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Research Article

Studies of the Crystallization of Amorphous Trehalose Using Simultaneous Gravimetric Vapor Sorption/Near IR (GVS/NIR) and "Modulated" GVS/NIR

Abigail Moran[1] and Graham Buckton[1,2]

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Abstract. The purpose of this research was to investigate the influence of changes in the amorphous state on the crystallization of trehalose. Amorphous trehalose is known to stabilize biomaterials; hence, an understanding of crystallization is vital. Amorphous trehalose, prepared by spray-drying, was exposed to either a single step (0–75%) in relative humidity (RH) or to modulated 0–75–0% RH to cause crystallization. For the single-step experiment, two samples crystallized in a predictable manner to form the dihydrate. One sample, while notionally identical, did not crystallize in the same way and showed a mass loss throughout the time at 75% RH, with a final mass less than that expected for the dihydrate. The idiosyncratic sample was seen to have a starting near infrared (NIR) spectra similar to that exhibited by anhydrous crystalline trehalose, implying that short-range order in the amorphous material (or a small amount of crystalline seed, not detectable using powder X-ray diffraction) caused the sample to fail to form the dihydrate fully when exposed to high RH. The modulated RH study showed that the amorphous material interacted strongly with water; the intensity of the NIR traces was not proportional to mass of water but rather the extent of hydrogen bonding. Subsequent crystallization of this sample clearly was a partial formation of the dihydrate, but with the bulk of the sample then shielded such that it was unable to show significant sorption when exposed to elevated RH. It has been shown that the nature of the amorphous form will alter the way in which samples crystallize. With oscillation in RH, it was possible to further understand the interactions between water and amorphous trehalose.

KEY WORDS: amorphous; crystallization; near IR; trehalose; water sorption.

INTRODUCTION

Trehalose has been found to be an excellent material to use to stabilize proteins in the amorphous state [e.g., (1)]. Indeed, trehalose has been shown to be present in desert plants where it is thought to provide protection against drought. It has been argued (2) that the mechanism of stabilization of proteins and peptides is due to the fact that trehalose can sorb water and then crystallize and phase separate from the protein or peptide, thus, acting as a desiccant removing large amounts of water that may otherwise result in detrimental crystallization of the protein or peptide itself. Raffinose which also forms a higher hydrate, in this case a pentahydrate, is equally known to have excellent stabilizing properties (3). Crowe *et al.* (4) found that increasing the water content of amorphous trehalose resulted in the glass transition temperature (T_g) remaining high, whereas amorphous sucrose was seen to have the T_g reduced by the sorbed water, the conclusion being that the trehalose partially crystallized to the dihydrate and the remaining material was essentially dry rather than plasticized by water sorption. As such, it is necessary to understand factors that impact on the ability of amorphous trehalose to crystallize.

The simultaneous use of gravimetric water sorption (GVS) and near infrared NIR spectroscopy was introduced as a method by which the water sorption and its impact on physical form can be studied simultaneously (5). This hyphenated approach has been used by Hogan and Buckton (6) to study the water sorption to amorphous raffinose and the subsequent formation of different hydrate levels. Moran and Buckton (7) have reported that there is variability in the crystallization behavior of amorphous trehalose depending upon the concentration used during spray drying, with lower feed concentrations giving greater variability in the subsequent crystallization process. In the current study, we report on variability in concentrated feed solution spray dried amorphous samples and for the first time report data from GVS–NIR measurements using a modulated humidity cycle during the crystallization experiment to attempt to shed light on the interaction between water and the amorphous trehalose.

MATERIALS AND METHODS

Trehalose (as the crystalline dihydrate) was obtained from Sigma and used as received.

