on the relative improvement of stability, calculated using equation 2.2 and rates from figures 2.4 and 2.6 (shown in parentheses). The formulation with the smallest improvement of stability was given the lowest rank, and vice versa.

Protein Specific Assay						
Sugar	**Insulin (6 kDa)**	**HBsAg (25 kDa)**	**LDH (140 kDa)**	**β-galactosidase (540 kDa)**	**Median rank**	**Figure 2.1**
Trehalose	1 (78%)	2 (82%)	1 (91%)	1 (79%)	1	A
Dextran 70 kDa + trehalose (1:1)	2 (65%)	3 (78%)	2 (66%)	2 (72%)	2	C
Inulin 4 kDa	3 (57%)	1 (83%)	3 (40%)	3 (49%)	3	B
Dextran 70 kDa	4 (41%)	4 (73%)	4 (20%)	4 (32%)	4	D
Dextran 6 kDa	6 (-25%)	5 (69%)	5 (17%)	5 (20%)	5	E
No sugar	5 (0%)	6 (0%)	6 (0%)	6 (0%)	6	F
Intrinsic Fluorescence Spectroscopy						
Trehalose	1 (61%)	1 (54%)	1 (70%)	3 (42%)	1	A
Dextran 70 kDa + trehalose (1:1)	2 (47%)	5 (-3%)	2 (58%)	1 (47%)	2	C
Inulin 4 kDa	3 (36%)	2 (46%)	3 (34%)	4 (22%)	3	B
Dextran 70 kDa	4 (9%)	3 (20%)	4 (32%)	2 (44%)	3.5	D
Dextran 6 kDa	6 (-15%)	6 (-21%)	5 (6%)	5 (15%)	5.5	E
No sugar	5 (0%)	4 (0%)	6 (0%)	6 (0%)	5.5	F

disaccharides, however, is their relatively low Tg. The visual appearance of the trehalose formulation did not change (no collapsed cake), which suggests that the Tg was well above 60 °C. To verify this observation, the Tg of placebo samples in hermetically sealed pans were measured after a week of storage at 60 °C, thus showing the actual Tg including moisture at that time. It was found that the Tg was still 25-30 °C above the storage temperature for the disaccharides and even more for the other sugars. As shown by Hancock *et al.*[33] there is still significant molecular mobility at temperatures above the Kauzmann temperature, which is estimated to be roughly 50 °C below the Tg. Recent work of Grasmeijer *et al.* [4] also showed that when the Tg of a sample is increased past 10 to 20 °C above the storage temperature, the stabilization mechanism that limits stability shifts from vitrification to water replacement (e.g. interaction between protein and sugar). This is in line with findings of Mizuno and Pikal, which show that proteins in the solid state display a transition in global protein mobility at temperatures well below the Tg.[34] Storage at higher relative humidities would further suppress the temperature at which these motions would occur, potentially critically lowering the Tgs of the disaccharide samples below this threshold. In that respect, sugars with a higher Tg (like oligosaccharides) are preferred.

Comparing both oligosaccharides in this study showed that proteins freeze-dried in the presence of the molecularly flexible inulin 4 kDa are far better stabilized than proteins freeze-dried in the presence of rigid dextran 6 kDa, even though the Tg of dextran 6 kDa is substantially higher than the Tg of inulin 4 kDa; for the dry sugars 192 °C versus 154 °C, respectively. Even though the molecular weights of the oligosaccharides are not identical, the differences in their stabilizing capacities are much larger than can be explained by size alone. As described in section *Degradation by reducing sugars*, the influence of the Maillard reaction was ruled out for these results.
Clearly, protein stabilization by sugars is not depending on the Tg of the sugar alone. This is illustrated by trehalose, which under these circumstances performed best, whilst having the lowest Tg of the used formulations. If vitrification of the sample is sufficient, the ability of a sugar to replace or maintain hydrogen bonds of the protein during drying, and with that its ability to minimize protein mobility, becomes the limiting factor for stabilization.[4] This translates to a close contact of the amorphous sugar with the irregular surface of the protein as described in the introduction. We hypothesized that flexibility of the backbone of oligosaccharides determines whether or not the sugar can do so. As expected, the more flexible inulin stabilized the proteins better during storage than the similarly sized rigid dextran, presumably by the better and more complete interaction at the surface of the protein.
Using in-line near-infrared spectroscopy during freeze-drying, it was shown that these protein-sugar interactions are formed during drying and that increasing amounts of stabilizers resulted in increasing amounts of both interactions and stabilization.[35] It is therefore most likely that the differentiation between the amount of interactions occurs mainly during drying, where the smaller and molecularly more flexible sugars form more tight interactions than their larger and more rigid counterparts.
Such protein-sugar interactions are not only needed for good protein stabilization but are also in a way necessary to prevent phase separation of the protein and sugar. Phase separation basically makes any sugar-protein interaction impossible. The size of the oligomers and proteins implies that the excluded volumes of these compounds are relatively large. For that reason the entropy of mixing is relatively low, implicating that the enthalpy rise caused by sugar-protein interaction should be low or preferably negative to avoid phase separation.[36] It might therefore be useful to investigate the miscibility of these or similar protein-sugar systems to further elucidate the mechanism by which various types of sugars stabilize proteins.
Proteins freeze-dried in the presence of a rigid oligosaccharide (dextran 6 kDa) were less stable than when a rigid polysaccharide (dextran 70 kDa) was used. Since dextran 6 kDa is a lot smaller than dextran 70 kDa, it was expected that proteins would be better stabilized by the smaller dextran. Again, the number of reducing groups could not provide an

explanation for this result. A possible explanation for the observed difference could lie in the branched structure of dextran, which is most likely more pronounced in the larger dextran. Potentially the different branches could allow for some accommodation to the surface of the protein. The low stabilizing effect of dextran 70 kDa compared to trehalose can be ascribed to the large and bulky nature of dextran 70 kDa.

Addition of trehalose to dextran 70 kDa greatly improved the stability of the proteins. The Tg of this formulation was higher than that of the formulation containing only trehalose. We hypothesize that the molecular mixture of trehalose and dextran combines the interaction capacity of trehalose with an increased Tg of dextran 70 kDa. Because of steric hindrance, cavities in the coating at the surface of the protein can appear when a large and bulky stabilizer like dextran 70 kDa alone is used. Trehalose limits this steric hindrance, resulting in a more complete and compact coating and a high glass transition temperature of the final sample. The finding that trehalose alone performs better than the combination suggests that there is still some sterical hindrance of dextran 70 kDa which trehalose cannot overcome.

Our results show that the small disaccharide trehalose is the best stabilizer for proteins during storage at 60 ºC for four weeks. It was illustrated that moisture can strongly lower the Tg of the formulations. Here the relative humidity was below 10% and vitrification was not the limiting factor for stability. In situations where protein formulations are exposed to higher relative humidities, increased water uptake may occur, resulting in a further increase in mobility. This may be critical when the Tg of the formulation is no longer significantly higher than the storage temperature, as the system is then no longer fully vitrified. For these situations, sugars with a higher Tg are required to obtain an optimally stabilized product. In these cases, oligosaccharides could provide a solution in two different ways. Firstly, a flexible oligosaccharide could be used to increase the Tg of the formulation, while still achieving an efficient coating of the protein or secondly, one could add an oligo- or polysaccharide to a formulation of smaller sugars. By varying the ratio between large and small sugars, a tailor-made adjustment of the Tg in combination with a maximization of the compactness of the coating of the protein by the sugars would be feasible.

This study confirms our mechanistic hypothesis that size and molecular flexibility of sugars affect their ability to stabilize proteins. As long as they maintain vitrification, smaller and molecularly more flexible sugars are less affected by steric hindrance and thus better capable at stabilizing proteins. Since the four model proteins showed the same trends, in spite of their difference in size and nature, it is likely that the conclusions from this study will be applicable broadly for other proteins as well.

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SUPPORTING INFORMATION

The figures below show the activity versus time profiles of insulin (figure 2.S1), HBsAg (figure 2.S2) and β-galactosidase(figure 2.S3) during storage. They are similar to figure 2.3 and support the information discussed in section *Protein specific assays*.

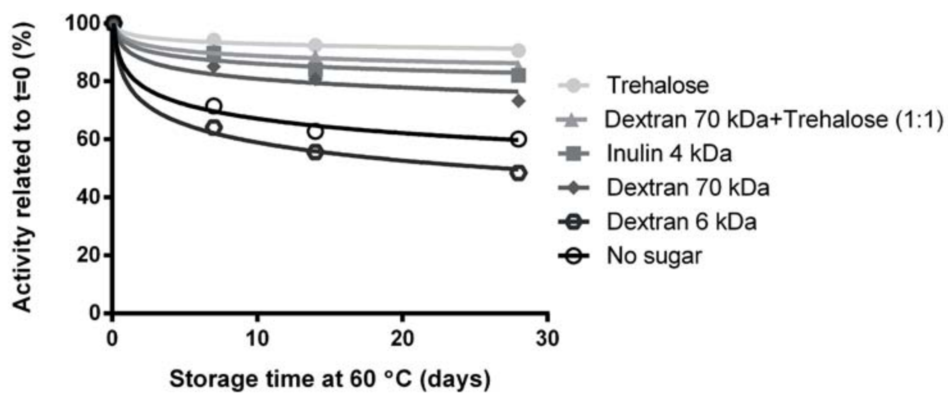

Figure 2.S1 Storage stability of insulin formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. Error bars are not shown for clarity purposes.)

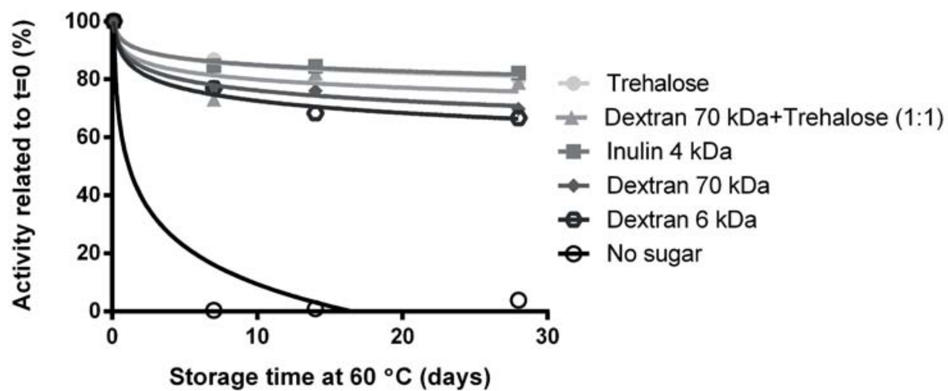

Figure 2.S2 Storage stability of HBsAg formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. Error bars are not shown for clarity purposes.)

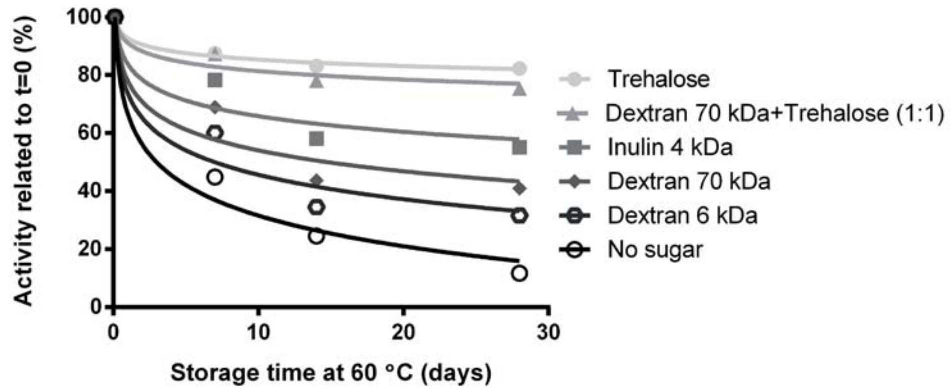

Figure 2.S3 Storage stability of β-galactosidase formulations by activity assay analysis after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per time point; samples were measured in triplicate. Error bars are not shown for clarity purposes.)

The figures below show the fluorescence intensity versus time profiles of insulin (figure 2.S4), HBsAg (figure 2.S5)en β-galactosidase(figure 2.S6) during storage. They are similar to figure 2.5 and support the information discussed in section *Intrinsic fluorescence spectroscopy*.

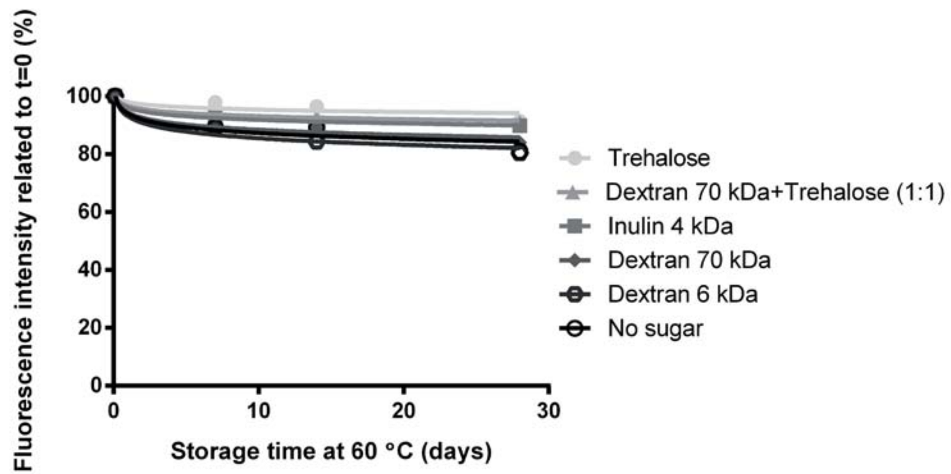

Figure 2.S4 Maximum fluorescence intensity of insulin formulations after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)

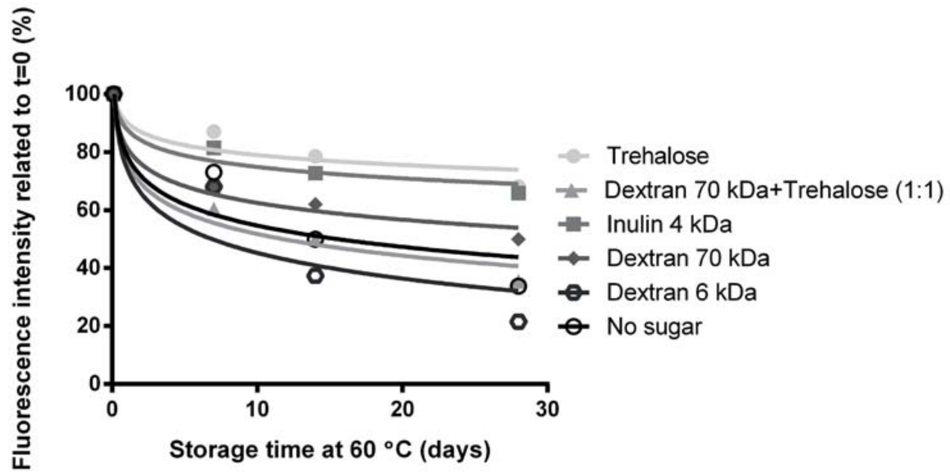

Figure 2.S5 Maximum fluorescence intensity of HBsAg formulations after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)

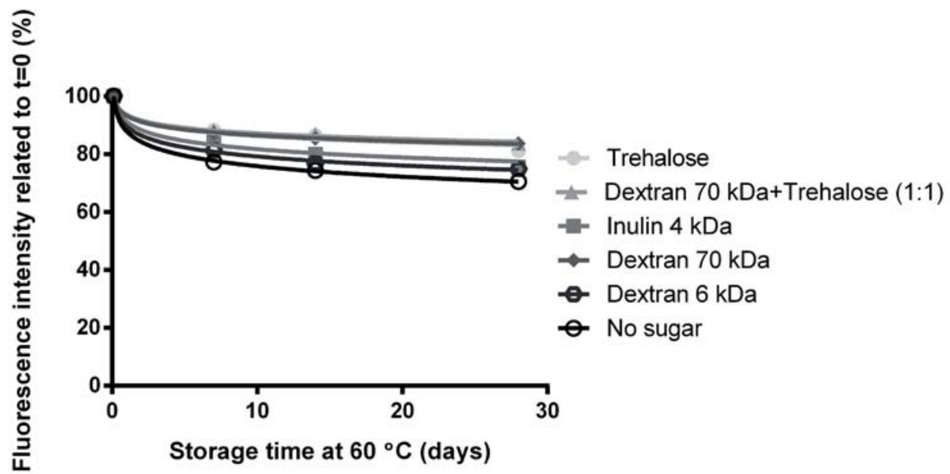

Figure 2.S6 Maximum fluorescence intensity of β-galactosidase formulations after 1, 2, and 4 weeks of storage at 60 °C. The results are normalized to day 0 (immediately after freeze-drying). (n=1 per timepoint, result is average of 5 scans)

Inulin, a flexible oligosaccharide I: Review of its physicochemical characteristics

3

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ABSTRACT

Inulin, a fructan-type polysaccharide, consists of (2→1) linked ß-D-fructosyl residues (n=2-60), usually with an (1↔2) α-D-glucose end group. The applications of inulin and its hydrolyzed form oligofructose (n=2-10) are diverse. It is widely used in food industry to modify texture, replace fat or as low-calorie sweetener. Additionally, it has several applications in other fields like the pharmaceutical arena. Most notably it is used as a diagnostic agent for kidney function and as a protein stabilizer. This work reviews the physicochemical characteristics of inulin that make it such a versatile substance. Topics that are addressed include morphology (crystal morphology, crystal structure, structure in solution); solubility; rheology (viscosity, hydrodynamic shape, gelling); thermal characteristics and physical stability (glass transition temperature, vapor sorption, melting temperature) and chemical stability. When using inulin, the degree of polymerization and processing history should be taken into account, as they have a large impact on physicochemical behavior of inulin.

INTRODUCTION

Inulin was discovered over two centuries ago by Rose[1] and since then its presence in many plants became apparent.[2] Some examples of plants containing large quantities of inulin are Jerusalem artichoke, chicory root, garlic, asparagus root, salisfy and dandelion root.[3] More commonly consumed vegetables and fruits containing inulin are onion, leek, garlic, banana, wheat, rye and barley. Daily intakes have been estimated to range from 1 to 10 g per day in the Western diet.[4,5] The average American diet contained between 1.3 and 3.5 g of inulin per day, with an average of 2.6 g.[5] The European consumption of inulin appears to be substantially higher at 3 to 11 g per day, which is below reported tolerances of at least 10 to 20 g per day.[6,7] Inulin has also been used safely in infant nutrition.[8] This has led to the American Food and Drug Administration to issuing a Generally Recognized As Safe notification for inulin in 1992.[9] Inulin is also used pharmaceutically, most notably as a diagnostic agent for the determination of kidney function.[10,11]

Over the past decades, a lot of research has been done showing that inulin is a versatile substance with numerous promising applications. Several reviews have been published on inulin, its characteristics and functionality in food and pharma.[12–16] This review aims to provide an overview of the relevant physicochemical properties of inulin, which make it such a useful excipient in food and pharma.

CHEMICAL STRUCTURE

Inulin, depending on its chain length, is classified as either an oligo- or polysaccharide and it belongs to the fructan carbohydrate subgroup. It is composed of β-D-fructosyl subgroups linked together by beta (2→1) glycosidic bonds and the molecule usually ends with a (1↔2) bonded α-D-glucosyl group.[12,17] The length of these fructose chains varies and ranges from 2 to 60 monomers. Inulin containing maximally 10 fructose units is also referred to as oligofructose.[18] In food, oligofructose is more commonly used a sweet-replacer and longer chain inulin is used mostly as a fat replacer and texture modifier.[12] Both inulin and oligofructose are used as dietary fiber and prebiotics in functional foods. Its longer chain length makes inulin more useful pharmaceutically than oligofructose.

Before processing, the degree of polymerization of inulin depends on the plant source, time of harvest, and the duration and conditions of post-harvest storage.[9,19,20] Processing itself also has a great influence on degree of polymerization of the obtained product as will be discussed in the section *Isolation and production*. Table 3.1 provides an overview of the structure and size of some carbohydrates frequently used in the pharmaceutical arena. The structures of a selection of those carbohydrates are shown in figure 3.1.

Like many oligosaccharides, inulin is heterodisperse. High performance anion exchange chromatography (HPAEC) with pulsed amperometric detection can be used to determine

the number average DP (DPn) and the weight average DP (DPw) of inulin.[36] Several chromatographic methods have been described, but HPAEC has a superior sensitivity and resolution.[30,36] The ratio between DPw and DPn is a measure of the molecular weight distribution (polydispersity) of a sample. The DP and polydispersity of an oligo- or polysaccharide influence the physicochemical properties to a large extent.[37,38]
Inulin is a unique oligo- or polysaccharide because its backbone does not incorporate any sugar ring, which can be seen in figure 3.1. The backbone is in essence polyethylene oxide.[30] This translates into a greater freedom to move and thus more flexibility of the molecule. Furthermore, inulin is built up mostly from furanose groups, which are more flexible than pyranose rings.[2,32]

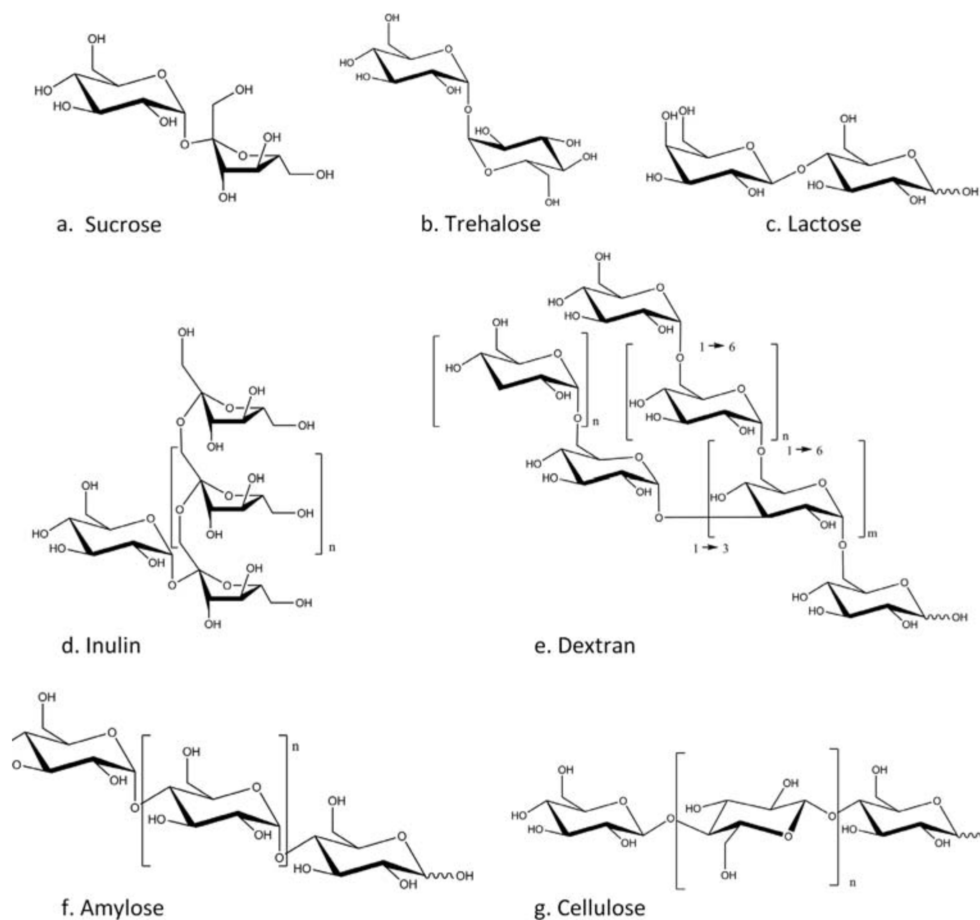

Figure 3.1 Chemical structures from a selection of the carbohydrates listed in table 3.1.

Table 3.1 Structure and size of some carbohydrates frequently used in food and pharma.

Carbohydrate	Building blocks & Linkages	Molecular weight (Da)	Backbone	Article cited
Glucose	αDGlc	1.8×10^2	-	[22]
D (+) Trehalose	αDGlc*p*(1↔1)αDGlc*p*	3.4×10^2	Linear	[22,23]
Sucrose	αDGlc*p*(1↔2)ßDFru*f*	3.4×10^2	Linear	[22]
ß (+) Lactose	ßDGal*p*(1→4)DGlc*p*	3.4×10^2	Linear	[22]
Maltodextrin	$[4)$-αDGlc*p*$(1\rightarrow]_n$	1.8×10^2 to 3.2×10^3	Linear	[24]
Amylose (α-Glucan)	$[4)$-αDGlc*p*$(1\rightarrow]_n$	5×10^5 to 2×10^6	Linear	[22,25,26]
Dextran (α-Glucan)	$[6)$-αDGlc*p*$(1\rightarrow]_n$ (Main) αDGlc*p*(1→3)αDGlc*p* (also (1→2) and (1→4) (Branches)	1.0×10^3 to ~ 10^7	Branched	[22,27,28]
Cellulose (ß-Glucan)	$[4)$-ßDGlc*p*$(1\rightarrow]_n$	3×10^5 to 2×10^6	Linear	[29]
Inulin (Fructan)	$[1)$-ßDFru*f*$(2\rightarrow]_n$ (Main) αDGlc*p*(1↔2) ßDFru*f* (End, usually)	5.0×10^2 to 1.3×10^{4}ǂ	Linear	[12,17,30,31]
Levan (Fructan)	$[6)$-ßDFru*f*$(2\rightarrow]_n$ (Main) ßDFru*f*(2→1) ßDFru*f* (Branches)	1×10^4 to 1×10^8	Branched	[31–34]

Glc*p* = Glucopyranosyl , Fru*f* = Fructofurananosyl , Gal*p* = Galactopyranosyl[21]
ǂBacterially produced inulin has been reported to be branched and have a significantly higher molecular weight than plant derived inulin, see also table 3.2.[35]

ISOLATION AND PRODUCTION

Inulin is predominately isolated from chicory root. The isolation process of inulin basically consists of 3 steps: (1) extraction of water soluble components, including inulins, from chicory root (2) purification to remove foreign compounds and optionally low DP inulins and (3) spray drying. Sometimes the extracted product is partially hydrolyzed to reduce the DP of the final product.[39] Here isolation and purification are only discussed briefly, for further reading on this topic the reader is directed to the review of Apolinário *et al.*[40]

Inulin extracted from chicory root contains up to 10% of sugars (mono- di- and small oligosaccharides).[5] Typically, extraction is done by boiling the cleaned and cut or ground up roots in water. Process conditions such as pH of the water, water-root ratio, boiling time *et cetera* may vary.[41,42] As will be described in section *Chemical stability*, pH and boiling time could affect the DP of the produced inulin. After extraction, the obtained mixture is condensed through evaporation.

Purification of inulin is mostly done by making use of the solubility difference of the DP fractions present in extracts. Heating and cooling in combination with filtration, decantation and (ultra)centrifugation have been described to produce different molecular weight fractions of inulin.[42–47] Alternatively (organic) co-solvents, such as methanol, ethanol and acetone, can be used to selectively precipitate long chain (DPn 25-40) inulin.[48] Inulin that

has not been precipitated in these processes can be turned into a solid by (spray) drying. Optimization of the spray drying process, by varying inlet air, solution temperature and feed pump speed, based on microstructure of the produced inulin and rheological behavior of concentrated inulin solutions have been described by Toneli *et al.*[47,49]
Ronkart *et al*.[50–53] investigated several aspects of the isolation and purification of inulin, with emphasis on the physical characteristics of the produced inulin. They investigated the influence of several parameters, such as feed and inlet temperature during spray-drying on the physicochemical characteristics of the produced inulin. It was found that at a feed temperature of 80 °C and higher, the produced inulin was completely amorphous. A high air inlet temperature (230 °C compared to 120-170 °C) also increased the amount of amorphous inulin produced. Next to that, they characterized oligofructose produced by hydrolysis of inulin from globe artichoke by endo-inulinase.[17]
Apart from extraction from plants, inulin can also be produced enzymatically. Inulosucrase type fructosyltransferase can synthesize inulin from sucrose by catalyzing both transglycosylation and hydrolysis of sucrose.[54] Several procedures to do so have been described, these mostly involve enzymes derived from bacteria. Enzymes from *Bacillus species 217C-11* have been used to produce inulin on a large scale[55] and *Escherichia coli* and *Streptococcus mutans* derived fructosyltransferase can produce very high molecular weight inulins.[56] Both these studies reported remarkably low polydispersity (around 1.1) of the produced inulin. Inulin producing fructosyltransferases from several *Lactobacillus* strains have also been characterized.[57,54] Inulosucrase from *Leuconostoc citreum CW* 28 was shown to produce different molecular weight inulin when it was cell associated compared to when it was free in solution.[58] The cell associated enzyme predominately produced inulin with a molecular weight between 1.35-1.60 × 10^6 Da and the free enzyme produced more inulin with a molecular weight between 2600 and 3400 Da.[58]
Isolation of two plant derived fructosyltransferases from *Helianthus tuberosus* and the production of inulin with those purified enzymes was described by Lüscher *et al.*[59] The fungus *Aspergillus oryzae KB* is also able to produce inulin type oligofructoses from sucrose, but additionally possesses another enzyme which simultaneously hydrolyzes sucrose. The first enzyme produces 1-kestose, nystose and fructosyl nystose, whereas the second one produces glucose and fructose.[60] Oligofructoses can be produced by partial enzymatic hydrolysis of polyfructoses. Enzymes from *Aspergillus niger* can produce oligofructose from both hydrolysis of inulin (by inulinase) and synthesis from sucrose (by β-fructosyltransferase) and its inulinases provided higher yields than inulinases from *Kluyveromuces marxianus.*[61] Beghin-Meiji, a commercial supplier of oligofructose, use ß-fructo-furanosidase from *Aspergillus Niger* to synthesize, rather than to hydrolyze, oligofructose from sucrose.[62] For more information on microbial enzymatic production of oligofructoses, either from synthesis from sucrose or from hydrolysis of inulin, the reader is directed to a recent review of

Mutanda *et al.*[63] on this topic. To the best of our knowledge, high molecular weight inulin from synthetic source is not yet commercially available on a large scale, most likely because of the high production costs.

Finally, a completely different method of production is the genetic modification of a potato to make it produce inulin like globe artichoke. However the inulin yield is low (5%) and inulin production goes at the cost of starch production.[64] Van Arkel *et al.*[65] recently published a review on plants that were genetically modified to produce inulin. They named modified sugar beet, sugarcane and rice as potential candidates for production of inulin, with possibilities to control certain characteristics (e.g. chain length) of the produced inulin by selectively controlling the expression of specific synthesizing enzymes.

USES

Inulin is widely applied in the food industry and it serves many purposes. It has been used as a (low calorie) sweetener, to form gels, to increase viscosity, to improve organoleptic properties, and as a non-digestible fiber. Mostly it is used as a sugar and fat replacer in dairy products and as a prebiotic. Examples of use in dairy are application in cheese, milk, yogurt and ice cream.[66] Some examples of use of inulin in non-dairy food are use in bread, biscuits, cereal and meat products.[67–70] Previous reports have already extensively reviewed the food applications of inulin,[9,12–14,30,39,66,71] as well as its prebiotic effects.[12,13,15,72,73]

Applications of inulin as pharmaceutical excipient are even more diverse and range from stabilization of protein-based pharmaceuticals,[74] through solid dispersions to increases dissolution rate,[75] to targeted colon delivery.[16] Moreover, as mentioned earlier, inulin itself is used as a diagnostic tool for measuring the kidney function (glomerular filtration rate).[10,11] Inulin is injected intravenously, after which it is excreted renally. As inulin is neither naturally present in nor metabolized in the circulation, the amount of inulin secreted in the urine provides information on kidney function. Less widespread is the use of inulin for industrial and chemical purposes. Stevens *et al.*[76] reviewed the derivatization of inulin and applications of these chemically modified inulins for a wide range of applications, from inhibiting calcium carbonate crystallization industrially to use in hair gel.

