Pharm Res (2012) 29:2684–2697
DOI 10.1007/s11095-011-0658-4

RESEARCH PAPER

The Effect of Water Plasticization on the Molecular Mobility and Crystallization Tendency of Amorphous Disaccharides

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Received: 29 September 2011 / Accepted: 13 December 2011 / Published online: 28 December 2011

ABSTRACT

Purpose To study how water plasticization affects the molecular mobility and crystallization tendency of freeze-dried trehalose, sucrose, melibiose and cellobiose.

Methods Freeze-dried disaccharides were subjected to different relative humidity atmospheres and their physical stabilities were evaluated. Lyophilizate water sorption tendencies and glass transition temperatures were modeled using Brunauer-Emmett-Teller (BET) and Gordon-Taylor (GT) equations, respectively. Sucrose and cellobiose crystallization tendencies were compared by using the concept of reduced crystallization temperature (RCT), and the molecular mobilities of trehalose and melibiose were compared by measuring their T_1H relaxation time constants.

Results Based on the BET and GT models, water sorption tendency and the resulting plasticizing effect were different in sucrose when compared to the other disaccharides. Trehalose and melibiose exhibited generally slower crystallization rates when compared to sucrose and cellobiose. Amorphous melibiose was shown to be particularly stable within the studied water content range, which may have partly been caused by its relatively slow molecular mobility.

Conclusions Slow amorphous-to-crystalline transition rate is known to be important for lyoprotecting excipients when formulating a robust drug product. The physical stabilities of amorphous trehalose and melibiose even with relatively high water contents might make their use advantageous in this respect compared to sucrose and cellobiose.

KEY WORDS amorphous · crystallization · disaccharide · mobility · water

INTRODUCTION

Physical stability of the amorphous state is very important for freeze-dried drug products containing protein-structured pharmaceuticals, where excipients in glassy state are used to inhibit protein degradation during processing and storage (1). Crystallization of such an amorphous excipient can lead to structural alterations, aggregation and activity loss in proteins, as the glassy matrix immobilizing the protein and forming hydrogen bonds with its hydrophilic surface is lost (2). This means that uncontrolled crystallization is never a desirable phenomenon in such drug products.

Disaccharides are often employed in commercial freeze-dried formulations as amorphous lyoprotecting excipients (3). However, the protein stabilization efficacies of different disaccharides have been shown to vary, especially when storing the lyophilizates at elevated temperature or humidity (4). This can be caused by differences in crystallization tendencies, which are more easily revealed under such conditions. For example, it was recently shown that trehalose and melibiose offer relatively good protection for β-galactosidase compared to sucrose and cellobiose during

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storage, when the excipient concentration was relatively low (5). High residual moisture contents of the lyophilizates may have facilitated the appearance of these differences.

The crystallization tendency of an amorphous compound is governed, amongst other factors, by its molecular mobility (6). Since crystallization takes place when the molecular structure of a glass becomes more ordered, increased mobility seems to be linked with decreased physical stability (i.e. susceptibility to crystallization). Mobility may be enhanced by plasticizers, which increase molecular rotation and movement in the mixture and as a result, lower the glass transition temperature (T_g) of the amorphous phase (7). If a solute and a solvent, here an excipient and a plasticizer, with differing T_gs are mixed without a change in the overall volume of the system, T_g of the mixture will change according to the following equation:

$$T_{g\,mix} = \frac{w_1 T_{g1} + K w_2 T_{g2}}{w_1 + K w_2} \tag{1}$$

where T_{g1} and T_{g2} are the glass transition temperatures of the solute and solvent and w_1 and w_2 their weight fractions, respectively, and:

$$K = \frac{\rho_1 \Delta\alpha_2}{\rho_2 \Delta\alpha_1} \tag{2}$$

where ρ_1 and ρ_2 are the densities of the solute and solvent and $\Delta\alpha_1$ and $\Delta\alpha_2$ their increments of thermal expansion coefficient at glass transition, respectively.

The equation (1), known as the Gordon-Taylor equation (GT), was originally formulated to describe glass transition temperature changes in polymer blends (8). However, it has since been successfully employed in modeling different kinds of amorphous mixtures as such or with small modifications (7,9). When considering freeze-dried products, the most important and potent plasticizer is water, which is present in all lyophilizates and has a glass transition temperature of approximately −135°C (10). With such a low T_g, it is evident from Eq. (1) that even a slight increase in water content will significantly reduce the overall glass transition temperature of an amorphous disaccharide-water mixture.

Water sorption characteristics of systems under isothermal conditions can be modeled using the Brunauer-Emmett-Teller equation (BET), which predicts the sorption isotherms well for water activities (a_w) up to 0.55 (11). The BET model was originally created to model the adsorption of nonpolar gases on nonpolar surfaces, but it has since been also employed to model the water sorption of different amorphous saccharides (12). The model can be used to calculate, for example, the amount of water necessary to generate a monolayer of water molecules on the solid surface, which gives an estimate of available water binding sites on that surface. The isotherm can be expressed as:

$$\frac{a_w}{(1-a_w)m} = \frac{1}{m_0} + \left(\frac{C-1}{m_0 C}\right) a_w \tag{3}$$

where m is the water content in grams per every 100 g of solids, m_0 is the amount of water in one monolayer in the same units and C is the heat constant of surface sorption.

When an amorphous compound is subjected to temperatures above its T_g, the probability of nucleation increases dramatically. The optimum crystallization temperature is somewhere between T_g and the melting temperature of the crystalline form (T_m), where both nucleation and crystal growth contribute to the overall rate of crystallization (6). One way to compare the crystallization tendencies of compounds is to use the concept of reduced crystallization temperature (RCT). It is defined by Zhou *et al.* (13) as a normalized estimate of how far above its T_g an amorphous compound can be heated before crystallization is induced, and it can be calculated according to the following equation:

$$RCT = \frac{T_c - T_g}{T_m - T_g} \tag{4}$$

where T_c is the nonisothermal crystallization temperature. Lower RCT values tend to indicate a faster onset of crystallization above T_g. RCT has been previously used to compare the crystallization tendencies of pure compounds not containing any plasticizers such as water (13), but it can also be calculated as a function of water content. It should be noted that RCT cannot be determined for all compounds, as in some cases crystallization does not occur under experimental time scale or the transition is so broad that assigning a temperature value for it is not feasible.

The aims of this study were to investigate the water sorption and crystallization tendencies of four different amorphous disaccharides, D-trehalose, D-sucrose, D-cellobiose and D-melibiose. Differences in these properties might affect the effectiveness of the disaccharides as lyoprotectants during storage. Of these four disaccharides, trehalose and sucrose have been accepted as pharmaceutical excipients, and they are used in commercial freeze-dried drug products (3). Melibiose has not been used in commercial drug formulations, but there are examples of it being used orally in the diagnostics of intestinal permeability in children with doses up to 5 g (14,15). Similarly, cellobiose has not been used in drug formulations, but it has been administered orally to humans with doses up to 30 g without significant side effects (16). Therefore there is reason to assume that the safety profiles of all four disaccharides could permit their use as pharmaceutical excipients, making them relevant study materials. It should be noted that both melibiose and cellobiose are reducing disaccharides, and their

free hemiacetal groups that are not partaking in glycosidic bond formation may participate in Maillard reaction with amino acids. The use of reducing disaccharides is limited but not unheard of in protein-structured pharmaceutical formulations. For example, Bexxar® (containing tositumomab and I 131 tositumomab) is a monoclonal IgG2λ antibody product that contains 10% w/v maltose (a reducing disaccharide) as an excipient (3). Therefore the use of reducing disaccharides as stabilizing excipients may be justified, if the protein is not very susceptible to Maillard reaction, and a_w of the formulation is either very low or very high, which slows down the reaction kinetics (17). Trehalose and sucrose were used as reference materials, because their stabilities in amorphous state have been well documented (18,19). However, there are fewer studies focusing on the physical stability of amorphous cellobiose (20) and virtually none for melibiose, meaning that very little is known about their crystallization and even less about their water sorption tendencies. However, based on previous results about their use as lyoprotecting excipients (5), it was hypothesized that the crystallization rates of trehalose and melibiose would be significantly slower than those of sucrose and cellobiose even with high water concentrations.