[1] Department of Pharmaceutics, School of Pharmacy, University of London, 29–39 Brunswick Square, London, WC1N 1AX, UK.
[2] To whom correspondence should be addressed. (e-mail: Graham.Buckton@pharmacy.ac.uk)

Production of Spray-Dried Amorphous Trehalose

Trehalose was spray-dried from a 10% solution in water (250 ml volume) using a Büchi 191 Mini Spray Dryer (Büchi, Switzerland). Parameters used are outlined in Table I. Spray-dried products were stored in a desiccator over phosphorus pentoxide (0% RH) until use. Samples were confirmed as amorphous using a Philips PW3710 X-ray powder diffractometer (XRPD; Philips, Cambridge, UK). Sample (<1 g) was loosely filled into the shallow well of the XRPD sample holder. A small purpose made block of Perspex (made in-house) was used to press carefully the sample into the sample holder cavity to create a smooth, level, finished powder surface. Any excess sample surrounding the circular sample holder cavity was carefully removed. The powder bed achieved by this method measured a depth of approximately 2 mm. The sample holder was loaded into the diffractometer and scanned between 5° and 50°2θ. Samples were measured at 45 kV and 30 mA.

Crystallization of Amorphous Spray-Dried Trehalose via Single Exposure to a 0% RH→75% RH→0% RH GVS Cycle

Samples of amorphous spray dried trehalose (approximately 30 mg) and crystalline trehalose dihydrate were subjected to a 0% RH→75% RH→0% RH cycle to induce recrystallization of amorphous content using the combined Dynamic Vapor Sorption (Surface Measurement Systems, UK) GVS/NIR apparatus. This novel method has been described previously [e.g., (6)]. Samples were checked for crystallinity before and after GVS experiments using X-ray powder diffraction as described above.

Crystallization of Amorphous Spray-Dried Trehalose via Exposure to a 0% RH→75% RH →0% RH GVS Cycle—"Modulated" GVS/NIR

In addition to exposing the amorphous trehalose to a single GVS cycle, an equivalent quantity of spray-dried trehalose was exposed to a repeated cycling program of 0% RH and 75% RH. Hour-long periods at 0% RH were divided by increasing periods of time at 75% RH. Amorphous spray-dried trehalose was exposed to 0% RH for 8 h (initial drying phase), 75% RH for 5 min, 0% RH for 1 h, 75% RH for 10 min, 0% RH for 1 h, 75% RH for 15 min, 0% RH for 1 h, 75% for 20 min, 0% RH for 1 h, 75% RH for 25 min, 0% RH for 1 h, 75% RH for 30 min, and finally, 0% RH for 1 h. NIR spectra were recorded every 150 s throughout the GVS experiment. Modulated methods (such as differential scanning calorimetry) are now frequently used to study reversing and nonreversing processes. Here, modulation of relative humidity was carried out in order to examine the reversibility of structural changes in the original single RH ramp GVS/NIR experiment and to analyze structural changes occurring during crystallization using NIR spectroscopy. In all cases, NIR data are plotted as second derivative of the standard normal variant, such that peaks point down in the figures.

Table I. Operating Parameters for the Preparation of Co-spray-dried Trehalose Powders using the Buchi 191 Mini Spray Dryer

Operating parameters	Settings
Inlet temperature (°C)	140–150
Outlet temperature (°C)	70–80
Feed rate (ml/min)	2
Pressure (bar)	3
Atomizer flow rate (normliter/h)	600
Machine settings (%)	
Aspirator	60
Feed rate	16–20

