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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

Maarten A. Mensink[a], Pieter-Jan Van Bockstal[b], Sigrid Pieters[b,1], Laurens De Meyer[b], Henderik W. Frijlink[a], Kees van der Voort Maarschalk[a,c], Wouter L.J. Hinrichs[a,*], Thomas De Beer[b]

[a] Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
[b] Laboratory of Pharmaceutical Process Analytical Technology, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium
[c] Process Technology, Corbion Purac, P.O. Box 21,4200 AA Gorinchem, The Netherlands

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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.



## 1. 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 (King, 2015). 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 (Chi et al., 2003). 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 (Carpenter et al., 1997; Wang, 2000). During these drying processes, however, proteins are subjected to several types of stresses, including thermal and dehydration stresses (Abdul-Fattah et al., 2007; Crowe et al., 1990). 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 (Arakawa et al., 1991; Chang and Pikal, 2009; Crowe et al., 1998). 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 (Hancock et al., 1995). 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 (Duddu and Dal Monte, 1997; Imamura et al., 2009). 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 (Carpenter and Crowe, 1989). These two theories are not mutually exclusive; both mechanisms

* Corresponding author. Fax: +31 50 363 2500.
*E-mail address:* w.l.j.hinrichs@rug.nl (W.L.J. Hinrichs).
[1] Present address: Pharma Technical Development Formulation, F. Hoffmann-La Roche, Grenzacherstrasse 124, 4070 Basel, Switzerland.

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play a role in protein stabilization (Grasmeijer et al., 2013; Randolph, 1997). Which mechanism is prevalent depends on several factors like formulation (e.g. type of stabilizer), residual moisture, presence of plasticizers and storage temperature (Grasmeijer et al., 2013). Protein stability has also been related to fast β-relaxation in the solid of these proteins (Cicerone and Douglas, 2012). 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 (Mensink et al., 2015). 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 (Allison et al., 2000; Duddu and Dal Monte, 1997; Lückel et al., 1997). Oligosaccharides, on the other hand, have higher Tgs, limiting their susceptibility to this problem (Allison et al., 2000; Hinrichs et al., 2001). Their size, however, imposes the problem of steric hindrance, potentially limiting their capacity to hydrogen bond with the protein (Allison et al., 1999; Tanaka et al., 1991; Taylor, 1998). 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 (Tonnis et al., 2015). 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 Fig. 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 (Tonnis et al., 2015). 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 (Pieters et al., 2012).

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 (Pieters et al., 2012). 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 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 paper mechanistic evidence explaining why size and molecular flexibility determine storage stability of lyophilized proteins is provided.

## 2. Materials and methods

### 2.1. 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.

### 2.2. 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,

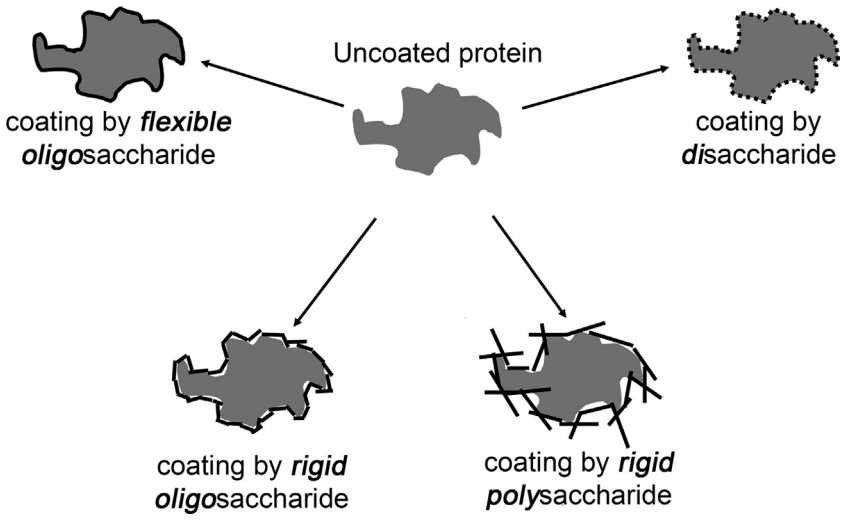


**Fig. 1.** Schematic overview of the compactness of coating of proteins by different types of sugars (Tonnis et al., 2015). Modified and reprinted with permission from American Chemical Society.