MATERIALS AND METHODS

Preparation of Amorphous Disaccharides

D-trehalose dihydrate (product number T9531), D-sucrose (product number 18219), D-cellobiose (product number C7252) and D-melibiose monohydrate (product number M5500) were all acquired from Sigma-Aldrich Co. (USA). The required amount of each disaccharide was dissolved in highly purified water (Milli-Q, Millipore Inc., USA) to produce solutions with 100 mg/ml disaccharide concentrations. The solutions were stored at 4°C overnight to reach an equilibrium state in mutarotation, after which 3 ml of solutions was added into pre-weighed 10 ml scintillation vials and frozen at −20°C for 24 h and at −80°C for additional 24 h. The samples were then freeze dried for 72 h at 0.2 mbar using Lyovac GT2 freeze drier (Amsco Finn-Aqua GmbH, Germany). Finally, the lyophilizates were stored under vacuum in desiccators containing phosphorous pentoxide (P_2O_5) for one week to further reduce the residual moisture content of the lyophilizates. Neither crystallinity nor crystalline regions were detected in the samples after lyophilization when analyzed with x-ray powder diffractometer (Bruker D8 Advance, Bruker AXS Inc., USA) and polarized light microscope (DAS Mikroskop, Leica Microsystems GmbH, Germany). No signs of deformation or microcollapse were evident when observing the lyophilizates with scanning electron microscope (FEI Quanta 250 FEG, FEI Inc., OR, USA).

Determination of Initial Water Content Using Karl-Fisher Titration (KF)

The scintillation vials containing lyophilizates were weighed after lyophilization and the initial average water contents of different amorphous disaccharides were measured (n=3) using volumetric Karl-Fisher titration (V30, Mettler Toledo, Switzerland). Each sample was taken from different scintillation vial. The measurement was carried out by grinding the lyophilizates in glovebox under low relative humidity (<5%) and transferring them into the KF titration vessel in closed containers.

Extended Storage of Amorphous Disaccharides and the Observation of Their Microstructure

The scintillation vials were stored at ambient temperature at RH 0%, 11%, 23%, 33% or 43% in desiccators under vacuum containing either P_2O_5 (RH 0%) or appropriate supersaturated salt solutions to produce the desired humidity conditions (RH 11–43%). Empty vials were also stored in the desiccators to account for the water adsorbed on vial surfaces. The vials were weighed periodically (n=4) and their water contents were calculated by subtracting the empty vial mass from the full vial mass and taking into account the initial average water content measured with KF. The relative humidity of the desiccators was continuously measured with humidity probes (Tinytag View 2, Gemini Data Loggers Ltd., UK), so that possible changes in desiccator moisture contents could be detected. The water sorption results of vials stored at RH 11%, 23% and 33% were fitted into the BET model (Eq. 3) to obtain the water monolayer (m_0) and sorption heat constant (C) values. Water sorption was considered to reach equilibrium state after 65 h in case of trehalose and melibiose, and 40 h in case of sucrose and cellobiose, as they tended to crystallize quickly at RH 33% and 43%. The microstructures of the lyophilizates were imaged before storage and in some cases after storage at different relative humidity by using scanning electron microscopy (SEM). The samples were coated with platinum and scanned using FEI Quanta 250 FEG (FEI Inc., OR, USA) under 60 Pa pressure with a secondary electron detector.

Crystallization Time and Crystal Structure Determination During Extended Storage

The solid state properties of the lyophilized disaccharides, that were stored at RH 33% and 43%, and in the case of cellobiose also at RH 23%, were periodically analyzed by

using x-ray powder diffractometry (XRPD) with Bruker D8 Advance (Bruker AXS Inc., WI, USA) (n=1). The lyophilizates were ground before storage and stored on zero background sample plates cut from a single silicon crystal along a non-diffracting plane. Such plates are used to minimize the diffraction caused by the sample support, and this way relatively good diffraction patterns could be measured even in the case lyophilizate macrostructures were lost due to collapse during storage. The diffraction patterns were collected through angular range (2θ) of 5° to 40° (step size 0.05°, with 1 second scan time/step). The measurements were carried out in ambient relative humidity (<40%). In case a crystal diffraction pattern was obtained, it was compared to those acquired from Cambridge Structural Database (CSD) to determine, which polymorph was produced. The quantitative analysis of the diffractograms was performed by utilizing the Rietveld refinement procedure with the MAUD software (21).

Accelerated Water Sorption Measurements

In addition to the desiccator experiments, lyophilizate water sorption rates were studied with isothermal microcalorimetry (IMC) using TAM 2277 (Thermometric AB, Sweden). The IMC included a sequential RH-unit setup, which was used to simultaneously determine the heat and quantity of vapor sorption (22). In this setup a gas flow with controlled relative humidity is lead to the first calorimeter cell containing the sample. The gas flux exiting the sample cell is then lead to a second cell where the gas is moistened to RH 100% and the moisturization heat is measured. The corresponding measurements without the sample were performed in order to investigate the blank effect. As a result, the sample water sorption and desorption could be observed in addition to other thermodynamic transitions occurring in the sample. The ground lyophilizate samples (m=1.5–2 mg, n=1) were ramped from RH 0% to RH 60% (to RH 70% in case of melibiose) at 25°C by 10% increases every 4 h, while measuring changes in heat flow.

Glass Transition and Crystallization Temperature Measurements

The glass transition temperatures of maximally freeze-concentrated aqueous solutions (T_g') were measured using DSC823e (Mettler-Toledo Inc., Switzerland) from 100 mg/ml solutions by cooling the samples to −50°C at 10°C/min, holding at that temperature for 5 min and then heating to 10°C at 10°C/min. The measurements were carried out under 50 ml/min N_2 purge, and the T_g' values were calculated from transition midpoints. The T_gs of lyophilizate samples were measured with DSC from dry samples or after 48 h storage at RH 6%, 11%, 23% and 33%. The samples (n=3) were prepared in glovebox in similar relative humidity (RH±5%) as they had been stored in by grinding them and sealing approximately 5 mg of sample hermetically in aluminum DSC pans. Each sample was taken from different scintillation vial. Samples were cooled at least 30°C below their T_g, equilibrated for 5 min, and then heated at least 30°C above their T_g at 10°C/min (in case of melibiose also at 5°C/min) while purging the measurement cell with N_2 at 50 ml/min. T_g was taken as the midpoint of the transition. The dry (RH 0%) samples were measured otherwise similarly, except that the lids of the DSC pans were pierced before insertion and they were further dried in the DSC at T=T_g−5°C for 15 min before T_g determination. In case of sucrose the drying was carried out by heating the sample to 100°C and then immediately cooling it to 25°C at 10°C/min, as drying at T=T_g−5°C was not sufficient due to its relatively low T_g. Rest of the samples in each vial not consumed by the DSC measurements were used to determine their respective water contents using KF (n=3) in order to calculate the GT parameters (Eq. 1). T_g of water was taken as −135°C (10). The RCT values were calculated similarly as the GT parameters for RH 0%, 11%, 23% and 33% for sucrose and cellobiose. However, T_c was taken as the onset of the transition, as the shapes of the crystallization exotherms sometimes varied between measurements even though there was only little variation in the onset temperatures in repeated measurements.