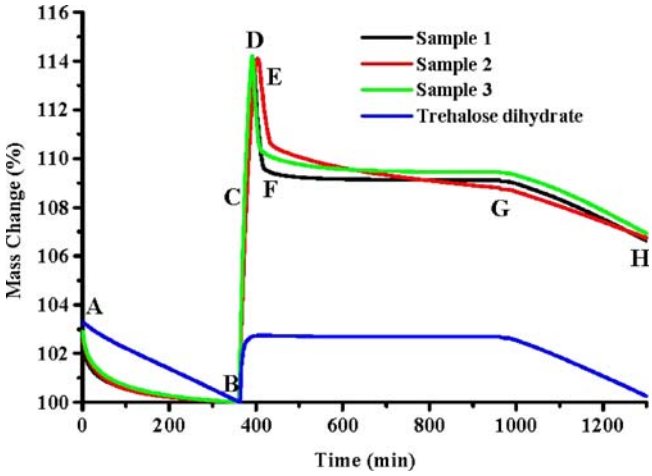

Fig. 1. GVS plots for three spray-dried trehalose samples (*1*, *2*, and *3*) and one trehalose dihydrate sample exposed to 0% RH for 6 h, 75% RH for 10 h, and then 0% RH for a further 6 h (at 25°C)

RESULTS AND DISCUSSION

Crystallization of Amorphous Spray-Dried Trehalose via Single Exposure to a 0% RH→75% RH→0% RH GVS Cycle

Water sorption isotherms for spray-dried amorphous trehalose are shown in Fig. 1 for three repeat samples, labeled 1, 2, and 3.

Sample mass values were determined at the most important positions; A–H of the GVS runs as shown in Fig. 1 for each spray-dried trehalose samples. These values are displayed in Table II. Samples showed an initial mass loss at 0% RH as drying occurred and then a rapid mass gain when moisture was absorbed by the sample on exposure to 75% RH, equating to ca. 14.5% mass gain over the dry weight. This was followed after an average of 35 min by a rapid mass loss as moisture was expelled from the sample due to crystallization. It was expected that samples would stabilize at 75% RH following crystallization to the dihydrate crystal form of trehalose (containing ~9.5% water). Samples labeled 1 and 3 in Fig. 1 showed stabilization at 75% RH whereas sample 2 continued to lose mass over the entire 75% RH stage, showing a move-away from the dihydrate water content. This continued loss of water indicated an unexpected trend to lose available water suggesting that sample 2 may not have formed a stable dihydrate.

Table II. Mass Changes (%) between Different Time-points of the GVS Plots for each Spray-dried Trehalose Sample as Described in the Discussion of Fig. 1

	B–A (%)	D–B (%)	F–D (%)	G–B (%)	H–B (%)
Sample 1	−2.30	14.08	−4.58	9.11	6.46
Sample 2	−2.58	14.10	−3.59	8.80	6.60
Sample 3	−2.91	14.20	−3.96	9.46	6.75
Average	−2.60	14.13	−4.04	9.12	6.60

On returning the three recently crystallized samples to 0% RH (at 16 h after the start of the GVS experiment), all three samples showed gradual mass loss at a faster rate than that shown by sample 2 during the 75% RH step, indicating diffusion of water from the samples, presumably due to loss of the dihydrate water. The sample that was in the crystalline form (trehalose dihydrate) at the start of the experiment lost mass during the final 0% RH stage of the experiment, at an equal rate to those samples that were originally in the amorphous state. This suggests that all four trehalose samples were likely to be tending towards an anhydrous state during the final drying stage.

From water sorption data alone, it was impossible to tell exactly why the crystal structure of sample 2 showed such instability (continued mass loss) as the amorphous trehalose samples crystallized at 75% RH. The combination of GVS with NIR spectroscopy (NIRS) allowed the crystallization process to be examined on a more structural level. During the GVS experiment (Fig. 1) for spray-dried trehalose sample 3, NIR spectra were recorded every 2.5 min in order to follow the crystallization process in more detail. In Fig. 2, NIR spectra between wavelengths of 1,300–1,500 nm are presented for spray-dried trehalose sample 3 as it absorbed and expelled moisture at 75% RH as crystallization occurred. Labels on the figure refer to the time points of the GVS–NIR experiment shown on Fig. 1, from which the spectra were recorded. The peak at 1,432 nm was reduced as moisture was taken up by the sample between points B to D (B being the end of the initial drying process and D being the point of maximum water content) and a shoulder at ~1,468 nm began to form. Peaks for water in the NIR region are well characterized (7–9). Peaks between 1,435 and 1,480 nm are referred to as first overtone bound –OH alcohol in tables of chemical group frequencies in the NIR region and refer to intermolecular hydrogen bonding (8). It is, therefore, likely that the peaks shown in Fig. 2 refer to changes in the bonding of water within the trehalose molecular structure as water is introduced into the system. In this region of the NIR spectrum, it is proposed that the water introduced to the sample changed from being free to becoming bound (−OH).

