Glass transition and time-dependent crystallization behavior of dehydration bioprotectant sugars

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ABSTRACT

It has been suggested that the crystallization of a sugar hydrate can provide additional desiccation by removing water from the amorphous phase, thereby increasing the glass transition temperature (Tg). However, present experiments demonstrated that in single sugar systems, if relative humidity is enough for sugar crystallization, the amorphous phase will have a short life. In the conditions of the present experiments, more than 75% of amorphous phase crystallized in less than one month. The good performance of sugars that form hydrated crystals (trehalose and raffinose) as bioprotectants in dehydrated systems is related to the high amount of water needed to form crystals, but not to the decreased water content or increased Tg of the amorphous phase. The latter effect is only temporary, and presumably shorter than the expected shelf life of pharmaceuticals or food ingredients, and is related to thermodynamic reasons: if there is enough water for the crystal to form, it will readily form.

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1. Introduction

The ability of amorphous sugars to preserve labile biomolecules in dried systems has been recognized for years in food, pharmaceutical, and biological sciences. The disaccharides sucrose and trehalose have been employed as protectants of labile biomolecules during drying and later storage, the nonreducing character of these sugars is crucial in biomolecule stabilization to avoid Maillard reactions. The action of saccharides as protectants can be ascribed to both specific and kinetic effects. At a specific level, they interact with biological structures and stabilize them during drying. Compared to trehalose, raffinose has similar Tg values at low water contents. In addition to existing in a stable pentahydrate structure, raffinose forms less stable intermediate crystalline states, including an anhydrous structure, which can be formed upon dehydration of the pentahydrated crystal. Amorphous sugars usually adsorb water into their bulk structure, and as a consequence Tg decreases. When the system reaches a Tg value lower than ambient temperature, the material may experience marked changes in the physical properties (i.e., plasticization, collapse, caking, and crystallization). With the aim of protecting biomolecules, solute crystallization from amorphous mixtures is considered to be of importance during both drying and storage, which is desirable or undesirable according to the solute function within the system and the process stage. In the case of protective solutes, they have the requirement to be amorphous during the drying stage, to form a single amorphous phase with the biomolecule, without phase separation or demixing. Crystallization causes the cessation of the interactions between sugars and the labile molecules and the protection is lost. Thus, crystallization of carbohydrates used as stabilizing excipients during storage is detrimental for biomolecules and biological structures. Also, when crystallization is accompanied by water release from the crystalline phase, it causes a decrease in the Tg value of the remaining amorphous material. Note that the generation of crystalline hydrate forms on storage of amorphous products is unacceptable from a pharmaceutical viewpoint, as the physical structure should remain unchanged over the shelf life of a product.
Sugar crystallization may, in certain cases, be of benefit in the enhancement of physical stability. Bulking crystallizing agents are commonly added to formulations, in order to improve the physical characteristics of the final lyophilized preparation, and to control the residual water content. Usually, crystallizing bulking agents (mannitol and glycine) provide physical stability but they are not effective as protein stabilizers, because they tend to phase separate from the protein.

Aldous et al. studying trehalose and raffinose matrices suggested that the crystallization of a sugar into hydrate forms can provide partial desiccation by removing water from the amorphous phase, thereby increasing the $T_g$ and the stability of the remaining fraction. The formation of hydrated crystalline forms by some sugars, and the desiccation value of extracting amounts of water into the hydrated structure would therefore seem to be vital in relation to the biostabilizing function of these materials. Although this desiccant action during crystallization has been accepted as a mechanism accounting for the high stability that trehalose confers to sensitive biological systems during drying and storage, there is not enough experimental evidence to substantiate the above statement. Several mechanisms, involving different crystalline forms, were suggested for the trehalose protecting action during storage following freezing or freeze-drying.

The purpose of the present work is to analyze the potential advantage of the use of sugars that form hydrated crystals as dehydration protectant excipients. Crystallization in freeze-dried trehalose and raffinose systems and its effect on the glass transition temperature of the remaining amorphous phase are analyzed.

### 2. Results

The formation of hydrated crystals from an amorphous phase has not only kinetic restrictions, that is, raising the temperature above $T_g$ might not be a sufficient condition to observe crystallization, but also a minimum amount of water required to form the hydrated stable crystals. Thus, the RH and temperature conditions were selected in order to assure the minimum water requirement to form the dihydrate crystal for trehalose and pentahydrate for raffinose (Table 1). Mass changes, $T_g$, and the area of the melting/crystallization peaks were periodically determined until a high degree of sugar crystallization was achieved and/or water uptake reached a quasi-equilibrium value.

Figure 1 shows DSC scans for selected freeze-dried amorphous samples of trehalose exposed to 75% RH at 37°C and raffinose exposed to 63% RH at 37°C. The endothermal baseline shift represents the glass transition. The endothermal peaks observed at about 97 and 80°C correspond to the melting of crystalline trehalose dihydrate and raffinose pentahydrate, respectively. The area of the endothermal peaks was then used to estimate the amount of hydrated crystal formed (degree of crystallization). Some trehalose samples also showed crystallization exotherms above $T_g$ prior to melting. This fact was taken into consideration when calculating the above-mentioned crystallization degree.

