Stability of Protein-Sugar Lyophilisates Investigated with Terahertz Spectroscopy

J. Kölbel[1], M. L. Anuschek[1,2], I. Seifert[2], W. Frieß[2] and J. A. Zeitler[1]
[1]Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK
[2]Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universität, Munich, Germany

***Abstract*—Understanding of the molecular mobility in freeze-dried matrices is of high importance for the assessment and prediction of the stability of lyophilised protein and peptide drugs. Using terahertz spectroscopy it is possible to measure the molecular mobility in such lyophilisates. Here we analyse samples composed of different ratios of the excipient sucrose to monoclonal antibody (mAb). Upon increasing the relative amount of mAb in the mixture we observe evidence for vibrational confinement which clearly originates from the protein molecules and not the excipient matrix or residual water molecules in the system.**

I. INTRODUCTION

To predict the stability of amorphous pharmaceuticals, including lyophilised (freeze-dried) drugs, an understanding of the molecular mobility in the sample is beneficial. Low mobility in the solid matrix, at the long-term storage temperature, is desirable as it is thought to result in slower physical and chemical degradation. It has been suggested that in protein formulations the internal protein dynamics are coupled to the dynamics of the surrounding excipient matrix, and that this coupling can be exploited to decrease the protein molecular mobility. Understanding the molecular mechanisms behind the stabilising effect of the matrix is crucial for optimising formulations suited to prevent degradation, denaturation, and aggregation of the protein [1].

In simple small organic molecular systems terahertz time-domain spectroscopy (THz-TDS) is a useful tool to observe two transition temperatures, namely $T_{g,\beta}$ at low temperatures and $T_{g,\alpha}$ at higher temperature, corresponding to the onset of local and global mobility, respectively. Previously, high-temperature macromolecular confinement was detected with THz-TDS in more complex systems of lyophilised protein formulations containing a range of excipients [2]. Here we use highly reproducible THz-TDS experiments on a well-controlled and simple two-component system containing different ratios of sucrose to monoclonal antibody (mAb) to measure the molecular mobility in the samples and investigate the effect of the sucrose matrix.

II. EXPERIMENTAL

Samples containing different ratios of sucrose to mAb were prepared using a standardised lyophilisation procedure at LMU Munich. Immediately before each measurement, the dry powder was pressed into a pellet under nitrogen atmosphere to avoid uptake of ambient water. The sample was analysed using a TeraPulse 4000 spectrometer (TeraView Ltd., Cambridge, UK) with a liquid nitrogen continuous flow cryostat attached. Terahertz spectra were acquired over a range of temperatures (80 to 420 K). Complementary thermal characterisation using differential scanning calorimetry (DSC) was carried out.

III. RESULTS AND DISCUSSION

The value of $T_{g,\alpha}$ measured for pure sucrose was found to be in good agreement with literature data [3]. In Fig. 1, the absorption coefficient of lyophilised pure sucrose is shown for different temperatures. For all samples containing < 50 % mAb three relaxation regimes, separated by $T_{g,\beta}$ and $T_{g,\alpha}$, were observed. The changes in absorption coefficient with temperature in these samples qualitatively resemble those observed in pure sucrose, i.e. the absorption coefficient increases strictly with temperature and $T_{g,\beta}$ and $T_{g,\alpha}$ indicate where the molecular mobility in the system changes. Crystallisation of sucrose occurs at 380 K (T_c), apparent in the spectrum.

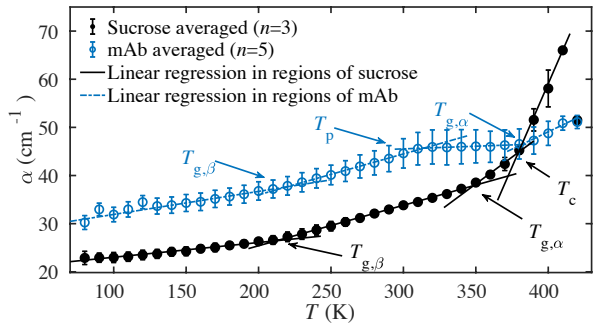

Fig. 1. Absorption coefficient of pure sucrose (black) and mAb (blue) measured at 1 THz. The average of n measurements is shown and each error bar indicates their standard error. Lines are drawn to highlight different absorption regimes. Arrows point to glass transitions of sucrose and mAb, respectively.

Upon adding mAb to the sucrose matrix we observe an increase of $T_{g,\alpha}$ and crystallisation temperature of sucrose for concentrations up to 40 % mAb as shown in Fig. 2. The DSC measurements result in lower values of $T_{g,\alpha}$ compared to THz-TDS for mAb concentrations between 2.8 % ≤ c[mAb] < 66%.

The potential energy surface (PES) model links energy barriers that surround local energy minima on the PES to glass transition temperatures that are high enough to leave those local minima through thermal activation of the molecule. For certain motions to take place, a correspondent energy barrier must be overcome first. It has been proposed that not only the depth of those minima, but also the rate by which excess motions become available upon increasing the temperature account for protein stability [4]. If the slope of the PES is low, an increase in temperature will result in more motions becoming activated compared to the same temperature increase applied to a PES with very steep minima. Conversely, when investigating a system at different temperatures with THz- TDS, we can track how the absorption coefficient and hence the dipole moment change. It is therefore possible to link the amount of absorption

change with temperature (gradient of linear regressions) to the steepness of the PES. The higher the gradient, the shallower the PES. A steep gradient indicates that the molecular mobility can be modified quite readily by a small change in temperature. For the mAb samples we observe that the change in absorption is lower at intermediary concentrations with a minimum at c[mAb] ≈ 44% and hence propose that stability is increased at intermediate c[mAb].