In Fig. 3, NIR spectra between wavelengths of 1,860–2,000 nm are presented for the same time points as shown in Fig. 2. The peaks in Fig. 3 are easier to explain in terms of the sorption and desorption of water into and out of the sample. The peak at 1,932 nm refers to free water in the sample (8). This peak increased in intensity as the sample was held at 75% RH and water was adsorbed onto and diffused into the trehalose structure. This peak began to reduce in intensity as the sample approached its threshold for moisture uptake prior to crystallization. At this point, the peak reduced in both intensity and shape, while a shoulder was formed at ~1,952 nm. This observation suggested that water was initially sorbed and then gradually rearranged within the sample to form the dihydrate by point E (just after the sample started to lose mass; Fig. 3). This information points towards the spectrum at point D being that of the collapsed form of amorphous trehalose, prior to recrystallization, because the peaks corresponding to the dihydrate form were not yet present.

Between points D and G on Fig. 1 (D being the maximum mass and G being the end of the 75% RH exposure), samples 1 and 3 both stabilized at a mass close to that expected if trehalose dihydrate had formed. Sample 2, however, continued to lose mass gradually over the entire 75% RH period, suggesting that this sample had not stabilized to the dihydrate mass. We have previously reported

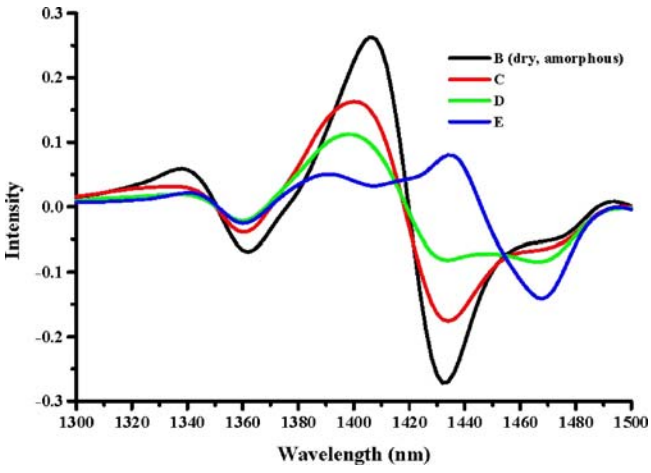

Fig. 2. NIR spectra of spray-dried trehalose sample 3 between 1,300 and 1,500 nm from different time-points through the GVS experiment described in Fig. 1

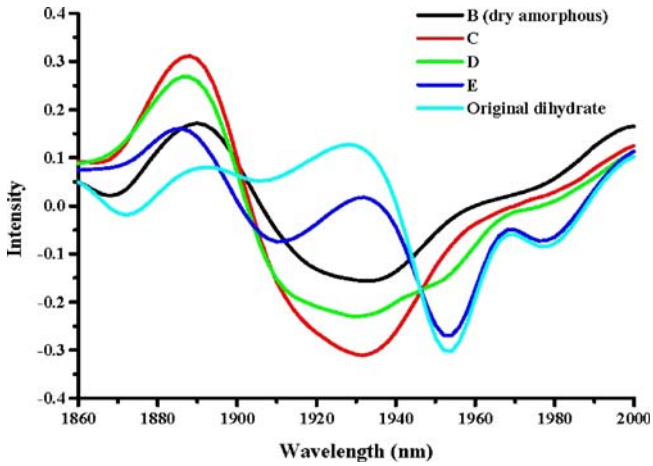

Fig. 3. NIR spectra of spray-dried trehalose sample 3 between 1,860 and 2,000 nm from different time-points through the GVS experiment described in Fig. 1 compared to the original crystalline trehalose dihydrate prior to spray drying