The section *Physicochemical characteristics* will address the physicochemical characteristics of inulin. These characteristics are what make inulin such a versatile substance. For example, inulin is used in food as a texture modifier and fat replacer because of its DP-dependent gel forming and viscous behavior (see section *Rheology*). The (2→1) glycosidic bonds of inulin make it indigestible to humans and it can therefore be used as a low-calorie sweetener, fat replacer and dietary fiber.[30] Colonic microorganisms such as lactobacilli, however, are capable of breaking down this bond, making inulin suitable for colonic targeting. The relatively high glass transition temperature of amorphous inulin (section *Thermal characteristics and physical stability)* in combination with its flexible backbone makes it a good stabilizer of proteins applied both pharmaceutically[77] and in food.[78] Lastly, specific crystalline morphologies (section *Morphology*) make inulin suitable as an adjuvant for vaccines.[79]

PHYSICOCHEMICAL CHARACTERISTICS

CHAIN LENGTH

As mentioned in the introduction the DP of inulin determines to a substantial extent its physicochemical characteristics. Table 3.2 provides an overview of the reported DPs of different types of inulin to serve as a frame of reference. It is, however, to be noted that the degree of polymerization alone oversimplifies reality, as it does not take into account the distribution of the different fractions. Also, in many cases no distinction is made between the DPw and DPn (thus nor between the weight and number based molecular weights (Mw and Mn)), which are only identical when the material is monodisperse. Where a degree of polymerization without further specification was reported, it was assumed to be the number based variety. For inulin the DPn can be converted into the average molar mass using equation 3.1. Similar can be done for DPw by substituting DPn by DPw and Mn by Mw. Table 3.2 contains reported DP and molecular weight values of inulin from various sources as reported in literature, it was not completed with calculated values for clarity purposes.

(3.1) $$Mn = 180 + 162 \times (DPn-1)$$

Wada *et al*.[55] reported that the main difference between the inulin they synthesized enzymatically and plant-derived inulin was the polydispersity. Synthetic inulin had a lower polydispersity, which they illustrated with chromatograms from HPAEC with pulsed amperometric detection. Unfortunately, however, the polydispersity was not quantified.

MORPHOLOGY

Crystal morphology

Lis and Preston[84] patented the production of obloid and needle-like shaped crystals of inulin. The needle-like crystals were 1-20 μm in length with the other axes being 10-30% of that. The obloid crystals were of the same length, yet the other axes were sized at 50-80% of the length. The different types of crystals were produced by cooling an aqueous liquid containing 10-50% of Fibruline Instant (DP 6-12). The crystal transition temperature of the two crystals was approximately 75-95 °C. If the solution was cooled form a temperature higher than the crystal transition temperature obloid crystals would be produced, if lower (given all inulin was previously dissolved) needle-like crystals were obtained.[84] It was argued that the mouth feel of the obloid shaped crystals is better than that of the needle shaped crystals. Viscosity could be altered by varying the ratio and sizes of the two types of crystals. Needle-like crystals predominately increased viscosity while obloid ones improved lubricity.

Table 3.2 Overview of size and origin of different inulins.

Manufacturer	Product name	Source	DP	Molecular weight	DPw/DPn	Article cited
Orafti	Raftilose P95	Chicory	DPn 4-5	Mn 624-679		[80,81]
	Raftiline ST	Chicory	DPn 10-12	Mn 1250		[80,82]
	Raftiline HP	Chicory	DPn 21-26, DPw 31	Mn 2499		[19,55,82,83]
	RS	Chicory	DPn 14.2; DPw 19.4		1.13	[74]
Cosucra	Fibrulose F97	Chicory	DPn 5.5			[81]
	Fibruline Instant	Chicory	DPn 9			[81,84]
	Fibruline LCHT	Chicory	DPn 20-22, DPw 26.4		1.3	[37,81]
	Fibruline XL	Chicory	DPn 20-23, DPw 27-30			[19,50,85]
Imperial Sensus	SC 95	Chicory	DPn 5.5, DPw 6.0		1.09	[74]
	Frutafit CLR	Chicory	DPn 7-9			[86]
	Frutafit	Chicory	DPn 9	Mn 832		[82]
	Frutafit IQ	Chicory	DPn 8-12			[86,87]
	Frutafit Tex!, EXL	Chicory	DPn ≥23, DPw 26.2		1.3	[74,86]
Sigma	Inulin	Chicory	DPn 25	Mn 4450, Mw 4620-6200		[55,80,88–90]
	Inulin	Jerusalem Artichoke	DPn 29	Mw 3400 ±150		[55,89]
	Inulin	Dahlia	DPn 26- 35			[55,88,91]
N.C.P.*	n/a	Jerusalem Artichoke		Mw 7200 ±100 Mn 6100 ±500	1.18	[92]
N.C.P.*	n/a	Jerusalem Artichoke	DPn 28-33	Mn 4900-5600 ±500		[41]
Beghin-Meiji	Actilight 950P	*Aspergillus niger*	DPn 3	Mn 579		[80,81]
N.C.P.*	n/a	*Bacillus sp. 217C-1*	DPn 16-18			[55]
N.C.P.*	n/a	Globe artichoke	DPn 80			[17]
N.C.P.*	n/a	*Aspergillus sydowi*		Mw 1.49 × 10^4-5.29 × 10^6	1.13-3.01	[93]
N.C.P.*	n/a	*Aspergillus sydowi*		Mw 26-28 × 10^6	1.7	[35]
N.C.P.*	n/a	synthetic FTF *Streptococcus mutans*		Mw 30-90 × 10^6	1.1	[35,56]

* N.C.P. = non-commercial product, purified or produced by the authors, n/a = does not apply

Hébette *et al*.[83] investigated the influence of cooling rate, molecular weight, concentration, and storage time on the crystallization of inulin using Raftiline ST (DP 10-12) and fractions thereof. The crystallization produced obloid, or more accurately eight-shaped, crystals which were 5-20 μm in size if they started forming at a high temperature (77 °C) and up to a tenfold smaller if they were formed at lower temperatures (65 °C). The thickness and perfection of the formed crystalline lamellae was inversely related to the amount of undercooling. By small angle X-ray scattering (SAXS), they found that the crystal structure was the same as the monohydrate form reported by André *et al.*[94] (see below). The periodicity of the crystals produced at higher temperatures was 110 Å and at lower temperatures 90 Å.

Crystal structure

Marchessault *et al.*[95] investigated the three-dimensional crystal structure of inulin. They reported it to have a 5-fold helix, being either left- or right-handed with a space of 2.16 Å per monomer and thus 10.8 Å per loop. Reported bond angles were Ψ130 °, φ75 ° and ω =60 ° (right-handed) or ω =180° (left-handed), see figure 3.2 for an illustration of which bond-angles are described. Large differences in crystal structure were shown between polyethylene glycol and inulin, which were explained by steric interactions between the substituents and the exo-anomeric effect.[95]

André *et al.*[94] claimed Marchessault's findings of an unusual 5-fold helix to be based on limited data and in fact incorrect and that the crystals they produced actually contained a 6-fold helix. They reported the formation an orthorhombic hemi-hydrate crystal with dimensions of a=16.70 Å, b= 9.65 Å, c=14.4 Å per 6 units and a pseudo-hexagonal monohydrate crystal with a=16.70 Å, b=9.80 Å, c=14.7 Å per loop. The hemi-hydrate contained one water molecule per two fructosyl residues while the mono-hydrate had one per fructosyl residue. The helical conformation of the hemi-hydrate was characterized by φ = 66 °, ψ = 154 °, and ω = -82 ° and the monohydrate's dimensions were very similar with the following bond angles φ = 68 °, ψ = 159 °, and ω =-87 °. André thus concluded that the progress per loop was 14.4 or 14.7 Å as opposed to 10.8 Å.[94,96] It should however be noted that the methods used to produce the crystals by André and Marchessault were not identical and the inulin used was not characterized apart from crystal structure. As described in section *Crystal morphology*, the method of production is of influence on the morphology of the produced crystals and thus it is possible that different isoforms might have been produced. Further down several isoforms of inulin monohydrate will be discussed based on classifications of solubility and size.

Structure in solution

French[32] calculated the theoretically allowed conformations for inulin in solution and concluded that the allowed conformations were similar to those of dextran. Of course the reported conformations are merely the allowed conformations based on specific assumptions, French also noted that there are a lot of factors influencing the favored structure of oligosaccharides. Vereyken *et al.*[91] also found many possible conformations for inulin in their models, including a zigzag conformation with the ω angle at 180° which stayed stable in their simulations. This multitude of possible conformations shows the molecular flexibility of inulin. Several reports have described the behavior of a broad range of inulins in solution. Models and measurements by Oka *et al.*[97] and Liu *et al.*[98] indicate that a helical conformation is possible for oligofructose of DP 5. This conformation would however not be possible for higher molecular weight inulins due to steric hindrance. Liu *et al.*[98] reported that for inulins sized up to DP 9 simple helical structures are not the predominant structure and Oka *et al.*[97] found that for a DP of 8 and higher the backbone would reach a more rigid conformation. It thus seems that an organized three-dimensional structure does not occur for oligosaccharides with a DP smaller than about 8 or 9.

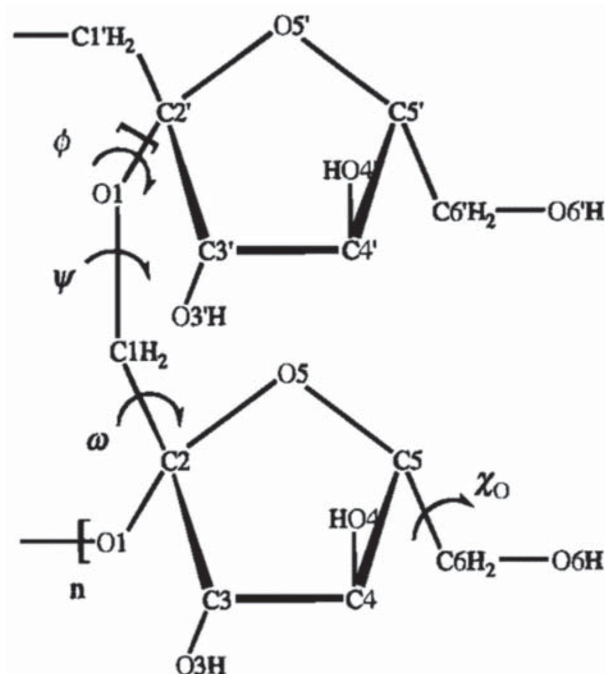

Figure 3.2 Representation of the atomic labeling scheme for the inulin chain. Reprinted with permission from André *et al.*[88]. Copyright 1996 American Chemical Society.

SOLUBILITY

Wada *et al.*[55] investigated the aqueous solubility at various temperatures of three different types of inulin, two Raftiline inulins which differed in size and an enzymatically produced synthetic inulin. Their results are depicted in figure 3.3, raftiline HP (DPn 23-25) displays lowest solubility, followed by Raftiline ST (DPn 10-12). What is remarkable, however, is that the enzymatically produced synthetic inulin (DPn 16-18) had a higher solubility than raftiline ST despite its higher DP. Normally the solubility of polymers decreases with increasing DP. As mentioned, the average DP of a polymer only tells part of the story and it is also relevant to consider the molecular weight distribution of the different DP fractions. The reader is directed to the cited article for molecular weight profile chromatograms of these inulins. The absence of highly polymerized fractions (no fraction with a DP larger than 30) in the enzymatically produced synthetic inulin could explain the higher solubility of the synthetic inulin.[55] Unfortunately, the method by which solubility was established was not described. Kim *et al*.[38] also investigated the solubility of Raftiline HP over a temperature range and also found a low solubility up to 50 °C from where on the solubility drastically increased until 35% at 90 °C. Reported aqueous solubilities of some other inulines are listed in table 3.3.
Bot *et al*.[101] reported hazing when dissolving Raftiline ST inulin in water. This was presumably the result of a small, high-DP crystalline fraction of inulin which did not dissolve readily. It was found that this fraction did not dissolve at room temperature, but typically would do so at temperatures of 60°C and higher.
Cooper *et al.*[102,103] initially identified four polymorphs of crystalline inulin (α, ß , γ and δ) based on their dissolution behavior. ß inulin, which was produced by addition of ethanol or by freeze-thawing, is readily soluble in water at room temperature. The other polymorphs, which could be interconverted into more stable versions (in the order ß, α, γ to δ), required

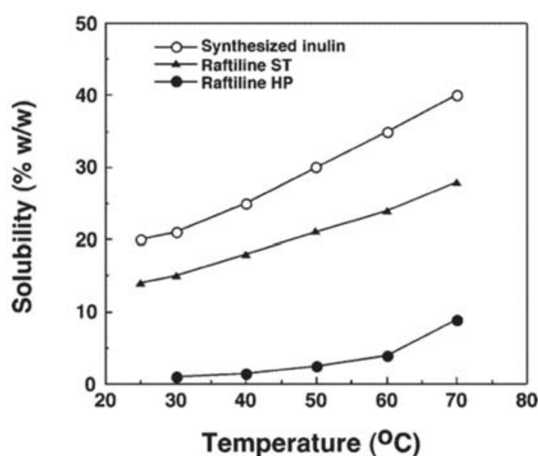

Figure 3.3
Differences of aqueous solubility between plant-origin (DPn10-12 and 23-25) and enzymatically synthesized inulin (DP 16-18). Reprinted with permission from Wada *et al.*[55]. Copyright 2005 American Chemical Society.

Table 3.3 Aqueous solubilities of different sizes of inulin at various temperatures

DPn or Mw (g/mol)	Solubility	Temperature (°C)	Article cited
4	>75% (w/v)	25	39
12	12% (w/v)	25	39
25	2.5% (w/v)	25	39
4468 g/mol	~10% (w/w)	30	99
8-12	17.4% (w/w)	37	100

higher temperatures to dissolve. The γ polymorph was made up only out of inulin with a molecular weight > 8000 g/mol, where the α and ß forms also contained lower molecular weight inulin fractions.[102] More recently the list of polymorphs was expanded to seven plus the amorphous form.[104] All the polymorphs, which differed in chain length, were monohydrate inulin crystals described earlier.[94,105] The monohydrate and hemi-hydrate only differ in the amount of water associated to the inulin, not in their crystal structure.[96,106] As suggested by André *et al.*[94], the fructose units of inulin formed helices with a 6-unit repeat. Cooper *et al.*[105] found that the different polymorphs increased in size by steps of 6 fructose units and concluded that these units formed additional helical turns. Surprisingly, these polymorphs were characterized by a degree of polymerization of 6n+1, rather than 6n. This additional fructosyl residue was shown to be able to link to glucose of another molecule through hydrogen bonding, allowing formation of tertiary structures of inulin.[107]
Ronkart *et al.*[50] found that increasing the feed temperature during spray drying reduced crystallinity and increased the Tg of the produced samples. As a higher Tg is correlated with a higher molecular weight (see section *Glass transition temperature (Tg)*), this too indicates that the crystals that dissolve at higher temperatures are made up out of higher molecular weight inulins.
In summary, inulin is poorly soluble in water, with decreasing solubility for higher molecular weight fractions. Solubility increases at higher temperatures for all different inulins. These characteristics enable a controlled production of several isomorphs, allowing modification of product characteristics such as rheology. Glibowski[108] however reported difficulties in controlling inulin crystallization.
Inulin is hardly soluble in ethanol,[87] explaining the use of ethanol in precipitating inulin,[102] it is freely soluble in dimethyl sulfoxide (DMSO) and very poorly to sparingly soluble in isopropanol.[99,90,89,109] Phelps *et al.*[110] reported that crystals produced using ethanol-recrystallization contained more low DP inulin compared to water-recrystallized samples. Considering that ethanol reduces the solubility of inulin so drastically, one would indeed expect that lower DP fractions of inulin are also affected and separate from solution.

RHEOLOGY

Viscosity

Multiple reports have appeared on the intrinsic viscosity of several inulins in different media, the results of which have been summarized in table 3.4. The intrinsic viscosity decreases by addition of salts and increases with increasing DMSO concentration and molecular weight. The dynamic viscosity of several types of inulin at specific concentrations and temperatures has also been reported, an overview can be found in table 3.5.

Like table 3.4, table 3.5 also shows an increase in viscosity with increasing molecular weight. With increasing temperature, the viscosity is reduced. Wada *et al.*[55] reported a slightly lower viscosity for enzymatically produced synthetic inulin (DPn 16-18) than for two commercial Raftiline samples (ST with a DPn of 10-12 and HP with a DPn of 23-25) despite the fact that it has a higher molecular weight than Raftiline ST. However, as explained in section *Solubility* the average molecular weight does not provide information about the size distribution. The enzymatically produced synthetic inulin lacks highly polymerized fractions, which could be an explanation for this difference in viscosity. *Wada et al*. [55] only presented the viscosity data graphically and they were thus not added to table 3.5.

Hydrodynamic shape

The Mark-Houwink equation (equation 3.2) defines the relationship between intrinsic viscosity ([η]) and molecular weight (M) for polymers, with two constants (K and a).[109,35]

(3.2) $$[\eta] = K \times M^{a}$$

The constant a in this equation is indicative for the shape of the polymer in the solution. The a-value for compact spheres is 0, whereas an a-value below 0.5 indicates branched structures, an a value between 0.5-0.9 is associated with a random coil, and an a-value over 2.0 with a rod structure.[35] Intermediate a values represent intermediate shapes.

The plots in figure 3.4 from the publication of Wolff *et al.*[35] show linear correlations between Mw and intrinsic viscosities for inulin species with a Mw > 5.0×10^4 and for species with a Mw < 5.0×10^4. They found that a = 0.71 for the ‘small’ inulins, showing a random coil structure and a=0.02 for the high molecular weights, indicative of a compact sphere. Remarkably these results are similar to those reported for levan, which does not have a polyethylene glycol-like flexible backbone. Apparently these bacterially produced fructans have similar characteristics, despite differences in their backbone structure, branching may explain the found similarities.[35] In addition, it should be noted that levan is still quite flexible compared to other polysaccharides like amylose, as it is linked via the C6 carbon (a primary alcohol) and not directly to the ring.

Table 3.4 Intrinsic viscosity ([η]) of inulin in several media at various temperatures (T)

Medium	[η] (mL/g)	Kh (-)	T(°C)	Mw (g/mol)	Source (manufacturer)	Article cited
Water	4.92	1.13	30	4450	Chicory root (Sigma)	[90]
Water	4.49	1.10	30	4478	Chicory root (Sigma)	[109]
Water	5.85	n.r.	25	1.49×10^4	*A. sydowi*	[93]
Water	6.97	n.r.	25	1.87×10^4	*A. sydowi*	[93]
Water	8.26	n.r.	25	2.38×10^4	*A. sydowi*	[93]
Water	10.5	n.r.	25	3.37×10^4	*A. sydowi*	[93]
Water	12.8	n.r.	25	7.52×10^4	*A. sydowi*	[93]
Water	16.3	n.r.	25	16.6×10^4	*A. sydowi*	[93]
Water	16.5	n.r.	25	60.4×10^4	*A. sydowi*	[93]
Water	16.5	n.r.	25	97.4×10^4	*A. sydowi*	[93]
Water	18.6	n.r.	25	178×10^4	*A. sydowi*	[93]
Water	19.1	n.r.	25	529×10^4	*A. sydowi*	[93]
Water	18	n.r.	25	54×10^6	FTF from *S. Mutans*	[35]
Water:DMSO(3:1)	5.86	2.12	30	4450	Chicory root (Sigma)	[90]
Water:DMSO(2:1)	6.63	1.50	30	4450	Chicory root (Sigma)	[90]
Water:DMSO(1:1)	7.96	1.27	30	4450	Chicory root (Sigma)	[90]
Water:DMSO(1:2)	11.0	1.09	30	4450	Chicory root (Sigma)	[90]
Water:DMSO(1:6)	14.9	1.75	30	4450	Chicory root (Sigma)	[90]
DMSO	18.8	1.30	30	4450	Chicory root (Sigma)	[90]
DMSO	15.2	0.48	30	4478	Chicory root (Sigma)	[109]
DMSO	9.1±0.2	n.r.	25	3400 ±150	Jerusalem artichoke (Sigma)	[89]
DMSO	10.7±0.2	n.r.	25	6200 ±200	Chicory root (Sigma)	[89]
0.5M NH_4SCN (in water)	3.65	2.40	30	4478	Chicory root (Sigma)	[109]
0.5M NaCl (in water)	4.30	2.16	30	4478	Chicory root (Sigma)	[109]
0.5M Na_2SO_4 (in water)	4.21	2.24	30	4478	Chicory root (Sigma)	[109]

Kh = Huggins constant (if the Huggins formula was used to calculate the intrinsic viscosity), n.r. = not reported.

Table 3.5 Reported dynamic viscosities of several sizes of inulin in water.

Viscosity (mPa.s)	T (°C)	Concentration (%)	DPn	Article cited
<1.0	10	5	4	[39]
1.6	10	5	12	[39]
2.4	10	5	25	[39]
1.21±0.06	25	5	28	[41]
1.27±0.08	25	5	30*	[41]
1.29±0.09	25	5	30*	[41]
1.31±011	25	5	33	[41]
1.12	37	10	8-12	[100]

*Samples are from two different subspecies of Jerusalem artichoke.

Next to viscosity, static light scattering was also used to determine the influence of molecular weight on the radius of gyration of the bacterially produced inulins. Those results too indicated a compact globular shape for high MW inulin, but more importantly showed that there might be a difference in branching architecture for inulins of different origins.[35] Using small angle x-ray scattering, Eigner *et al.*[92] showed that inulin from *Jerusalem Artichoke* with a Mw of 7200 had a rod-like formation in aqueous solution. This is not consistent with the above mentioned conclusions for bacterially produced inulins. The most likely explanations for this are the enormous difference in molecular weight between bacterially produced and natural inulin (see table 3.2) combined with the amount of branching of the bacterially produced inulins and the lack thereof in natural inulins.
Azis *et al*.[89] investigated characteristics of inulin extracted from Jerusalem artichoke and Chicory root (Mw 3400 ±150 and 6200 ±200, respectively) in DMSO. They differed significantly in size, but a lot less in intrinsic viscosity, indicating a conformation between a random coil and a compact sphere in that solvent. Naskar *et al.*[90] concluded that inulin forms globular aggregates in aqueous solutions and rod-like or spindle-like assemblies in DMSO. In summary hydrodynamic shape and behavior of inulin are influenced by molecular weight, solvent and branching (depending on the inulin source).
De Gennaro *et al.*[80] investigated the hydrodynamic behavior of several inulins (ranging from oligofructose with Mn 579 to inulin with Mn 4620) by looking at apparent specific volume (ASV), isentropic apparent specific compressibility [$K_{2(s)}$] and spin-lattice relaxation times (T_1). ASV, a measure of hydrostatic packing with water molecules, was found to increase with degree of polymerization, indicating that low DP inulin had better hydrostatic packing and interacted with water more. Isentropic compressibility values can be interpreted as a measure for the compatibility between water and inulin. $K_{2(s)}$ increased with DP and concentration, showing reduced solute-water affinity. Inulin was found to be more water

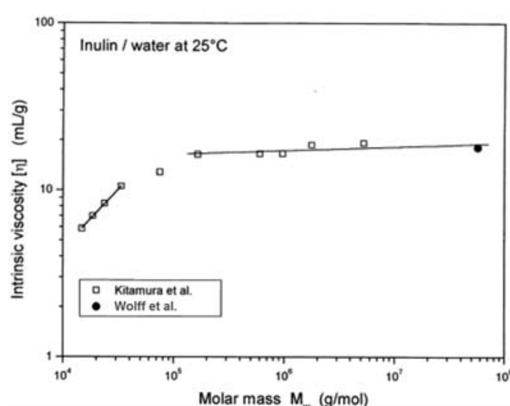

Figure 3.4
Molar mass dependence of intrinsic viscosity for high Mw bacterially produced inulin, data from Kitamura *et al.*[93] and Wolff *et al.*[35] Lines represent the linear regression of the Mark-Houwink equation (equation 3.2).[93,35]

compatible than other tested carbohydrates except at high concentrations (>15% (w/w)) and/or for a DP of 9 or higher. In the light of the discussion above the latter could mean that the formation of three-dimensional helical structures reduces inulin's water compatibility. Lastly, due to an increased order of protons and reduced water mobility, T_1 values decreased with increasing Mn and concentration.[80]

Gelling

In general inulin gels are based on the interactions occurring between dissolved inulin chains. However, inulin gels may also still contain undissolved microcrystals. These microcrystals can be interconnected, forming a network that is able to interact with both the solvent and other inulin particles thereby increasing gel strength.[38,39,101,85,111] As described earlier, temperature and molecular weight influences the formation of microcrystals and thereby also gel formation. Based on this and their higher viscosity, high molecular weight inulins are better gel formers than their lower molecular weight counterparts. This also explains why hydrolysis, which reduces the degree of polymerization, reduces gel formation by disturbance of the network.[112] Using nuclear magnetic resonance spectroscopy, Van Duynhoven *et al.* [111] showed that lower inulin concentrations lead to lower concentrations of crystalline material. This results in a reduction in the network formation, explaining lower mechanical strength of the gel.

Inulin gels can be formed either thermally, through heating and cooling, or by applying shear forces.[38] Kim *et al.*[38,112] have investigated both methods of gel production extensively. Thermally produced gels were found to be stronger and smoother than shear induced ones. Gel production was dependent on temperature, heating time, concentration, pH and addition of other solvents. Addition of other solvents (ethanol or glycerol) reduced polarity of the solution causing less solvent-inulin interactions, resulting in faster gel formation but with similar gel strengths. The minimal concentration of inulin needed for gel formation differed with temperature. The solution needed to be heated up to at least 40 °C to achieve gelling. However, heating to temperatures of 80 °C and higher, and acidic conditions (pH < 3) lead to substantial hydrolysis of inulin, resulting in reduced gel formation.[38] In these studies, only Raftiline HP (DPn 23-25) was used, the influence of molecular weight was thus not taken into account. Meyer *et al.*[66] did investigate the influence of DP and concentration on gel strength. They found that higher molecular weight inulins produce stronger gels and are able to form gels at lower concentrations as can be seen in figure 3.5.[66]

Chiavaro *et al.*[113] specifically investigated the influence of DP on thermal gelation and found that by using inulin of different molecular weight gels could be produced with different characteristics due to a difference in balance between solid-solid and solid-liquid interactions. Using texture profile analysis, higher molecular weight inulins were found to form gels that were harder, more adhesive and less cohesive both after production and after storage at

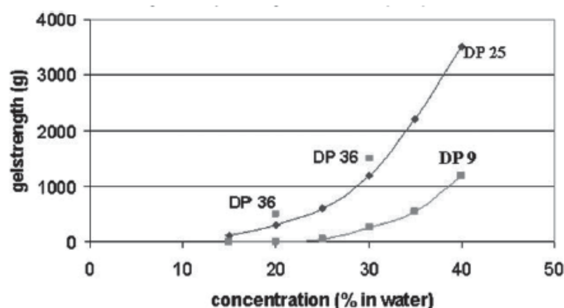

Figure 3.5
Gel strength in relation to concentration of different inulin types. The gels were prepared by heating the solutions at given concentrations to 85°C and allowing them to cool overnight at 4°C.[66]

4 °C for 4 weeks. This means that higher molecular weight gels required more force to be deformed, would stick to surfaces more and had weaker internal bonds between components.[114] The gels prepared from higher molecular weight inulin had more freezable water in comparison to gels prepared from low molecular weight inulin.[113] These observations were ascribed to an increase in inulin-inulin interactions and a decrease in inulin-water interaction with increasing molecular weight. As solid-solvent interactions were needed for storage stability, lower molecular weight inulin gels maintained their textural characteristics better during storage. Here too, the average DP does not tell the complete story and the polydispersity should be taken into account as well. It seems that for a stable gel a fraction of the inulin needs to be of high enough DP for micro-crystallization and solid-solid interactions to form a network, and another part needs to be smaller to interact with the solvent.[113] This is in line with the findings Glibowski *et al.*[115] that addition of low concentrations (≥0.02%) of seeding crystals allowed heated inulin solutions to form gels instead of precipitating during cooling. At a higher concentration of seeding crystals (≥0.4%) stronger and more stable gels were obtained.

Shear-induced gels were reported to become smoother when the applied shear stress was increased.[38] This is because low shear caused the formation of larger aggregates; at higher shear stresses a better dispersion was achieved. In comparison to thermally produced gels, shear gels contain larger particles with a broader particle size distribution and with that the gels have a reduced yield stress. Ronkart *et al.*[85] found that repeated application of high shear stress reduced particle size, facilitating the formation of a finer network of particles and textural modifications. In addition, the reduction in particle size might have resulted in more inulin dissolving, increasing viscosity and also modifying gel behavior. Bot *et al.*[101] investigated how several methods of crystallization influenced the large deformation rheology of inulin gels and found that shape and size of the produced crystals play an important role in the formed network and thus the texture of the produced gel.

Using high-pressure homogenization, Alvarez-Sabatel *et al.*[116] related gel characteristics to pressures used during this process. It is important to note here that the product temperature increases during processing and that this temperature increase is much larger for higher processing pressures. Caution should therefore be taken in relating processing pressures to gel characteristics directly, as this heating also influences the characteristics of the formed gel.[108] Nonetheless, by varying this pressure and therewith the product temperature, inulin gels with specific characteristics can be produced.

Gelling and texture modifying properties of inulin in more complex systems have been reported. Some reports suggest that inulin has a synergistic effect on gelation with other gelling agents (e.g. gelatin, alginate, maltodextrins and starch) and proteins whilst others actually report inulin competing with them.[39,66,71,117,86,118] It seems that for some excipients a competition for water occurs whilst with others a combined network is formed, but it goes beyond the scope of this review to discuss this behavior in detail here.

Lastly, several reports described the synthesis and behavior of (meth)acrylated inulin gels for controlled release of drugs in the colon.[119–127] Gels of these chemically modified inulins were produced by formation of covalent cross-links between the added side-chains using free radical polymerization. In terms of rheological behavior, a higher degree of substitution resulted in a faster gelation process and higher rigidity of the obtained gels for methylacrylated inulin due to more inter-molecular crosslinking.[124] Different cross-linkers were investigated and found to modify rate of crosslinking and elasticity of produced gels differently, allowing for control of mechanical properties of these gels.[119] Controlling the amount of swelling of the hydrogels is critical. High swelling of the gel is needed to allow degradation in the colon by bacteria,[122] however, to prevent premature drug release before the colonic environment is reached, low swelling is key.[125] Recently, chemically crosslinking of inulin molecules using divinyl sulfone was used to produce microgels intended for controlled release in the stomach.[128]

THERMAL CHARACTERISTICS AND PHYSICAL STABILITY

Glass transition temperature (Tg)

Most commercially available types of inulin are amorphous and can thus be characterized by a glass transition temperature (Tg). Above the glass transition temperature molecular mobility is strongly increased and crystallization can occur. Molecular weight influences the Tg of anhydrous carbohydrates and the Tg of the maximally freeze concentrated fraction (Tg’) of carbohydrates.

The Tg’ is of interest when freeze-drying is used as a production process. The Tg’ should not be surpassed during the first part of freeze-drying (primary drying) in order to achieve an amorphous product. The Fox-Flory equation (equation 3.3) describes the relationship between Tg and molecular weight.[129]

(3.3) $$T_g = T_{g,\infty} - \frac{C}{M}$$

With Tg,∞ being the Tg at infinite molecular weight, M molecular weight, and C a constant. The Tg,∞ and constant C were calculated for inulin using data of Hinrichs *et al.*[74] and unpublished data. The maximal Tg (Tg,∞) was 175 °C, with a fitting constant of 75 kDa. The maximal Tg' (Tg,∞) was -14 °C with a fitting constant of 11.3 kDa. Compared to smaller carbohydrates like sucrose and fructose, inulin has a much higher Tg. At similar weights glucans have even higher Tg values. For the Tg' values the same trends apply.[130]
Water acts as a plasticizer on amorphous carbohydrate samples, meaning it decreases the Tg. The Gordon-Taylor equation (equation 3.4) describes Tg of an ideal mixture of two amorphous components, in this case a mixture of water and inulin. Water has a very low Tg of approximately 165K, explaining why even small amounts strongly decrease the Tg.[131,132]

(3.4) $$T_{g,\,mix} = \frac{f_a * T_{g,a} + K * f_b * T_{g,b}}{f_a + K * f_b}$$

f_x is the weight fraction of component x (with x being either a or b), and K is usually considered as a fitting parameter.[133]
Several papers have reported measurements of the influence of the water content on the Tg of inulin. Figure 3.6 shows the results of water uptake of up to 12% on the Tg of inulins of various molecular weights. The Gordon-Taylor equation was used to fit the curves. For all inulins, a water content of just 2% decreased the Tg with around 30 K and at a moisture content of 10% the Tg of the mixture had gone down by nearly 100 K.