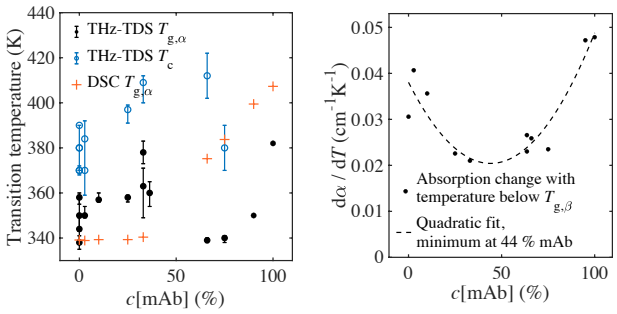

Fig. 2. Left: Transition temperatures above room temperature extracted from the absorption coefficient at1 THz (black and blue). Error bars are derived from the fitting algorithm. For comparison, the calorimetric $T_{g,\alpha}$ as measured with DSC is shown. Right: Change of absorption with temperature below $T_{g,\beta}$. A quadratic fit yields a minimum change at 44 % mAb.

The DSC measurements were in good agreement for pure sucrose as well as for c[mAb] = 75% but diverge at intermediary concentrations (Fig. 2). Above c[mAb] = 75%, the changes in heat flow as measured with DSC are very low, hindering an accurate determination of transition temperatures. Crystallisation is no longer observed with THz-TDS. It is important to note that the DSC method can only determine $T_{g,\alpha}$ but not the other transition points.

For c[mAb] > 66% the response appears to be dominated by the mAb as the absorption coefficient does no longer strictly increase with temperature. Further, Tg,α and $T_{g,\beta}$ differ considerably from those found in samples containing less mAb. As previously observed in complex formulations of bovine serum albumine (BSA) the absorption reaches a local maximum at 300 to 320 K in some samples [2]. This peak showed pseudo irreversible behaviour. In these samples, upon direct reheating the flattening of the gradient of the absorption coefficient with increasing temperature vanished and instead a continuous increase in absorption with temperature was observed. This is also consistent with [2].

However, the secondary drying during the lyophilisation process was carried out at higher temperatures (323 K). Given that we still observed this flattening of the absorption coefficient in the first heating run, the molecular mechanism behind this observation must be reversible on longer timescales. Water desorption as a potential origin of the peak could be excluded since i) at no point did the spectra indicate the presence or emergence of water vapour, which exhibits clear spectral features at terahertz frequencies; and, ii) so far this phenomenon has only been observed in samples containing proteins and not in e.g. pure sugar matrices or linear polymers.

These data provide further evidence that the protein molecules can be locked, or jammed, into a conformation in the solid state. During the initial heating process the increase in molecular mobility is hindered at a critical temperature due to the jamming until the temperature is high enough to overcome the associated potential energy barrier of the confinement and changes in dihedral angles become available again. This has also been hypothesised in [2]. Our new experiments clearly show that this process must be associated with the macromolecular structure of the protein itself and is not dependent on the presence of any excipient or the formation of a specific protein-excipient matrix. The experimental data furthermore highlight that this process is not dependent on the presence of water molecules. The thermal barrier for the mAb samples is similar to that previously measured for BSA (≈ 2.67 kJ mol−1) [2].

A transition below 300 K is detected by THz-TDS in all samples, implying that rotational and translational motions are present in the samples at and above room temperature. The confined state at 320 K is therefore likely to be related to intermolecular degrees of freedom. The most pronounced confinement was observed for the pure mAb sample for which no excipient matrix was present. This suggests that protein-protein interactions play a crucial role.

Ageing could result in more stable mAb conformations which would be in accordance with storage time as an influential factor for the confinement. High sucrose content might inhibit the confinement by suppressing conformational flexibility and protein-protein interactions. Without stability studies and conformational studies on the investigated formulations it is uncertain whether the confinement of the protein is increasing or decreasing its stability.

IV. Conclusions

Mobility changes and stabilisation by sucrose in freeze- dried sucrose-mAb mixtures were investigated. Below 40% mAb content, the sample behaviour is similar to that of small molecular systems. At about 50% mAb content, the absorption changes the least with temperature, potentially indicating increased stability, whereas above 60% mAb, the sample behaviour is dominated by the mAb dynamics. In some of those systems, the absorption plateaus or peaks at temperatures between 300 and 320 K which might be due to the protein being locked into a confined state with sucrose acting as void filler.

Acknowledgment

JK would like to thank the EPSRC Cambridge Centre for Doctoral Training in Sensor Technologies and Applications (EP/L015889/1) and AstraZeneca for funding. MLA would like to thank Erasmus+ for funding. All authors would like to thank the Cambridge-LMU Strategic Partnership for funding.

References

[1] M. T. Cicerone et al. “Stabilization of proteins in solid form.” Adv. Drug Del. Rev., vol. 93, pp. 14-24, 2015.
[2] T. Shmool et al., “Observation of high-temperature macromolecular confinement in lyophilised protein formulations using terahertz spectroscopy”, Int. J. of Pharmaceutics: X., vol. 1, 1:100022, 2019.
[3] K. D. Roe and T. P. Labuza “Glass Transition and Crystallization of Amorphous Trehalose-sucrose Mixtures”, Int. J. of Food Prop., vol. 8, pp. 559-574, 2005.
[4] M. Batens et al. ”Advancing predictions of protein stability in the solid state”, Phys. Chem. Chem. Phys., vol. 22, pp. 17247-17254, 2020.