(7) that variability in crystallization behavior is related to the concentration of the feed solution used in spray drying (which links to the morphology of the particles produced). It was found that the lower the concentration of trehalose in the solution to be spray-dried, the more variable the crystallization behavior of the final spray-dried product. As all three samples shown in Fig. 1 were spray-dried from 10% *w/v* solutions, it would be expected that the variability would be fairly low between these samples (7). It seems, therefore, that sample 2 is tending towards a form other than the dihydrate, perhaps an anhydrous form. The NIR spectrum of sample 2 from the end of the 75% RH stage (time-point G) was compared with those of samples 1 and 3. The only major difference between the spectra is shown in Fig. 4.

In Fig. 4, both samples 1 and 3 showed a single peak in the region at 1,360 and 1,366 nm, respectively, whereas sample 2 exhibited a double peak with minima at 1,358 and 1,376 nm. The spectrum of the original crystalline trehalose sample, prior to spray-drying, showed a single peak in this region at 1,360 nm. Although the crystalline spectra of samples 1 and 3 were not identical to that of the original crystalline form, they displayed similar peaks in the same region and had water sorption behavior suggestive of crystallization to the dihydrate form. These data may well suggest that the dihydrate did not form perfectly during the experiment (as the peaks are not identical in Fig. 4 for samples 1, 3, and the original dihydrate sample). Interestingly, in the spectra from time-point B in Fig. 1 (end of the first drying stage), samples 1 and 3 showed a single peak at 1,362 nm whereas sample 2 showed a double peak again at 1,358/1,376 nm (Fig. 5). As these "amorphous" peaks were still present in the crystalline spectrum at point G (end of the 75% RH stage) for sample 2 (Fig. 4), it suggests that the sample had not entirely crystallized to the dihydrate form. The double peak at 1,358/1,376 nm prior to crystallization of the sample compared well with spectra of crystalline anhydrous trehalose (data not shown). Given that the samples all appeared amorphous by X-ray (which is not proof of absolute absence of crystallinity but shows substantial absence), the retention of peaks that correspond to anhydrous crystalline material may well relate to short-range order in the amorphous sample. The crystallization of the sample that had short-range order similar to that of the anhydrous form (sample 2) did not give rise to 100% crystalline dihydrate. The other (seemly identical in terms of history) amorphous samples, however, were not confined in structure and formed the dihydrate in the plentiful water that was available in the GVS experiment. These data indicate that short-range order in the amorphous state can potentially alter the form that is produced when the sample crystallizes. The alternative explanation is that NIR is more sensitive than X-ray diffraction to the presence of small amounts of crystalline anhydrous trehalose, which may then have acted as a seed for crystallization in the one sample where it was present.

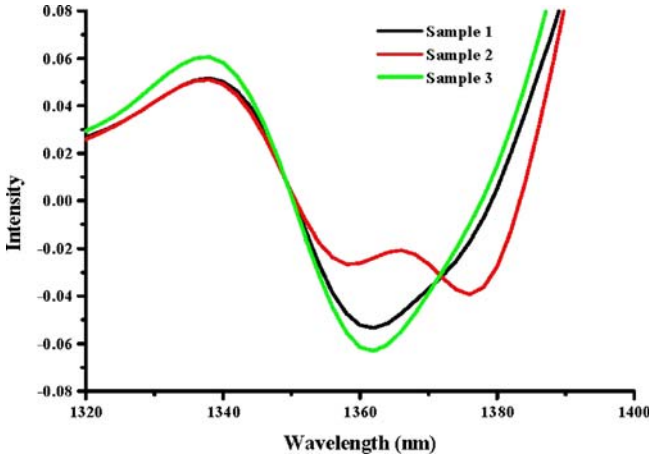

Fig. 5. Comparison of NIR spectra of samples *1*, *2*, and *3* from time-point B on Fig. 1