Vapor sorption

Knowing that water can strongly reduce the Tg of a mixture, it is important to determine the water sorption of inulin in relation to relative humidity in the atmosphere. Using dynamic vapor sorption, water uptake of several inulins and trehalose was studied as a function of relative humidity (RH).[74] Water sorption was similar for all sizes of inulin and was similar to that of other amorphous carbohydrates. Trehalose crystallized at a RH above 50%, whereas the inulin samples remained amorphous on the timescale of the dynamic vapor sorption experiments (hours), even though they all surpassed their Tg during the measurement. This shows that inulin crystallizes less easily than trehalose. Incidental (short term) exposure to high relative humidity of amorphous inulin does therefore not necessarily lead to immediate crystallization. In two other studies where inulin was stored at controlled relative humidities for weeks, crystallization was found.[82,134]
Ronkart *et al.*[51–53] described the consequences of moisture sorption for inulin samples with different degrees of crystallinity. Depending on the molecular weight of the inulin, the

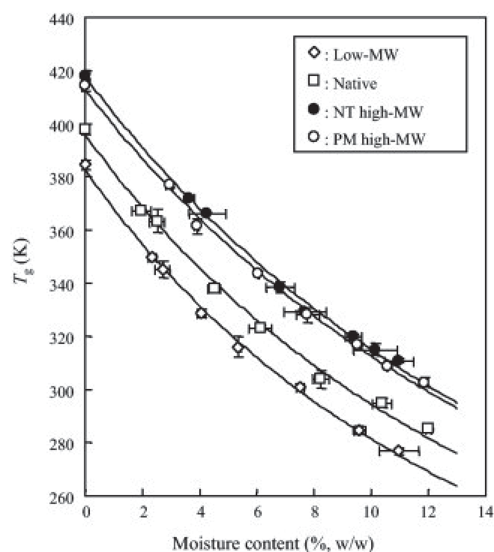

Figure 3.6
Effect of moisture content on T_g of several inulin samples. The low molecular weight (low-MW), native and high molecular weight (high-MW) samples had degrees of polymerization of respectively 7, 13 and 27. PM denotes pre-melted, meaning the sample had been heated in solution, quench-cooled and subsequently freeze-dried to make the sample completely amorphous, NT denotes not treated.[130]

amorphous particles fused at RH of >56%[51] or at RH over >75% at 20 °C [53] (corresponding to a water uptake of 12-15 g/100g dry inulin at >75% RH). This lead to caking, i.e. sticking together of the powder particles resulting in reduced flowability. The presence of crystals in the amorphous matrix limited the caking.[53] This behavior is not uncommon for polysaccharides.

They then defined three regions based on water uptake and crystallinity at 20 °C, as shown in figure 3.7.[52] In region I inulin remained completely amorphous, in region III inulin was completely crystallized (and caked). Region II represents an intermediate region where inulin's macroscopic and thermal properties were changing. In region I the Tg of the samples was at least 10 °C above storage temperature, in region III the Tg was room temperature or lower. This shows that if the Tg drops below storage temperature +10 °C, mobility will increase and lead to crystallization and caking, which is nearly always undesirable. Therefore, storage conditions should be carefully chosen and exposure to high relative humidities and temperatures should be avoided.

Similarly, Schaller-Povolny *et al.*[82] defined a critical moisture content (and corresponding critical relative humidity) based on macroscopical changes to inulin morphology, above which inulin would be crystalline. These large macroscopical changes are only truly apparent when crystallization is widespread and are therefore not a good measure for determination of a critical moisture content.[52] The study does however show that inulins of different molecular weight pass through this critical point at different amounts of water uptake. Inulins with a higher molecular weight can withstand more water uptake before they reach

the critical point and thus be stored at higher RH. Higher molecular weight inulins may therefore be used to improve processability and storage stability in food or other products.[82]

Melting temperature

Melting temperatures of fractions of Fibruline LCHT with different degrees of polymerization were determined and are shown in figure 3.8.[37] Two groups with different degree of crystallinity could be distinguished. The higher DP fractions were insoluble in water (obtained by precipitation in aqueous solutions at various temperatures), while the low DP fractions were produced by freeze-drying water soluble fractions.[37] Low DP fractions had a lower melting enthalpy, which is indicative for crystallinity, of 7-9 J/g and the higher fractions 17-19 J/g.[37] Even higher melting enthalpies ranging up to 47.6 J/g have also been reported.[134] Melting temperatures reported elsewhere were similar to the ones shown in figure 3.8, with melting temperatures being reported between 165 and 183 °C.[41,56,109,134] The melting temperature of a enzymatically produced synthetic inulin as determined by Heyer *et al.*[56] was only 183 °C despite its much larger size (70 × 10^6 g/mol), which is common for polymers.[135] Inulin started degrading after melting, when heated above 200-225 °C.[56,106,109] The hemi-hydrate of inulin (produced by water sorption of amorphous inulin) had a melting temperature of around 155-160 °C and the mono-hydrate (seeding crystals) had a melting point between 170-180 °C.[106] Similar melting temperatures were reported for the different monohydrate polymorphs described in section *Solubility*, which differed from each other in molecular weight.[104] It is therefore likely that the two different fractions shows in figure 3.8 are mono-hydrate and hemihydrate forms of inulin.

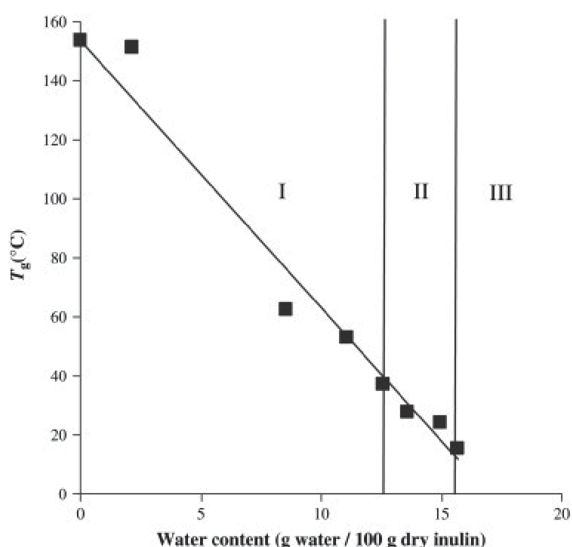

Figure 3.7
Glass transition temperature-water content relationship for inulin DPn 23/ DPw 30 with three regions of different crystallinity.[52]

CHEMICAL STABILITY

Inulin with a glucose end group does not have or form any reactive aldehyde or ketone groups and is therefore non-reducing. However, inulin molecules lacking this glucose end group, thus ending with a fructose group, are reducing.[136] Furthermore, as discussed previously, inulin is a polydisperse mixture and can also contain mono- and disaccharides which are more reactive. These inulins without glucose end group can thus take part in reactions with other components, such as the amino group of proteins in the Maillard reaction. In the light of the above, it could be useful to distinguish between inulin with and without glucose end groups. If reducing groups are present and the Maillard could potentially occur, formulation modifications such as the addition of sulfite, or adjusting the pH could be used to reduce the risk of the Maillard reaction occurring.[137]

Several reports discussed the amount of reducing groups of inulin, some supplied more details than others.[74,76,80] Stevens *et al.*[76] found a residual reducing activity of 0.5-2.5% after removal of mono- and disaccharides from 'native inulin'. Hinrichs *et al*.[74] found that the percentage of carbohydrate units containing reducing groups was much higher for small inulins in comparison to larger inulins. Oligofructose synthesized from sucrose had fewer reducing groups than oligofructose produced by hydrolysis of inulin.[80] Hydrolyzed inulin will contain fructose chains both with and without glucose end group, whereas inulin synthesized from glucose only contains fructose chains with a glucose end group. The relative abundance of fructose chains without glucose can explain the difference in amount of reducing groups between these two production methods.

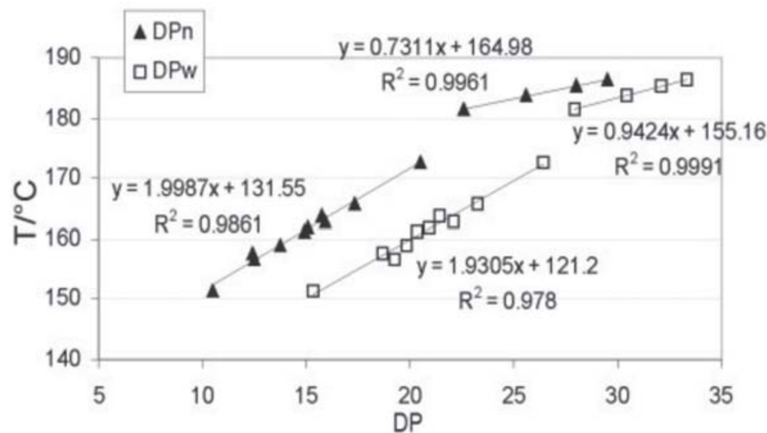

Figure 3.8 Relations between degree of polymerization (DP) and inulin's melting temperature.[37]

Influence of several processing parameters on the amount of reducing groups of inulin were reported.[38,112] Reducing sugar content of aqueous inulin solutions increased with increasing temperature and with lower pH due to hydrolysis of inulin.[38] At neutral pH, the percentage of reducing groups increased from <0.1% to only 1.2% after heating a concentrated solution to 100 °C for 5 minutes. At pH values of 3 or lower the amount of reducing sugars formed increased drastically, up to 25% at pH 1.[38] Reducing groups were formed as a result of hydrolysis, which followed pseudo first-order kinetics with reducing activity increasing continuously over time during heating.[112] Since hydrolysis was the cause of the increase in reducing activity, it was indirectly indicative of a reduction of DP. This is because hydrolysis cleaves the end fructosyl group of inulin, reducing its DP. Which, as explained above, in turn influences several other characteristics of inulin.[38]
For oligofructose, the influence of various processing parameters on hydrolysis have also been reported.[138,139,81,140,141] Hydrolysis of oligofructose also follows pseudo first-order kinetics.[139,81,140] Little hydrolysis was found up to 60 °C, this changed at 70 °C and above.[138] Hydrolysis mainly took place at acidic rather than neutral or alkaline conditions, where low molecular weight oligofructose reacted faster than high molecular weight ones.[139] It was also found that fructose was produced at a higher rate than glucose.[140] Sucrose, containing only a (1↔2) linked β-D-glucosyl and β-D-fructosyl group, reacted more slowly than the oligofructose carbohydrates. Combined these results indicate that the terminal β-D-fructosyl-(2→1)-β-D-fructosyl glycosidic bond is most susceptible to acidic hydrolysis.[139,81,140] At lower degrees of polymerization this terminal bond is relatively more abundant and they thus have a lower chemical stability. At a pH of around 3, changes in pH of 0.3 units were found to have a large impact on hydrolysis.[138] At pH 2.7 and a temperature of 90-100 °C nearly complete degradation of oligofructose into monomers was achieved in 30-40 minutes.[138] At a pH ≥ 5, relevant for food applications, no degradation was found regardless of thermal processing (up to 100 °C for 55 minutes).[142]
Inulin and oligofructose thus show similar trends with respect to pH, temperature and molecular weight dependent hydrolysis.[81] The kinetics of the reactions are however different.[140] For higher molecular weight inulins, the rate of hydrolysis is initially low, but increases as hydrolysis progresses.[81] An explanation for this could be the amount of end-chain fructosyl groups. Initially, they are scarce, meaning hydrolysis of mid-chain glycosidic bonds will be more pronounced. Mid-chain hydrolysis in turn increases the amount of more reactive end chain fructosyl groups, resulting in an increase in reaction rate.[81] It was also suggested that the helical structure of inulin, or the lack thereof for oligosaccharides, influences their reaction rate and how those are influenced by temperature.[140]

OVERVIEW

Here the physicochemical characteristics of inulin, an oligosaccharide widely used in food and pharma, have been reviewed. The average DP of inulin is often used when describing the physicochemical properties such as solubility and thermal and rheological properties. This generally works well but can potentially also be misleading as the average DP only provides an average and does not provide information on the actual size distribution. Inulin consists of a mixture of polymers of different chain length, its physicochemical properties are to a great extent dependent on the size distribution of this mixture. This means that two different batches of inulin with the same average DP can have different size distributions and therewith different characteristics. Higher DP inulin fractions are less soluble in water, possess higher melting temperatures if crystalline or higher glass transition temperatures if amorphous, are chemically more stable (less sensitive to hydrolysis), form stronger gels and are more viscous when dissolved.

Inulin is used to modify texture or replace fat in food, its DP-sensitive gel forming and viscous behavior make it suitable for that purpose. Additionally, the (2→1) linked fructosyl residues of inulin are not hydrolyzed by the human digestive enzymes, enabling low-calorie replacement of fat. The partially hydrolyzed form of inulin, oligofructose (DP≤10), has this same feature and is more sweet, and is therefore used as a low-calorie sugar replacer. Their indigestibility makes both inulin and oligofructose suitable as dietary fibers. As microbiota in the colon are capable of breaking down inulin, it is also used as a prebiotic and to prepare gels for targeted drug release in the colon.

Inulin's backbone is relatively flexible compared to other polysaccharides, as it does not incorporate the sugar ring. This combined with a relatively high Tg makes inulin a suitable stabilizer of proteins in the dry state for both food and pharma applications. Some specific pharmaceutical applications are its use as a diagnostic agent for kidney function and as an adjuvant for vaccines. Again, the size distribution of the inulin is relevant for these applications. Therefore, regardless of its application both the average DP and the size distribution of inulin should be taken into account. Information on how the molecular weight of inulin and other factors affect its characteristics relevant for its various applications can be found in this chapter.

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Inulin, a flexible oligosaccharide II: Review of its pharmaceutical applications

4

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ABSTRACT

Inulin is a flexible oligosaccharide which has been used primarily in food for decades. Recently new applications in the pharmaceutical arena were described. In a previous review[1] we described the physicochemical characteristics of inulin, characteristics which make inulin a highly versatile substance. Here, we review its pharmaceutical applications. Applications of inulin that are addressed are stabilization of proteins, modified drug delivery (dissolution rate enhancement and drug targeting), and lastly physiological and disease-modifying effects of inulin. Further uses of inulin include colon specific drug administration and stabilizing and adjuvating vaccine formulations. Overall, the uses of inulin in the pharmaceutical area are very diverse and research is still continuing, particularly with chemically modified inulins. It is therefore likely that even more applications will be found for this flexible oligosaccharide.

INTRODUCTION

Inulin is a fructan-type oligosaccharide that can be found in a wide range of plants.[2] It was discovered in the 19th century[3] and since then its characteristics has been investigated extensively. Most commercially available inulin is extracted from chicory root, which contains a relatively high concentration of this carbohydrate.[4] Inulin has proven to be a versatile substance with a large number of different applications mainly in food and pharma. In a previous review, we addressed the physicochemical properties of inulin that make it such a widely applicable compound.[1] Processing history and degree of polymerization have a large impact on the physicochemical behavior of inulin. As physicochemical behavior governs functionality for different applications, these characteristics should be taken into account when using inulin.

Some examples of saccharides frequently used as excipient in pharma are glucose, sucrose, trehalose, lactose, dextran and cellulose.[1] Inulin differs from these saccharides in molecular weight and/or the type of glyosidic bond between monomers. Inulin has a higher molecular weight than mono- and disaccharides, with that it has a higher glass transition and melting temperature, and is more viscous when dissolved. The higher molecular weight also correlates with a lower solubility. Compared to other oligo- and polysaccharides, inulin has a high molecular flexibility because of its (2→1) linked-d-fructosyl backbone. Therefore inulin has relatively low glass transition and melting temperatures compared to other oligo- and polysaccharides. These characteristics can be both advantageous and disadvantageous depending on the application at hand. Unlike most of the other mentioned saccharides, inulin is not metabolized by humans. This allows for unique applications such as determination of kidney function and colonic targeting, which makes use of metabolization by microbiota present in the colon. Reducing groups of saccharides are undesired for many pharmaceutical applications. When presence of reducing groups is a concern, inulin is more suitable as an excipient than for example glucose and lactose.[1,5]

Over the past decades, more and more research on food and pharmaceutical applications of inulin has been published. The approval of the Generally Recognized As Safe (GRAS) status of inulin by the United States Food and Drug Administration in 2002 seems to have provided a boost to research into applications of inulin.[6] As inulin's food applications have been reviewed before we will not discuss them here.[1] To our knowledge, only one review has been published in which pharmaceutical applications of inulin are addressed amongst other topics.[7] In this chapter, an overview will be given of the current pharmaceutical applications of inulin in the light of its physicochemical characteristics. Applications which will be addressed are stabilization of proteins, modified drug delivery (dissolution rate enhancement and drug targeting), and physiological and disease-modifying effects of inulin.

PHARMACEUTICAL APPLICATIONS

STABILIZATION

Anhydrobiosis

In nature, inulin has been associated with drought protection in several plants, with increased levels of inulins found in stress resilient plants.[8,9] Membrane stabilization is believed to be an important part of the drought protection of inulin.[8] Vereyken *et al.*[10–13] have published several papers on the mechanism of stabilization of fructans on membranes. They investigated the effects of inulin on model systems, such as: phospholipid monolayers, bilayers and liposomes in states varying from dry to completely hydrated. Fructans (inulin- and levan-type) were found to increase lamellar repeat distance and reduce vesicle fusion during air drying, indicating they were present between the lipid bilayers.[11] Fructans insert between the headgroups of several glyco- and phospholipids even if the lipid packing is very tight.[10,12,13] Fructan immobilized the lipid headgroups through direct interaction, but increased mobility of the acyl chains because the insertion of fructan caused them to be spaced further apart, in turn leading to a lower order-disorder phase transition temperature.[10] Additionally, fructan reduced the accessibility of the membranes, indicative of some form of coating of the membrane.[13]

The effect of fructans on the membranes was much stronger than that of other polysaccharides such as dextran, which was explained by inulin's more hydrophobic character.[13] Inulin showed more interaction with the headgroups than levan-type fructan.[12] This is probably because inulin inserts more deeply into the membrane compared to levan.[10] Increasing molecular weight of inulin was found to correlate with stronger interactions with the membranes.[12] Levan-type fructans perform worse than inulin-type fructans despite the fact that they are much larger. Apparently, some other trait of inulin must be the reason for its success. The flexibility of inulin's molecular backbone, conformation of the backbone as well as hydrophobicity were suggested as explanations for inulin's effects on these membranes.[11,12] Additionally, inulin's furanose groups are also smaller and more flexible compared to pyranose groups found in dextrans.

Hincha *et al.*[8,14–16] also investigated membrane stabilization by oligosaccharides including inulin using liposomes as model systems. During freeze-drying inulin stabilized large unilamellar vesicles of egg phosphatidylcholine, whilst hydroxyethylstarsch (HES) did not stabilize them at all. A combination of inulin and glucose was especially effective.[15] Based on findings with raffinose-family oligosaccharides, it was concluded that a higher degree of polymerization (DP) provides increased protection of liposomes against fusion at elevated temperatures and against leakage after rehydration, because of the higher glass transition temperature (Tg) of longer chain oligosaccharides.[14] A larger chain length resulted in better membrane stability during air drying when inulin-type fructans were used, but not when

glucans were used.[16] Glucans were however increasingly effective at reducing membrane fusion with increasing chain length, contrary to inulin.[16] The limited solubility of larger inulin (DP >10) caused it to precipitate during the used slow air-drying and prevented it from stabilizing the liposomes.[8] In agreement with the findings of Vereyken *et al.* discussed above, Hincha *et al.* also found a direct interaction of inulin with the phospholipid in the dry state and a reduction of the gel to liquid-crystalline phase transition temperature.[15,16]
Livingston *et al.* (2007)[8] have provided an in-depth review on abiotic stress tolerance in plants by fructans. It shortly describes current, more general theories on how (oligo)saccharides stabilize membranes, mainly the vitrification theory and the water replacement theory. The water replacement theory explains how sugars stabilize membranes by replacing the hydrogen bonds of water with the membrane during drying and thereby inserting into the lipid bilayer.[10] Overall, it seems that inulin's characteristics, the structural flexibility of the backbone and furanose groups, conformation, and hydrophobic-hydrophilic balance allow it to interact with the membranes better than other oligosaccharides as described above. Inulin's flexibility in particular allows it to overcome the steric hindrance which usually inhibits sugar-membrane interactions.[8,11]
The vitrification theory states that sugars form glasses instead of crystals during drying. The viscosity of a glass is extremely high, resulting in practically no diffusion and restricted molecular mobility of the membranes. A kinetically stable glass is the result. The glass should remain amorphous, as crystallization would lead to a loss of protein-sugar interactions. Clearly, a higher Tg then correlates with better stabilization.[8] Therefore, vitrification alone cannot explain the stabilizing effect of fructan[17–19], as other oligosaccharides with even higher Tgs, such as dextran and hydroxyethyl starch, did not provide better stabilization. Both vitrification and water replacement are needed for stabilization.[19] Inulin is unique in its ability to vitrify as an oligosaccharide, whilst maintaining a good interaction with the membrane as if it was a smaller saccharide, thus combining requirements for both the vitrification and water replacement theories.

Protein stabilization

The mechanisms behind membrane stabilization in nature and protein stabilization in the pharmaceutical arena are very similar, with the vitrification theory and water-replacement theory also being applicable to protein stabilization.[19,20] Recently, the role of these two different mechanisms under different conditions was investigated.[21] It was concluded that if the Tg of a sample is at least 10-20 °C above storage temperature, water replacement is the predominant mechanism of stabilization. However if the Tg is lower, vitrification limits stability.[21] In other words, if the Tg is high enough to immobilize the protein, the amount of interaction between the sugar and the protein determines its stability, yet if the Tg is too low, the (lack of) immobilization becomes the limiting factor.

Other physico-chemical characteristics of sugars required for good stabilization are low hygroscopicity, low crystallization rate and little or no reducing groups. For freeze-drying a high Tg of the maximum freeze-concentrated solution (Tg’) is also desired. Compared to trehalose, which is a frequently used protein stabilizer, inulin has similar hygroscopicity, it crystalizes less rapidly and the longer chain inulins have higher Tg and Tg’ values.[1] Additionally, higher molecular weight inulin has fewer reducing groups, a higher glass transition temperature and with that a lower tendency to crystallize for example when exposed to ambient moisture. This being given, it is not surprising that inulin was shown to be a good stabilizer of proteins under various conditions and stresses. Inulin has been successfully used to stabilize proteins during spray-drying[21–23], freeze-drying (lyophilization)[5,24–28] and spray-freeze drying.[23,28,29] However, inulin does have some reducing groups, limiting its applicability to some extent.[30] The number of reducing groups is dependent on processing and DP and is generally limited.[1]

Inulin, trehalose and dextran have been identified as suitable cryo- and lyoprotectants for the major constituent of influenza subunit vaccine, i.e. haemagglutinin (HA), meaning they all stabilized HA during freezing and freeze-drying.[31] During storage of 4 different model proteins after lyophilization, inulin was a better protein stabilizer than a similar-sized dextran.[5] The storage temperature was well below the Tg of the formulations to allow for optimal vitrification. Water-replacement was therefore considered to be dominating protein stability. It was suggested that inulin encountered less steric hindrance than dextran when interacting with the protein, because of inulin’s higher molecular flexibility. Lyophilization of alkaline phosphatase without stabilizer reduced its activity to around 5%, but when either inulin, trehalose or glucose was added, full activity was maintained.[24] When added to bovine plasma protein, inulin also reduced denaturation during freeze-drying similar to sucrose and glucose, with an optimal concentration of 10% w/v.[26,27]

During spray-freeze-drying, spraying a solution into liquid nitrogen followed by freeze-drying, HA stabilized with inulin in hepes buffer was not structurally altered and it did not lose its antigenetic properties.[23,28] When PvdQ, acyl-homoserine-lactone (AHL) acylase, was spray-freeze-dried, mannitol, trehalose and inulin all fully maintained its activity.[29] Inulin has also been applied as a protein stabilizer during hot-melt extrusion, in which a polymer based product for the controlled release of protein is created. As lysozyme is a relatively thermostable protein[32], no degradation was found during extrusion at 55 °C. It was however suggested that for a more labile protein, inulin could also provide protection to the heat stress induced by this process.[33]

The main goal of the various processes described above is to achieve a product that is stable over time. Table 4.1 provides an overview of reports on inulin’s stabilizing effect on dried proteins during storage under various conditions. Tonnis *et al.*[5] reported degradation kinetics of their 4 model proteins during storage (60 °C, <10% RH, 28 days), but not the remaining

Table 4.1. Overview of reported storage stabilities of proteins dried with inulin and other sugars.

Protein	Protein: Sugar Ratio (w/w)	Prepa-ration	Storage conditions			Activity/Potency loss after storage		Article cited
			T (°C)	RH (%)	t (days)	Stabilizer	Loss *	
PvdQ	1:100	SFD	20	<10%	28	Inulin DPn 23; trehalose	None	29
						Mannitol	Some	
						Liquid control (after 7 days)	None	
AP	1:9	FD	20	0	28	inulin DPn 6**,14,23	None	24
						glucose, trehalose	None	
						No stabilizer	Complete	
AP	1:19	FD	20	0	105	Inulin DPn 23, trehalose	None	34
HA	1:47	FD	20	0	182	Inulin DPn 6, DPn 14	<20%	31
						Trehalose, dextran56kDa	<20%	
						No stabilizer	Complete	
HA	1:200	SD,SFD	20	10	1085	Inulin DPn 23	None	23
						Liquid control 4°C	Complete	
AP	1:9	FD	20	45	28	Inulin DPn 14,23; trehalose	No	24
						Inulin DPn 6	~25%	
						Glucose	~50%	
						No stabilizer	Complete	
AP	1:9	FD	20	60	28	Inulin DPn 14,23; trehalose	None	24
						inulin DPn 6; glucose	~50%	
						No stabilizer	Complete	
PstI	n.r.	AD	37	n.r.	7	Trehalose	none	35
						Maltotriose	80-90%	
						Inulin; dextran ***	complete	
HA	1:47	FD	45	11	28	Inulin DPn 14	38%	31
						Inulin DPn 6	24%	
						Trehalose	23%	
						Dextran 56kDa	65%	
						No stabilizer	89%	
HA	1:47	FD	45	11	182	Inulin DPn 6,14; dextran 56kDa; no stabilizer	Complete	31
						Trehalose	20%	
PvdQ	1:100	SFD	55	<10%	28	Inulin DPn 23;trehalose	No	29
						Mannitol	Complete	
						Liquid control (after 7 days)	Complete	
AP	1:9	FD	60	0	6	Inulin DPn 14,23	~50%	24

Table 4.1. *Continued*

Protein	Protein: Sugar Ratio (w/w)	Prepa-ration	Storage conditions			Activity/Potency loss after storage		Article cited
			T (°C)	RH (%)	t (days)	Stabilizer	Loss *	
						Inulin DPn 6; trehalose; glucose; no protectant	Complete	
AP	1:1-1:20	SD	60	≤6%	19	Inulin DPn 23	30%	21
						Trehalose	10%	
DNAse	1:11	FD	60	0	42	Sucrose; trehalose; inulin DPn 14, 23	~20%	30
						No protectant	~65%	
AP	1:19	FD	60	0	90	Inulin DPn 23	20%	34
						Trehalose	None	
AP	1:19	FD+T	60	0	105	Inulin DPn 23	25%	34
						Trehalose	Complete	
DNAse	1:11	FD	85	0	42	Trehalose	~50%	30
						Inulin DPn 14, DPn 23	~70%	
						Sucrose; protectant	Complete	

AP=alkaline phosphatase, DNase = recombinant human DNase, HA = Haemagglutinin, Lys=Lysozyme, PstI = restriction enzyme PstI, PvdQ = acyl-homoserine lactone acylase PvdQ ; AD = air dried, FD=Freeze-drying, SD= Spray-drying, SFD=Spray-Freeze-Drying, T= Tableting, C= Crystallization. n.r. = not reported
*If loss is listed as none, no significant loss was detected
** Inulin DPn 6 can also be referred to as oligofructose
*** Degree of polymerization/molecular weight was not reported

activity after storage. Their results are therefore not included in this table, the reader is directed to that article for further information.

At room temperature inulin proved to be an equally good or better stabilizer than other saccharides. With increasing relative humidity, inulins with a higher DP perform better. This is likely because those inulins have a higher intrinsic glass transition temperature and can thus absorb more water before this lowers the Tg of the mixture to below the storage temperature. The relatively small inulin with DP 6, also called oligofructose, caused a loss of activity of around 50% in alkaline phosphatase. That degradation may have been induced by the relatively high amount of reducing groups in that particular inulin sample. This level of degradation was not found with the other, larger inulins tested.[24] When lyophilized alkaline phosphatase formulations with trehalose were compressed into tablets, the very brief exposure to atmospheric moisture and compacting forces resulted in formation of trehalose anhydrate crystals, leading to a complete loss of activity of the incorporated protein within 1 week of storage at 60 °C, 0% RH. In contrast, inulin remained amorphous

and maintained 75% activity of the protein after 3 months of storage at 60 °C, 0% RH. In fact, inulin did not even crystallize when stored at 60 °C, 33% RH for two days.[34] PvdQ spray freeze dried with mannitol stored at 55 °C also resulted in crystallization of the sugar, leading to a complete loss of activity.[29] Inulin and dextran were unable to maintain activity of air-dried samples of restriction enzyme PstI stored at 37 °C.[35] It is most likely that limited solubility of inulin caused it to precipitate during this slow drying process, in turn preventing it from stabilizing the protein as was also seen with air-dried liposomes.[8] Unfortunately, the authors did not report the DP of the inulin, which influences its solubility.[35]

At elevated temperatures, the stabilizing capacities of different saccharides vary. As mentioned above, if vitrification is sufficiently achieved, the stabilizer with most interactions with the protein is likely to stabilize best. Oligosaccharides, however, are more sterically hindered than smaller mono- or disaccharides, reducing their interactions with the protein.[5] Inulin's molecular flexibility is able to compensate this to some extent, unlike the more molecularly rigid dextran.[5,31] Thus depending on the storage temperature and the Tg of the sample, a longer chain length of the oligosaccharide (resulting in a higher Tg) could be beneficial if vitrification is lacking, yet it could also have the opposite effect if more protein-sugar interaction is needed. As mentioned inulin has a small amount of reducing groups, meaning some protein degradation because of the Maillard reaction might occur. Yet even under extreme storage conditions (85 °C, 0% RH, 42 days) this only played a minor role in protein stability of rhDNase, a protein that is sensitive to this type of degradation.[30] In summary, inulin is a good stabilizer of proteins in the dry state, and depending on the formulation and storage conditions it can be better than smaller sugars.

Other stabilization

Apart from membranes and proteins, inulin has also been used to stabilize several pharmaceutically relevant systems. For example, (PEGylated) liposomes, polyethylenimine based polyplexes, lipoplexes[36,37], polymersomes[38], influenza virosomes with and without encapsulated plasmid DNA[39], whole inactivated influenza virus[40], recombinant adenovirus[41], and Δ^9-tetrahydrocannbinol (THC)[42,43].