T_1H Relaxation Time Measurements

Solid state cross polarization magic angle spinning (CPMAS) NMR experiments were carried out using Bruker Avance III 500 spectrometer (Bruker BioSpin GmbH, Germany) with magnetic flux density of 11.7 T, and with a 4 mm double resonance broad band VTN CPMAS probehead. Trehalose and melibiose lyophilizates produced earlier were further dried for 3 h at 40°C and 0.15 mbar and measured as such or after 72 h equilibration at RH 11%, 23% and 33%. Sample glass transition temperatures were calculated from their water contents by using the GT models determined earlier. Samples (n=1) were packed into ZrO_2 rotors plugged with KEL-F endcaps, and the sample spinning rate was set to 10 kHz. Longitudinal relaxation time constants for proton (T_1H) were measured using a saturation recovery experiment with cross polarization step and carbon detection. Contact time for cross polarization was 500 μs, and the signal acquisition time was 10 ms, during which proton decoupling (SPINAL-64) with a rf-field strength of 63 kHz was used. The spectra were referenced externally via adamantane by setting the low field resonance at 38.48 ppm. The intensity of the largest signal found in the spectrum at close to 72 ppm for trehalose

(70 ppm for melibiose) was measured at eight delay times after the saturation step, and an exponential fit of the obtained data was carried out by using TopSpin 2.1 software (Bruker BioSpin GmbH, Germany).

RESULTS

Water Sorption and Crystallization During Extended Storage

The glass transition temperatures of maximally freeze-concentrated solutions (T_g') were measured before freeze drying using DSC, and the values acquired were −31°C for trehalose, −34°C for sucrose, −32°C for melibiose and −31°C for cellobiose. The freeze drier used did not allow sample temperature measurement during drying, but under such conditions the lowest possible primary drying temperature would be approximately −36°C based on the equilibrium vapor pressure over ice (for samples with negligible sublimation resistance). Taking into account that there were no signs of viscous flow in the fresh sample when analyzed with SEM, it is unlikely that the formulations were subjected to significantly higher temperatures than their collapse temperatures during primary drying. The initial average water contents (w/w) that were measured using KF were for 1.4% trehalose, 0.9% for sucrose, 1.6% for melibiose and 1.2% for cellobiose lyophilizates after 1 week of storage under vacuum in desiccators containing P_2O_5, before initiating the water sorption studies. Water contents of lyophilized disaccharides that were stored in desiccators under different relative humidity atmospheres are shown in Fig. 1. Storage times when crystalline diffraction patterns were first observed with XRPD are marked with circles. Crystallization generally leads to a clear decrease in water content, if the resulting polymorph is an anhydrate. If a hydrate is formed, the amount of water loss depends on the stoichiometric ratio between solvent and solute in the hydrate structure. Amorphous sucrose lyophilizates contained generally less water compared to the other disaccharides when stored at RH 11% or 23%. According to the XRPD analysis results shown in Fig. 2, cellobiose crystallized at RH ≥23%, sucrose at RH ≥33%, whereas trehalose yielded a crystal diffractogram only at RH 43% and melibiose showed only amorphous halos when stored up to 500 h. Some peak were visible in the diffractogram of melibiose after 687 h at RH 43%, and a diffraction pattern closely resembling that of melibiose monohydrate was acquired after 1100 h (Fig. 2). Still, it is likely that nucleation was taking place in trehalose and melibiose even before 300 h at RH 33%, as the water contents of the lyophilizates decreased measurably. However, the nuclei were too small to cause clear diffraction patterns in

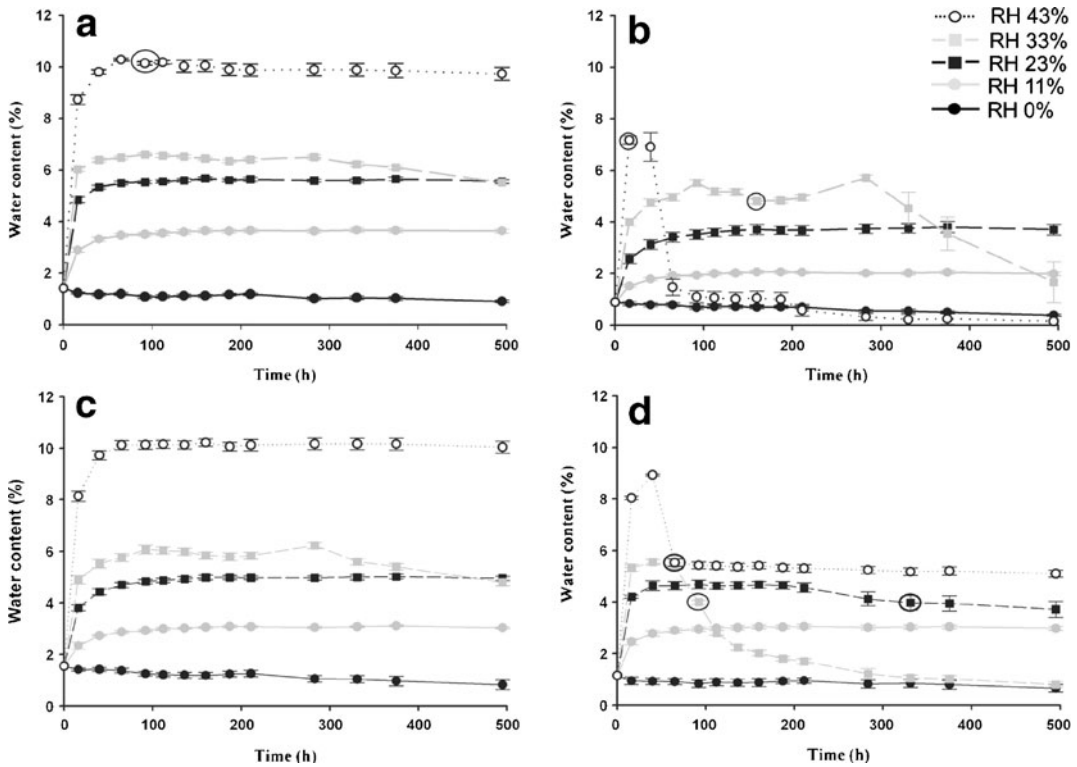

Fig. 1 The water contents (%-w/w) of trehalose (**a**), sucrose (**b**), melibiose (**c**) and cellobiose (**d**) lyophilizates, when they were stored at RH 0–43%. The points where crystalline diffraction patterns were observed with XRPD have been circled.