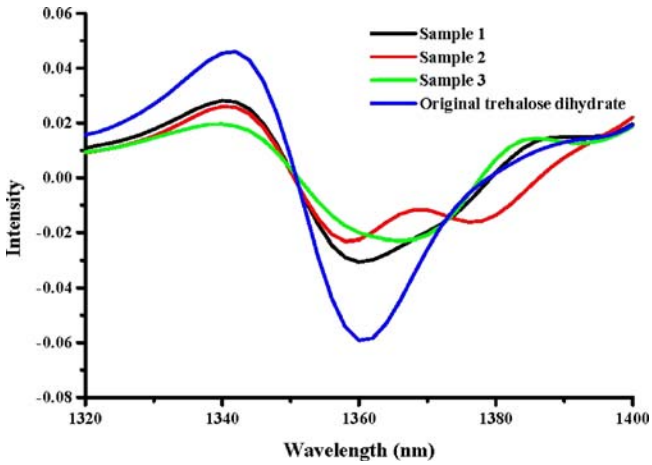

Fig. 4. Comparison of NIR spectra of samples *1*, *2*, *3*, and *trehalose dihydrate* from time-point G on Fig. 1

Crystallization of Amorphous Spray-Dried Trehalose via Modulated Exposure to a 0% RH→75% RH→0% RH GVS Cycle

The GVS plot of the crystallization of amorphous spray-dried trehalose via repeated exposure to a 0% RH→75% RH→0% RH cycle is shown in Fig. 6. It was expected that as sorption and desorption occurred, there would be some peaks

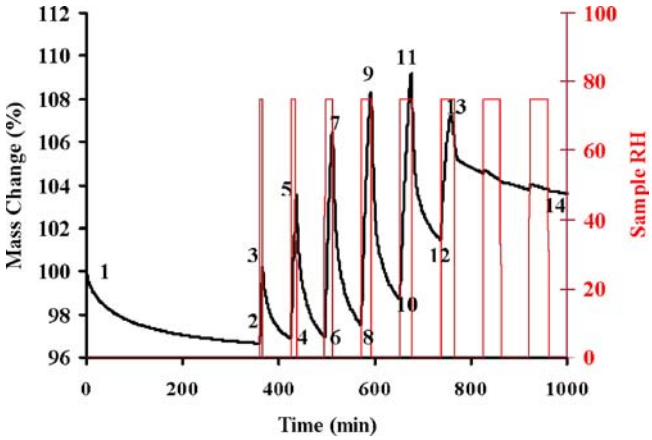

Fig. 6. GVS plot of the recrystallization of amorphous spray-dried trehalose via repeated exposure to a 0% RH→75% RH→0% RH DVS cycle. Time-points from which NIR spectra were collected are numbered for ease of discussion

that would reversibly change with the water going into and out of the system and some peaks that would not reverse to signify structural changes within the sample.

Between time-points 2 and 6 of the GVS experiment shown in Fig. 6, repeated water sorption followed by water desorption resulted in mass gain, and loss with the mass after each desorption returned to approximately the same as it was prior to the previous water sorption. In each case, however, the mass loss was at a much slower rate than the mass gain, indicating an affinity between water and the amorphous sample. This being so, it would be likely that major changes within the NIR spectra between points 2 and 6 would be due to this reversible water movement. Figure 7 shows a section of the spectra recorded at time-points 2–6 of the DVS plot shown in Fig. 6.