This variety of applications shows that under the appropriate conditions inulin may provide stabilization against chemical degradation (e.g. THC) as well as against physical degradation (e.g. lipoplexes). Inulin fully protected PEGylated lipoplexes against aggregation during lyophilization and 3 months of storage thereafter, whilst dextran did not.[36] Inulins were also preferred as stabilizer over dextran for various other types of PEGylated nanoparticles due to their compatibility with PEG.[37]

In preserving physicochemical characteristics of doxorubicin-loaded $(PEG)_3$-PLA nanopolymersomes during lyophilization, inulin was found to be superior over trehalose, sucrose, mannitol, lactose, polyvinylpyrrolidone, and several other excipients.[38] Inulin also

fully preserved influenza virosomes during lyophilization. Compared to a liquid dispersion, the powder had substantially prolonged preservation of potency and in case of influenza virosomes with encapsulated plasmid DNA transfection activity.[39] A combination of mannitol and inulin improved the therapeutic applicability of a recombinant adenovirus during freeze-drying and storage.[41] Lastly, incorporation of THC in an inulin matrix by lyophilization strongly increased its stability.[43] Spray-freeze drying THC with inulin turned out to provide even better stabilization than freeze-drying, likely because of the much faster cooling rate.[42] Inulin might therefore also be an interesting stabilizer for other unstable drugs.

DRUG DELIVERY

Solution behavior alteration

Solution rate enhancement

For oral drug administration, drugs need to be dissolved before they can be absorbed by the intestinal membrane. Therefore, the bioavailability of drugs with low aqueous solubility (Biopharmaceutics Classification System class II and class IV drugs) is usually poor because they slowly dissolve in the gastro-intestinal track. One of the strategies that can be applied to increase the dissolution rate is the production of a solid dispersion that is composed of a hydrophilic carrier in which the drug is finely dispersed. When this soluble carrier dissolves rapidly, the poorly soluble drug is hydrated faster, resulting in faster dissolution. Inulin has been used to produce solid dispersions of various poorly soluble drugs.[43–46] Solubility of inulin is much better than these drugs and is dependent on the molecular weight of inulin, with smaller inulins having a higher solubility.[47]

Dissolution behavior of solid dispersion tablets made of diazepam in inulin was significantly better than those based on dispersions with disaccharides like sucrose or trehalose.[44] It was found that the dissolution rate of the disaccharides was extremely high, resulting in supersaturation of diazepam in the near environment of the dissolving tablet, causing it to subsequently precipitate into large crystals with obviously detrimental consequences on the release profile. This uncontrolled crystallization did not occur for inulin included diazepam, due to its lower dissolution rate.[44] Figure 4.1 illustrates the difference in release profiles of the sugars and diazepam for trehalose and sucrose formulations, and the similar release profiles for the solid dispersions of inulin and diazepam.

However, using fenofibrate as model drug, the dissolution behavior of the inulin based solid dispersion tablets at high drug load (50% (w/w)) was poor, probably also due to recrystallization of the incorporated drug during dissolution. This disadvantage could be circumvented by the incorporation of superdisintegrants, croscarmellose sodium or sodium starch glycolate, in solid dispersion.[46] Similar solid dispersion tablets based on polyethylene glycol 20K and and polyvinylpyrrolidone K30 also exhibited excellent dissolution behavior, but were outperformed by those based on inulin after they were stored for 3 months at

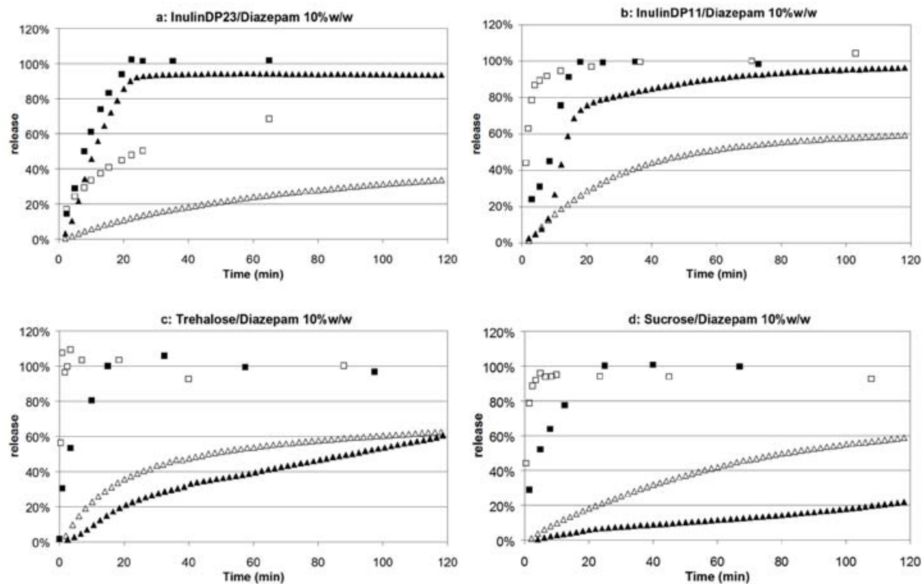

Figure 4.1 Dissolution of tablets prepared from solid dispersions and physical mixtures containing 10% w/w diazepam prepared with different sugars: (a) inulin DP23, (b) inulin DP11, (c) trehalose, (d) sucrose. (Key: ■: sugar from solid dispersion, ▲: diazepam from solid dispersion □: sugar from physical mixture, △: diazepam from physical mixture) (n=3 tablets, all s.d.< 10%)[44]

20 °C, 45% RH or 40 °C, 75% RH.[46] Release of TMC240, an HIV protease inhibitor, was improved both *in vitro* and *in vivo* after oral administration due to incorporation in a solid dispersion of inulin.[45] Lyophilization of nifedipine with relatively low concentrations (up to 10%) of inulin directly in gelatin capsules increased dissolution rate, but as dissolution measurements were not carried out under sink conditions the overall dissolution enhancement was limited.[48]

Inulin improved the dissolution of irbesartan in a physical mixture, with gradually increasing dissolution rates with increasing inulin content. Chemically modified poly(acrylic acid) grafted inulin was less successful at increasing dissolution behavior for irbesartan.[49] Another chemically modified form of inulin tested for dissolution rate enhancement is Inutec SP1. It is an inulin with alkyl groups grafted on primary or secondary hydroxyl groups of the polyfructose backbone and acts as a polymeric surfactant.[50,51] Itraconazol was incorporated in a solid dispersion of Inutec SP1 via spray drying and hot-stage extrusion. Even though phase separation was found and part of the itraconazol was crystalline, the dissolution behavior of the solid dispersion was better than that of itraconazol alone.[50] It was later shown that spray dried itraconazol and Inutec SP1 showed no interaction and addition of

an itraconazol compatible polymer was needed to obtain a molecular dispersion.[51] Contrastingly, when solid dispersions with high drug loads were produced by spray freeze-drying, Inutec SP1 was found to have superior dissolution behavior compared to inulin and polyvinylpyrrolidone.[52]
Amorphous unmodified inulin has been shown to be a suitable filler-binder for tablet production, with chain length dependent disintegration time.[53] It was also possible to produce tablets from freeze-dried inulin formulations, in which case preconditioning of the inulin powder at 45% RH prior to tableting produced the best tablets.[34] It was also possible to make a tablet for sublingual administration of poorly soluble THC based on a solid dispersion with inulin.[43] For regular delivery to the GI tract, dissolution of 80% of the drug in around 20-30 minutes is normal, whereas sublingual formulations require a dissolution time of a few minutes at the most. The inulin based sublingual formulation showed complete release within around 3 minutes.

Modified release
Inulin could be used to alter the release profile of lysozyme from a hydrophilic multiblock copolymer aimed at at prolonged release, because inulin acted as a pore-former.[33] Some studies have also been published in which drugs are encapsulated with inulin or chemically modified inulin for prolonged release.[54,55] Acetylation of inulin strongly reduces its solubility (in a buffer of pH 7.4). Additional succinylation can then be used to increase solubilty, allowing control over solubility and with that the release profile of microspheres from this material.[54] Microspheres of this modified inulin produced by solvent precipitation showed a porous interior and different particle size distributions for the different drugs used.[54] Chymotrypsin containing microspheres, with a particle size distribution from 0.5 to 4 μm, showed release for up to a week with a release of nearly 70% at the first time point of 24 hours. Chlorhexidine containing particles were significantly larger, i.e. 90-130 μm, and showed a similar burst release, followed by a release rate that was slower than the chymotrypsin microspheres.[54] Overall, the prolonged release is limited, since most of the drug is released in the initial burst.
Inulin, inulin acetate (inac) and inulin acetate associated with 1,12-dodecanedicarboxylic acid (inac-dia) were used in the encapsulation of serine protease inhibitors using coacervation, producing particles with a size ranging from 0.5 to 5 μm.[55] Inulin and inac microspheres showed a burst release of around 60% in the first minutes of dissolution, with a controlled release of the rest of the dose over a period of 4 days. Inac-dia displayed an initial burst release of only 32% after 15 minutes, but showed an additional burst release of around 30% after about 1.5 days due to erosion, followed by prolonged release similar to the inuline and inac spheres.[55] Another example of controlled release with a chemically modified inulin is the chemical coupling of inulin to ibuprofen using N,N'-cabonyldiimadazole.

Nanoparticles consisting of methylprednisolone and this ibuprofen-inulin could be produced, providing an interesting combination therapy for spinal cord injury.[56] Lastly, it was recently shown that inulin conjugated with diethylenetriamine might be suitable as a carrier for therapeutic delivery of small interfering RNAs.[57]

Local drug delivery

Colon targeting

Chemically modified inulin was also applied in controlled delivery to the colon. The mechanism behind colon targeting with inulin is based on the fact that inulin is only significantly hydrolyzed by inulinases produced by bifidobacteria in the colon and not by the digestive enzymes in the upper parts of the gastro intestinal tract.[50,58,59] This means that gels and coatings of inulin are not hydrolyzed until these reach the colon where they are fermented, resulting in a colon-specific release of incorporated drug. Several reports have described chemically modified methacrylated inulin (IN-MA) for colonic targeting.[60,61] These modified inulin chains can be covalently cross-linked to each other and optionally in combination with other monomers using free-radical polymerization resulting in the chemical formation of a gel.[62]

Hydrogel characteristics such as swelling need to be well chosen for controlled release. If the gel swells too much, premature release will occur. On the other hand to achieve a fast enough release in the colon, sufficient swelling is needed to allow for inulinase to access and hydrolyze the inulin.[61,62] Finding this balance has been proven difficult, but not impossible.[62] Gels which were co-polymerized with N,N'-bis(methacryloylamino)azobenzene could be degraded both through breakdown by inulinase as well as reduction of the introduced azo group by bacterial strains present in the colon.[63] The molecular weight of inulin used is also relevant here, as higher molecular weight inulins form stronger gels and have higher viscosity, in turn influencing their hydrogel characteristics.[1]

IN-MA was used for the colonic delivery of bovine serum albumin, with release being mainly dependent on degree of substitution (DS, defined as the amount of methacryloyl groups per 100 fructose units) of inulin and concentration of IN-MA used.[50,61] Two formulations, one with 27% w/w IN-MA and a DS of 8.1 and another with 22% w/w IN-MA and a DS of 12.1 were most promising for colonic delivery.[50] Apart from varying DS and concentration of polymer, copolymerization with other monomers, either more hydrophobic or more hydrophilic, could also modify release profiles.[62] Another approach was to use a two-step chemical modification of inulin with first methacrylic anhydride (MA), followed by succinic anhydride (SA) for a pH-sensitive release. Hydrogels of inuline-MA-SA showed a pH-responsive delivery of ibuprofen and diflunisal.[64,65] It was also possible to incorporate IgG during the formation of an inuline-MA_1-SA hydrogel, achieving controlled colonic release of the protein.[66]

Also several other attempts have been made to combine the metabolic degradation dependent release of inulin with a pH sensitive release, but without chemical modification and crosslinking. Free films of inulin with several varieties of Eudragit® were produced and evaluated for colonic delivery. Combinations of inulins with Eudragit RS and RL had more potential for colonic delivery than combinations of inulin and other Eudragit types.[67] An example of a tablet formulation for the delivery of aceclofenac to the colon that has been shown to be successful in an *in vivo* study is based on a combination of the pH independent Eudragit® RS100 and inulin.[68]
Recently some non-gel colonic delivery systems based on modified inulin have been described. Inulin chemically bonded with cinnamate was used to form drug containing vesicles for colonic targeting. The cytostatic methotrexate was successfully encapsulated in these inulin cinnamate vesicles. However, this system showed a methotrexate release of 30-40% in 24 hours even in the absence of inulinase.[69] This is undesirable for a cytostatic agent. Another way to produce inulin particles for colonic targeting is by electrospraying inulin. To make inulin suitable for electrospraying it was first acetylated. Particles containing indomethacin produced using this method showed colonic-specific release (by inulinase) in vitro.[70]

Pulmonary delivery

Pulmonary administration of drugs is often desirable for pulmonary diseases and it can also be a good route for systemic drug administration. The aerodynamic particle size (APS) of the inhaled aerosol is of great importance for pulmonary delivery. An APS of 1-5 μm is considered essential to obtain an adequate lung deposition.[28] Both spray drying and spray-freeze drying can be used to produce particles with a broad APS distribution, allowing targeting of the airways. As mentioned in section *Protein stabilization*, inulin containing formulations have been used to stabilize a vaccine and other proteins during spray drying and spray-freeze drying.
A spray-freeze dried influenza subunit vaccine stabilized by inulin was suitable for inhalation and induced systemic, mucosal, humoral and cell-mediated immune responses in mice after pulmonary administration.[28] Inhaled spray dried and spray-freeze dried influenza subunit vaccines formulations induced higher serum IgG titers than intramuscular administration.[23] Influenza whole inactivated virus vaccine spray freeze-dried with inulin was also safe and efficient for pulmonary immunization.[40] Combined with the improved storage stability pulmonary administration of inulin stabilized dry powder influenza vaccines seems promising.[23,40] Inulin is thus a suitable stabilizer for dry powder formulations of vaccines for pulmonary administration.[71]
Spray freeze-drying of acyl-homoserine lactone acylase PvdQ, an enzyme that can be used in the treatment of *Pseudomonas aeruginosa* infections, with either trehalose or inulin

produced stable formulations. The powder particles had an average aerodynamic diameter of ~1.8 μm, indicating they would be suitable for inhalation and might be used in the treatment of chronic infections in cystic fibrosis patients.[29] Another example of a therapeutic spray-freeze dried with inulin is cyclosporine A, which might be used to prevent allograft rejection for lung transplant patients.[72] Using spray-drying, an inulin-based formulation of rhDNase was produced with a mass mean aerodynamic diameter of 2.3 μm, making it a suitable therapeutic for patients with cystic fibrosis.[30] Lastly, inulin-stabilized spray freeze-dried particles containing THC were also suitable for inhalation.[42]

PHYSIOLOGICAL AND DISEASE-MODIFYING EFFECTS

Systemic

Vaccine adjuvant

Apart from being a suitable stabilizer of vaccines, several papers have reported an adjuvant role of inulin in obtaining an immune response upon vaccination. Mostly the crystalline types of inulin, γ- and δ-inulin, which are virtually insoluble at 37 °C, were used. These crystalline forms consist of inulin with a relatively high molecular weight, the more soluble α and β forms also contain lower molecular weight inulin fractions.[73] More information on the different subtypes of crystalline inulin can be found in the first part of this review.[1] It was shown that γ-inulin enhances both humoral and cell-mediated immune responses in various animal models for a wide variety of antigens, making it a very interesting adjuvant for vaccines.[74,75] Cooper & Carter (1986)[76] reported γ-inulin specifically activates the alternative complement pathway, whereas the more soluble isoforms of inulin, α and β, were found to be biologically inactive and even hindered pathway activation by γ-inulin. The δ-inulin isoform had not been discovered at that time, but most likely acts as an adjuvant according to the same mechanism as γ-inulin.

It was found that δ-inulin was more immunoactive than γ-inulin.[77] Thus far, it has for example been applied to enhance the potency of a trivalent human seasonal influence vaccine in mice[78], split-virion H5N1 influenza in ferrets[79], a pulmonary formulation of whole inactivated H1N1 influenza vaccine in mice[80], and hepatitis B in mice, guinea pigs and humans.[81,82] It was also shown that co-administration of δ-inulin with inactivated H1N1 influenza vaccine achieved a better immune response in pregnant mice, both for the mother and the pups.[83]

In contrast to the above, Kumar & Tummala (2013)[84] have reported that it is possible to use soluble inulin for both vaccine stabilization and as an adjuvant. They developed soluble inulin microparticles which they loaded with ovalbumin and achieved a robust immune response, outperforming antigens adjuvated by traditionally used aluminum salts. It would be interesting to see further research to confirm that soluble inulin can also be an adjuvant and if confirmed, further investigation into the mechanism behind turning soluble inulin into an adjuvant.

Kidney function

A widely established application of inulin is as a diagnostic for kidney function testing, for this application inulin is intraveneously injected. Inulin is highly suitable for this application because it is distributed over the extracellular volume only and it is not metabolized. Furthermore it is only excreted via glomerular filtratrion and not resorbed by renal tubules. This makes it the most accurate substance for determination of glomerular filtration rate.[85] The test can be executed by administering inulin as a bolus or constant infusion and measured concentrations of inulin in both urine and plasma can be used for the determination of the filtration rate.[86] Because inulin is filtered out freely because it is relatively small, the excretion rate is directly proportional to the glomerular filtration rate.[85] As the kidneys do resorb water, the ratio between the concentrations of inulin in the tubular fluid plasma can be used to determine water resorption in the kidney.[87] However, a drawback of this method is the detection of inulin in biological matrices.[85] Several analytical techniques have been described in literature, but care should be taken in comparing results from different analytical methods as differences are not always negligible.[88]

Gastro-Intestinal tract

Constipation

Inulin is widely used as a dietary fiber and prebiotic in so-called functional foods. These uses are worth mentioning in this context, but are not pharmaceutical applications and we will thus suffice by referring to some reviews on this topic. Flamm *et al.*[59] reviewed inulin as a dietary fiber, Kolida, Tuohy, & Gibson (2007)[89] made a comprehensive overview of the prebiotic effects of inulin and oligofructose and lastly Kelly (2008, 2009)[90,91] produced an extensive two-part review about inulin-type prebiotics also in relation to some physiological effects. Nutrition can be used to provide health benefits by modification of gut microbial flora.[92] In fact, inulin's effect on gut flora and gut mobility has been linked to a variety of beneficial effects, both local and systemic.[93] Inulin, in particular high molecular weight inulin, increased stool frequency and can thus be used against constipation.[94] It has been shown to improve stool frequency in formula fed newborns, creating a gut microbiota closer to that associated with breastfeeding.[95] Inulin was also able to relieve constipation in elderly patients, indicating that the stool promoting effect of inulin is present for all ages.[96]

Inflammatory bowel disease & colon cancer

Oral administration of inulin has been reported to achieve both local and systemic immune modulation. A review of the supporting evidence concluded that the local immunomodulatory effect is apparent, but systemic effects are less substantiated.[97] The local effect is possibly indirect through the prebiotic action of inulin, stimulating growth of beneficial bacteria in the gut.[98,99] In a later in vitro study, it was found that inulin also possesses direct signaling

capacity on human immune cells, mainly through the toll-like receptor 2.[100] It is conceivable that these local effects could be beneficial to patients suffering from inflammatory bowel disease and irritable bowel syndrome.[101] Thus far, investigations with small numbers of patients have reported mixed results in this regard and there is thus a need for further investigation of this potential application of inulin.[91,102]

Inulin was able to reduce chemically induced pre-neoplastic lesions or tumors in the colon of mice and rats.[103,104] Long chain inulins were more potent than smaller chain inulins in this sense. This reduction was associated with the gut flora-mediated fermentation of inulin.[103] In a recent study in rats, inulin showed to have a bigger prophylactic potential to colon carcinogenesis compared to lactulose.[104] Inulin reduced cytotoxicity and genotoxicity *in vitro* in human colon adenocarcinoma cells.[105] Again these results seem promising, but further clinical research is required to see if these results can be reproduced *in vivo* in humans.

OVERVIEW

In this chapter the pharmaceutical applications of inulin, an oligosaccharide being increasingly used in food, pharma and other fields, have been reviewed. The most widely established use of inulin in pharma is as a diagnostic to determine kidney function. Inulin's chemical structure makes that it is not metabolized by the body and excreted completely via glomerular filtration. Gut microbiota, however, are able to metabolize inulin. This makes inulin suitable for colon specific drug delivery and as a prebiotic. There is some evidence that inulin's prebiotic characteristics also lead to health benefits, particularly for patients with inflammatory bowel disease or in prevention of colon cancer. These claims, however, need further evidence.

Solid dispersions of amorphous inulin can be used to improve the dissolution behavior of lipophilic drugs. Amorphous inulin was shown to be a suitable stabilizer for membranes and proteins, protecting them against drought and elevated temperatures. Inulin has not yet been used in commercial formulations for this purpose but as the number of new biopharmaceuticals keeps increasing, it could be an interesting new stabilizer. In addition, insoluble isoforms of inulin have an adjuvant effect on the immune response achieved with several vaccines. Inulin could therefore serve two purposes as an excipient for vaccines, achieving both stabilization and an increased effectivity. Overall the uses of inulin are already diverse. Additionally more and more research is being done with chemically modified inulins, making it likely that more applications will be found for this flexible oligosaccharide.

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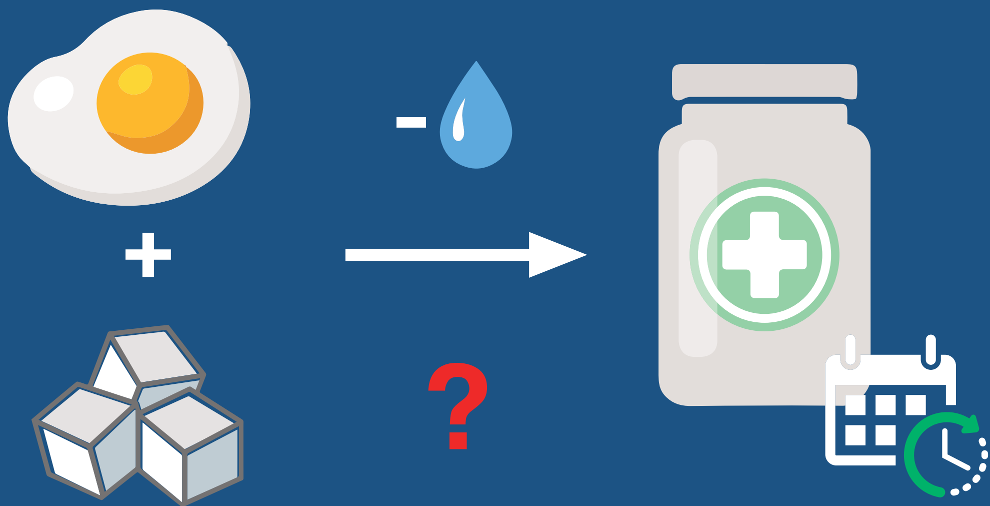

In-line near infrared spectroscopy during freeze-drying as a tool to measure efficiency of hydrogen bond formation between protein and sugar, predictive of protein storage stability

5

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ABSTRACT

Sugars are often used as stabilizers of protein formulations during freeze-drying. However, not all sugars are equally suitable for this purpose. Using in-line near-infrared spectroscopy during freeze-drying, it is shown here that hydrogen bond formation during freeze-drying, under secondary drying conditions in particular, can be related to the preservation of the functionality and structure of proteins during storage. The disaccharide trehalose was best capable of forming hydrogen bonds with the model protein, lactate dehydrogenase, thereby stabilizing it, followed by the molecularly flexible oligosaccharide inulin 4 kDa. The molecularly rigid oligo- and polysaccharides dextran 5 kDa and 70 kDa, respectively, formed the least amount of hydrogen bonds and provided least stabilization of the protein. It is concluded that smaller and molecularly more flexible sugars are less affected by steric hindrance, allowing them to form more hydrogen bonds with the protein, thereby stabilizing it better.

INTRODUCTION

Over the past decades protein drugs have gradually grown to become important players in the pharmacological treatment of diseases. In fact, there are seven biopharmaceuticals among the ten top-selling drugs of 2014.[1] Proteins as such are not stable in solution and require refrigerated storage and transport, the so-called cold chain, to limit loss of functionality and formation of immunogenic degradation products.[2] Cold chain handling is expensive and often impractical, creating serious logistical problems particularly in tropical developing countries. Therefore, protein formulations are frequently dried e.g. by spray-drying or freeze-drying (lyophilization), to create a powder that is less sensitive to degradation and does not require a cold chain.[3,4] During these drying processes, however, proteins are subjected to several types of stresses, including thermal and dehydration stresses.[5,6] To protect the proteins against these and storage stresses, stabilizers are required. For this purpose, small sugars (e.g. disaccharides) are often used.

Currently, there are two predominant theories regarding how lyoprotectants stabilize proteins, namely the vitrification and the water replacement theories.[7–9] Vitrification theory states that protein stabilization is achieved by the formation of a glass, in which mobility is reduced so strongly that molecular mobility needed for degradation does not take place on the timescale of storage.[10] A characteristic of glasses is the glass transition temperature (Tg), above which molecular mobility increases dramatically, with potentially detrimental effects on protein stability. Therefore, glassy (amorphous) formulations should not be subjected to temperatures above their Tg.[11,12] Water replacement theory encompasses the idea that the sugar molecules replace the hydrogen bonds of water with the protein during drying and thus stabilize the protein conformation.[13] These two theories are not mutually exclusive; both mechanisms play a role in protein stabilization.[14,15] Which mechanism is prevalent depends on several factors like formulation (e.g. type of stabilizer), residual moisture, presence of plasticizers and storage temperature.[15] Protein stability has also been related to fast β-relaxation in the solid of these proteins.[16] This could explain how water replacement and vitrification together result in protein stabilization by reduction of the detrimental protein mobility in the solid state.

The above-presented theories lead to specific predictions about the behavior and limitations of various sugar types. Frequently used disaccharides (sucrose and trehalose) are characterized by relatively low Tg values.[17] This means that plasticizers (e.g. residual water, atmospheric water and buffers), which lower the Tg, can critically increase molecular mobility with detrimental consequences for protein stability.[11,18,19] Oligosaccharides, on the other hand, have higher Tgs, limiting their susceptibility to this problem.[18,20] Their size, however, imposes the problem of steric hindrance, potentially limiting their capacity to hydrogen bond with the protein.[21–23] Thus, in general, small sugars (e.g. disaccharides) are not ideal

in the light of the vitrification theory and larger sugars (e.g. oligo- and polysaccharides) have their limitations in relation to the water replacement theory.
Recent work confirmed that smaller sugars stabilize proteins better than larger sugars.[24] In addition, it was shown that the molecularly flexible oligosaccharide inulin stabilized four model proteins better than the more molecularly rigid oligosaccharide dextran. The authors hypothesized that molecular flexibility can reduce the steric hindrance associated with the relatively large size of oligosaccharides. The molecular flexibility allows the sugars to accommodate to the protein structure, forming a tight coating around it, as illustrated in figure 5.1. This tight coating should allow the molecularly flexible oligosaccharides to form more hydrogen bonds with the protein than do molecularly rigid oligosaccharides, overcoming the main limitations of oligosaccharides.[24] Unfortunately, mechanistic evidence supporting this flexibility hypothesis is still lacking. A new in-line near-infrared (NIR) spectroscopy method allows monitoring of hydrogen bonding between proteins and lyoprotectants (e.g. sugars) during lyophilization, and is therefore very suitable to test this flexibility hypothesis.[25]
In short, the method uses a non-contact NIR probe to monitor water elimination and the amide A/II band (near 4850 cm^{-1}), indicative of protein-excipient hydrogen bonding.[25] Correlating these two parameters throughout the drying process allows one to see which sugars effectively take over the hydrogen bonds from water during drying and which do

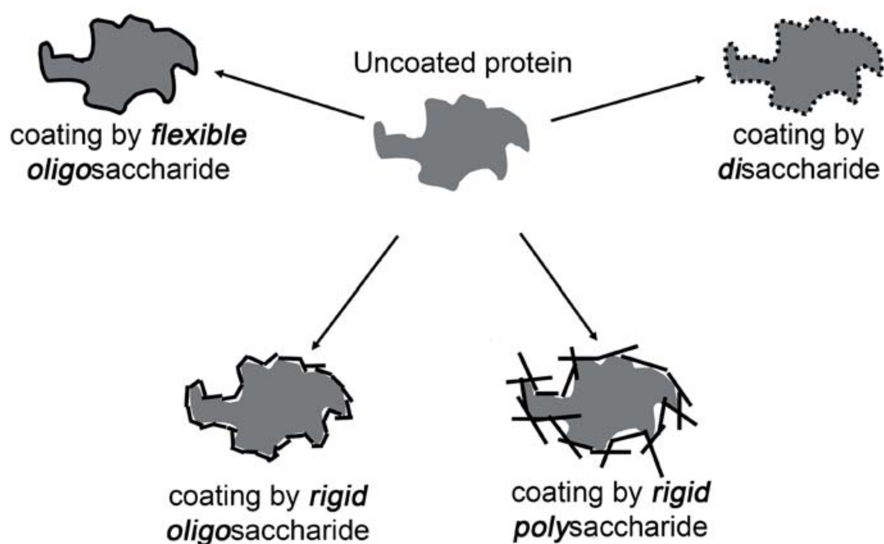

Figure 5.1 Schematic overview of the compactness of coating of proteins by different types of sugars.[24] Modified and reprinted with permission from American Chemical Society.

not. Based on the flexibility hypothesis, it is expected that small sugars and molecularly flexible oligosaccharides form hydrogen bonds more efficiently than their larger and more molecularly rigid counterparts, thereby stabilizing the proteins better. In this chapter mechanistic evidence explaining why size and molecular flexibility determine storage stability of lyophilized proteins is provided.

MATERIALS & METHODS

MATERIALS

The model protein used in this study, L-Lactic Dehydrogenase (LDH) from rabbit muscle, was obtained as a lyophilized powder from Sigma-Aldrich (Zwijndrecht, The Netherlands). The chemicals required for the activity assay of LDH (sodium pyruvate, a reduced disodium salt hydrate of β-nicotinamide adenine dinucleotide (NADH) and bovine serum albumin (BSA)) were also purchased there. Inulin 4 kDa was a generous gift from Sensus (Roosendaal, The Netherlands). Trehalose was obtained from Cargill (Amsterdam, The Netherlands) and dextran 5 kDa and 70 kDa from Pharmacosmos (Holbaek, Denmark). Lastly the buffer components, HEPES free acid and sodium phosphate (monobasic as a dihydrate, and dibasic as a dodecahydrate) were supplied by MP Biomedicals (Illkirch, France) and Merck (Darmstadt, Germany), respectively.

SAMPLE PREPARATION

Solutions containing 100 mg/g of sugar in 2 mM HEPES buffer (pH 7.5) were produced. Subsequently, LDH was weighed and dissolved in the appropriate amount of this stabilizer solution to achieve a protein concentration of 10 mg/g. The protein:sugar ratio was thus 1:10 (w/w). For inulin, the stabilizer solution was mildly heated to achieve complete dissolution, and the solution was allowed to cool again before addition of the protein. For the NIR-monitored lyophilization runs, 1.5 mL of this solution was placed in 4 mL vials of type 2R (type I glass, Fiolax clear, Schott, Müllheim, Germany) with corresponding lyophilization stoppers (West, Eschweiler, Germany). The samples for stability testing and Fourier transform infrared spectroscopy (FTIR) measurements were also lyophilized in 2R type 4 mL vials, yet in 200 μL aliquots. The smaller fill volume is assumed to cause faster drying, but not influence the storage stability. Also solutions with different protein-sugar ratios were prepared and lyophilized as described above. The combined concentration of LDH and sugar of these solutions was 50 mg/mL in 2 mM HEPES buffer (pH 7.5). Protein-sugar ratios ranged from 1:7 to 7:1 (w/w), each ratio was produced in duplicate and those were measured in triplicate.