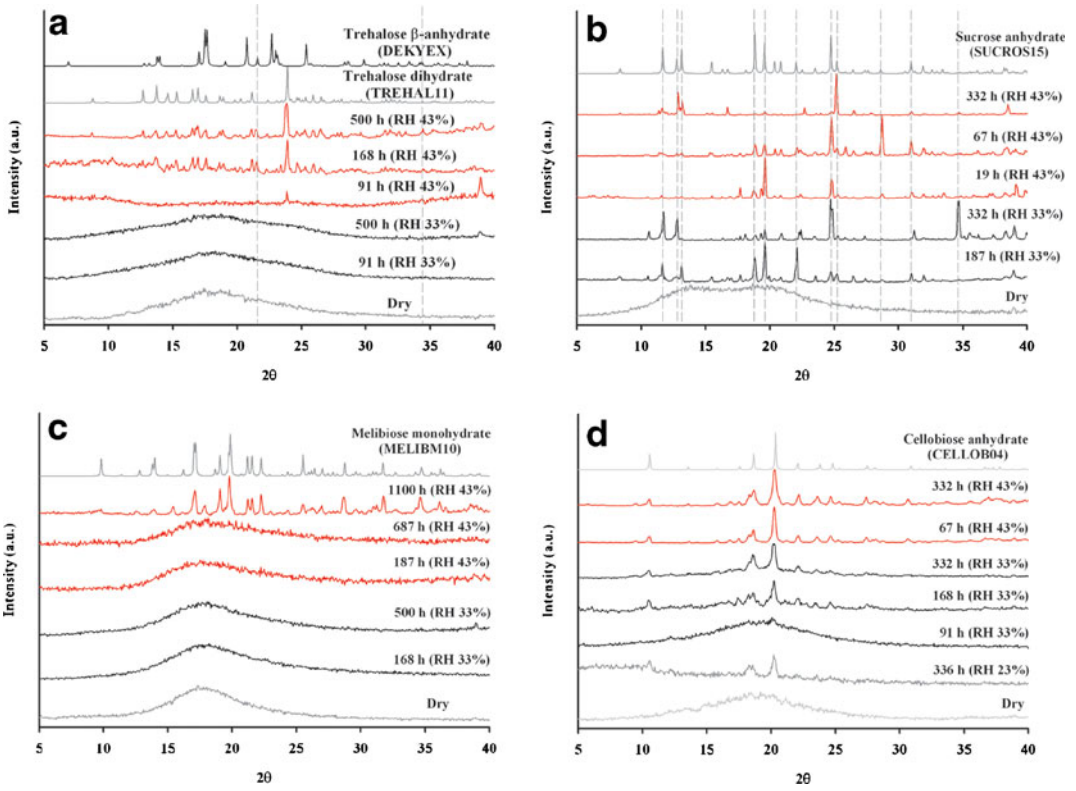

Fig. 2 The x-ray powder diffractograms of trehalose (**a**), sucrose (**b**), melibiose (**c**) and cellobiose (**d**) lyophilizates when they were stored at RH 23–43%. Calculated diffractograms from CSD are shown as reference, and some diffraction angles of interest have been marked with dashed lines.

XRPD analyses at that time, as seen in Fig. 2. Lack of mass decrease due to crystallization in trehalose stored at RH 43% (Fig. 1) can be explained by the fact that all lyophilizates collapsed when stored under those conditions (sucrose collapsed even at RH 33%). Once the porous lyophilizate has collapsed, water penetrates the viscous matrix more slowly, which impedes evaporation. Furthermore, the water content for pure trehalose dihydrate is approximately 9.5% (w/w), meaning that even complete crystallization to dihydrate would not lower the mass dramatically when stored at RH 43%.

Trehalose was shown to crystallize mostly as dihydrate at RH 43%, even though two diffraction peaks around $2\theta = 21.6°$ and 34.4° appear in only in the diffractogram of the β-anhydrate (Fig. 2). DSC experiments verified the presence of two different crystal structures, as a melting endotherm was observed at approximately 175°C in trehalose samples stored at RH 43% for 168 h in addition to the dihydrate melting endotherm around 100°C (data not shown). No trehalose polymorph has been reported to melt at this temperature (23), and the melting point of the β-anhydrate has been approximated as 200°C. This may indicate that the crystallized component contained similar structures as β-anhydrate, but was less stable. Nonetheless, assuming that the crystal structure formed was β-anhydrate, estimating its amount during storage with the Rietweld refinement procedure gives a result that its amount was 8% of the total crystalline content after 168 h and 2% after 500 h. This seems to indicate that the amount of the unstable component decreased slowly during storage at RH 43%. Cellobiose crystallized quickly as anhydrate without any further apparent change in the crystal structure at RH 33% and 43%. On the other hand, sucrose diffractograms changed more slowly as a function of time, and storage at RH 33% and 43% seemed to produce slightly different diffraction patterns. Still, practically all the same peaks were observed in samples stored at RH 33% and 43% and the main differences between the two were intensity related, suggesting that the differences were due to changes in preferred orientation.

It can be seen from Figs. 1 and 2 that cellobiose crystallization was well underway after 336 h of storage at RH 23% and after 91 h at RH 33%. However, these lyophilizates did not shrink or collapse visibly at any time during extended storage, meaning that the viscosity of the amorphous form did not decrease in such an extent that the macroscopic lyophilizate structure would have been lost. SEM images taken from lyophilizates before and after storage at RH 23% or 33% confirm that small scale viscous deformation had occurred at RH 33% which had been caused by the decrease in viscosity, but it was too small to be detected without stronger magnification (Fig. 3). At RH 23% the changes in lyophilizate surface structure were less

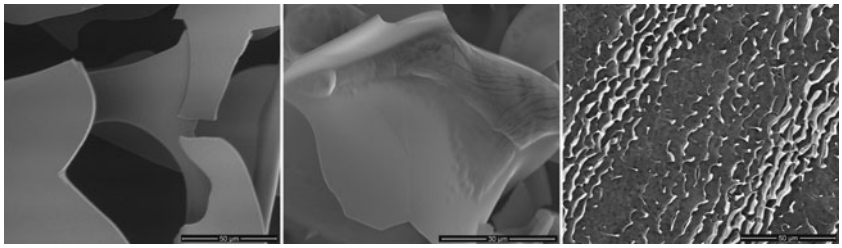

Fig. 3 SEM images of cellobiose before storage (left) and after 336 h at RH 23% (center) or after 500 h at RH 33% (right), with ×2000 magnification.

significant, and clear signs of viscous flow were not observable.

Accelerated Water Sorption and Crystallization

Figure 4 shows the accelerated water sorption results, which were acquired with IMC by increasing the relative humidity in 10% steps from RH 0% to 60%, or to RH 70% in case of melibiose. The IMC heat flow curve displayed an exothermic peak every time RH was increased, when water was absorbed by the sample. Heat flow baseline shifts between sorption steps were most likely caused by changes in sample heat capacity (24). Crystallization itself is an exothermic phenomenon, but water desorption creates an endothermic shift which sometimes overlaps it. The change in mass was calculated from the signal of the second measurement cell, where the exiting gas was moistened to RH 100%. The change in mass measured in this fashion showed a stepwise increase up to a point where crystallization occurred and a portion of water was expelled. Trehalose crystallized almost immediately after increasing the relative humidity to 60%, as shown by the sharp exotherm that overlaps the adsorption exotherm. At the same time, the water content decreased to roughly 9.5% (w/w), what corresponds to the calculated water content of trehalose dihydrate, after which desorption continued more slowly. On the other hand, cellobiose crystallized at RH 50% just before the shift to RH 60% and the crystallization exotherm was overlapped by the evaporation endotherm. The water content stabilized to approximately 9% (w/w), possibly due to water being trapped in the collapsed viscous sample. Sucrose crystallization began already at RH 50%, as the heat signal baseline started to