Figure 2 shows the water content corresponding to the amorphous and crystalline fractions as a function of humidification time at 75% RH and 37°C, for trehalose (a) and raffinose (b) samples. The water content of the amorphous phase initially increased giving rise to a water mass which is sufficient to: (a) lower $T_g$ below the storage temperature and consequently give the material enough molecular mobility to allow crystallization (Fig. 3) and (b) fulfill the requirements for hydrate formation. Upon water adsorption by the amorphous sugar, crystallization can occur when the material reaches the water content needed to form trehalose dihydrate (10.5 g water/100 g solids) or raffinose pentahydrate (17.8 g water/100 g solids). Upon trehalose crystallization, the water content of the amorphous fraction remains almost constant and close to that obtained for the crystalline material, within the analyzed experimental time (Fig. 2a). Thus, trehalose crystallization did not produce a dehydration effect on the remaining amorphous phase. After reaching the water content needed for crystallization, raffinose sample showed a decrease of 4% in the water content of the amorphous phase (Fig. 2b). Although partial

### Table 1: Temperature and relative humidity (RH) for storage conditions

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Temperature (°C)</th>
<th>Saturated salt solution</th>
<th>RH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>6</td>
<td>NaBr</td>
<td>63</td>
</tr>
<tr>
<td>R</td>
<td>16</td>
<td>NaCl</td>
<td>76</td>
</tr>
<tr>
<td>R</td>
<td>26</td>
<td>Mg(NO₃)₂</td>
<td>56</td>
</tr>
<tr>
<td>R</td>
<td>37</td>
<td>NH₄NO₃</td>
<td>68</td>
</tr>
<tr>
<td>Tr</td>
<td>26</td>
<td>K₂CO₃</td>
<td>43</td>
</tr>
<tr>
<td>R</td>
<td>37</td>
<td>Mg(NO₃)₂</td>
<td>53</td>
</tr>
<tr>
<td>R</td>
<td>37</td>
<td>NaBr</td>
<td>58</td>
</tr>
<tr>
<td>Tr</td>
<td>37</td>
<td>Mg(NO₃)₂</td>
<td>50</td>
</tr>
<tr>
<td>R</td>
<td>37</td>
<td>NH₄NO₃</td>
<td>62</td>
</tr>
<tr>
<td>R</td>
<td>37</td>
<td>KI</td>
<td>67</td>
</tr>
<tr>
<td>R</td>
<td>37</td>
<td>NaCl</td>
<td>75</td>
</tr>
<tr>
<td>Tr</td>
<td>37</td>
<td>NaCl</td>
<td>75</td>
</tr>
</tbody>
</table>

R: raffinose, Tr: trehalose.
The changes in the amorphous fraction and the corresponding $T_g$ values during humidification time at different RH and temperature conditions are shown in Figure 3 for trehalose (a and b) and raffinose (c–f) samples. Due to water uptake, $T_g$ of the samples showed an initial decrease, reaching a minimum value which remained almost constant (Fig. 3a–d). After certain storage time, the amount of amorphous raffinose fraction was negligible, and therefore $T_g$ was not observed in the corresponding thermograms (Fig. 3d–f). In raffinose samples at some conditions (Fig. 3e and f), the initial $T_g$ decrease was followed by a temporary increase. It has been claimed that the increase of $T_g$ is responsible for the high stability that sugars form hydrated crystals confer to sensitive biological systems during drying and storage.

3. Discussion

Given that crystallization may compromise the preservation of dry biomolecules, the use of sugars that crystallize as hydrates was proposed to be an advantage. These matrices can be stable at higher relative humidities than those formed with sugars that form anhydrous crystals providing the possibility of broader storage conditions, keeping the sample free from crystallization. In this respect, trehalose and raffinose, which form di- and pentahydrates, respectively, have potential stabilizing properties.

Raffinose has the unique ability among oligosaccharides to crystallize as pentahydrate, rendering a stabilizing matrix that does not crystallize up to 53% RH (17.8% d.b.) at 25 °C.

Although Sussich et al. proposed that the protective action of trehalose is based on the formation of different crystalline forms, crystallization of sugars is considered a deleterious process in anhydrobiotic organisms. However, no evidence of sugar crystallization in cells or tissues has ever been reported. Crystallization was not observed in dehydrated yeast cells containing 15% trehalose, even at 80% RH. X-ray diffraction studies failed to detect the presence of cytoplasmic crystallization in dry seeds. Espinosa et al. analyzed freeze-dried model systems consisting of yeast extracts and trehalose humidified at various RHs. They did not observe trehalose crystallization at any of the analyzed relative humidities, even at 75% RH, at which the samples...
contained 36.2% water content (three times higher than the amount of water required for trehalose crystallization) and a $T_g$ value of 98°C (at which instantaneous crystallization could be expected for a pure sugar). Mixtures of sucrose and other oligosaccharides were found to inhibit crystallization, and it was suggested that this could be the reason for the lack of sugar crystallization in seeds.38 The addition of polymers, sugars, or salts to sugar-based matrices allowed to delay sugar crystallization of trehalose;39,40 lactose;41 and sucrose.42