LYOPHILIZATION

Samples were lyophilized using an Amsco FINN-AQUA GT4 freeze-dryer (GEA, Cologne, Germany) for the NIR-monitored runs and using a Christ model Epsilon 2-4 lyophilizer (Salm en Kipp, Breukelen, The Netherlands) to prepare the samples for stability testing and FTIR measurements. Initially the shelf was precooled at 3 °C. Subsequently the samples were frozen by lowering the shelf temperature to -40 °C at 0.5 °/min. The samples were kept at this temperature for 150 minutes whilst the pressure was lowered to 150 μBar after which the shelf temperature was increased to -20 °C at 0.5 °/min. This temperature was maintained for 23 hours, completing primary drying. Secondary drying was initiated by raising the temperature to 40 °C in 350 minutes, maintaining the pressure of 150 μBar. After 6 hours, or more for some of the NIR-probed lyophilization cycles (figure 5.2), the cycle was ended. The samples for storage and FTIR were prepared using a similar cycle and the vials of those runs were stoppered at 150 μBar inside the freeze-drier. It should be noted that here the terms primary and secondary drying are used to describe the phase of the freeze-drying process as is common in literature. The terms primary and secondary drying usually reflect removal of free ice and more tightly adsorbed water, respectively. Therefore, a clear distinction will be made between primary and secondary drying conditions, which refer to the process parameters during freeze-drying, and sublimation of free ice (primary drying) and removal of tightly bound water (secondary drying), referring to the drying of the product.

NEAR-INFRARED SPECTROSCOPY

NIR Measurement

The in-line Near-Infrared measurements were conducted as described previously.[25] Briefly, a fiber-optic non-invasive probe and a Fourier transform NIR spectrometer (Thermo Fisher Scientific, Nicolet Antaris II near-IR analyzer) were used to collect diffuse reflectance NIR spectra of the sample every minute during lyophilization. Spectra were collected between 10 000 and 4 500 cm^{-1} with a resolution of 8 cm^{-1} and were the average of 32 scans. The probe measured the vial from the side at the bottom, measuring over a detection area of around 28 mm^2. The probe thus did not measure the complete cake, but it was assumed the exposed part was representative for the entire sample.

NIR Data processing

Data were processed using in-house written m-files in Matlab 7.1 (The Mathworks, Natick, MA, United States of America) as previously reported.[25] In brief, spectra were preprocessed by offset correction, vector normalization and the second derivative with 17 point Savitzky-Golay smoothing. The frequency of the minimum of the second derivative of the amide A/II band ($V_{A/II}$) (near 4850 cm^{-1}) was monitored to evaluate changes in the hydrogen bonding strength of the protein's amide groups. A decrease in this frequency is indicative of a relative

increase in hydrogen bonding, and vice versa.[26,27] Using spline interpolation, missing frequency values were predicted, achieving a data spacing of 1 cm^{-1} to allow better accuracy. The frequency values of 10 previous measurements were averaged to reduce noise. The intensity of the band near 5160 cm^{-1} in the baseline-corrected and normalized spectra was used to calculate an apparent water absorbance (AWA) value.[25,28] The band at that frequency is the product of OH-stretching and HOH bending vibrations and indicates unfrozen water moiety and provides an estimation of the apparent water loss. During the first hours of primary drying conditions, present ice interfered with the spectra. These spectra were therefore not taken into account and the obtained $V_{A/II}$ and AWA values thus represent the second part of primary drying conditions and secondary drying conditions. Effectively this means that the actual primary drying (e.g. removal of free ice) is not measured, instead secondary drying (e.g. removal of tightly absorbed water) is monitored under the conditions commonly referred to as primary (e.g. low shelf temperature, low pressure) and secondary freeze-drying (high shelf temperature, very low pressure). Logically, the water removed under secondary freeze-drying conditions is the most tightly bound water.

NIR Data Interpretation

The change in AWA intensity and the frequency of the amide A/II band were plotted against process time. This plot provides information protein-environment interactions after the bulk of the free ice has been removed and how these change during the process. A low A/II frequency at the start of the process, by definition at 0 change in AWA, indicates good initial interactions between the protein and its environment (e.g. sugars, water). No conclusions can be drawn from the final A/II frequency nor from the exact A/II values, because the AWA has not been quantitatively related to remaining water activity and residual moisture after lyophilization and it is not the same for the different formulations. Comparing the relative changes in A/II and AWA curves during the different parts of drying, however, do provide useful information.

The part of the curves up to 1656 minutes corresponds to primary drying conditions, followed by a part corresponding to secondary drying conditions, in which the temperature is increased relatively fast, ending with an isothermal phase. If the hydrogen bonds taken up by water are replaced effectively by sugar, the curve of the A/II frequency tracks the AWA curve, meaning the A/II frequency rises when water is removed. Diverging AWA and A/II curves on the other hand point at incomplete water replacement. Water substitution is most important during removal of tightly bound water (secondary drying), because degradation of LDH during lyophilization mainly occurs under secondary drying conditions.[29] How the A/II and AWA curves track each other under secondary drying conditions is therefore particularly interesting.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

In addition to the in-line NIR measurements, FTIR was used to monitor interactions between protein and sugar in the solid state and to investigate whether conformational changes occurred during storage. The samples were measured in solid form after lyophilization. Between lyophilization and analysis, samples were stored at -20 °C in the stoppered vials to prevent any degradation. For these measurements, a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) was used, equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The spectrometer was placed inside a glove bag (Sigma AtmosBag), which was purged with dry nitrogen gas to avoid moisture sorption of the samples. The vials were only opened inside the glove bag, where the relative humidity was below 5%. Spectra were collected between 4 000 and 850 cm^{-1} at a resolution of 4 cm^{-1}, and for every measurement 256 scans were obtained and averaged. Additionally, the equipment itself was purged using dried nitrogen gas to eliminate the influence of atmospheric gasses. Samples were measured at room temperature without further preparation using a high temperature golden gate mark II attenuated total reflectance accessory (Specac, Slough, United Kingdom). A few milligrams of sample were used for each measurement. Opus software (Bruker, Ettlingen, Germany) was used for processing of the data. Spectra were baseline corrected and triplicate measurements were averaged. The storage stability samples were vector normalized between 1720 and 1590 cm^{-1} (amide I region) and this region was plotted. Additionally, the second derivative of this band was calculated using 17 point Savitzky-Golay smoothing and plotted. Using the same second derivative, frequencies of peak and shoulders in the Amide I regions were determined for the various protein-sugar ratio samples. These frequencies (averaged of n=2, each measured in triplicate) were plotted against sugar content for all formulations. The absorption of the various sugars in the used amide I region was negligible.

STORAGE STABILITY

To investigate the stabilizing effects of the different excipients, a storage stability test was performed. Samples were stored at 60 °C for up to 28 days in closed vials and LDH functionality was tested at various time points (after 1, 3, 7, 14 and 28 days) during that period, using an enzymatic activity assay. After storage at 60 °C, up until the moment of analysis, samples were stored at -20 °C. It was assumed that during storage at -20 °C, no significant degradation of LDH occurred. Samples were reconstituted with 2 mL of a solution of 0.01% bovine serum albumin in 0.1 M phosphate buffer (pH=7.5) and diluted 40 times twice to reach a concentration suitable for the assay. The assay is based on the rate of conversion of pyruvate into lactate by LDH by measuring the decrease in absorption at 340 nm caused by a decreasing amount of substrate NADH. The assay was carried out as previously described.[24]

RESULTS

NEAR-INFRARED DURING LYOPHILIZATION

The AWA and A/II frequencies were monitored during lyophilization for each formulation, the results are depicted in figure 5.2. The top right figure shows the progress of the shelf temperature during the cycle and illustrates which part of lyophilization occurs at which time.

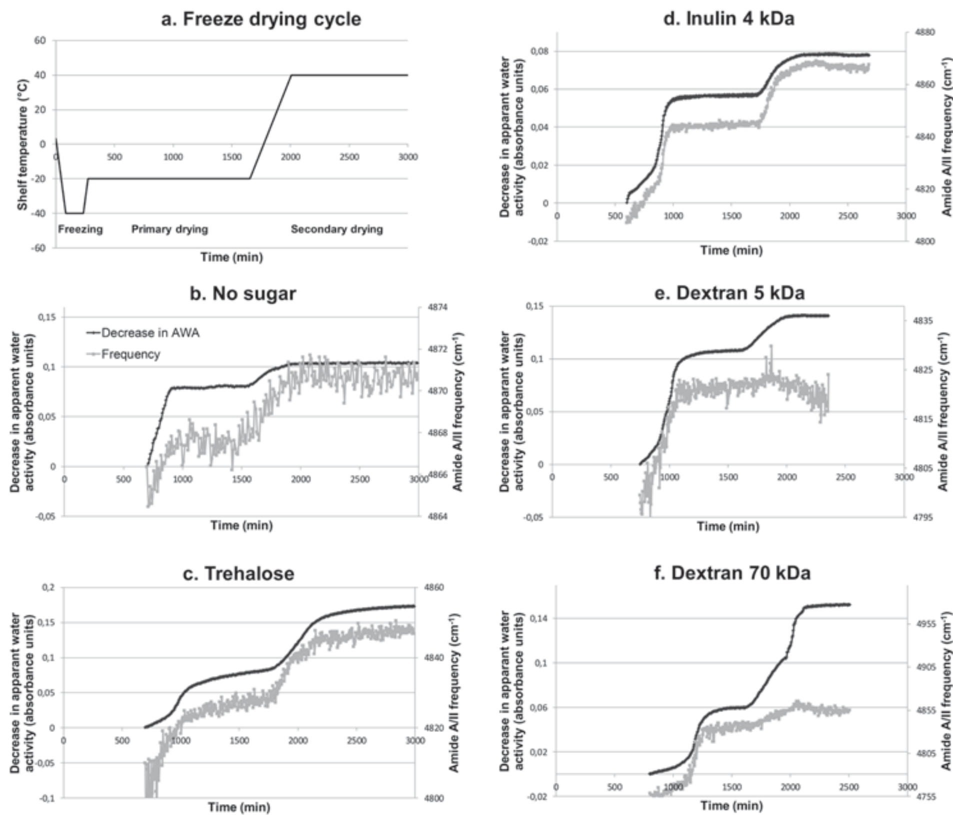

Figure 5.2 An overview of the shelf temperature used during lyophilization (a) and the Amide A/II frequency and decrease of Apparent Water Absorption (AWA) of the different formulations during lyophilization (b-f).

The AWA and A/II curves of the sample without sugar show similar changes throughout the process. The A/II frequency (tracking hydrogen bonds formed with the protein amide groups) increases with a further decrease of the AWA (tracking water loss), which indicates that the protein gets partially dehydrated as a result of the drying process. This effect is larger under secondary drying conditions than under primary drying conditions, as more

energy is provided for the removal of bound water when the temperature is raised. The total change in $V_{A/II}$ is only 6 cm^{-1} for the sample without sugar, four times smaller than that of the sugar containing formulations, showing less changes in hydrogen bonding during the process. The A/II frequency at 0 AWA (start of the monitoring) is also much higher than the other formulations, indicating that less intermolecular interactions between protein and surroundings are present at that moment. For the formulations containing trehalose and inulin the curves show very similar profiles, with the curves running parallel both under primary and secondary drying conditions. For both dextran formulations (of 5 and 70 kDa respectively), the AWA and A/II curves run parallel under primary drying conditions, but under secondary drying conditions the decrease in AWA goes up further, whilst the A/II frequency decreases several cm^{-1} instead of increasing. Compared amongst each other, the larger dextran shows a larger overall shift in A/II frequency.

FTIR OF LYOPHILIZED SAMPLES

The frequencies of the peak and shoulder in the amide I region of the formulations with varying protein-sugar ratio are plotted against sugar content in figure 5.3. In most cases, the frequency of both the peak and shoulder go up with increasing sugar content, indicative of a reduction of the density of hydrogen bonding of the protein in the formulation. In the shoulder area this seems a linear process, whereas for the amide I peak there is less increase or even a small decrease in frequency up to 25% sugar content. Trehalose has the lowest frequencies, indicative of strongest hydrogen bonding, followed by inulin, dextran 5 kDa and lastly dextran 70 kDa. The differences in frequencies between formulations are relatively insensitive to sugar content, showing similar spacing for different types of sugar at all contents.

STORAGE STABILITY: ACTIVITY

The activity of LDH of the various formulations was monitored both immediately after lyophilization and after subsequent storage at 60 °C (figure 5.4). Immediately after lyophilization, the differences in activity of the different formulations were small. It should be noted that the left panel is a zoomed in portion of the right panel, exaggerating small differences. The activity of the dextran 70 kDa formulation was lower than the other formulations. However, this difference was not significant (t-test, $p = 0.12$) compared to the reference without sugar. To prevent skewing of the data, the activity during storage (figure 5.4b) is therefore represented as measured (in arbitrary units) rather than relative to the activity at t=0. Trehalose was best capable of maintaining protein stability, with only a 10 ±3% overall loss in activity compared to t=0. Inulin 4 kDa was the second best stabilizer showing a loss of 26 ±2% after 28 days. The dextrans lost 47 ±1% (5 kDa) and 36 ±1% (70 kDa) activity after 28 days of storage. Dextran 5 kDa basically does not act as a stabilizer at all.

STORAGE STABILITY: SECONDARY STRUCTURE

Figure 5.5 depicts the amide I bands of LDH before and after storage of the various formulations. For all formulations, there is a peak around 1660 cm^{-1}, indicating alpha helix structures, and a shoulder near 1640 cm^{-1}, showing beta-sheets.[30] The shoulder increases in size during storage for all formulations. The peak at 1660 cm^{-1} remains similar during storage for trehalose, increases slightly for inulin stabilized LDH and decreased for dextran based formulations. The band also shows a small shift to lower wavenumbers during storage, more so for both dextrans than for the other formulations. Overall trehalose shows least spectral changes, followed by inulin 4 kDa, whereas dextran 5 kDa and dextran 70 kDa displayed most changes during storage.

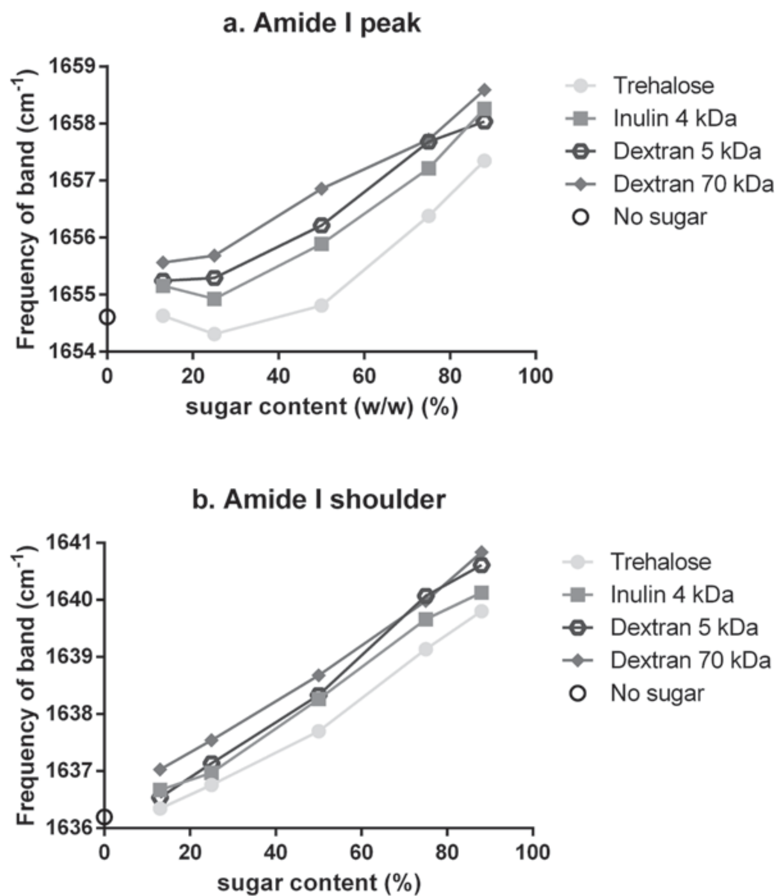

Figure 5.3 Frequency of amide I peak (a) and shoulder (b) of LDH of the different formulations at varying protein-sugar ratios.

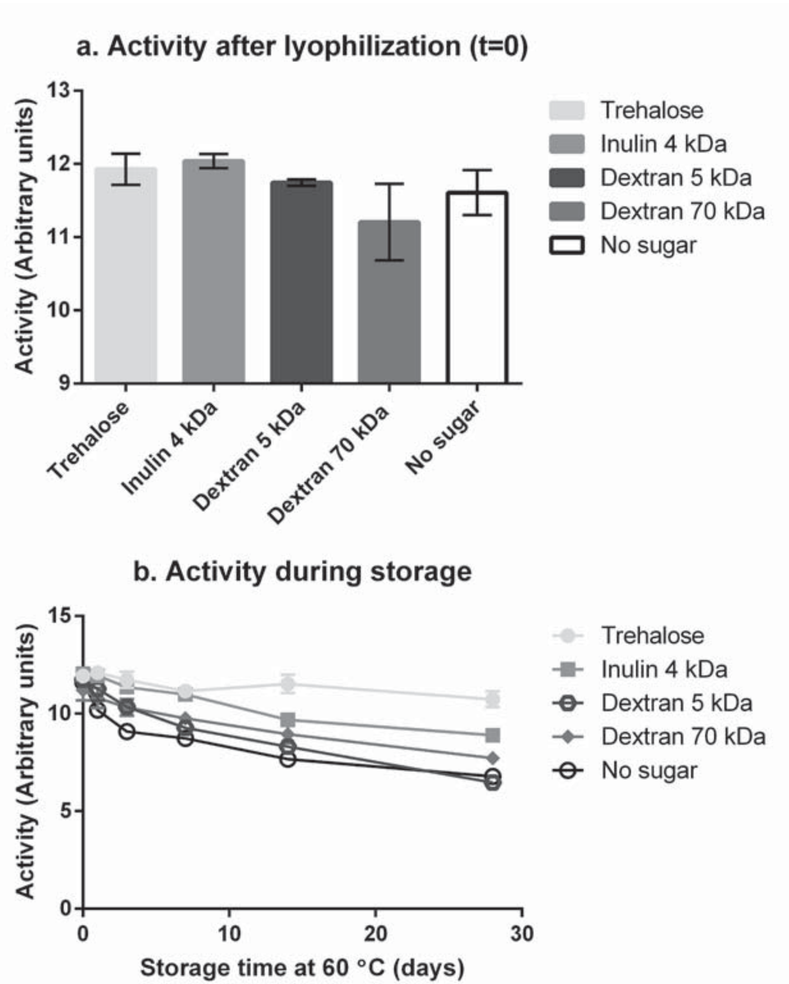

Figure 5.4 Activity of lactate dehydrogenase samples immediately after lyophilization (a) and during storage at 60 °C for 28 days (b).

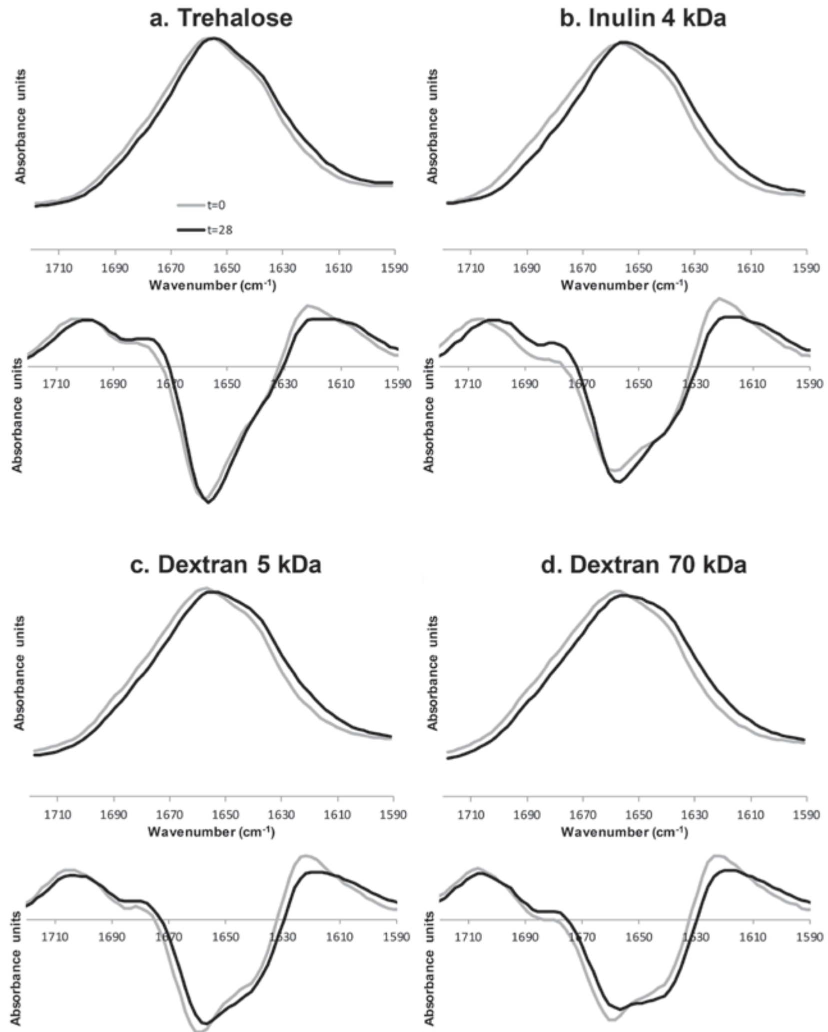

Figure 5.5 Normalized amide I band (top) and second derivative (bottom) of that band of the different formulations (a-d) before and after storage at 60°C for 28 days. Gray lines represent before storage, black after storage.

DISCUSSION

In this study, the influence of hydrogen bond formation between protein and sugars during lyophilization in relation to the storage stability of a model protein, LDH, was investigated. Previously, it was hypothesized that smaller and more molecular flexible sugars are better stabilizers of proteins during storage as they were less affected by steric hindrance in interacting with the protein, provided that they remained vitrified.[24] Therefore, smaller and more molecularly flexible sugars are expected to be better capable of forming hydrogen bonds with the protein. The sugars used were a disaccharide (trehalose), two similarly sized oligosaccharides: molecularly flexible inulin 4kDa and rigid dextran 5 kDa, and a large molecularly rigid polysaccharide (dextran 70 kDa).

As described in the *methods* section, the NIR results provide information about the formation of hydrogen bonds between the protein and surroundings during the different stages of lyophilization, in particular during the removal of tightly bound water (secondary drying). By comparing the AWA and A/II throughout the process, one can see during which phase loss of hydrogen bonds is prevented, indicating lyoprotection. The relatively high starting A/II frequency of the formulation without sugar, compared to the sugar containing formulations, indicated either little intramolecular hydrogen bonding at the start or somestructural loss during the freezing and primary drying stage (prior to monitoring). Small structural changes should result in only little loss of activity, as was confirmed by activity tests directly after freeze-drying. It seems unlikely that the structural changes were completely reversible upon reconstitution, therefore it is most likely that the A/II frequency was high due to little hydrogen bonding between the protein and its environment. In addition to that, the change in Amide A/II frequency was small for the formulation without sugar compared to the sugar formulations, meaning there was only a small amount of hydrogen-bond formation during drying. As there is no stabilizer present, these few interactions are most likely hydrogen bonds between the protein molecules. The changes in frequency occur simultaneously with the changes in AWA, thus these few bonds are formed throughout the entire lyophilization cycle. For the formulations with sugar, the initial amide A/II frequencies were lower, indicating that they were more able to compensate for the loss of protein-environment hydrogen bonds during earlier phases, i.e. freezing and primary drying.

The good correspondence between AWA and A/II signals and large overall shift in A/II frequency for formulations with trehalose and inulin are indicative of the formation of new hydrogen bonds between the protein and these sugars both under primary and secondary drying conditions. It should be noted that under primary and secondary drying conditions, only secondary drying was monitored as it was technically impossible to monitor the spectral changes during primary drying. Both dextrans are capable of replacing hydrogen bonds

under primary drying conditions, but seem to fail to do so under secondary drying conditions, where the most tightly bound water is removed. The larger dextran has a larger shift in amide A/II frequency during removal of tightly absorbed water (secondary drying) compared to the smaller dextran, which could indicate a better interaction with the protein for the larger dextran.

The amide I band, mainly associated with C=O stretching of the amide group, is sensitive to both inter- and intramolecular hydrogen bonding.[31] Generally a lower frequency is indicative of more or stronger hydrogen bonds.[30] Figure 5.3 thus indicates that the small disaccharide trehalose has the best hydrogen bond forming potential with the protein, followed by oligosaccharides inulin 4 kDa, dextran 5 kDa and lastly polysaccharide dextran 70 kDa. This is true for each protein-sugar ratio tested. Remarkably, however, the amide I frequencies shift up with an increasing amount of sugar. A possible explanation for this is that the protein-sugar interactions are weaker than the protein-protein interactions, resulting in weaker overall hydrogen bonding and therewith higher amide frequencies.

Storage stability results show similar trends compared to previously published results with 4 model proteins, despite the substantially higher protein-sugar ratio (1:10 versus 1:249) used here for the spectroscopic analyses.[24] The results show that disaccharide trehalose conserves the activity of LDH best, followed by molecularly flexible oligosaccharide inulin 4 kDa. The two molecularly rigid dextran formulations perform worst, with dextran 5 kDa only slightly outperforming the formulation without sugar. Overall, dextran 5 kDa and the formulation without sugar lost nearly half of their activity during 4 weeks of storage, whereas the formulation with trehalose only lost around 10% activity. The FTIR spectra show lowest change during storage for trehalose, with more changes for inulin and most changes for both dextrans. Generally, spectral changes are undesired as the aim is to preserve the proteins native structure. Here too the smallest change to the amide I region correlate with the best conservation of activity. The changes in secondary structure, a decrease in alpha helix content and an increase in unordered structures or β-sheets, indicate aggregation.[30]

The sugars which maintained protein activity best, i.e. disaccharide trehalose and molecularly flexible inulin, distinguished themselves from the other tested sugars by their ability to replace hydrogen bonds throughout the lyophilization process. The more molecularly rigid dextrans, regardless of their size, were unable to efficiently replace hydrogen bonds under secondary drying conditions, leading to the formation of less or weaker hydrogen bonds than smaller or molecularly more flexible sugars. These findings are in line with the flexibility hypothesis (figure 5.1), showing that smaller and more molecularly flexible sugars are less affected by steric hindrance and can therefore interact with the protein better. The effect of size is larger than the effect of molecular flexibility. It should be noted that all sugars used here had sufficiently high glass transition temperatures to maintain vitrification, which is also required for protein stabilization.[21,24] The larger dextran 70 kDa is a better stabilizer

than dextran 5 kDa. Dextran 70 kDa has a higher Tg than dextran 5 kDa meaning its ability to vitrify is bigger. However, as long as sufficient vitrification is achieved, a higher glass transition temperature does not further increase stability.[15] A possible explanation for the difference between the differently sized dextrans could be the branching of dextran, which will have a greater influence at larger molecular weight. This could also be an explanation for the relatively large shift in amide A/II frequency for dextran 70 kDa. Alternatively, the Maillard reaction could be a reason for this difference, as the reducing end groups of dextran are relatively more present in lower molecular weight dextrans. Previous results, however, showed this same trend and ruled out the Maillard reaction as cause and additionally to that no browning was observed.[24]

Interestingly, during lyophilization there is only very limited divergence in loss of activity, despite the clear differences in hydrogen bonding of the different excipients. It is during storage that these differences in stabilizing capacity become evident. As mentioned in the introduction, it is likely that the water replacement (protein-excipient hydrogen bond formation) translates to a reduced molecular mobility of the protein during storage, resulting in increased storage stability. In-line NIR measurements can therefore be a valuable addition in estimating storage stability of proteins, which is not apparent directly after lyophilization. Additionally, these results show that hydrogen bonding under secondary drying conditions is particularly important. Removal of the hydrogen bonds of this tightly bound water has a larger impact on the protein than the removal of less tightly bound water which is removed under primary drying conditions. A good stabilizer should thus be capable of forming hydrogen bonds both under primary as well as secondary drying conditions. Again it should be noted that primary drying was not investigated due to interference of ice and these results thus describe hydrogen bond replacement during removal of tightly bound water (secondary drying) under primary and secondary drying conditions. This paper shows that in-line NIR during lyophilization is preeminently suitable to determine how the excipients behave during each phase of the lyophilization cycle and can provide useful insights in protein-excipient interactions.

CONCLUSION

This manuscript shows that, compared to larger and molecularly more rigid sugars, smaller and molecularly more flexible sugars are better able to form hydrogen bonds with a model protein during lyophilization. This is in support of the previously described flexibility hypothesis, which states that the sugars can overcome steric hindrance more effectively. The differences in hydrogen bonding mainly occur under secondary drying conditions, during which the most tightly bound water is removed. It should be noted that all sugars

used here had sufficiently high glass transition temperatures to maintain vitrification, which is also required for protein stabilization. The differences in hydrogen bonding do not result in different activities after lyophilization for the different formulations, but they only become apparent during storage. During storage less hydrogen bonding results in a greater loss of activity. This is most likely the result of increased molecular mobility of the protein in the solid state, resulting in more aggregation. A good stabilizer should thus be capable of forming hydrogen bonds both under primary as well as secondary drying conditions. Lastly, in-line NIR can be a useful tool in gaining a deeper understanding of protein-excipient interactions during different phases of lyophilization.

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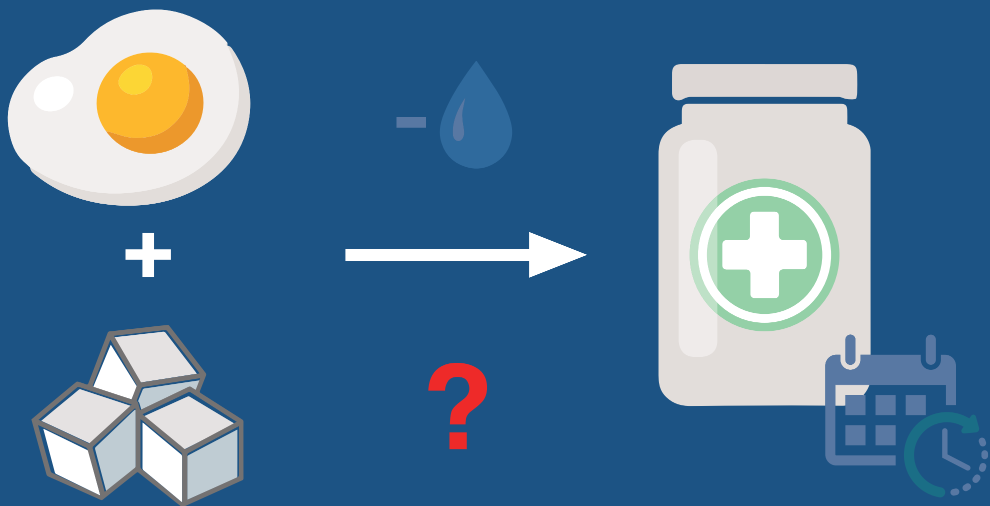

6

Terahertz time domain spectroscopy as a tool for measuring intermolecular protein-sugar interactions in the solid state

Manuscript in preparation for submission

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ABSTRACT

Protein drugs have become increasingly important in modern day medicine. They are generally produced as solutions dependent on refrigerated storage and handling to maintain their functionality. To circumvent this need for refrigeration, proteins can be dried in the presence of sugars to obtain thermally more stable formulations. To maintain functionality of the protein during drying and storage, sugars have to be able to effectively hydrogen bond with the protein. Smaller sugars generally are better able to do so compared to larger sugars. Terahertz time-domain spectroscopy (THz-TDS) has previously been shown to be sensitive to hydrogen-bonding in binary liquid mixtures. In this chapter we show that THz-TDS is also able to qualitatively measure protein-sugar hydrogen bonding in the amorphous solid state using freeze-dried protein-sugar samples with sugars of different sizes and molecular flexibility. The THz-TDS data confirm that smaller sugars interact with the protein better, explaining why they are better stabilizers for proteins.