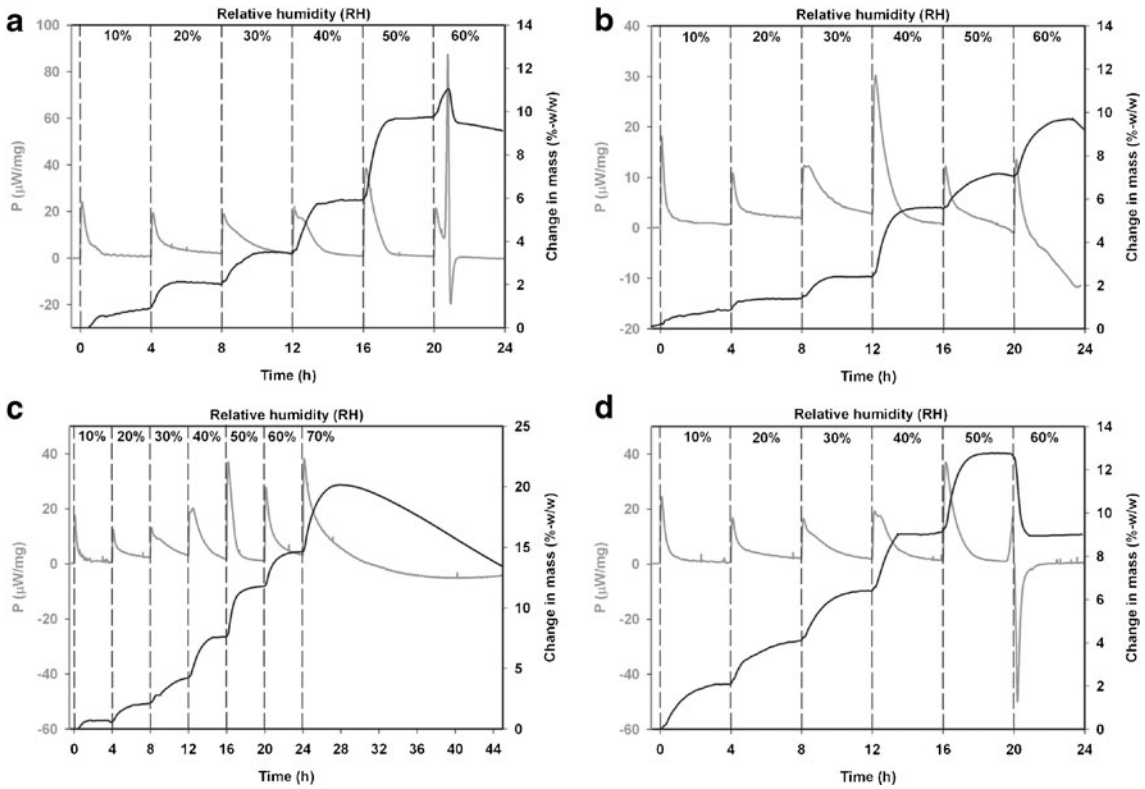

Fig. 4 Accelerated water sorption and heat flow results of amorphous trehalose (**a**), sucrose (**b**), melibiose (**c**) and cellobiose (**d**), measured using IMC. The gray line represents the sample heat flow in the first measurement cell and the black line the change in mass (%-w/w) that has been calculated from the signal of the second measurement cell.

decrease and desorption began before the humidification to RH 60%. The desorption endotherm was broad, and the water content decrease due to crystallization was partly overlapped by additional water sorption at RH 60%. As in the extended storage sorption experiments, amorphous melibiose was shown to crystallize very slowly. Broad endotherm appeared in the heat flow curve when the sample was held at RH 70% for extended period of time and no crystallization exotherm was visible. Similarly, decrease in mass due to crystallization occurred very slowly.

Modeling the Glass Transition Temperatures and Water Contents

The BET parameters of amorphous disaccharides were calculated from the extended storage studies at equilibrium mass or from the largest mass points in case crystallization took place quickly. Also, the water contents and T_g values measured using KF and DSC, respectively, were used to calculate the GT parameters. The results are shown in Fig. 5 and Tables I and II. The water content values of RH 43% were not used in the BET-model, as including them resulted in poor model fit. In three-point models (RH 11–33%) R^2 was 0.99 for trehalose and melibiose, 0.98 for cellobiose and 0.86 for sucrose, whereas in four-point models (RH 11–43%) R^2 was 0.82 for trehalose, 0.80 for cellobiose, 0.76 for sucrose and 0.62 for melibiose. This indicates that a change occurred in the water sorption behavior of freeze-dried disaccharides when RH33% was exceeded, after which the model was no longer predicted the sorption tendencies accurately. It should be noted that this coincided with the critical relative humidity where collapse occurred for trehalose, melibiose and cellobiose. Furthermore, the sorption results could not be reliably used to model the sorption tendencies at RH 43%, as all disaccharides discounting melibiose started to crystallize relatively quickly.

Trehalose, melibiose and cellobiose showed characteristic type II sorption isotherms in the BET plots (12), which is commonly observed when the heat constant of surface sorption (C) resides between 2–50 (30). Such sorption isotherms are often observed with amorphous compounds. Type III isotherms, where $C<2$, are more common for crystalline materials. In those isotherms the amount of adsorbed water is relatively little at low a_w, but the water content increase becomes more notable at higher a_w. Table I shows that there are considerable differences in the C-values between disaccharides. Notably, the water sorption isotherm of sucrose differed from the other disaccharides, as the water content increased less with increasing a_w (Fig. 5). The shape of its sorption curve bears resemblance to that of a type III isotherm, which is also depicted by its relatively low C-value (Table I). The m_0 values differed very little between disaccharides, implying that there were no large differences between the amounts of water binding sites on lyophilizate surfaces.

The T_g values for dry disaccharides (Table II) corresponded well with those acquired from literature, discounting that of cellobiose, where the measured value was higher than what has been previously reported (2,20). When calculating the glass transition temperatures with GT models, trehalose and melibiose T_g values were predicted fairly accurately, but the calculated values were somewhat higher than measured ones in the case of sucrose $a_w>0.2$, and slightly lower in case of cellobiose (Fig. 5). The GT model

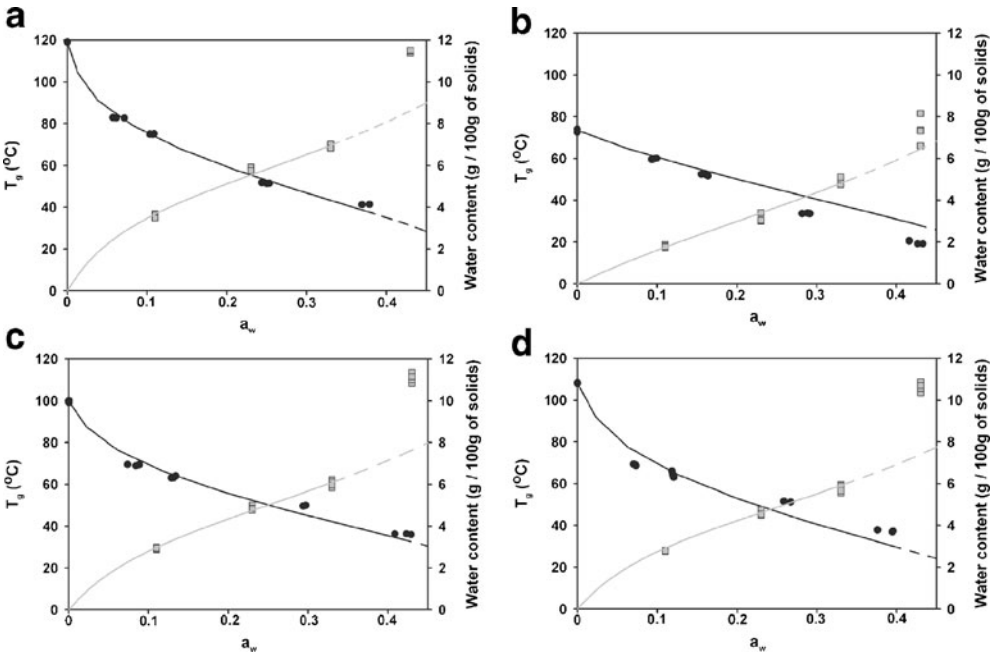

Fig. 5 Measured and predicted glass transition temperatures and water contents as functions of water activity for trehalose (**a**), sucrose (**b**), melibiose (**c**) and cellobiose (**d**). The black circles represent measured T_g values, the black lines their GT models, whereas the gray squares represent measured water contents and the gray lines their BET models. The predicted GT and BET lines are dashed after exceeding the a_w range used in model fitting.

