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

Manuscript in preparation for submission

Maarten A. Mensink*, Juraj Šibík*, Henderik W. Frijlink,
Kees van der Voort Maarschalk, Wouter L. J. Hinrichs, J. Axel Zeitler

* authors contributed equally

ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

METHODS

MATERIALS

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

FREEZE-DRYING

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

TERAHERTZ TIME-DOMAIN SPECTROSCOPY

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

RESULTS

TERAHERTZ SPECTROSCOPY

Frequency-dependent spectra

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

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

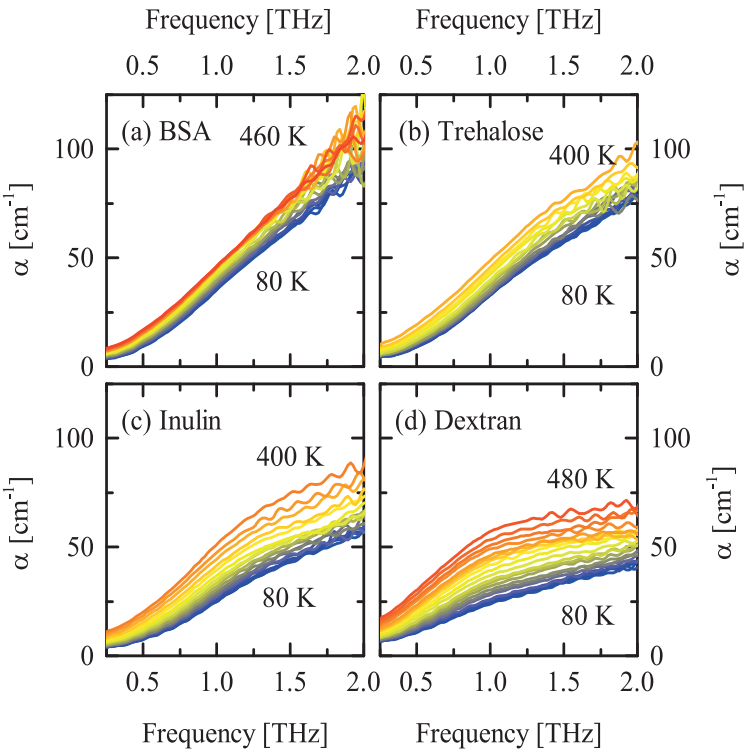

Figure 6.1 Terahertz absorption coefficient (α) of amorphous (a) BSA, (b) trehalose, (c) inulin 1.8 kDa and (d) dextran 70 kDa over the temperature range 80 K – 480 K. Data significantly above Tg are not shown as the amorphous pellets became structurally unstable and collapsed.

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

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

Temperature-dependent spectra

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

DISCUSSION

PROTEIN-SUGAR INTERACTIONS

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

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

components have phase separated. This matches our previous findings, where we found a decreasing protein-sugar miscibility with increasing protein size.[18]
These THz-TDS data thus confirm that smaller sugars interact more with the protein than larger sugars do, which is in line with the hypothesis that smaller sugars are less affected by steric hindrance, and explains why they are better capable of maintaining protein functionality during storage.[4,5]

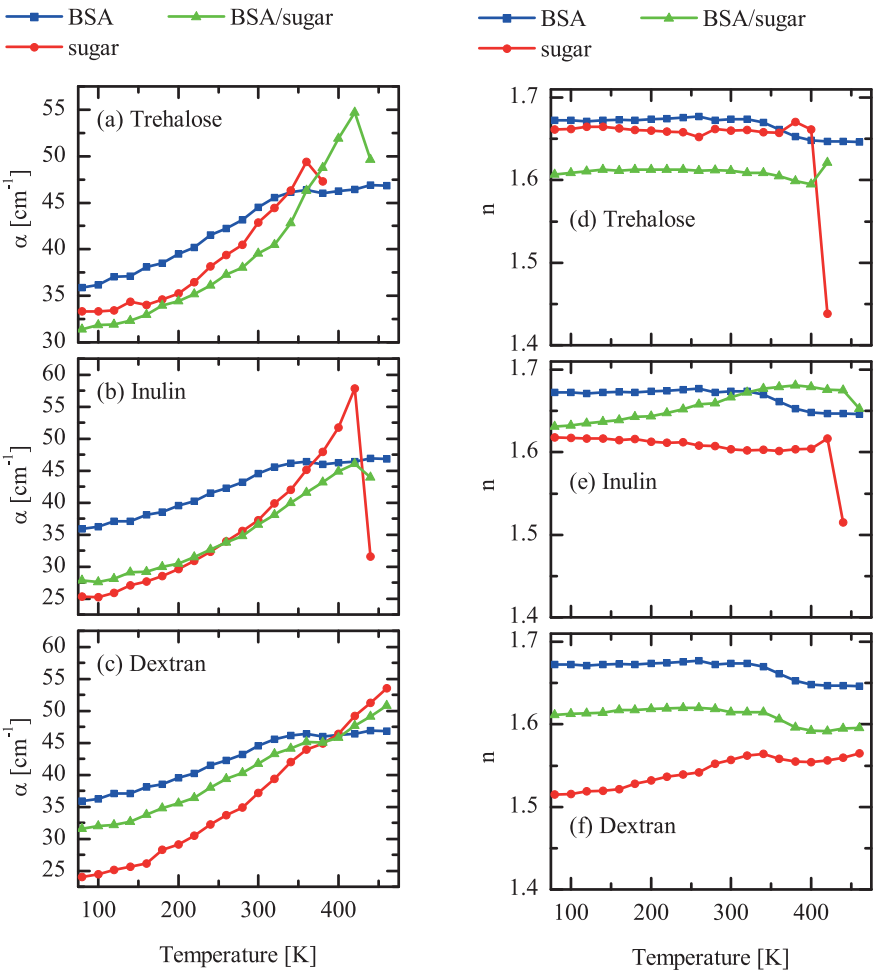

Figure 6.2 LEFT COLUMN: Absorption coefficient (α) at 1.0 THz for (a) trehalose, (b) inulin 1.8 kDa and (c) dextran 70 kDa, together with the absorption spectra of BSA and 1:4 mixture of BSA and respective sugar as a function of temperature. RIGHT COLUMN: Refractive index (n) at 1 THz for (d) trehalose,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

EFFECT OF MOLECULAR SIZE ON TEMPERATURE DEPENDENCE OF TERAHERTZ ABSORPTION

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

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

GLASS TRANSITION

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

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

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

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

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

CONCLUSION

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

ACKNOWLEDGMENTS

This research was jointly financed by Royal FrieslandCampina, the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the Municipality of Groningen, the Provinces of Groningen, Fryslân and Drenthe as well as the Dutch Carbohydrate Competence Center.

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