INTRODUCTION

Over the past decades, the proportion of biopharmaceuticals amongst new drugs has gradually increased. In 2014, seven of the 10 best-selling drugs were proteins.[1] These proteins are typically formulated as solutions which require cold chain processing. Cold storage and transportation are both costly and impractical, particularly in developing countries. To overcome the need for refrigeration, proteins can be dried in the presence of one or more stabilizing excipients to produce a more thermally stable formulation. Drying is mostly done by either spray or freeze drying and sugars are frequently used as stabilizers during processing. Sugars need to meet certain criteria to be good stabilizers: First they need to be able to replace water during drying by replacing the hydrogen bonds between water and the protein[2,3]; secondly the sugar should form an amorphous matrix that exhibits a high enough Tg such that the mobility of the protein is strongly reduced. Finally, the protein should be non-reducing.

Recently, it was shown that smaller and molecularly more flexible sugars stabilize proteins better than larger and more rigid sugars do, provided they maintain sufficient vitrification.[4] This is most likely because the more flexible sugars are less affected by steric hindrance and therefore better suited to form hydrogen bonds with the protein.[5]

Terahertz time-domain spectroscopy (THz-TDS) can be used to study changes in intermolecular interactions, such as hydrogen bonding, in liquids and solids.[6] THz-TDS allows obtaining qualitative information on intermolecular protein-sugar interactions as has been shown for binary liquid mixtures.[7] When two compounds in a mixture do not interact with each other and the mixture is effectively phase separated, the terahertz response of the mixture can be to a good extent described as a linear combination of the terahertz response of the pure constituents. On the other hand, when the constituents form a rich intermolecular network, the system behaves as one unit and the overall terahertz absorption is lower than that of the constituents due to a disrupted original hydrogen-bonded network that exists in the pure constituents.[8,9]

In addition to being able to measure intermolecular interactions, terahertz spectroscopy is sensitive to both α and β relaxations of the proteins, responsible for global mobility above the glass transition temperature and local mobility which can already be detected well below the glass transition temperature (Tg), respectively.[10–12] These relaxations cannot be measured directly by THz-TDS, as they are typically observed at much lower frequencies (MHz frequencies and below). They can, however, be observed indirectly by measuring over with THz-TDS over a range of temperatures.

Amorphous samples do not exhibit specific spectral features in the far-infrared region, and mostly show a broad absorption peak from ~1-5 THz, commonly described as the vibrational density of states (VDOS), which is the result of libration-vibration motions.[12,13] Dielectric losses

in the VDOS region generally change linearly with temperature, provided mobility in the sample is constant. When dielectric losses of the VDOS of melted small polyalcohol samples were monitored as a function of temperature, three distinct linear regions with sequentially increasing slope could be distinguished.[12] The first region was from the lowest measured temperature upon approximately 0.65 Tg (in Kelvin), the second from that point up until the Tg and the third above the Tg. The first increase in slope could be attributed to the onset of local, fast β relaxations and the second increase was the result of global α relaxations.[12,14]
In this chapter we examine the ability of THz-TDS to measure protein-sugar hydrogen bonding in the amorphous solid state using freeze-dried protein-sugar samples with sugars of different sizes and molecular flexibility. Additionally, we evaluate if onsets of mobility can also be observed for these mixtures.

METHODS

MATERIALS

Bovine Serum Albumin (BSA) was acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands), trehalose was purchased from Cargill (Amsterdam, The Netherlands), dextran 70 kDa was obtained from Pharmacomos (Holbaek, Denmark) and inulin 1.8 kDa was a generous gift from Sensus (Roosendaal, the Netherlands).

FREEZE-DRYING

The sugars and BSA were dissolved in ultrapure water to a concentration of 100 mg/mL. 10 mL vials of type 6R (type I glass, Fiolax clear, Dedecke, Königswinter, Germany) were filled with 1 mL of the different formulations. For the protein-sugar mixtures 200 µL of BSA solution and 800 µL sugar solution were used, achieving a 1:4 (w/w) protein-sugar ratio. The vials were placed on the precooled (278 K) shelf of a Christ Epsilon 2-4 freeze-dryer (Salm & Kipp, Breukelen, the Netherlands). Freezing was done by cooling the shelf to 233 K with intermediate isothermal periods of 30 minutes at 278 K and 268 K. All cooling and heating was done at a rate of 1 K/min unless otherwise mentioned. The shelf was kept at 233 K for an hour, after which the pressure was lowered to 87 µbar whilst the temperature was raised to 248 K. Primary drying was done under these conditions during 24 hours. For secondary drying, the shelf temperature was increased to 313 K at 0.1 K/min after which the temperature was maintained for 6 hours. The vials were closed using rubber stoppers at 87 µbar and additionally crimped with aluminum seals upon removal from the freeze-drier. Samples were stored at ambient temperature for 3 months followed by another 3 months storage at 278 K. The samples were stored in closed vials and the vacuum was maintained in the vials for all the samples up until the moment of analysis.

TERAHERTZ TIME-DOMAIN SPECTROSCOPY

Particular care was taken to minimize influence of atmospheric moisture on the results of the terahertz measurements, as the lyophilized samples are hygroscopic. Water exhibits very strong terahertz and the presence of water molecules is well known to have significant effect on the molecular mobility in the sample. The final sample preparation, from opening the vials to mounting the sample in the sample holder, took place in a glove bag (Sigma-Aldrich AtmosBag®) purged with dry nitrogen gas (relative humidity <1%) to avoid moisture sorption from atmospheric water vapor. The lyophilized cake was broken up using a spatula and the powder was pressed into a pellet of 13 mm diameter using a load of 2 metric Tons. The resulting pellets were wedged between 2 z-cut quartz windows of 3 mm thickness each and placed in a copper sample holder. Another slot on the same copper sample holder was loaded with a set of two reference z-cut quartz windows. The sample holder was attached to a cryostat with a motorized linear translation stage that allowed to switch between the sample and reference slots, and placed into a vacuum chamber of a home-built THz-TDS setup, as described elsewhere.[11] The cryostat was first cooled to 80 K and then heated to either 460 K or 500 K, depending on sample, with 20 K increments. The temperature was controlled using a LakeShore 331 Temperature Controller. We allowed 10 minutes for thermal equilibration at each temperature point. The THz-TDS setup allows to measure absorption spectra and refractive index spectra between 0.3 – 3.0 THz. The terahertz data were analyzed following the procedure proposed by Duvillaret *et al.*[15]. When reporting thermal change in terahertz absorption as a function of temperature, we use the frequency of 1 THz as it provides the best signal-to-noise ratio as outlined previously.[12]

RESULTS

TERAHERTZ SPECTROSCOPY

Frequency-dependent spectra

The terahertz absorption spectra for pure BSA and pure sugars are shown in figure 6.1. Generally, the absorption increases with both temperature and frequency. The increase of absorption with frequency is expected, as it has been shown previously that the terahertz absorption is roughly proportional to the square of the frequency up to the Ioffe-Regel limit (I-R limit),[16,17] above which phonon-like excitations become dispersed by disorder.[13] Qualitatively, the terahertz spectra suggest that the larger the molecule is, the lower the I-R limit is. This would be expected intuitively, as in fully disordered systems any crossover between global and local disorder shifts to larger length scales, and hence can be observed at lower wavelengths. In addition, the intra-molecular motions are expected to couple to the intermolecular motions, resulting in a stronger scattering of phonon-like intermolecular

excitations. The most pronounced example is the case of dextran, where it may be concluded that the I-R limit is below 1.0 THz, given the strong flattening of the absorption above 1.0 THz. Surprisingly, BSA (66 kDa) does not show such a shoulder despite having a molecular mass similar to dextran (70 kDa). We presume these differences originate from the differences in molecular structure of the two molecules.

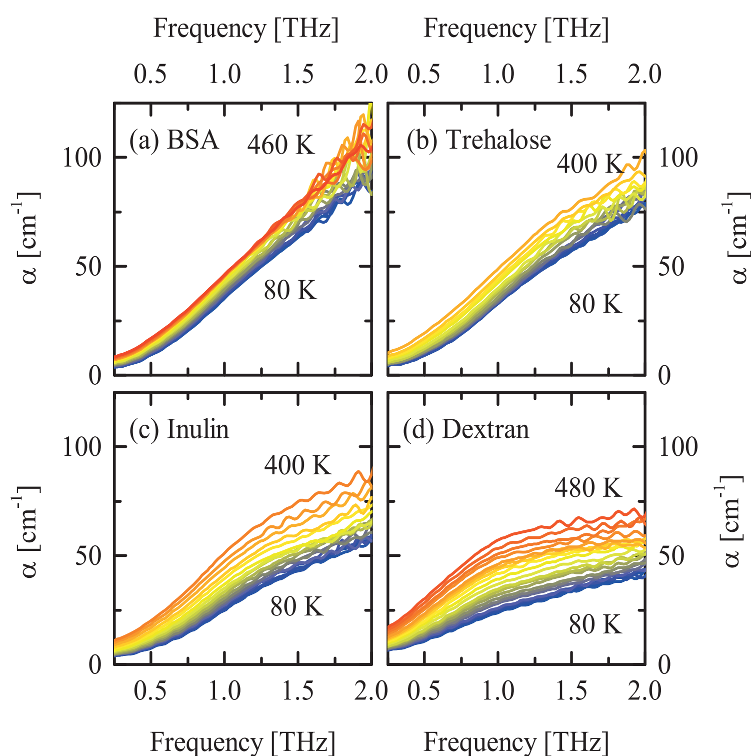

Figure 6.1 Terahertz absorption coefficient (α) of amorphous (a) BSA, (b) trehalose, (c) inulin 1.8 kDa and (d) dextran 70 kDa over the temperature range 80 K – 480 K. Data significantly above Tg are not shown as the amorphous pellets became structurally unstable and collapsed.

In order to advance the interpretations it would normally be sensible to convert the terahertz absorption coefficient into the dielectric losses (i.e. imaginary part of the dielectric function). While the quality of the current data is of high standard, we choose not to do this given some systematic discrepancies in the data. First, from the low-frequency low-temperature limits it is apparent that there is some background absorption (at the level of a few cm^{-1}). In principle, this background absorption can be subtracted as it is not expected to vary

extensively with temperature. However, the precise value of the background could not be determined at the higher temperatures with sufficient confidence. Second, both absorption coefficient and refractive index values are skewed as a result of the porosity of the pellets. The porosity of the samples was not measured, but can be assumed to be the same for all samples as these were all produced from lyophilized powder using the same sample preparation. Third, given the porosity some scattering is possible and unaccounted for. We estimate the magnitude of the absolute error on absorption coefficient to be 1-10%, with the measurements at the higher frequencies suffering from larger relative error. We therefore do not attempt to apply any corrections to the measured data, to avoid introducing artefacts. It should however be emphasized that the relative temperature and frequency variation of the spectra are almost unaffected by these systematic errors.

Temperature-dependent spectra

The temperature induced changes in the terahertz absorption coefficient and refractive index at 1.0 THz are shown in figure 6.2. Given that in all cases the same protein was used, the data for BSA in subfigures a-f is identical and is plotted repeatedly for clarity.

DISCUSSION

PROTEIN-SUGAR INTERACTIONS

As discussed in the introduction THz-TDS can provide qualitative information on intermolecular protein-sugar interactions by comparing absorption coefficients of separate components and mixtures over temperature. For systems lacking interactions, the absorption coefficient of the mixture is expected to be the sum of the absorption of the individual components and thus fall between the absorption of the separate components, as would be expected for a physical mixture. For systems with tight interactions it is expected that the absorption would be lower than for such a physical mixture.

For the smallest sugar used here, trehalose, the absorption coefficients of the protein-sugar mixture were lower than of the separate components (figure 6.2, left side). This shows that there are strong interactions between trehalose and BSA. The larger inulin, a flexible oligosaccharide, shows this behavior to a lesser extent, and for the largest sugar, polysaccharide dextran 70 kDa, this effect is completely absent. The absorption coefficients of the mixture of dextran and BSA are between those of the separate components at all temperatures, implying the two components behave independently of each other like a physical mixture. Additionally, the dextran-BSA mixture shows a depression around 360 K as can also be seen for pure BSA yet is absent in the other protein-sugar mixtures. This shows independent behavior of dextran and BSA in the mixture, suggesting that the two

components have phase separated. This matches our previous findings, where we found a decreasing protein-sugar miscibility with increasing protein size.[18]

These THz-TDS data thus confirm that smaller sugars interact more with the protein than larger sugars do, which is in line with the hypothesis that smaller sugars are less affected by steric hindrance, and explains why they are better capable of maintaining protein functionality during storage.[4,5]

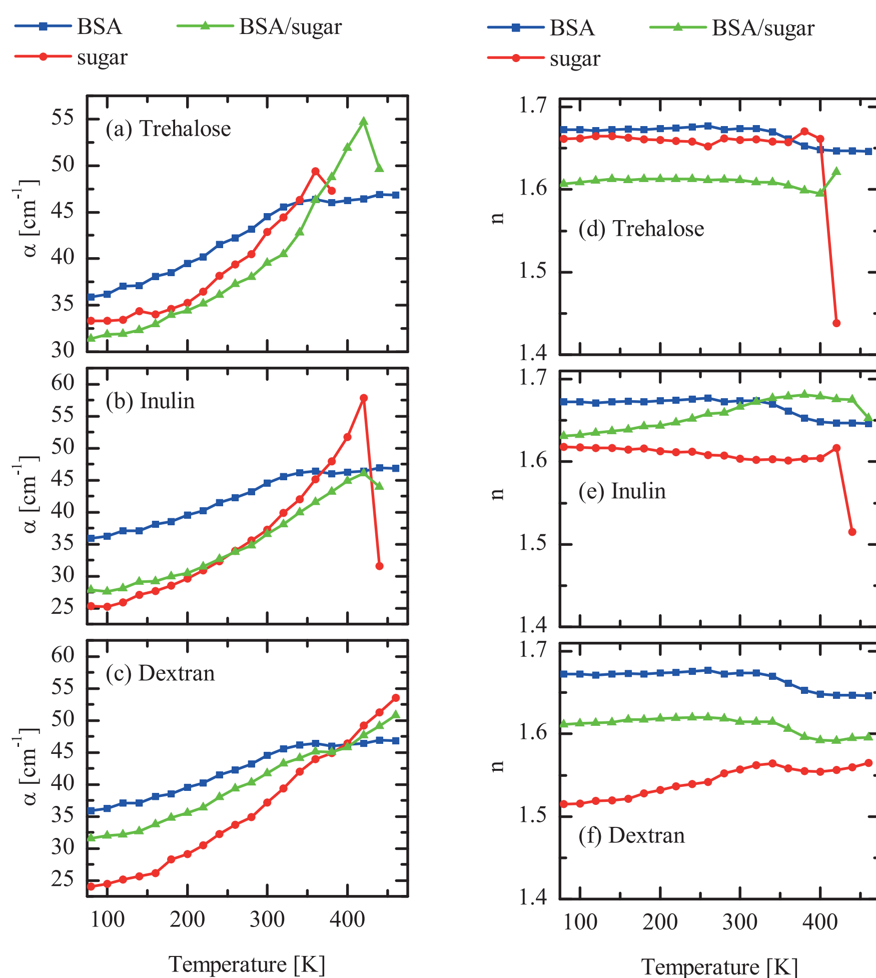

Figure 6.2 LEFT COLUMN: Absorption coefficient (α) at 1.0 THz for (a) trehalose, (b) inulin 1.8 kDa and (c) dextran 70 kDa, together with the absorption spectra of BSA and 1:4 mixture of BSA and respective sugar as a function of temperature. RIGHT COLUMN: Refractive index (n) at 1 THz for (d) trehalose, (e) inulin 1.8 kDa and (f) dextran 70 kDa, together with that of BSA and 1:4 mixture of BSA and respective sugar as a function of temperature.

EFFECT OF MOLECULAR SIZE ON TEMPERATURE DEPENDENCE OF TERAHERTZ ABSORPTION

In general, the terahertz absorption coefficient of all formulations is observed to increase with temperature. Such behavior is expected as the molecular mobility in disordered systems increases with temperature. This is valid both at temperatures above and below the Tg, albeit for different reasons. Below the Tg the increase in terahertz absorption is linked to the local mobility originating from β relaxations.[19] Above the Tg the temperature dependent increase in absorption becomes even stronger due to α relaxation.[19] In our measurements this increase in mobility above the Tg was sufficient for the sample pellets to collapse causing a drastic decrease in absorption and refractive index and thus we do show no data above the Tg.

For most samples absorption increases gradually from 80K, but in the case of pure trehalose the increase in absorption is pronounced only above 220 K. This behavior is likely linked to the different molecular size of the formulations. Trehalose is a relatively small disaccharide. As described in the introduction, in amorphous systems of small molecules the onset of β relaxation takes place only above a certain temperature.[14] On the other hand, for large to very large molecular systems such as BSA, inulin and dextran it may be expected that the intermolecular mobility is strongly coupled with intra-molecular mobility. The latter may be responsible for the observed increase in the absorption coefficient in those temperature regions. These spectra thus indicate a gradually increasing mobility in the larger molecules over temperature, lacking a clear onset of mobility as can be observed in small molecules.

GLASS TRANSITION

THz-TDS spectroscopy is very sensitive to the changes in molecular dynamics associated with surpassing the glass transition. Generally, terahertz absorption is expected to increase significantly when the molecules regain their mobility corresponding to the primary dielectric relaxation, allowing an easy determination of Tg.[11,12] Here the opposite is observed in figure 6.2. This can be ascribed to the sample format used here. The previous studies considered homogeneous non-porous amorphous sample. In this study, lyophilized cakes were pressed into porous tablets. Upon heating beyond the Tg the sample softens, becomes mechanically unstable and collapses. This effectively lowers the beam path for the terahertz radiation and appears as a drop in the absorption coefficient and refractive index. Hence, rather than deducting Tg from the molecular response, in this case one can determine it from the bulk sample response.

Table 6.1 shows the Tg values determined by THz-TDS in this fashion (i.e. temperature at drop in absorption coefficient and refractive index), with previously obtained results by the more conventional technique of differential scanning calorimetry (DSC) for the different sugars. The THz-TDS and DSC measurements match well considering the limited resolution

of 20 K of the THz-TDS measurements. The Tg for dextran could not be clearly deducted from the THz-TDS results as it occurs in the proximity of the maximum temperature reachable by the used cryostat (500 K).

Table 6.1 Comparison of glass transition temperature as established by traditional method (DSC) and the results obtained here using THz-TDS.

Method	**THz-TDS**	**DSC** [4,20]
Effect	Steep change in absorption and ref. index	Change in heat capacity
Sugar	**Tg (K)**	**Tg (K)**
Trehalose	400	395 ± 1
Inulin 1.8 kDa	420	413 ± 2
Dextran 70 kDa	n/a	496 ± 1

CONCLUSION

We show that THz-TDS is capable of detecting protein-excipient interactions in the solid state and it could thus be used in solid-state protein formulation development. The THz-TDS data confirm that smaller sugars interact with the protein better, explaining why they are batter stabilizers for proteins. Additionally THz-TDS shows a gradual increase in mobility in the sample over temperature below the Tg, lacking a clear onset point for increased mobility.

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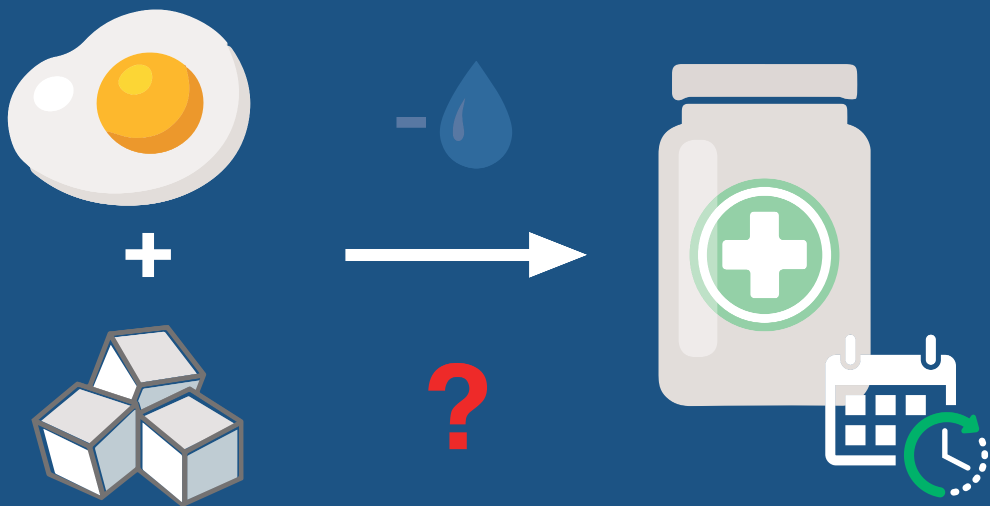

Influence of miscibility of protein-sugar lyophilizates on their storage stability

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ABSTRACT

For sugars to act as successful stabilizers of proteins during lyophilization and subsequent storage, they need to have several characteristics. One of them is that they need to be able to form interactions with the protein and for that miscibility is essential. To evaluate the influence of protein-sugar miscibility on protein storage stability, model protein IgG was lyophilized in the presence of various sugars of different molecular weight. By comparing solid state nuclear magnetic resonance spectroscopy relaxation times of both protein and sugar on two different timescales, i.e. ^1H T_1 and ^1H $T_{1\rho}$, miscibility of the two components was established on a 2-5 and a 20-50 nm length scale, respectively, and related to protein storage stability. Smaller sugars showed better miscibility with IgG and the tendency of IgG to aggregate during storage was lower for smaller sugars. The largest sugar performed worst and was phase separated on both length scales. Additionally shorter protein ^1H T_1 relaxation times correlated with higher aggregation rates during storage. The ELISA assay showed overlapping effects of aggregation and Maillard browning and did not correspond as well with the miscibility. Because of the small scale at which miscibility was determined (2-5 nm) and the size of the protein domains (~2.5 x 2.5 x 5 nm) the miscibility data give an indirect measure of interaction between protein and sugar. This reduced interaction could be the result of steric hindrance, providing a possible explanation as to why smaller sugars show better miscibility and storage stability with the protein.

INTRODUCTION

Protein pharmaceuticals have become increasingly important in the pharmaceutical industry over the past decade, with 10 out of the 41 drugs approved by the American Food and Drug Administration (FDA) in 2014 being proteins.[1] Most protein formulations are produced as solutions requiring refrigerated storage and handling, the so-called cold chain. This is both costly and impractical. The cold chain can be circumvented by drying the protein formulation in the presence of a stabilizer, using methods such as lyophilization and spray drying.[2,3] For these situations, sugars are often used as stabilizers.

Much research has been done into the mechanism by which these sugars stabilize proteins both during drying and storage,[4–6] producing several widely discussed theories regarding these mechanisms (e.g. vitrification theory, the water replacement theory and refinements focusing on local mobility of the protein).[5,7] For each of those theories interactions between protein and sugar are needed, and for that miscibility of the protein and sugar is a necessary condition.

To qualify solids as miscible or immiscible, it is important to define the scale of this miscibility and the technique used to quantify miscibility. Two substances can appear miscible using a technique that measures a larger domain size, whilst showing phase separation using a technique that measures a smaller domain size. Therefore, a definition of miscibility should be accompanied by a qualification of the scale at which it was established. In this chapter, miscibility was determined using solid state Nuclear Magnetic Resonance spectroscopy (ssNMR). The method uses the concept of spin diffusion to determine the homogeneity of mixed systems.[8] In homogeneous systems spin diffusion causes magnetization transfer between neighboring nuclei, resulting in similar relaxation times for both nuclei. For phase separated systems magnetization transfer does not occur and the relaxation times of different components are not averaged.

Spin diffusion is a time dependent phenomenon and the length scale of spin diffusion L is correlated to the relaxation time as follows:

(7.1) $$L=\sqrt{6Dt}$$

D is the spin diffusion coefficient, which is typically assumed to be 10^{-12} cm^2/s [9,10] and t is the relaxation time. When relaxation times of different timescales are compared, this principle allows differentiation of homogeneity on different length scales. In particular, a spin-lattice relaxation time (^{1}H T_1) is typically 1-5 s, corresponding to a spin diffusion over a distance of approximately 20-50 nm, while a rotating frame spin-lattice relaxation time (^{1}H $T_{1\rho}$) is usually 5-50 ms during which spin diffusion occurs over a distance of around 2-5 nm.

By comparing ^{1}H T_1 and ^{1}H $T_{1\rho}$ relaxation times of different components in a mixture, in this

case protein and sugar, it is thus possible to establish the intimacy of mixing on two different length scales (figure 7.1). If a system is fully miscible, both the relaxation times will be similar for both components. If a system is phase separated, both ^1H T_1 and ^1H $T_{1\rho}$ values will be different for the sugar and protein. When the ^1H $T_{1\rho}$ values are different, but the ^1H T_1 values are similar, this indicates phase separation on the 2-5 nm scale, but miscibility on the 20-50 nm scale (partial phase separation). Using these relaxation times, it is thus possible to determine miscibility of these systems on two different length scales.

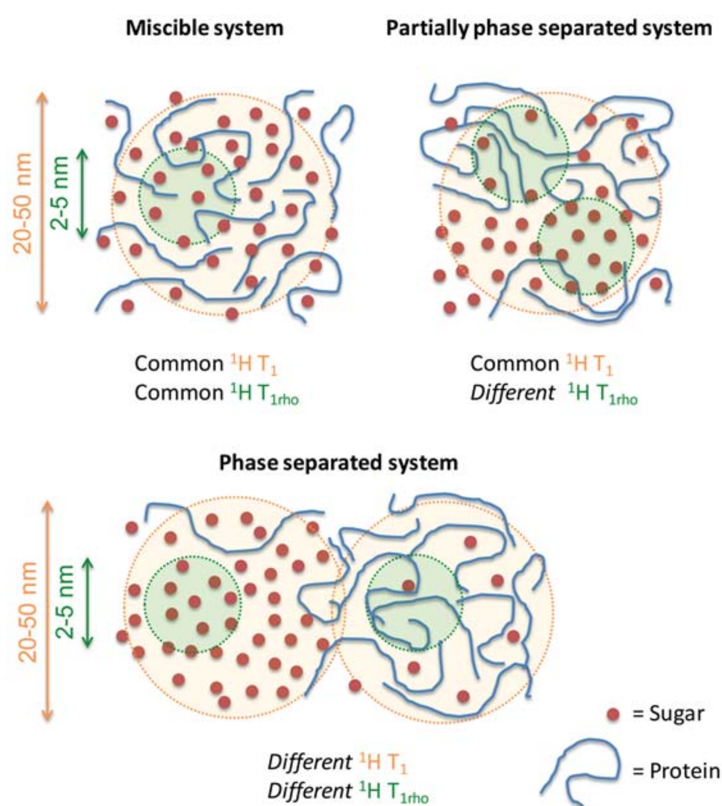

Figure 7.1 Illustration of the effect of spin diffusion on measured area for three different degrees of miscibility. Modified with permission from Yuan *et al.*[8]. Copyright 2015 American Chemical Society.

Recently it was shown that larger and molecularly more rigid sugars (e.g. oligo- and polysaccharides with a backbone through the sugar ring) are less efficient stabilizers of proteins than smaller sugars (e.g. disaccharides).[11] It was found that these larger sugars form fewer hydrogen bonds with the protein during the last part of lyophilisation.[12] This

lack of interactions could be responsible for phase separation in these protein-sugar mixtures. Therefore, the influence of protein-sugar miscibility on protein stability will be investigated in this chapter.

MATERIALS & METHODS

MATERIALS

Immunoglobulin G (IgG, from bovine serum) was obtained from Sigma-Aldrich U.S.A. (St. Louis, MO, United States of America). Trehalose was acquired from Ferro Pfanstiehl (Waukegan, IL, United States of America) and dextrans 1.5, 5 and 70 kDa from Pharmacosmos (Holbaek, Denmark). Inulins 1.8 kDa and 4 kDa were generous gifts from Sensus (Roosendaal, The Netherlands). Sodium phosphate buffer components used for the formulations and dialysis (monobasic as monohydrate, dibasic as heptahydrate) were purchased at Fisher Scientific.

FORMULATION PREPARATION

IgG was received frozen and thawed slowly on ice prior to use. The protein solution (IgG) was dialyzed at least 3 times for a minimum of 3 hours against 2 mM sodium phosphate buffer (pH 7.5, SPB) at 4 °C using a Spectra/Por® 6 dialysis tube membrane with a molecular weight cut-off of 2000 Da. After dialysis, the protein solution was filtered using Millex-GV low-protein binding PVDF syringe filters with a 0.22 µm pore size and 33 mm diameter. Subsequently protein concentration was determined spectroscopically at 280nm, using the absorption coefficient $A_{1cm}^{1\%}$ = 14.[13] The protein solution concentration was adjusted to 10.71 mg/mL by dilution with filtered SPB. Sugar solutions were prepared by dissolving sugars in SPB to a concentration of 100 mg/mL. If needed, solutions were heated to dissolve the sugar. All formulations were also filtered using 0.22 µM PVDF filters. Protein and sugar solutions were mixed to achieve an end concentration of 6 mg/mL protein and 44 mg/mL sugar (12% protein on dry solids basis). When IgG and dextran 70 kDa were mixed, some hazing was observed in the resulting solution. The solutions were transferred to Schott Fiolax® tubular vials of 10 mL with a 13 mm opening. For samples for physico-chemical testing a fill volume of 5 mL was used, for stability testing a fill volume 1 mL was used.

LYOPHILIZATION

Freeze-drying was conducted using a SP Scientific LyoStar 3 (Warminster, PA, USA) equipped with Praxair ControLyo™ controlled ice nucleation technology. Samples were placed on a shelf pre-cooled at 5 °C and kept at that temperature for 30 minutes. The temperature was then lowered to -5 °C at a rate of 1 °C/min and held at that temperature for 30 minutes.

Controlled nucleation was then induced, followed by further cooling to -40 °C at a rate of 1 °C/min. After 60 minutes at that temperature, the pressure was lowered to 87 μBar and the temperature was raised to -25 °C at 1 °C/min. Sample temperature of at least 5 vials was monitored using thermocouples. Shelf temperature was lowered to -28 °C if primary drying was not done after 30 hours or if the product temperature would exceed -33 °C. The end of primary drying was established by comparative pressure measurement (i.e. Pirani *vs.* MKS Baratron).[14] After primary drying was completed, the shelf temperature was slowly raised to 40 °C at a rate of 0.1 °C/min and secondary drying was continued for 6 hours at this temperature. Lastly, the vials were stoppered in the freeze-drier at 87 μBar using Flurotec™ stoppers and were sealed with aluminum crimpers upon removal from the freeze-drier. All samples were stored at -20 °C up until the moment of analysis. Water content of the lyophilizates was determined using Karl Fisher coulometry and was found to be ≤0.25% for all tested formulations.

SOLID STATE NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

All solid state NMR spectra were acquired using a Tecmag Redstone HF3 (Tecmag, Houston, TX) spectrometer operating at 100.6 MHz for ^{13}C (9.4 T static magnetic field). The lyophilized powders were packed into 7 mm zirconia rotors and sealed with Kel-F end-caps (Revolution NMR, LLC, Fort Collins, CO) in a glovebox purged with dry nitrogen gas with a relative humidity of <1%. Experiments were performed using a 7 mm double resonance magic angle spinning (MAS) probe (Agilent, Palo Alto, CA). All ^{13}C spectra were acquired under magic angle spinning (MAS)[15] at 4 kHz, using ramped-amplitude cross polarization (CP),[16] total sideband suppression (TOSS)[17] and spinal64 decoupling[18] with a ^{1}H decoupling field of about 66 kHz. A 2 ms contact time was used in all experiments. 3-methylglutaric acid was used to optimize spectrometer settings and the methyl peak was referenced to 18.84 ppm.[19] All spectra were acquired at ambient temperature.