Table I BET Parameters for the Amorphous Disaccharides Calculated from RH Range 11–33% (n=4). When Previously Reported, Literature Values are also Shown, along with the Methods Used to Acquire them

	m_0 (g / 100 g of solids)		C	
	Measured	Literature	Measured	Literature
Trehalose	5.4	6.4 (12)[a]	12.2	5.0 (12)[a]
Sucrose	5.0	7.5 (25)[b]	3.7	–
Melibiose	5.0	–	9.3	–
Cellobiose	4.8	–	9.5	–

[a] Method used: Gravimetric water sorption in desiccators, T=30°C, RH range 0–45%, equilibration time 2–4 weeks

[b] Method used: DVS, T=25°C, RH range 0–40%, stepwise RH increase of 8–10% when $dm/dt < 2.5\ \mu g$ / 5 min

K-values differed slightly from those reported in literature, but the measurement methods were often different as well. The critical water content values [C_{crit} (H_2O)], where T_g=25°C, are also shown in Table II. These values were calculated from the GT models for other disaccharides except for sucrose, in which case a 2nd order polynomial fit (with $R^2 \approx 0.996$) of the experimental values was used to predict C_{crit} (H_2O) instead of the GT model due to its poor fit when $a_w > 0.2$.

The Effect of Water Content on RCT

The effect of water content on the reduced crystallization temperature (RCT) is shown in Fig. 6. Reproducible results could only be measured for sucrose and cellobiose. Trehalose did not crystallize during the experiments when C (H_2O)=0% (w/w), and with higher water contents two melting temperatures were sometimes observed due to crystallization as dihydrate and possibly as unstable β-anhydrate—resembling form. Melibiose did not crystallize under experimental time scale even with relatively high water content (up to approximately 8%-w/w), when heated either with 10 or 5°C/min. In case of sucrose and cellobiose, there was a decrease not only in T_c as a function of water content but in T_m as well, indicating that the crystals produced with higher water contents contained more structural errors (data not shown). RCT values of sucrose were generally lower than those of cellobiose at low water contents, but this order changed as C(H_2O)→C_{crit}. This means that sucrose appears to crystallize more easily than cellobiose at $T > T_g$ when C (H_2O)<4%, but with higher water content amorphous cellobiose becomes more susceptible to crystallization.

Table II GT Parameters and the Critical Water Content [C_{crit} (H_2O)] where T_g=25°C for the Amorphous Disaccharides (n=3), along with T_g- and K-values Obtained from Literature. GT Models were Used to Predict the C_{crit} H_2O Value for other Disaccharides Except for Sucrose, where a 2nd Order Polynomial Fit of the Measured T_g Values was Used Instead

	T_g (dry)	Literature	K	Literature	C_{crit} (H_2O) (w/w)
Trehalose	119°C	121°C (26)[a]	6.0	5.4–5.8 (27)[c]	8.9%
Sucrose	74°C	70°C (28)[b]	4.2	5.1–5.2 (27)[b]	5.4%
Melibiose	100°C	95°C (28)[b]	5.4	6.1 (29)[d]	8.0%
Cellobiose	108°C	77°C (2)[b]	7.1	–	6.9%

[a] Method used: DSC, heating rate 20°C/min, T_g value calculated from glass transition midpoint

[b] Method used: DSC, heating rate 10°C/min, T_g value calculated from glass transition midpoint

[c] Method used: DSC, heating rate 20°C/min, T_g value calculated from glass transition onset

[d] Method used: DSC, heating rate 5°C/min, T_g value calculated from glass transition onset

The Effect of Water Content on T_1H Relaxation Time

Figure 7 shows the change in the T_1H relaxation times in trehalose and melibiose, when increased water plasticization depressed their T_g (shown as an increase in T / T_g). An increase in molecular mobility is depicted by a decrease in T_1H, and both disaccharides displayed a similar decrease due to increased water plasticization. The relaxation constant values were also slightly higher for melibiose than for trehalose at a given T / T_g along the water content range studied.

DISCUSSION

Analysis of the BET and GT Equation Results

The results of the BET model were quite different depending on the a_w range used (RH 11–33% or RH 11–43%). One reason for this might be that the lyophilizates collapsed when stored at RH 43% (discounting sucrose, which collapsed at RH 33%), meaning that water previously in the surface was trapped within the closing pores of the rubbery matrix. Collapse may also have affected the amount of surface water binding sites, as new surface area was exposed due to viscous flow. This may have created the difference between the measured and predicted water content values in the sorption studies, as seen at $a_w > 0.4$ in Fig. 5. Therefore, BET models that included water content values above the lyophilizate collapse point tended to give different m_0- and C-values compared to those that did not. The accelerated sorption experiments were not used to form BET models, because the initial water contents could not be measured as accurately as in the extended sorption studies due to inhomogeneous water distribution (only 1.5–2 mg from each vial was used in the IMC studies, compared to 300 mg in the desiccator studies). Also, the stabilization time of 4 h after RH 10% increase that was used in the IMC

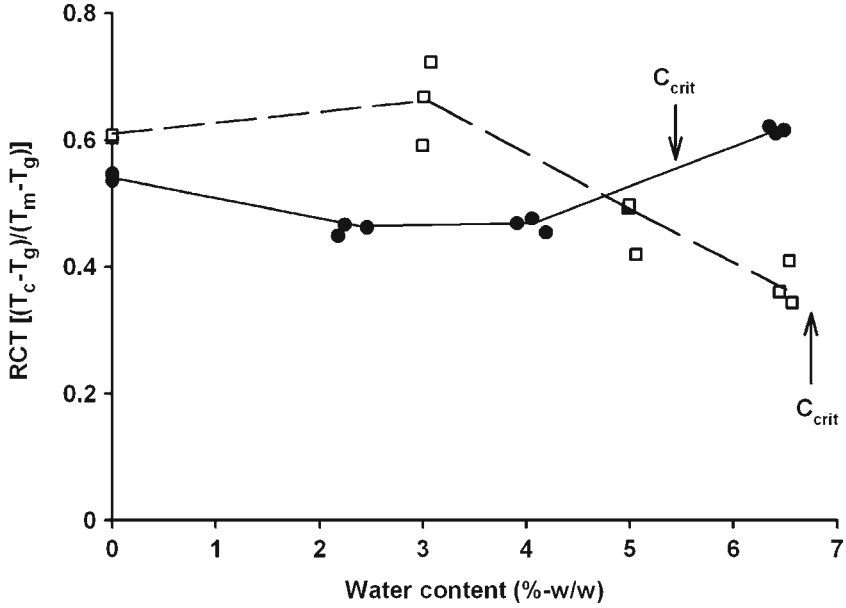

Fig. 6 The effect of water content on the reduced crystallization temperature (RCT) of sucrose (black circles) and cellobiose (white squares) measured with DSC at 10°C/min heating rate. The lines connecting the cluster midpoints are shown as visual aid, and the critical water contents (C_{crit}) where T_g=25°C have been marked with arrows.

experiments may not always have been sufficient. This is corroborated by Fig. 1, where it is seen that equilibrium mass after transfer to RH 11% desiccator is reached only after roughly 48–60 h. Even though the samples in IMC experiments were considerably smaller than those in the desiccator experiments and their macroscopic structure had been broken by grinding, it seems that equilibrium in water sorption had not always been reached before the next RH 10% increase.