As indicated by the arrow, the peak at 1,932 nm increased in intensity as water sorption took place and reduced in intensity with water desorption. A peak at this wavelength is likely to have arisen from the combination of O–H stretch and O–H bend vibrations in free water in the sample, corroborating the theory that this peak movement was due to free water sorption/desorption. The magnitude of the peak in Fig. 7 (peak points down) is interesting as initially there is a correlation with the water content (Fig. 6), i.e., point 2 (end of first drying) is the lowest peak, point 3 (peak of first short exposure to 75% RH) has a similar magnitude to that seen for the original spray-dried sample before drying in the GVS (point 1; Fig. 7), which is in keeping with the mass data (Fig. 6). Point 4 (end of second drying stage; Fig. 7) has a higher peak than point 1, in keeping with the mass data (Fig. 6), where it is clear that there is a small retained mass of water. Point 5 (end of second sorption process) has the highest peak (Fig. 7), in keeping with the highest water content (Fig. 6), however, point 6 (end of third drying process retains a large NIR peak; Fig. 6) despite a mass that is very similar to that at point 4 (Fig. 6). It follows that the water has a stronger bonding retention on the second cycle than on the first, such that the NIR peaks are not quantitative by mass but indicative of the strength of interaction of that retained water.

Between time-points 6–10 of the DVS experiment shown in Fig. 6, the GVS plot showed repeated water sorption followed by water desorption, similar to that shown between time-points 2–6, except that the mass did not return to "baseline" upon desorption. While the kinetics of desorption were not noticeably different from those seen following the earlier sorption steps, the drying stages between time-points 6–10 were not long enough to allow water to fully desorb from the sample due to the longer sorption times allowing a higher water content to sorb. The NIR data in the region 1,900–1,950 nm showed modest increases at points 6, 8, and 10, once again showing the strength of the bonding of retained water.

Spectra from time-points 11–13 during the GVS plot (Fig. 6) are shown in Fig. 8. Crystallization of the amorphous trehalose had begun by the point at which spectrum 12 was recorded. This could be determined by tracking the peak for O–H first overtone absorption (for free water) at 1,432 nm. This peak decreases in intensity from time-point 11 of the GVS experiment onwards, shifting to become a peak at 1,468 nm, corresponding to bound −OH alcohol first overtone vibrations. This indicated an increase in intermolecular

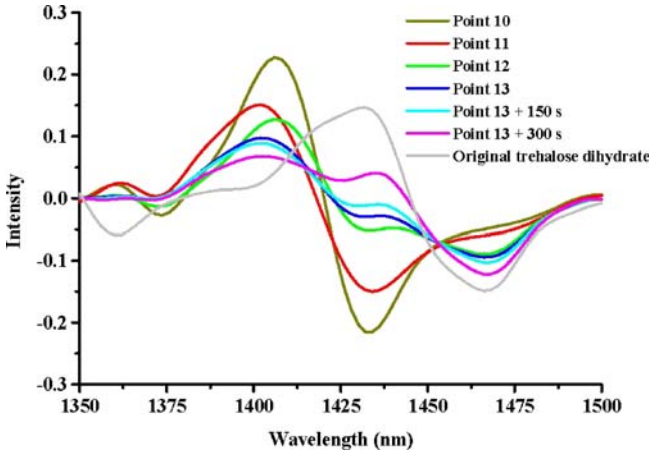

Fig. 8. NIR spectra between 1,350 and 1,500 nm, recorded at numbered time-points over the trehalose recrystallization event during the GVS experiment shown in Fig. 6

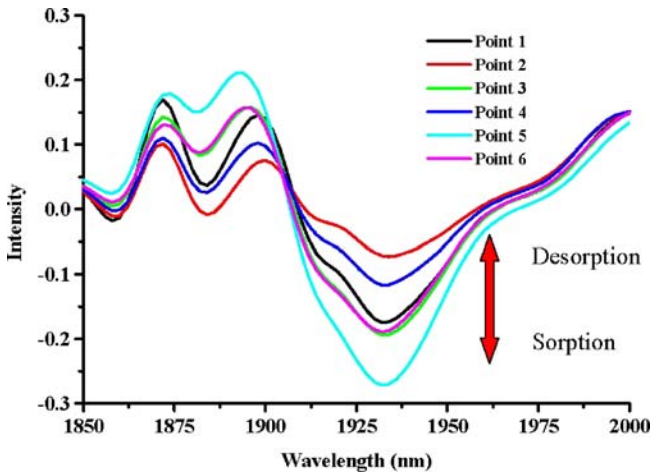

Fig. 7. NIR spectra recorded at numbered time points during the GVS experiment shown in Fig. 6. The *arrow* indicates the reversible nature of the peak at 1,932 nm