It has been considered that sugar crystallization may be beneficial. If a sugar could crystallize as a hydrate, it would provide additional desiccation by removing water from the amorphous phase, and thereby increase the storage stability by increasing $T_g$ of the remaining amorphous matrix.15–22 Our results showed that when water content and $T_g$ values are adequate, crystallization occurred with short induction times (0–15 days depending on storage conditions). Although in some cases $T_g$ values of the remaining amorphous phase increased temporarily, the crystallization process proceeded in short time rendering samples with little or none remaining amorphous phase. Note that these samples were completely collapsed, so the recovery of labile biomaterials embedded in a matrix with those characteristics may be difficult. The incorporation of additives that prevent or delay sugar crystallization may be an alternative to this problem.

The good performance of sugars that form hydrated crystals (trehalose and raffinose) as stabilizing agents for labile biomaterials in dehydrated systems is related to the need of a relatively high amount of water to form the crystal, but not to the decreased water content or increased $T_g$ of the amorphous phase. The latter effect is only temporary, and presumably shorter than the expected shelf

![Figure 3. Amorphous fraction and $T_g$ changes during humidification time for trehalose and raffinose samples exposed to different conditions of RH and temperature: trehalose at 53% RH and 37°C (a), trehalose at 75% RH and 37°C (b), raffinose at 58% RH and 26°C (c), raffinose at 75% RH and 6°C (d), raffinose at 63% RH and 6°C (e), and raffinose at 75% RH and 37°C (f).](image-url)
life of pharmaceuticals or food ingredients, and is related to thermodynamic reasons: if there is enough water for the crystal to form, it will readily form. Thus, if the maintenance of partially crystalline non-equilibrium states is required, the formulation of bioprotectant matrices should be specifically designed by combination of solutes or by other ways of kinetically ‘freezing’ those unstable states.

4. Experimental

4.1. Preparation of model systems

Raffinose pentahydrate (Pfanstiehl Laboratories, Inc., Waukegan, Illinois, USA) and trehalose dihydrate (Hayashibara Co., Ltd, Shimoishii, Okasama, Japan/Cargill Inc., Minneapolis, Minnesota, USA) were used without further purification. Distilled water was used for all experiments.

The amorphous systems were obtained by freeze-drying solutions containing 20% (w/w) of the selected sugars. Aliquots of 1 mL of the solution were placed in 5 mL capacity vials, frozen 24 h at −26 °C, and immersed in liquid nitrogen before freeze-drying. A Heto-Holten A/S, cooling trap model CT110 freeze-drier (Heto Lab Equipment, Denmark) was used which operated at −110 °C and at a chamber pressure of 4 × 10⁻⁶ mbar.

The freeze-dried samples were transferred into desiccators and kept at 6, 16, 26, and 37 °C over saturated salt solutions that provided constant relative humidities (RHs), as described in Table 1.

4.2. Determination of water content

The total water content of the humidified samples was determined by the difference in weight before and after drying in a vacuum oven at 96 °C for 48 h in the presence of a desiccant. In samples in which part of the sugar crystallized, the water content of the amorphous phase (Wa) was calculated through the following equations:

\[ Xa + Xc = 1 \]
\[ W_1 = XcWc + XaWa \]
\[ Wa = \frac{(W_1 - XcWc)}{(1 - Xc)} \]

where \( Xc \) indicates mass fraction of crystalline sugar (trehalose dihydrate or raffinose pentahydrate); \( Xa \), mass fraction of amorphous sugar; \( W_1 \), total water content (% dry basis); \( Wa \), water content of the amorphous phase (% dry basis); and \( Wc \), water content of the crystalline phase (% dry basis).

To obtain \( Xc \), the area of the melting peak of a given sample (obtained by DSC) was related to the area of the melting peak of totally crystallized sugar samples. In the case of samples showing crystallization exotherms above \( T_0 \) prior to melting, this area was subtracted from the corresponding melting peak area to obtain \( Xc \).

4.3. Differential scanning calorimetry (DSC)

DSC was used to determine glass transition temperatures (\( T_g \)) and endothermal peak areas (related to the amount of crystalline sugar present). Glass transitions were recorded as the onset temperature of the discontinuities in the curves of heat flow versus temperature. The area of the endothermal peak is proportional to the amount of sugar crystallized, and the degree of crystallization was obtained by comparing this value with the calorimetric enthalpy of the melt in pure crystalline samples (139 J/g and 148 J/g for trehalose dihydrate and raffinose pentahydrate, respectively). The instrument used was a Mettler-Toledo equipment model 822 (Mettler Toledo AG, Switzerland), and STARe Thermal Analysis System version 3.1 software (Mettler Toledo AG) was used for all the measurements. The instrument was calibrated with indium and zinc. All measurements were made at 10 °C/min, using hermetically sealed aluminum pans (Mettler, 40 µL capacity), and an empty pan was used as a reference. An average value of two replicate samples was reported.

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