Fairly similar m_0 values were calculated for all disaccharides. This value gives an estimate of the available water binding sites, and a higher value may indicate a stronger interaction between solvent and solute (12). The lack of difference between disaccharides could be explained by the fact that the compounds have identical molar masses,

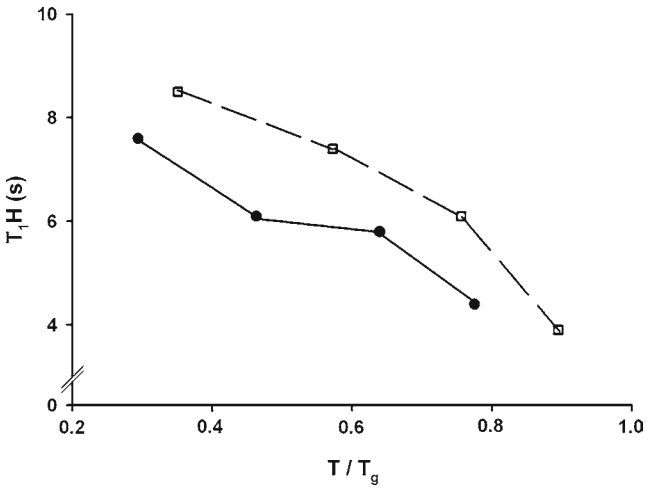

Fig. 7 The change in T_1H relaxation rate constant of trehalose (black circles) and melibiose (white squares) as a function of glass transition temperature.

very similar molecular structures and the samples were all prepared with the same method. The heat constant of surface sorption (C) values are not commonly reported in literature, and the value reported here differed quite clearly from what has been reported elsewhere for trehalose (Table I). However, when calculating the C-value by using the RH range of 11–43% which was quite similar as the RH range (0–45%) of the reference study (12) in Table I, the model gave C=5.4, which was close to the reference value of 5.0. Still, when modeling water sorption at $a_w \leq 0.3$, the use of RH 11–33% range can be justified by the clearly higher BET model regression coefficients. When comparing the extended sorption experiment C-values between disaccharides, the rank order was the same as with the dry T_g values (sucrose<melibiose<cellobiose<trehalose). Similar observation about the relation between T_g and C-values has been previously reported by Zhang *et al.* (12) for several mono-, oligo- and polysaccharides. In that study, the authors concluded that the C-value is affected by the effect of water plasticization on the free energy of the amorphous system, as well as the chemical affinity of water on the solids. In our study, the C-value of sucrose was relatively low compared to the other disaccharides, which was also evident from the relatively low water content of sucrose at low a_w. The aqueous solubility of sucrose is very good, which means that the affinity of water on sucrose should also be quite high. In this light, the explanation that the low C-value of sucrose compared to the other disaccharides is caused by a difference in water plasticization effect on the free energy of the amorphous phase seems more plausible.

The glass transition temperature of amorphous cellobiose has been reported to reside around 50–77°C (2,20), but in our study the measured value was clearly higher (Table II). However, in both of those previous studies the amorphous samples have contained significant amounts of water, which

has most likely lowered the measured glass transition temperatures. There was a good fit between the measured water contents and T_g values with the predicted GT model in case of trehalose and melibiose. However, the K-value of melibiose deviated somewhat from what has been reported in literature, probably due to different measurement parameters (Table II). It has been previously shown by Frank (27) that there is a significant amount of variation between K-values of disaccharides found in literature due to different measurement and calculation methods used. In the case of sucrose, the measured T_g values were systematically lower than what was predicted by the GT model, especially at higher a_w (Fig. 5). This has also been observed by other researchers attempting to model the T_g of sucrose-water systems, who concluded that the nature of the interactions between sucrose and water affects the free volume of the mixture differently than with other disaccharides, which results in a lower T_g value than what is predicted by the GT model (7). The K-value of sucrose of this study also differs somewhat from a previously reported value, even though the measurement methods were similar (27). Even though the differences can sometimes be explained by the use of different T_g values for water in the calculations (27), it still seems that the T_g of sucrose-water systems is not as straightforward to predict as that of the other disaccharides. Furthermore, taking into account the relatively low C-value of sucrose in the BET model, it does indeed seem likely that the effect of water plasticization in sucrose differs from the other disaccharides. Due to this, amorphous sucrose adsorbs generally less water compared to the other disaccharides and the plasticizing effect of water is greater than predicted.

The relatively high K-values of trehalose- and cellobiose-water systems indicate that their T_g decreases rapidly with increasing water content. This means that cellobiose has a lower C_{crit} (H_2O) value than melibiose, even though its T_g is higher than that of melibiose when both are completely dry. There was also a statistically significant difference ($p<0.05$) between measured and GT-predicted T_g values for cellobiose-water mixtures at $a_w>0.2$ (seen also in Fig. 5). This may have been partly caused by uneven water distribution in the lyophilizate. As Fig. 3 shows, viscous deformation had taken place during storage in some parts of the lyophilizate at RH 33%, meaning that T_g has been exceeded. However, if the water content of the whole lyophilizate is taken into account, T_g of the cellobiose-water mixture should be higher than room temperature under RH 33% conditions (Fig. 5), making viscous flow under experimental time scale unlikely. It is possible that solute concentration gradients were formed during the freezing step of lyophilization by the slowly advancing freezing front, which has produced a density gradient in the resulting lyophilizates. This in turn may have lead to uneven water distribution during sorption studies. Indeed, crystal nucleation may have been initiated in regions where the water content was higher and T_g had been exceeded, and these nuclei have then acted as crystallization seeds for the surrounding amorphous matrix.

Differences in Crystallization Tendency

Figure 2 shows that, apart from sucrose, the disaccharide x-ray diffraction patterns changed very little during extended storage after the onset of crystallization. The differences in the case of sucrose were most likely due to changes in preferred orientation, as similar conclusion for sucrose has also been drawn by Kawakami *et al.* (31). This is further corroborated by the fact that all sucrose anhydrate diffractograms in CSD database are very similar and no stable hydrate structures have been reported. This means that sucrose anhydrate crystal growth and reorientation continues long after nucleation has been initiated, even though nucleation starts fairly quickly both in RH 33% and 43%. In contrast, it seems that when trehalose was stored at RH 43%, two different crystal structures were formed. According to the XRPD and DSC analyses, the dihydrate polymorph was the prevalent structure, but a small amount of crystal that resembled β-anhydrate was also formed. The melting point of trehalose β-anhydrate has been reported to be approximately 200°C (23), and here an endotherm at 175°C was observed after 168 h storage at RH 43%. It is therefore possible that the endotherm was caused by the melting of an unstable form bearing some resemblance to trehalose β-anhydrate. This was then gradually converted to trehalose dihydrate when stored further at RH 43%. Similar behavior has been previously reported with amorphous lactose, which has been shown to crystallize as both mono- and anhydrate when stored at RH 54%, but eventually to convert towards fully monohydrate form (32). All in all, the dehydration / rehydration kinetics of trehalose seem relatively complex. It has been previously shown that dehydration of trehalose dihydrate gives α-anhydrate, which readily converts back to dihydrate in the presence of water more readily than amorphous trehalose due to a continuum in the lattice order (33). Also, trehalose is known to form crystals that contain both anhydrous and hydrous regions, for example the so-called trehalose γ form (23), which means that its melting point might depend on the ratio of these regions. It is therefore possible that when crystallizing as a dihydrate, small amounts of intermediate anhydrous crystal structures of trehalose are also produced that may facilitate the overall crystallization process by acting as crystal seeds and/or by improving hydration.