DETERMINATION OF PHASE SEPARATION

To evaluate phase separation of the protein and sugar, the ^{1}H T_1 and ^{1}H $T_{1\rho}$ values were determined. The ^{1}H T_1 relaxation values were measured using a saturation recovery experiment through ^{13}C observation. The peak areas were integrated, and plotted in Kaleidagraph (Synergy Software, Reading, PA) and fit to the following equation to determine ^{1}H T_1 times for the protein and the sugar peaks individually:

(7.2) $$M = M_0(1\text{-}e(-\tau/T_1))$$

Where M is the integrated signal intensity, M_0 is an amplitude parameter from the fit, τ is the recovery delay time, ^{1}H T_1 is the obtained spin-lattice relaxation time. The protein peak

was integrated in the ~164 – 180 ppm region, whereas for the sugar peak the ~90 - 106 ppm region was used. Only the central 1/3rd of the peaks were used. These peaks were chosen because of the lack of overlap between the two species in those areas. For the ^{1}H $T_{1\rho}$ experiment the spin locking time was varied from 1 to 30 ms. Similar to the ^{1}H T_1 experiments, Kaleidograph was used for integration and fitting of the data using equation 7.3.

(7.3) $M = M_0 e(-\tau / T_{1\rho})$

The symbols are the same as used in equation 7.2. ^{1}H $T_{1\rho}$ is the rotating frame spin lattice relaxation time. Data acquisition parameters for the ^{1}H T_1 experiments were a 1 s recycle delay, 2048 acquisition points (51.2 ms acquisition time), a 3.8 μs ^{1}H 90° pulse, (~66 kHz decoupling field), a 2 ms contact time, and a 4 kHz MAS frequency. 12 time points (0.1, 0.3, 1.2, 1.8, 3.0, 4.0, 9.0, and 15 s) were used in the ^{1}H T_1 determination, and 500 acquisitions were acquired per T_1 point. Data acquisition parameters for the ^{1}H T_{1rho} experiments were a 4 s recycle delay, 2048 acquisition points (51.2 ms acquisition time), a 3.8 us ^{1}H 90° pulse, (~66 kHz decoupling field), a 2 ms contact time, and a 4 kHz MAS frequency. 8 spin locking times (1, 2, 3, 5, 10, 15, 20, and 30 ms) were used, with 500 acquisitions per T_1 point. Data acquisition for the ^{13}C spectra were a 4 s recycle delay, a 2 ms cross polarization contact time, 2048 acquisition points (51.2 ms acquisition time) and 1000 acquisitions.

Data processing was done the same way for all spectra, and was done in the TNMR software package (Tecmag, Houston, TX). The first 512 points (13.3 ms acquisition time) was used, and Fourier transformed with 20 Hz of line broadening. The spectra (supporting information) were individually phased and integrated using abovementioned limits and the fit to the equations above. The central 1/3rd of the peaks was used for the integration to obtain the best data, and minimize error of the peak integrations. The error presented for the peaks is the error of the fit from the Kaleidagraph fitting. Protein and sugar relaxation times were considered different if they were more than two fitting errors apart.

7

STORAGE STABILITY

To evaluate the stabilizing effects of the different sugars, the different formulations were subjected to an accelerated storage stability test. Samples were stored at 60 °C up to 90 days. Samples were taken from the freezer at different time points, so they could all be analyzed together, eliminating assay variations. The storage temperature was below the Tg of all samples tested and they therefore remained physically as original during storage.

HIGH PRESSURE SIZE EXCLUSION CHROMATOGRAPHY (HPSEC)

HPSEC was conducted to provide information on aggregation of the IgG. The samples which were reconstituted with 1 mL ultrapure water were diluted further to a concentration of 1

mg/mL IgG with 20 mM NaH_2PO_4 with 150 mM NaCl (pH 7.5). The samples were filtered using a 0.45 μm filter before being injected on a Superose 6 GL 10/30 column in 100 μL aliquots. The mobile phase was the same as the dilution buffer and the flow used was 0.5 mL/min. Detection was done spectrophotometrically at 214 nm.
Baseline corrections based on the signals between 6 and 7 minutes and 26-27 minutes were carried out for the obtained chromatograms. The area under the curve of the monomer peak (~16 minutes) was determined from half height to half height of the peak. Degradation kinetics were established by correlating monomer peak area and the square root of storage time and carrying out a linear regression analysis, the slope of that curve was used as a measure for the aggregation rate. To limit aggregation after reconstitution of the samples, each sample was measured only once.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Since HPSEC only provides information about the aggregation of IgG but does not give information about functionality, an ELISA was conducted on the same samples to provide additional information. The integrity of the epitope of IgG was used as a measure for its stability. This was established using a bovine IgG ELISA kit supplied by Bethyl (Montgomory, TX, United States of America). Lyophilized samples were reconstituted using 1 mL of ultrapure water and diluted to concentrations between 500 and 7.8 ng/mL using diluent buffer, which consisted of 50 mM Tris with 0.14 M NaCl and 0.05% Tween 20 (pH 8.0). Analysis was carried out on 96-wells plates, which were pre-incubated with affinity purified bovine IgG coating antibody for an hour at room temperature and subsequently blocked with blocking solution, using the diluent buffer, at the same temperature for 30 minutes. The diluted samples were incubated for an hour at room temperature, followed by incubation of horseradish peroxidase conjugated bovine IgG detection antibody for an hour. Lastly a substrate solution containing 3,3',5,5'-tetramethybenzidine (TMB) was added and incubated in the dark during 15 minutes at room temperature. The reaction was stopped by addition of 0.18 M H_2SO_4, after which the conversion of TMB by peroxidase was measures spectrophotometrically at 450 nm. The plates were washed 5 times with diluent buffer between all steps up to the addition of TMB. The absorbance was related to an unprocessed reference sample, giving an indication of the amount of epitopes still intact. Results were not related to t=0 as some formulations already showed loss of epitopes during lyophilization. Each sample was measured in 4 dilutions, results from the two optimal dilutions were averaged. A duplicate analysis of a large part of the formulations provided nearly identical results.

RESULTS

SOLID STATE NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

For the formulations of trehalose, dextran 1.5 kDa, and both inulins the ^{1}H T_1 relaxation times of protein and sugar are indistinguishable (figure 7.2 (top)). For dextran 5 kDa and 70 kDa the values of the protein and sugar are clearly different, with a bigger difference in ^{1}H T_1 relaxation times for the formulation of dextran 70 kDa. This is indicative of phase-separation at the 20-50 nm scale for formulations with dextran 5 kDa and 70 kDa and IgG. For the formulations with IgG and trehalose, both inulins, and dextran 1.5 kDa there is no indication of phase separation on this scale.

In addition to phase separation, there is a trend in the ^{1}H T_1 relaxation time, where the lower molecular weight sugar systems had the longest relaxation time, and the protein relaxation time decreases as the sugar molecular weight increases. Since ^{1}H T_1 relaxation times are indicators of molecular mobility, the longer relaxation times would correlate with a lower molecular mobility.[20,21] The absolute ^{1}H T_1 relaxation time of IgG is highest for the smallest sugar trehalose and decreases when the molecular weight of the combined sugar increases, suggesting less molecular mobility of the protein when incorporated in the low molecular weight sugar.

The ^{1}H $T_{1\rho}$ relaxation times of IgG do not show an increase with lower molecular weight (figure 7.2 (bottom)). ^{1}H $T_{1\rho}$ relaxation times are indistinguishable for trehalose and IgG, somewhat different for the inulin formulations, and, even more different for the formulations containing dextran. This indicates miscibility down to the 2-5 nm scale for trehalose. The other formulations show immiscibility on this scale. Therefore, combinations of IgG with both inulins and dextran 1.5 kDa are classified as partially phase separated, whereas dextran 5 kDa and 70 kDa are phase separated and trehalose and IgG are fully miscible.

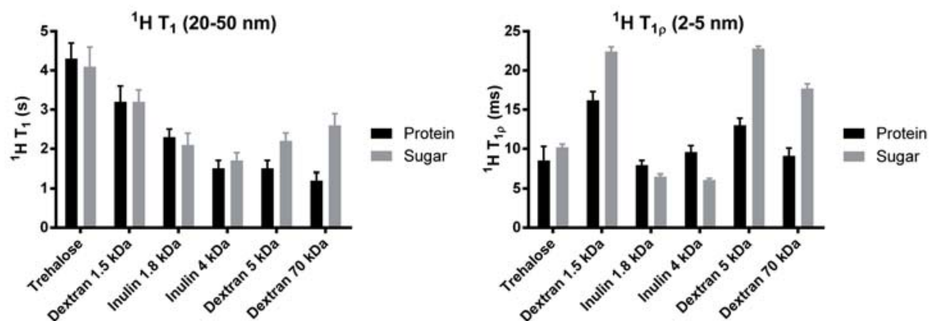

Figure 7.2 Spin-lattice relaxation times (^{1}H T_1, top) and rotating-frame spin-lattice relaxation times (^{1}H $T_{1\rho}$, bottom) of both protein and sugar from lyophilizates of IgG with various sugars. The formulations are ordered by molecular weight of the sugar, increasing from left to right.

STORAGE STABILITY - HPSEC

During storage at 60 °C the formulation containing trehalose shows nearly no aggregation of IgG (figure 7.3). For the other sugars, there is an increasing degree of IgG aggregation with increasing molecular weight of the sugar, with the exception of dextran 5 kDa. The formulation with dextran 1.5 kDa shows ~20% of aggregation of IgG after 90 days of storage, for inulin 1.8 kDa and dextran 5kDa this is ~40%. Inulin 4 kDa shows ~50% loss of monomer and lastly there was no clear difference in aggregation between the formulation without sugar and that with dextran 70 kDa. The aggregation results correlate with the ^{1}H T_1 relaxation times of the protein, and are consistent with the more stable samples being fully or partially miscible, and the phase separated samples having the poorest stability.

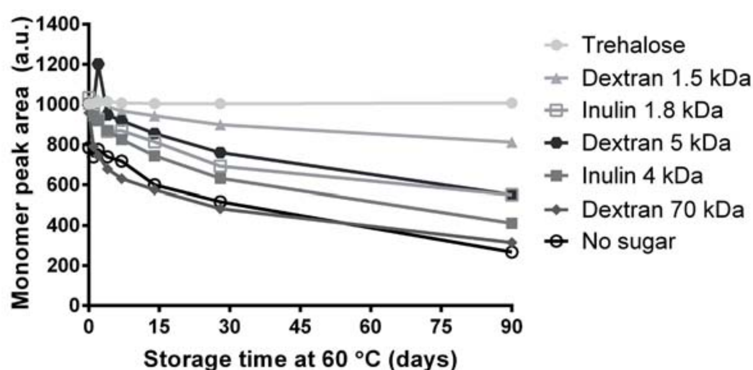

Figure 7.3 Amount of unaggregated IgG (soluble monomer) of IgG lyophilized with various sugars during storage at 60 °C during 90 days.

STORAGE STABILITY – ELISA

As with aggregation, the formulation of IgG and trehalose shows little or no change in amount of intact epitopes during storage (figure 7.4). Inulin 4 kDa shows a loss of around 30% of epitopes, the formulations with dextran 70 kDa and the formulation without sugar lost ~ 40% of epitopes. Remarkably, the smaller inulin 1.8 kDa and the two smaller dextrans 1.5 and 5 kDa show a bigger loss of intact eptiopes than the formulation without sugar, a total loss of ~50%, ~70% and ~60% respectively. This indicates a deteriorative effect of the sugar on the protein, which could indicate that the reducing groups of the sugar reacted with the amine groups of the protein, the so-called Maillard reaction. Initial Maillard browning could affect the functional epitopes of IgG and therewith the ELISA data, but because of the molecular weight of these sugars compared to that of IgG, it would likely not impact HPSEC results signficantly. Some discoloration was observed in the samples with

dextran 1.5 kDa, dextran 5 kDa and inulin 1.5 kDa after 90 days. Most discoloration was found in the formulation containing dextran 1.5 kDa. There was no observable discoloration for formulations with trehalose, inulin 4 kDa and dextran 70 kDa.

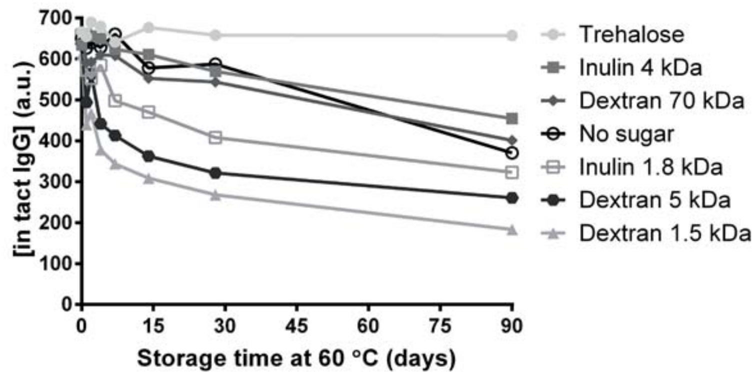

Figure 7.4 Amount of intact epitopes of IgG lyophilized with various sugars during storage at 60 °C during 90 days.

DISCUSSION

In this chapter, the influence of miscibility of protein-sugar lyophilizates on the storage stability was investigated using model protein IgG. Miscibility was evaluated by comparing ssNMR relaxation times at two timescales, providing information on miscibility on 2-5 nm and 20-50 nm length scales. When ^{1}H T_1 relaxation times of protein and sugar were different for a single sample, this indicated immiscibility of the system already on the larger 20-50 nm scale and these combinations were classified as immiscible. When ^{1}H T_1 relaxation times were similar, but ^{1}H $T_{1\rho}$ relaxation times, with a diffusion length scale of 2-5 nm, were different, the combination was considered partially phase separated. If both ^{1}H T_1 and ^{1}H $T_{1\rho}$ relaxation times were similar, there was no indication of phase separation and the sample was deemed miscible. Table 7.1 shows the classifications of all the tested combinations, based on this rationale.

Previously we reported that smaller and molecularly more flexible sugars were better capable of stabilizing proteins during lyophilization,[11] as they were less inhibited by steric hindrance and configurational inflexibility and therewith better able to form hydrogen bonds with the protein during lyophilization.[12] Here, it was found that miscibility and absolute ^{1}H T_1 relaxation times decreased with increasing molecular weight of the sugar used. ^{1}H T_1 relaxation times have been shown to be predictive for physical stability of amorphous

systems[22] and protein storage stability,[20] presumably because the molecular mobility involved in generating the ^{1}H T_1 relaxation process is similar to the molecular mobility that causes degradation. Here too, protein ^{1}H T_1 relaxation times correlate with protein aggregation rates (figure 7.5).

Table 7.1 Classification of miscibility based on ssNMR relaxation times of protein-sugar lyophilizates.

Sugar	MW (kDa)	20-50 nm	2-5 nm	Classification
Trehalose	0.3	● (grey)	● (grey)	Miscible
Dextran	1.5	● (grey)	●	Partially phase separated
Inulin	1.8	● (grey)	●	Partially phase separated
Inulin	4	● (grey)	●	Partially phase separated
Dextran	5	●	●	Phase separated
Dextran	70	●	●	Phase separated

MW = molecular weight; ● (grey) = similar relaxation times; ● = different relaxation times

The overall size of IgG is significantly larger than 2-5 nm, with a total maximum length of ~16 nm.[23] However, the protein is not a sphere and its subdomains (chains) are smaller, with dimensions of approximately 2.5 by 2.5 by 5 nm.[24] Therefore, these miscibility data likely describe the ability of sugars to enter empty spaces in the protein's three-dimensional structure and form intimate contact. Thus, the miscibility we describe here could possibly indirectly indicate steric hindrance of the protein and sugar. This is in line with our previous findings, as a close proximity of the sugar to the protein is prerequisite for hydrogen bonding and therewith stabilization. It is therefore not surprising that the smallest sugar tested, disaccharide trehalose, is the best stabilizer of IgG as represented by both the HPSEC and

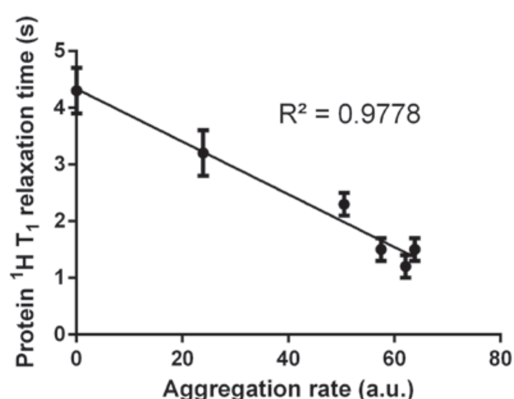

Figure 7.5
Correlation between protein aggregation rate (loss of monomer as determined with HPSEC per the square root of time during storage) versus protein ^{1}H T_1 relaxation times for the different formulations.

ELISA storage stability data, and that stabilizing capacity diminishes with increasing molecular weight of the stabilizer.

The ELISA data do not correlate as well with the miscibility data as the HPSEC results do. Most likely, this is because of an additional mechanism of degradation, being the Maillard reaction. Inulin without the glucose end group and dextran both have a reducing end group, which is relatively more abundant in lower molecular weight sugars.[25] This explains why the smaller dextrans (1.5 and 5 kDa) and smaller inulin (1.8 kDa) maintain less functional epitopes than the protein without sugar. As mentioned previously, chemical bonding of a sugar of 1.5-5 kDa, as happens in the initial steps of the Maillard cascade, does not have a large impact on the total mass and three-dimensional size of IgG, which has a molecular weight of approximately 150 kDa. Therefore, HPSEC is insensitive to Maillard reactions with relatively small oligosaccharides, unlike the ELISA assay which will detect these changes if they impact the functional epitope directly or indirectly do so by causing conformational changes.

Considering the above, smaller non-reducing sugars are preferred for stabilizing proteins as they are less limited by steric hindrance and are thus more miscible with the protein. However, these sugars are generally characterized by a lower glass transition temperature (Tg) and with that care should be taken that vitrification of the formulation is maintained during storage.[26] That is, one should not exceed the glass transition temperature. This is particularly a problem in case of higher levels of residual moisture or moisture sorption, as water has a strong plasticizing effect on sugar glasses.[27] In cases where the Tg would not be adequate for the planned storage conditions (i.e. approximately 10-20 °C higher than the storage temperature),[28] combining a large polysaccharide with a non-reducing disaccharide is a suitable alternative.[29,30] This limits steric hindrance as much as possible, whilst achieving a high enough Tg.[11]

CONCLUSION

In summary, ssNMR is able to detect miscibility on a level close to the size of the protein molecules and the obtained miscibility classifications and $^{1}T\ H_{1}$ relaxation times correlate well with aggregation rates of IgG during storage. The ELISA storage stability data show degradation by both aggregation and Maillard browning, which cannot be deconvoluted accurately. Smaller sugars show better protein-sugar miscibility as they are less influenced by steric hindrance and this makes them more suitable as stabilizers of proteins.

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SUPPORTING INFORMATION

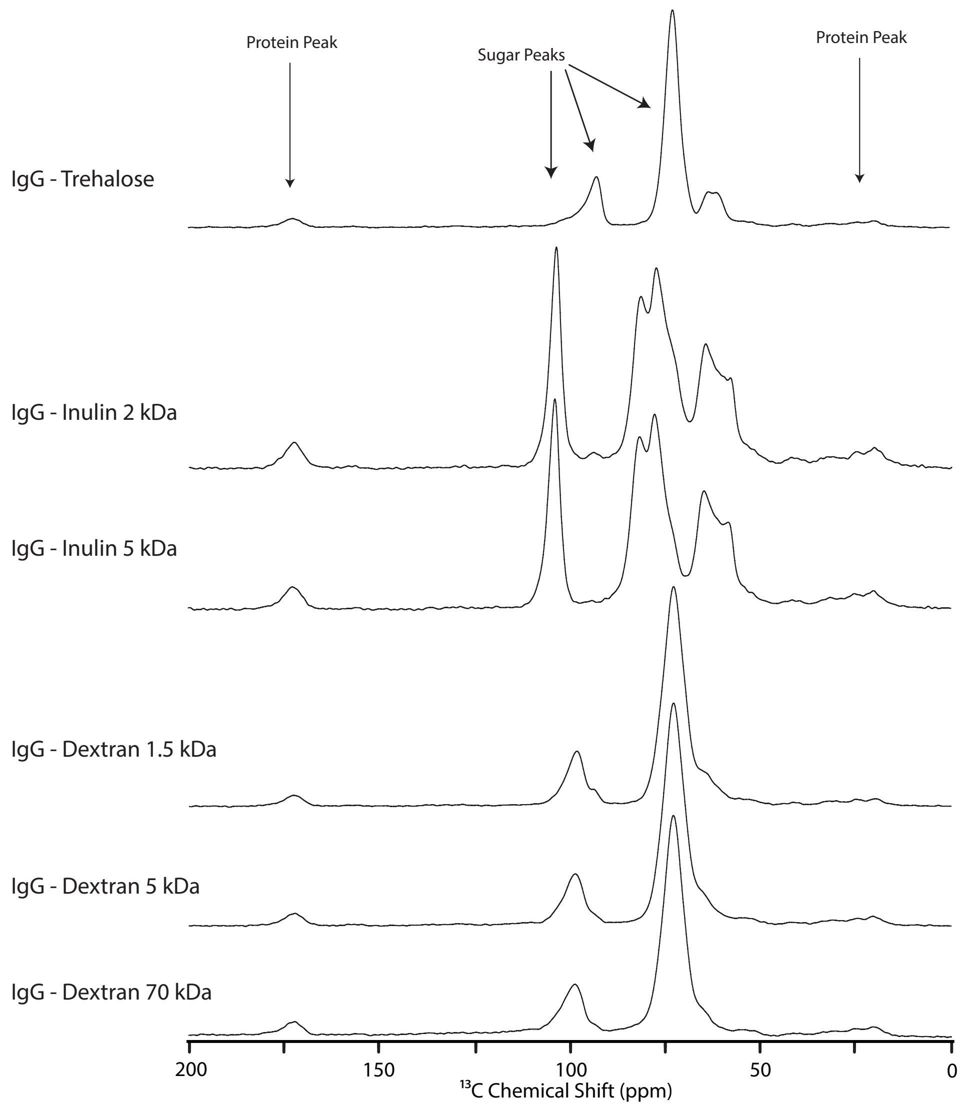
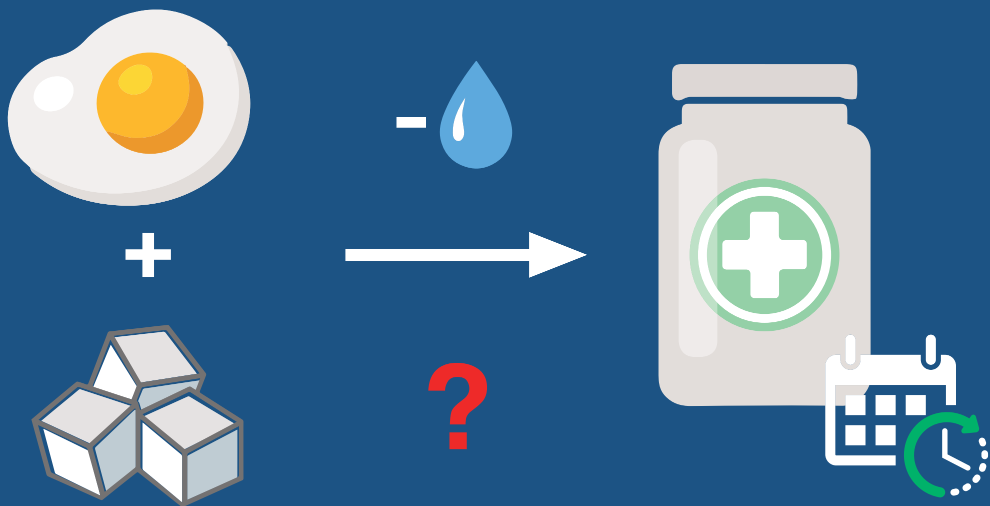


Figure 7.S1 Solid state Nuclear Magnetic Resonance spectra of the various formulations.



How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions

8

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ABSTRACT

This review aims to provide an overview of current knowledge on stabilization of proteins by sugars in the solid state in relation to stress conditions commonly encountered during drying and storage. First protein degradation mechanisms in the solid state (i.e. physical and chemical degradation routes) and traditional theories regarding protein stabilization (vitrification and water replacement hypotheses) will be briefly discussed. Secondly, refinements to these theories, such as theories focusing on local mobility and protein-sugar packing density, are reviewed in relationship to the traditional theories, and their analogies are discussed. The last section relates these mechanistic insights to the stress conditions against which these sugars are used to provide protection (i.e. drying, temperature, and moisture). In summary sugars should be able to adequately form interactions with the protein during drying, thereby maintaining the protein in its native conformation and reducing both local and global mobility during storage. Generally smaller sugars (disaccharides) are better at forming these interactions and reducing local mobility as they are less inhibited by steric hindrance, whilst larger sugars can reduce global mobility more efficiently. The principles outlined here can aid in choosing a suitable sugar as stabilizer depending on the protein, formulation and storage condition-specific dominant route of degradation.

INTRODUCTION

Over the past decades, the importance of protein therapeutics for the pharmaceutical industry has grown from a nearly negligible role to being a primary focus. As proteins are generally not stable for prolonged periods of time, formulation scientists faced many challenges in achieving sufficient shelf life for these protein therapeutics.[1,2] A lot of these challenges have been overcome, as is illustrated by the fact that in 2015 nearly 30% of drugs newly registered at the United States Food and Drug Administration (FDA) were protein drugs.[3] However, all but 1 of these protein drugs are liquid formulations which require refrigerated (2-8 °C) storage and transportation, the so-called cold chain, whereas the remaining dry powder formulation (mepoluzimab, Nucala®) must be stored and transported below 25 °C, see table 8.1.

Table 8.1 Overview of protein drugs newly registered at the United States Food and Drug Administration (FDA) in 2015, their type, physical form, and storage temperatures.[3]

Protein	Trade name	Type	Form	Storage temperature
alirocumab	Praluent	monoclonal antibody	liquid	2-8°C
asfotase alfa	Strensiq	enzyme	liquid	2-8°C
daratumumab	Darzalex	monoclonal antibody	liquid	2-8°C
dinutuximab	Unituxin	monoclonal antibody	liquid	2-8°C
elotuzumab	Empliciti	monoclonal antibody	liquid	2-8°C
evolocumab	Repatha	monoclonal antibody	liquid	2-8°C
idarucizumab	Praxbind	monoclonal antibody	liquid	2-8°C
insulin degludec	Tresiba	hormone	liquid	2-8°C
mepolizumab	Nucala	monoclonal antibody	lyophilized powder	>0°C;<25°C
necitumumab	Portrazza	monoclonal antibody	liquid	2-8°C
recombinant human parathyroid hormone	Natpara	hormone	liquid	2-8°C
sebelipase alfa	Kanuma	enzyme	liquid	2-8°C
secukinumab	Cosentyx	monoclonal antibody	liquid	2-8°C

Table 8.2 Lyophilized protein drugs approved for marketing by the FDA (Biologics License Agreement (BLA)) between 2011 and 2016. Approval and product information from www.fda.gov and product labels [8].

Trade name	Protein	Class	Protein quantity
Adcetris	Brentuximab vedotin	Antibody-drug conjugate	52.5 mg
Benlysta	Belimumab	Monoclonal antibody	120 or 400mg
Blincyto	Blinatumomab	Monoclonal antibody	35 mg
Entyvio	Vedolizumab	Monoclonal antibody	300mg
Erwinaze	Asparaginase *Erwinia chysanthemi*	Enzyme	10.000 IU
Inflectra	Infliximab	Monoclonal antibody	100 mg
Kadcyla	Ado-trastuzumab emtansine	Antibody-drug conjugate	100 or 160mg
Keytruda	Pembrolizumab	Monocolonal antibody	50 mg
Myalept	Metreleptin	Hormone	11.3 mg
Nucala	Mepolizumab	Monoclonal antibody	100 mg
Nulojix	Belatacept	Fusion protein	250 mg
Sylvant	Siltuximab	Monoclonal antibody	100 or 400mg
Voraxaze	Glucarpidase	Enzyme	1000 units

Table 8.2 *Continued*

Excipients	Company	Protein size (kDa)
735 mg trehalose 58.8 mg sodium citrate dihydrate 2.21 mg citric acid 2.1 mg polysorbate 80	Seattle genetics	153
For 400 mg formulation 400 mg sucrose 13.5 mg sodium citrate 2.0 mg citric acid 0.8 mg polysorbate 80	GSK	147
95.5 mg trehalose dehydrate 23.23 mg lysine hydrochloride 3.35 mg citric acid monohydrate 0.64 mg polysorbate 80	Amgen	54
500 mg sucrose 131.7 mg L-arginine hydrochloride 23 mg L-histidine 21.4mg L-histidine monohydrochloride 3 mg polysorbate 80	Takeda	147
5.0 mg glucose monohydrate 0.5 mg sodium chloride	Jazz Pharmaceuticals	35
500 mg sucrose 6.1 mg disodium hydrogen phosphate dihydrate 2.2 mg sodium dihydrogen phosphate monohydrate 0.5 mg polysorbate 80	Pfizer	149
For 160mg formulation 480 mg sucrose 1.6 mg polysorbate 20 0.08 mmoles sodium succinate	Genentech	149
140 mg sucrose 3.1 mg L-histidine 0.4 mg polysorbate 80 Optional sodium hydroxide or hydrochloric acid	Merck	149
44 mg glycine 22 mg sucrose 3,23 mg glutamic acid 0.2 polysorbate 20	Bristol-Myers Squibb	16.15
160 mg sucrose 7.14 mg sodium phosphate dibasic heptahydrate 0.67 mgPolysorbate 80	GSK	149
500 mg sucrose 34.5 mg monobasic sodium phosphate 5.8 mg sodium chloride	Bristol-Myers Squibb	90
For 400mg formulation: 14.9 mg L-histidine (from L-histidine and L-histidine monohydrochloride monohydrate) 3.2 mg polysorbate	Janssen	145
10 mg lactose monohydrate 0.6 mg Tris-HCl 0.002 mg Zinc acetate dihydrate	BTG International	83

Maintaining the cold chain regime is costly and particularly provides difficulties in remote areas of developing, often tropical, countries.[4] One of the proven strategies to overcome this, is to dry proteins in the presence of stabilizers like sugars.[5–7] The number of licensed lyophilized protein drugs has also grown steadily; table 8.2 provides an overview of lyophilized protein drugs which received a biological license approval by the FDA since 2011. A plethora of research on the topic of drying proteins with sugars has been published by scientists from food and pharmaceutical sciences, describing various aspects of how these sugars stabilize proteins. This review aims to provide an overview of the current knowledge regarding the mechanisms behind stabilization of proteins by sugars in the solid state in relationship to stress conditions commonly encountered during production and storage. First protein degradation mechanisms and traditional theories regarding protein stabilization will be briefly discussed; secondly refinements to these theories and how they come together will be reviewed. The last section will relate stress conditions to how sugars protect against them.

DEGRADATION

Degradation of proteins commonly leads to a loss of functionality and formation of potentially immunogenic products.[9] To understand stabilization of proteins an understanding of how proteins can degrade is important. Therefore, the main mechanisms of degradation of proteins, classified as either physical or chemical degradation, will be addressed here briefly. For more in-depth information the reader is directed to several extensive reviews on this topic.[2,10–12]

The most common physical degradation mechanisms are denaturation and noncovalent aggregation. Denaturation is the unfolding of the three-dimensional structure of the protein. This can be caused by various stresses such as heat, shear stress, exposure to interfaces, or chemical factors.[2,10] Denaturation can occur in the solid state but is more likely to happen when the protein is dissolved in a liquid and during drying.[5,6,10,13,14] Generally in the native conformation hydrophobic parts of the protein are folded inward and unfolding/ denaturation results in these groups being exposed on the outside of the protein's three-dimensional structure.[10] The increased surface area and exposed hydrophobic groups of unfolded or partially refolded proteins increase the risk of adsorption and non-covalent aggregation.[1,15] Therefore, non-native proteins have a higher tendency to aggregate than native proteins.[16,17] Aggregation is in most cases irreversible.[1] Furthermore, aggregates in liquid formulations can be qualified as either soluble or insoluble and when aggregate size increases, sedimentation (or floating) will eventually occur.[13]

Important chemical degradation mechanisms include covalent aggregation, deamidation, oxidation, and Maillard browning. Chemical covalent aggregation, rather than physical non-covalent aggregation, is the predominant route of aggregation in the solid state.[18,19] Chemical aggregation is in most cases linked to a thiol-disulfide interchange in the protein, and is accelerated by residual moisture or exposure to atmospheric water. Many other chemical degradation mechanisms (i.e. oxidation, deamidation, Maillard browning) are also dependent on moisture content (see section *Moisture*). Other factors affecting these chemical degradation reactions include storage temperature, excipients, the physical state of the excipients (e.g. liquid, amorphous, crystalline), and obviously the chemical composition of the protein.[12] In the context of stabilizing proteins with sugars, Maillard browning is of particular interest as it involves reducing sugars. Maillard browning starts with a reaction between the aldehyde or ketone group of the reducing sugar and the amino group of the protein forming a Schiff's base and is followed by a cascade of reactions eventually leading to the formation of covalent aggregates.[20]

THEORIES ON STABILIZATION BY SUGARS

Two theories on the mechanism of stabilization of sugars on proteins in the solid state, the vitrification theory and water replacement theory, have been around for several decades and have been widely discussed in literature.[21,22] More recently, refinements and new theories focusing on global and local mobility of the protein, molecular flexibility of the sugar, and protein-sugar miscibility on a molecular level have been published.