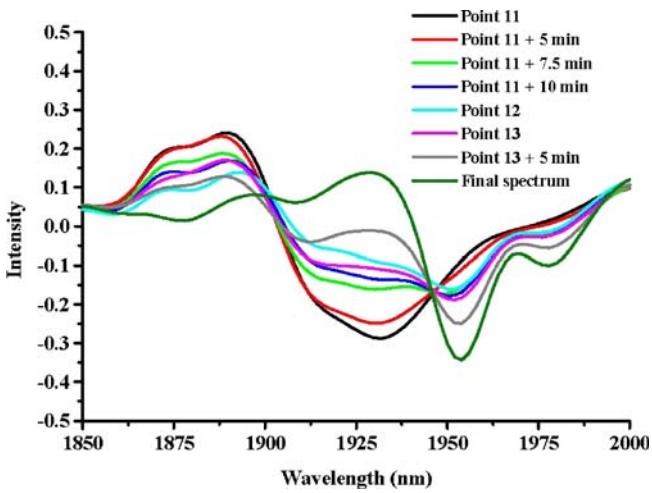

Fig. 9. NIR spectra between 1,850 and 2,000 nm, recorded at numbered time-points over the trehalose recrystallization event during the GVS experiment shown in Fig. 6

hydrogen bonding in the sample and, hence, the onset of recrystallization (8).

Between spectrum 11 and 12, a drying stage at 0% RH meant that no extra water was taken up by the sample and mass loss occurred during this period. The spectra in Fig. 9 show a decrease in intensity of the peak at 1,932 nm (relating to free water in this sample according to previous assignment of peaks). This peak appeared to shift and transform into the peaks for bound water at 1,954 and 1,978 nm between time-points 11–12 rather than simply reducing in intensity as would be expected if only water desorption were taking place. This observation would suggest that the sample had absorbed enough water at point 11 to crystallize but had yet to rearrange fully to the crystal form. NIR peaks corresponding to the dihydrate form developed and increased in intensity after time point 13, indicating that the material was reordering with time. During each drying stage after point 13 (Fig. 6), the sample lost mass at a much slower rate than had been observed prior to the crystallization, in keeping with a slow loss of the dihydrate water rather than the loss of water bound in an amorphous form. It is clear in the sorption/desorption cycles after point 13 that the sample actually exhibits mass loss during both sorption and desorption. The NIR data show no change in the magnitude of the peaks representing the dihydrate between the end of the penultimate and last sorption processes, despite a lower mass. As the final mass gain is well under the 9% mass gain expected to form the dihydrate, it must be that the surface of the sample has crystallized and prevented the central regions from sorbing more water and forming any further dihydrate material; indeed, only mass loss is possible whether at high or low RH.

CONCLUSIONS

Crystallization of amorphous trehalose when exposed to 75% RH proved to be variable, with the short-range order in the amorphous state being related to the behavior during crystallization. Modulation of the exposure of amorphous trehalose to RH showed strong hydrogen bonding. It was further shown that the changes in water exposure resulted in the formation of partial dihydrate with a core that was unable to crystallize to the dihydrate form. Both the nature of the amorphous state and the mechanism by which the material is exposed to water can alter the outcome of a crystallization process.

DVS–NIR is a valuable tool for the study of the amorphous state and its crystallization, with the modulated method of water interaction providing valuable data.

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