The isothermal crystallization rate of sucrose was shown to be fairly similar as in previous study by Carstensen *et al.* (18), where the water content of freeze-dried sucrose held at room temperature in RH 33% started to decrease after approximately 10 days (compared to approximately 300 h,

or 12.5 days in Fig. 1). According to the IMC experiments, both trehalose and cellobiose crystallized very quickly once the appropriate water content was reached. The fast crystallization of cellobiose at $T>T_g$ has also been noted by Hatakeyama *et al.* (20), who showed that cellobiose crystallizes more quickly than for example glucose under similar conditions. The fast crystallization rate combined with the uneven distribution of water may explain why cellobiose crystallized without collapse at RH 33%. The water solubility of cellobiose is also fairly low (34), and poor solubility has been previously linked with rapid crystallization rate (35). At RH 23% there was no clear sign of viscous flow in the SEM images (Fig. 3), meaning that crystallization occurred most likely below T_g of the water-disaccharide mixture. Trehalose crystallization rate at RH 43% was also shown to be similar as reported elsewhere in literature (36), where trehalose that was freeze dried with 5% (w/w) of protein showed significant crystalline content after 1 week, or 168 h of storage at RH 44% (identical to that shown in Fig. 1). According to our knowledge, there are no previous studies published about the crystallization tendency of amorphous melibiose. However, its behavior appears similar to that of lactose, a disaccharide also consisting of galactose and glucose but with 1→4 glycosidic linkage compared to 1→6 bond of melibiose. Freeze-dried lactose has been shown not to form observable crystalline regions when stored at RH 44% up to 500 h (37), meaning that melibiose is not the only amorphous disaccharide that exhibits delayed crystallization under such conditions. Based on this, it can be hypothesized that the crystallization rate of hydrates is slower than that of anhydrates, because the hydrate crystal lattice is more complex than the anhydrate one, and a certain stoichiometric ratio of solute and solvent is required. Hydrate crystallization may occur when water has been distributed properly throughout the matrix, whereas anhydrate crystallization may result as soon as the amorphous phase has gained sufficient mobility. Therefore, crystal formation and growth might be impeded in hydrates by the requirement of proper water molecule arrangement.

When assessing the physical stabilities of the amorphous disaccharides at $T>T_g$ based on their RCT values as a function of water content (Fig. 6), cellobiose was shown here to be more stable at low water contents than sucrose, but to become less stable when $C(H_2O, w/w)>4\%$. This supports the results of the desiccator studies, where crystallization proceeds more slowly in sucrose than in cellobiose when stored at RH 33% (crystalline diffraction pattern obtained with XRPD after 91 h in cellobiose and after 187 h in sucrose). However, at RH 23% the maximum measured C (H_2O, w/w) was 3.7% for sucrose and 4.7% for cellobiose, and crystallization occurred in cellobiose under those conditions but not in sucrose even though the predicted RCT values at those water contents were fairly similar. This implies that crystallization rates should not be predicted based only on RCT values at $T < T_g$. It also seems that a low RCT value indicates faster crystal nucleation at $T>T_g$, but it does not give an idea how fast the nuclei grow. This is because the shape of the crystallization exotherm is not taken into account when measuring RCT by using calorimetric methods.

Measuring RCT for a compound with several polymorphs is difficult because it is possible that more than one polymorph is formed during crystallization. For example, in case of trehalose the calculation of RCT would not yield useful results due to more than one melting point. It is no surprise that no RCT values could be determined for melibiose with the experimental setting in question. The IMC water sorption experiments showed that melibiose crystallization was still ongoing after 12 h at RH 70% (Fig. 4). With such water contents (increasing up to 20% w/w at RH 70%) the T_g of the melibiose-water mixture may have been as low as −35°C based on its GT model, assuming that water was distributed evenly. This means that even at $T >> T_g$ the crystal growth rate was still very slow. This distinguishes melibiose from the other disaccharides that were studied.

Differences in T_1H Relaxation Rate Constants

Upon crystallization, intra- and intermolecular hydrogen bond networks of the crystallizing molecules tend to form repeating motifs based on the most stable structure available (38). In amorphous state this requires some degree of mobility, as molecules lack long range order. It has been previously shown that amorphous-to-crystalline transformation may sometimes be initiated at $T < T_g$ (6), meaning that hydrogen bond forming moieties may possess sufficient mobility to align properly and form stable motifs even in the glassy state. Therefore it can be hypothesized that proton longitudinal relaxation time constants (T_1H) could be used to assess a form of molecular mobility that has an influence on crystallization rates when samples are stored under their T_g. If this were the case, a change in water content should also affect T_1H. According to Fig. 7, increase in the extent of water plasticization indeed decreases T_1H, and it also seems to slightly decrease the difference in mobility between trehalose and melibiose when approaching T_g. This is reasonable, as the significant viscosity depression at glass transition temperature may even out any differences in T_1H between disaccharides. Based on the differences in T_1H at $T < T_g$ it may be concluded that mobility of melibiose is slower than that of trehalose, which may have an impact on crystallization rates. Under the experimental time scale used, crystallization was apparently initiated at RH 33% between 200–300 h in both trehalose and melibiose (Fig. 1), even though the nuclei were too small to be

detected by XRPD. This corresponded roughly T / T_g=0.8 and 0.9 and T_1H of 4.4 and 3.9 for trehalose and melibiose, respectively (Fig. 7). Therefore similar crystallization rates corresponded with relatively similar relaxation rate constants. Crystallization of trehalose or melibiose was not observed with lower water contents than this under the experimental time scale, so it is not known whether the differences in T_1H could be related to crystallization rates at T / T_g<0.8. However, based on their T_1H values, proton mobility appears to be slower in melibiose than in trehalose under such conditions, possibly resulting in slower crystallization rates.

CONCLUSIONS

After storing freeze-dried disaccharides under different RH atmospheres, significant differences in crystallization rates were observed. There were differences in the predicted BET model constants between disaccharides, and the heat constant of surface sorption (C) of sucrose was clearly lower than that of the other disaccharides. Overall, the rank order of the C-values was the same as the order of glass transition temperatures of dry disaccharides. Predicting sucrose- and cellobiose-water mixture glass transition temperatures using GT model was sometimes proven to be inaccurate. The water concentration needed to lower the glass transition temperature to 25°C increased in the order sucrose<cellobiose<melibiose<trehalose, but nucleation and crystal growth were sometimes observed with lower water concentrations than this. This may have been partly caused by the inhomogeneous distribution of water, as there were signs of viscous flow in cellobiose at RH 33%, even though the overall glass transition temperature of the disaccharide-water mixture was higher than the storage temperature. However, it is likely that in some cases crystallization also took place at T < T_g, as was shown in cellobiose when it was stored at RH 23%. At T>T_g both trehalose and cellobiose crystallized quickly, which appears to be due to high rate of crystal growth. Melibiose was shown to possess a relatively high glass transition temperature, relatively low K-value of water plasticization and relatively slow overall rate of crystallization even at T>T_g. Furthermore, its molecular mobility (measured as T_1H) was slower than that of trehalose at T < T_g, which might impede its crystallization under such conditions. Therefore, it appears to be physically more stable in amorphous form than the other disaccharides studied. Even though lyophilized pharmaceuticals should not contain as much water as sometimes seen in this study, a sudden water influx due to faulty stoppers and suboptimal storage conditions might result in a momentary inhomogeneity in water distribution. This in turn might be enough to cause nucleation in physically unstable amorphous formulations. In such a case, the formulation where excipients with lowest possible crystallization tendency were used would be the most robust. Given the results presented here, trehalose and melibiose would be more stable under such aggravated conditions than sucrose or cellobiose.

ACKNOWLEDGMENTS & DISCLOSURES

The authors wish to acknowledge The Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki for providing laboratory facilities.

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