CLASSIC THEORIES: VITRIFICATION AND WATER REPLACEMENT

Stabilization of bioactive proteins is traditionally based on two approaches: the vitrification theory which describes alterations in reaction kinetics and the water replacement theory which is based on (equilibrium) thermodynamic considerations. The vitrification theory is based on the concept of immobilizing the protein in a rigid, amorphous glassy sugar matrix and by doing so drastically slowing down degradation.[21] Unfolding and most other modes of degradation require molecular mobility of the protein and are thus slowed down by vitrification.[23] A striking example of vitrification in nature is the preservation of insects in amber for up to tens of millions of years.[24] Thus, the vitrification theory describes stabilization from a kinetic perspective. Sugar glasses are characterized by a glass transition temperature (Tg), above which the kinetic immobilization and therewith also the stabilizing power of the sugar are largely lost.[25] In addition, crystallization of small molecules such as the sugar can occur when the system is in the rubbery state (i.e. above the Tg) and this can have detrimental effects on proteins.[26,27] Water plays a vital role as it drastically reduces the Tg of sugar glasses.[28]

The water replacement theory describes stabilization from a thermodynamic point of view.[10] It encompasses the concept that during drying the hydroxyl groups of the sugar form hydrogen bonds with the protein, thereby replacing hydrogen bonds between water and the protein. By this replacement of hydrogen bonds the protein's native conformation is maintained.[22,29–32] Carpenter and Crowe showed that the ability of a sugar to prevent shifts of the amide II band, indicative of protein secondary structure and hydrogen bonding, during drying correlated with their ability to stabilize enzymes during drying.[22]
Vitrification and water replacement both result in preservation of the structure of the protein, by preventing molecular mobility and by preventing changes in protein structure, respectively.[33,34] Grasmeijer and coworkers showed that as long as there is sufficient vitrification, i.e. a Tg of at least 10-20 °C above the storage temperature, water replacement is the predominant mechanism of stabilization.[35] However, when the storage temperature is closer to or over the Tg, vitrification becomes the limiting factor for stability. This illustrates that both theories have their merit, but also that both cannot fully explain protein stabilization on their own, leaving room for further refinements.

REFINEMENTS OF THEORIES

Local versus global mobility

Recently, it has been shown that local mobility (β relaxation) of specific groups of the protein can be more predictive of protein stability than the global mobility (α relaxation), on which the vitrification theory is based.[36–38] This was further confirmed using anti-plasticizers, additives which can increase global mobility whilst reducing local mobility, and plasticizers, which increase both global and local mobility.[39] The predictive capacity of β relaxations are presumed to be derived from coupling of β relaxations to local molecular mobility of the protein, and coupling to diffusion rates of small molecule reactive species in the glass.[39] β relaxations can be measured with neutron backscattering, but unfortunately facilities for neutron backscattering measurements are not available for routine testing. A potential alternative benchtop method using time-resolved fluorescence Stokes-shift has been proposed, which is currently under development.[40,41]
One could hypothesize that global mobility correlates with physical degradation and local mobility correlates with chemical degradation, as for physical degradation mobility on a larger scale is needed and for chemical degradation mobility of specific groups of the protein is more relevant. As a rule of thumb, this concept seems to hold some truth, but it was also shown that this distinction cannot be made so generally as different routes of chemical degradation require mobility on different length scales and global mobility can also play a role there.[42,43] However, when the degradation routes of a protein and the therefore required types of mobility are known, it should be possible to come up with targeted strategies to prevent these degradations based on the concepts illustrated above.

Packing density and interactions

Several research groups have explored why some sugars are better water replacers than other sugars and/or further looked into the concept and consequences of water replacement. It was shown that smaller and molecularly more flexible oligosaccharides (i.e. those with more flexible backbones) were better able to stabilize four model proteins during storage after lyophilization than their larger and molecularly more rigid counterparts.[44] Additionally, these smaller and molecularly more flexible sugars formed more hydrogen bonds with the protein during freeze-drying.[32] This was proposed to be due to the fact that these sugars are less sterically hindered in interacting with the protein and could therefore achieve a tighter packing with the protein.[44] A logical consequence of stronger interactions and a tighter packing are increased density and thus a decreased free volume of these formulations. Using positron annihilation lifetime spectroscopy (PALS) it was shown that addition of maltose to a maltopolymer reduced free volume and molecular dynamics simulations showed that free volume increased with increasing molecular weight for amorphous maltodextrins.[45,46] Additionally, density of amorphous dextran powders was found to increase with decreasing molecular weight of the polysaccharide as measured by gas pycnometry.[47] It is most likely that the same principles apply for protein-sugar mixtures, with smaller sugars reducing free volume by filling smaller ‘cavities’ of the protein structure. It is evident that protein-sugar interactions are essential for protein stabilization. Lesser known is the fact that next to replacing hydrogen bonds, sugars can also interact with aromatic protein residues via their CH groups.[48] These so-called CH-π interactions are highly relevant for protein-ligand binding but they also play a (modest) role in protein stabilization.[49] Given that interactions are essential, miscibility of protein and sugars on a molecular level is an absolute requirement for successful stabilization. Phase separation between protein and sugar after freezing and lyophilization has been reported.[50,51] Protein-sugar miscibility decreased with increasing sugar size for formulations with a model protein, IgG, and reduced miscibility correlated with increased protein aggregation.[50] Factors of the freezing process, such as sugar concentration and degree of supersaturation prior to freezing, can also be of influence on phase separation.[51] When phase-separation or partial phase-separation occurs during freeze-drying, one might expect a change in the amount of protein found on the solid-air interface. Using surface analysis it was shown that the estimated amount of protein present at the solid-air interface varied for different protein-sugar combinations and correlated with storage stability of the protein.[52,53] For spray-dried protein-sugar formulations it was shown that proteins are relatively more abundant on the dried particle surface compared to in the center because they are surface active and because during drying the protein’s relatively large size inhibits them from diffusing away from the drying interface as fast as smaller sugars.[54,55] Increasing the amount of sugar in the formulation or adding surfactants to the formulation could reduce the relative presence of proteins on the particle surface.

Phase separation can also occur in the solid state by crystallization of one of the components. As mentioned in section *Classic theories: vitrification and water replacement*, it is widely recognized that crystallization of the sugar in an amorphous protein-sugar formulation is detrimental for protein structure. This is because the crystallization process causes a loss of interactions and induces shear stresses on the protein.[26,56,57] Crystallization can occur when the storage temperature is higher than the glass transition temperature, yet then still some sugars have a lower tendency to crystallize than other sugars.[56] Therefore a low tendency to crystallize is desired in addition to a native glass transition temperature high enough to achieve a glass transition temperature of the formulation higher than the storage temperature for protein stabilizing sugars.

COMMON GROUNDS

The above illustrates that stabilization of proteins by sugars is a complex puzzle which cannot be solved by a single hypothesis and that the different theories each describe stabilization from a different perspective. Moreover, one stabilization approach has more than one effect from a mechanistic perspective. For example, water replacement describes how hydrogen bonding is responsible for protein stabilization, yet hydrogen bonding also implies close contact of the sugar with the protein by which a reduction in local mobility of (reactive) protein groups is achieved.[25,58] Similarly, the preservation of protein structure is ascribed to the water replacement hypothesis, but is equally relevant in the vitrification theory. Because if vitrification is lost and crystallization occurs, hydrogen bonds between sugar and protein will also be broken, resulting in loss of stabilization according to both mechanisms. Conversely, with sufficient vitrification but limited or no protein-sugar interactions, protein structure is also lost.[32,37,59] In that sense, the sugar could simplistically be seen as a scaffolding around the protein, inhibiting protein movement locally (water replacement, reduction of local mobility) and more globally by its rigidity (vitrification).[44] Not surprisingly, this representation also does not completely explain protein stabilization, as it does not account for reactions of the protein with other molecules (i.e. oxygen, water, sugar, impurities) which could be present in and/or diffuse through the sugar matrix [60]. Therefore, it is important to relate the protein stabilizing capacity of the sugars to specific stress conditions and degradation pathways.

STRESSES DURING PRODUCTION AND STORAGE

Proteins are produced as solutions and thus require drying to become solids. The most important stress factors against which sugars can be used as stabilizers are dehydration stress, temperature and moisture. In this section, those stresses will be discussed within

the framework of the above presented theories. Stresses against which sugars do not provide specific protection, such as shear stress, acidity and photodegradation will not be discussed here. For more information regarding those topics, the reader is directed to the reviews of Manning *et al.* (2010), Chang *et al.* (2009), and Cicerone *et al.* (2015).[2,10,58]

DRYING

It is essential that stabilization is effective during drying, as degradation of the protein during drying can accelerate degradation during subsequent storage.[43] Proteins can be dried using a range of techniques, with lyophilization (freeze-drying) and spray drying being the most frequently used techniques.[61,62] In pharmaceutical industry, lyophilization is the most used technique as it is generally less stressful to the protein and can be part of an aseptic process, whereas spray-drying is more frequently used for food purposes for economic reasons.[61,63] In spray-drying a solution is atomized by pumping it through a nozzle and exposing it to hot air, causing evaporation of the moisture and thus drying. In freeze-drying, the solution is frozen and water is subsequently removed by sublimation under a vacuum. These two processes subject the protein to fundamentally different stresses. Spray-drying exposes the protein to shear (during atomization), heat, air-liquid interfacial, and dehydration stresses; where lyophilisation is associated with freezing, dehydration and solid-liquid interfacial stresses.[61,64] It can be assumed that sugars are not effective against shear and interfacial stresses, for which surfactants are frequently used as protectant.[2,65] Sugars can be more useful in protecting against dehydration, freezing and thermal stress.[29,66–69] Here again, stabilization depends on the characteristics of the sugars, as the stabilization is based on forming interactions with the protein and reducing global and local mobility. It deserves extra mention here that drying conditions, e.g. the freezing rate in lyophilization, are also very important to maximize protein stabilization and these conditions should thus be chosen carefully.[14,68]

Additionally, differences in solubility of different components form a potential issue during drying. For example, when sodium phosphate buffer is frozen one of the components can precipitate, resulting in a pH drop of up to 3 units, which is clearly problematic for protein formulations.[70] The same principle possibly also explains the observed reduced miscibility of larger sugars (i.e. polysaccharides) with proteins described in section *Packing density and interactions*. Of course, the processing parameters of drying (drying rate, time, temperatures etc.) influence the stresses of drying and thus how much degradation occurs during drying.[71–73] Therefore, an optimized combination of formulation and processing should be chosen to maximize protein stability.[74] The effectiveness of different sugars as stabilizers probably also depends on the drying process used and other formulation choices such as the used protein-sugar ratio. It was for example recently shown that the stabilizing capacity of disaccharide sucrose depended on the protein-sugar ratio used, contrasting to a

homologous disaccharide trehalose which did not show such a dependency.[75] The stabilizing capacity of sugars is commonly compared by drying proteins with different sugars in the same protein-sugar ratio with the same drying regime for all formulations. It is possible that if optimized drying regimes and protein-sugar ratio were to be used for the different sugars, different results could be obtained. We therefore think that further elucidation of the interplay between drying regime and other formulation aspects with stabilization by sugars with different characteristics would be valuable.

TEMPERATURE

Thermal stress is considered a major stress factor for dry protein formulations, as degradation generally increases with temperature and one of the reasons to dry proteins in the first place can be to circumvent the cold chain. In the liquid state, proteins are characterized by a 'melting' temperature (Tm), above which they rapidly unfold and lose their functionality.[76] For solid state proteins dried in the presence of sugars the Tg is the temperature above which the degradation rate increases rapidly. In general degradation is not as rapid after surpassing the Tg as it is after surpassing the Tm in the liquid state. Above the Tm the free energy change associated with the transition from folded to unfolded is negative, making the unfolded state thermodynamically favorable. Surpassing the Tg is characterized by an increase in global mobility; it is a kinetic process. Hence, it does not necessarily lead to immediate degradation.[56,76]

Degradation can also occur below the Tm and Tg, albeit much more slowly and not mainly by unfolding. Both chemical and physical degradation (see section *Degradation*) can potentially occur below these transition temperatures.[2,23] The mechanism of stabilization of sugars against thermal stress has largely been explained in section *Common grounds* and relies on forming an immobilizing matrix around the protein, which ultimately reduces local and global mobility, resulting in protein structure preservation.[44,77] For physical degradation the concept of reduced mobility can be easily imagined as for those routes of degradation molecular mobility of the protein is required and this is strongly reduced by vitrification. For chemical degradation this link is not as obvious. It has been suggested that chemical degradation is reduced by sugars through a reduction of solubility and reduction of diffusion of small molecule reactive species in the glass as well as by a reduction of local protein motions.[25,39]

Unfortunately, just adding any sugar to a protein and drying does not guarantee protein stabilization, as not all sugars are equally good at stabilizing proteins. To maximize stabilization, the choice of sugar and the method of drying are of particular interest. As mentioned, to achieve more interactions (i.e. hydrogen bond) and therewith a reduction of local mobility, smaller sugars usually are more suitable.[32] However, for vitrification, which is also required, larger sugars (oligo- and polysaccharides) are generally more suited.

Therefore, a balance should be found where sufficient vitrification is maintained (i.e. a formulation Tg of around 10-20 °C above storage temperature), whilst maximizing reduction of local mobility.[35] Ideally, one would therefore use a relatively small sugar with a relatively high glass transition temperature and a good ability hydrogen bond with the protein, such as trehalose. If a higher Tg is desired, this can be achieved by combining large polysaccharides with smaller disaccharides (anti-plasticization of the polysaccharide) or by using oligosaccharides of a desired chain length.[25,44,78,79] During drying, the molecules are immobilized in a random orientation, in which free volume is relatively high. As a tighter packing is better for reduction of molecular mobility, this is not ideal. Raising the temperature close to the Tg without surpassing it to remove strain in a glass (annealing), can be used to reduce this free volume and with that local mobility.[80,81]

MOISTURE

Next to temperature, moisture generally has a major impact on protein stability in the dry state. Water acts as a strong plasticizer, drastically reducing the Tg (indicative of global mobility) and increasing local mobility.[37,82,83] As a consequence, it can accelerate degradation below the Tg or cause a formulation to surpass its Tg with previously explained detrimental consequences.[12,84] Below the Tg, increasing moisture levels mostly speed up chemical degradation reactions, like covalent aggregation, deamidation and oxidation.[12,19] Above the Tg, the increase in global mobility has a larger impact on physical stability.

Since different proteins have different physicochemical characteristics and thus different modes of degradation, a generalized prediction of how exactly moisture affects protein degradation rates cannot be made. However, the presented mechanism of stabilization by sugars (i.e. reducing protein local and global mobility) is applicable for most of these degradation routes. Following the same strategies as described in the section *Temperature*, if vitrification (global mobility) is lost due to moisture exposure, addition of high molecular weight sugars may be beneficial; whereas when moisture increases chemical reactivity below the Tg, lower molecular weight sugars are likely to be more efficient at stabilizing by reducing local mobility.

As not all reactions are equally sensitive to moisture, some even need water as a reactant, different water contents can also change the dominant mechanism of degradation, particularly below the Tg. The Maillard degradation deserves special mention here, as it involves a set of moisture depending reactions involving reducing sugars. The degradation rate due to the Maillard reaction generally increases with increasing moisture content, with little or no degradation below a water activity of ~0.25 and with a maximum around ~0.75.[85] Above this maximum, dilution effects and an increased global mobility are given as explanation for a decreased reaction rate.[12] Because of the Maillard reaction, non-reducing sugars are preferably used for protein stabilization. Should sugars with reducing groups be used, moisture content should be minimized and factors like pH should also be carefully chosen.[86]

In general, drying more is thus beneficial, however, reduced moisture levels are not always better for protein stability. Multiple studies found that when stabilized protein formulations were dried beyond a critical point (e.g. <1% moisture content) degradation rates would increase again.[12,87] When several enzymes were dried without stabilizers, drying below ~10% water content resulted in complete loss of functionality.[88] Using the previously presented hypotheses, over-drying can be interpreted as a critical loss of protein hydrogen bonds and with that an increased local mobility and reduced stability. Over-drying in the presence of sugars might therefore be an indication of inefficient hydrogen bonding of the carbohydrate, either because of the characteristics of the carbohydrate (e.g. size and molecular flexibility) or by the drying process used. Over-drying is thus a potential risk for protein formulations, particularly when large molecularly rigid polysaccharides are used.

CONCLUSION

At this point it should be clear that there is not one single unifying theory which completely explains how sugars stabilize proteins. This is mostly because there is a multitude of potential degradation routes, which are different for each protein, and which on top of that are affected differently by various stress conditions. Therefore it is important to first identify potential routes of degradation and identify the expected processing and storage conditions.[89] When the primary routes of degradation and storage conditions are known, a strategy can be defined to protect against specific stresses and degradation pathways. Sugars can decelerate both chemical and physical degradation pathways of protein in the solid state by a reduction of local and global mobility through tight interactions (i.e. hydrogen bonding with the protein). Sugars are generally effective in protecting against dehydration, freezing and thermal stress, but cannot overcome all protein instabilities. Therefore, other factors such as drying and other formulations aspects (e.g. pH, buffer strength, purity of excipients and other excipients such as surfactants) should also be included in the stabilization strategy. For good stabilization, a sugar should form as much interactions (i.e. hydrogen bond) with the protein as possible, thus reducing local mobility, and it should have a sufficiently high glass transition temperature to maintain vitrification of the formulation under the planned storage conditions. Furthermore, the sugar should be miscible with the protein on a molecular level; preferably be non-reducing or otherwise be stored under conditions that limit the Maillard reaction; and preferably have a low tendency to crystallize or be stored under conditions that prevent surpassing of the Tg. Practically, this will often mean that disaccharides such as trehalose will be ideal stabilizers as they are good at forming interactions and reducing local mobility. If however moisture content cannot be kept low, vitrification is likely to become problematic. In such situations, larger

oligosaccharides or a combination of disaccharides and polysaccharides can be used to increase the glass transition temperatures. Effectively the amount of interactions (i.e. reduction of local mobility) should be maximized, whilst preventing loss of vitrification. Additionally, at elevated moisture levels, the use of non-reducing sugars becomes more critical.

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Appendices

I Summary
II Samenvatting
III List of publications

I SUMMARY

Protein therapeutics play an important role in modern day medicine. Their introduction provided immense improvements to various therapeutic areas for diseases such as diabetes, breast cancer, and rheumatoid arthritis (chapter 1). However, an important limitation of many protein therapeutics, which are often in aqueous solution, is their limited shelf life. Proteins can degrade and lose their functionality in a multitude of ways. How and how fast proteins degrade depends on the characteristics of the protein as well as on the stresses the protein is exposed to. Drying proteins in the presence of sugars can increase protein shelf life, even though the drying process itself can lead to protein degradation. Sugars therefore have to provide protection during drying and sufficiently increase storage stability of the dried product. How sugars stabilize proteins has been researched extensively over the past decades (chapter 8). For a sugar to act as a good protein stabilizer, the sugar needs to be able to closely interact with the protein (by forming intermolecular hydrogen bonds), inhibit mobility of the protein on a molecular level (known as vitrification), and the sugar should not react with the protein (contain no or a limited amount of reducing groups). Not all sugars meet all of these criteria and therefore not all sugars are equally suitable as protein stabilizers.

The ability of a sugar to stabilize proteins is related to the size and molecular flexibility of the sugar (chapter 2). This was demonstrated by freeze-drying four model proteins with diverse characteristics with sugars of varying sizes and molecular flexibility. Smaller (disaccharides versus oligo- and polysaccharides) and molecularly more flexible (similarly sized inulin versus dextran) sugars better stabilized the proteins during storage at an elevated temperature (60 ºC), where vitrification was maintained and the number of reducing groups of the sugars did not influence degradation. When a dried formulation surpasses its glass transition temperature (Tg) molecular mobility drastically increases (vitrification is lost) and with it the protein degradation rate also increases strongly. Moisture and other excipients such as buffer components can drastically lower the Tg of a formulation. In a situation where maintaining vitrification is problematic, larger sugars might provide benefit as their Tgs are generally higher. However, this inherently reduces protein stabilization as smaller sugars are better at providing stabilization to proteins as long as vitrification is maintained. This trade-off might be minimized by using a mixture of smaller (disaccharides) and larger sugars (polysaccharides), or by using flexible oligosaccharides.

The reason why smaller and molecularly more flexible sugars are better protein stabilizers was explored in more detail. The hypothesis was that such sugars are able to interact more closely with proteins as they would be less affected by steric hindrance. First, the ability of

the sugars to form hydrogen bonds with the protein during drying was investigated (chapter 5). This was done by making use of in-line near infrared spectroscopy during freeze-drying. Smaller and molecularly more flexible sugars were better able to form hydrogen bonds with the protein, particularly during the latest stage of drying where the most tightly bound water is removed. Secondly, protein-sugar interactions were evaluated in the solid state using far infrared/terahertz-time domain spectroscopy (chapter 6). Again the smallest sugar tested (disaccharide trehalose) showed most protein-sugar interactions, followed by the flexible oligosaccharide (inulin), whereas the large polysaccharide (dextran) behaved independently of the protein, indicating little or no protein-sugar interactions. A prerequisite for good protein-sugar interaction is a molecular distribution of the protein and sugar molecules: the compounds have to be miscible on a scale that matches the scale of the protein. Protein-sugar miscibility was evaluated on two length scales (2-5 and 20-50 nm) using solid state nuclear magnetic resonance spectroscopy (chapter 7). Miscibility at the 2-5 nm length scale can be interpreted as the ability of sugars to enter empty spaces in the three-dimensional structure of the protein. Miscibility at the 20-50 nm scale is an indication of more global miscibility. The smallest sugar tested sugar (trehalose) was the only sugar which was miscible on the 2-5 nm scale and protein-sugar miscibility decreased with increasing sugar size. Larger, molecularly more rigid sugars showed phase separation on both scales, where a similarly sized molecularly more flexible sugar (inulin 4 kDa versus dextran 5 kDa) was still miscible on the 20-50 nm scale. Overall these results show that smaller and molecularly more flexible sugars are better protein stabilizers because they are better able to closely interact with proteins.

To allow investigation of the impact of molecular flexibility of sugars on their ability to stabilize proteins, a molecularly flexible sugar, inulin, was used. Inulin is more flexible than other sugars, primarily due to the fact that the backbone of the sugar does not pass through the sugar rings, allowing more freedom for movement. The physicochemical properties of inulin were reviewed to provide further information in support of the presented comparison of various sugars (chapter 3). Furthermore, how those properties translate into pharmaceutical applications, including stabilization of proteins, was also discussed (chapter 4).

II SAMENVATTING

Eiwitgeneesmiddelen zijn niet meer weg te denken uit de hedendaagse geneeskunde. Hun invoering leverde een immense verbetering op in diverse therapeutische gebieden voor ziektes als diabetes, borstkanker en reuma (hoofdstuk 1). Een belangrijke beperking van veel eiwitgeneesmiddelen, die meestal als waterige oplossingen worden toegepast, is echter hun beperkte houdbaarheid. Eiwitten kunnen op velerlei manieren degraderen en hun activiteit verliezen. Hoe en hoe snel eiwitten degraderen, hangt af van de kenmerken van het eiwit en van de stresscondities waaraan het eiwit wordt blootgesteld. Opslagstabiliteit van de eiwitten kan worden verbeterd door ze tezamen met suikers te drogen, hoewel het droogproces zelf ook tot degradatie kan leiden. Derhalve moeten suikers de eiwitten beschermen tijdens het drogen en de houdbaarheid van het gedroogde product voldoende verlengen. De afgelopen decennia is veel onderzoek gedaan naar hoe suikers eiwitten stabiliseren (hoofdstuk 8). Om eiwitten te stabiliseren, moet een suiker in staat zijn om interacties met de eiwitten aan te gaan (door intermoleculaire waterstofbruggen met het eiwit te vormen), beweging van het eiwit op moleculaire schaal tegen te gaan (vitrificatie) en het suiker moet niet reageren met het eiwit (geen of zo min mogelijk reducerende groepen bevatten). Niet alle suikers voldoen aan deze criteria en daarom zijn niet alle suikers even geschikt als eiwitstabilisator.

Het vermogen van suikers om eiwitten te stabiliseren is gerelateerd aan de grootte en moleculaire flexibiliteit van het suiker (hoofdstuk 2). Dit werd aangetoond door vier modeleiwitten met uiteenlopende eigenschappen te vriesdrogen met suikers van verschillende grootte en moleculaire flexbiliteit. Kleinere (disaccharides tegenover oligo- en polysaccharides) en moleculair meer flexibele (inuline tegenover dextran van vergelijkbare grootte) suikers waren beter in staat de eiwitten te stabiliseren tijdens opslag bij een verhoogde temperatuur (60 ^{0}C), waarbij vitrificatie behouden bleef en het aantal reducerende groepen van de suikers geen invloed had op eiwitdegradatie. Wanneer een gedroogde formulering de glasovergangstemperatuur (Tg) passeert, neemt de moleculaire beweeglijkheid drastisch toe (vitrificatie wordt verloren) en daarmee neemt de eiwitdegradatiesnelheid ook sterk toe. Vocht en andere ingrediënten in de formulering zoals buffercomponenten kunnen de Tg van een formulering drastisch verlagen. Als het behouden van vitrificatie een probleem is, zouden grotere suikers uitkomst kunnen bieden omdat hun Tgs veelal hoger zijn. Dit resulteert echter tegelijkertijd in verminderde eiwitstabilisatie, omdat kleinere suikers beter in staat zijn eiwitten te stabiliseren zolang vitrificatie behouden blijft. Dit nadeel zou mogelijkerwijs beperkt kunnen worden door gebruik te maken van flexibele oligosaccharides of van een mengsel van kleinere (disaccharides) en grotere suikers (polysaccharides).

Waarom kleine en moleculair meer flexibele suikers betere eiwitstabilisatoren zijn werd verder onderzocht. De veronderstelling was dat zulke suikers beter in staat zijn om met eiwitten interactie aan te gaan omdat ze minder last hebben van sterische hindering. Eerst werd het vermogen van de suikers om waterstofbruggen te vormen met de eiwitten onderzocht tijdens het drogen (hoofdstuk 5). Dit werd gedaan door gebruik te maken van nabij-infrarood spectroscopie tijdens het vriesdroogproces. Kleinere en moleculair meer flexibele suikers waren beter in staat om waterstofbruggen te vormen met het eiwit, in het bijzonder gedurende de laatste fase van het droogproces, waar het meest vast gebonden water wordt verwijderd. Vervolgens werden de eiwit-suiker interacties geëvalueerd in de vaste vorm met behulp ver-infrarood/terahertz-tijdsdomein spectroscopie (hoofdstuk 6). Ook daar werd gevonden dat het kleinste geteste suiker (disaccharide trehalose) de meeste eiwit-suiker interacties had, gevolgd door de flexibele oligosaccharide (inuline). De grote polysaccharide (dextran) gedroeg zich onafhankelijk van het eiwit, hetgeen duidt op beperkte interacties of zelfs afwezigheid van interacties. Een voorwaarde voor goede eiwit-suiker interactie is een moleculaire verdeling van de eiwit- en suikermoleculen: de stoffen moeten gemengd zijn op een schaal die overeenkomt met de grootte van het eiwit. Met vaste stof kernspin resonantie (hoofdstuk 7) is gekeken naar eiwit-suiker mengbaarheid op verschillende schalen (2-5 en 20-50 nm). Mengbaarheid op een schaal van 2-5 nm kan worden gezien als het vermogen van suikers om vrije ruimtes in de driedimensionale structuur van het eiwit op te vullen. Mengbaarheid op een schaal van 20-50 nm is een maat voor meer globale mengbaarheid. Alleen het kleinste geteste suiker (trehalose) was mengbaar op een schaal van 2-5 nm en de eiwit-suiker mengbaarheid nam af met toenemende suikergrootte. De grotere en moleculair meer rigide suikers waren op beide schalen ontmengd, terwijl een moleculair meer flexibele suiker van vergelijkbare grootte (inuline 4 kDa tegenover dextran 5 kDa) nog wel mengbaar was op een schaal van 20-50 nm. Alles bij elkaar tonen deze resultaten aan dat kleinere en moleculair meer flexibele suikers beter in staat zijn eiwitten te stabiliseren omdat ze beter in staat zijn om interacties aan te gaan met de eiwitten.

Om de invloed van moleculaire flexibiliteit van suikers op hun vermogen om eiwitten te stabiliseren te kunnen onderzoeken, werd inuline als moleculair flexibel suiker gebruikt. Inuline is moleculair meer flexibel dan veel andere oligo- of polysaccharides, voornamelijk omdat de ruggegraat van het oligomeer niet door de suikerringen loopt, waardoor er meer bewegingsvrijheid is. De fysisch-chemische eigenschappen van inuline werden op een rij gezet om verdere informatie te geven ter ondersteuning van de vergelijking van de verschillende suikers (hoofdstuk 3). Verder werd ook besproken hoe die eigenschappen zich vertalen naar farmaceutische toepassingen, waaronder het stabiliseren van eiwitten (hoofdstuk 4).

III LIST OF PUBLICATIONS

2017 **How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions**
M. A. Mensink, H. W. Frijlink, K. van der Voort Maarschalk, and W. L. J. Hinrichs
European Journal of Pharmaceutics and Biopharmaceutics, volume 114, pages 288-295

2016 **Influence of Miscibility of Protein-Sugar Lyophilizates on Their Storage Stability**
M.A. Mensink, M.J. Nethercott, W.L.J. Hinrichs, K. van der Voort Maarschalk, H.W. Frijlink, E.J. Munson, and M.J. Pikal
The AAPS Journal, volume 18, issue 5, pages 1225-1232

2015 **In-line near infrared spectroscopy during freeze-drying as a tool to measure efficiency of hydrogen bond formation between protein and sugar, predictive of protein storage stability**
M.A. Mensink, P.J. Van Bockstal, S. Pieters, L. De Meyer, H.W. Frijlink, K. van der Voort Maarschalk, W.L.J. Hinrichs, and T. De Beer
International Journal of Pharmaceutics, volume 496, issue 2, pages 792-800

2015 **Inulin, a flexible oligosaccharide II: Review of its pharmaceutical applications**
M. A. Mensink, H. W. Frijlink, K. Van Der Voort Maarschalk, and W. L. J. Hinrichs
Carbohydrate Polymers, volume 134, pages 418-428

2015 **Inulin, a flexible oligosaccharide I: Review of its physicochemical characteristics**
M. A. Mensink, H. W. Frijlink, K. Van Der Voort Maarschalk, and W. L. J. Hinrichs
Carbohydrate Polymers, volume 130, pages 405-419

2015 **Size and Molecular Flexibility of Sugars Determine the Storage Stability of Freeze-Dried Proteins**
W. F. Tonnis*, M. A. Mensink*, A. de Jager, K. van der Voort Maarschalk, H. W. Frijlink, and W. L. J. Hinrichs
Molecular Pharmaceutics, volume 12, issue 3, pages 684-694

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