View Article Online / Journal Homepage / Table of Contents for this issue
Soft Matter
Dynamic Article Links

Cite this: *Soft Matter*, 2012, **8**, 2983
www.rsc.org/softmatter
PAPER

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

β-Relaxation governs protein stability in sugar-glass matrices†

Marcus T. Cicerone* and Jack F. Douglas

Received 17th October 2011, Accepted 2nd January 2012
DOI: 10.1039/c2sm06979b

The stabilizing effect of sugar-glass matrix materials for freeze-drying proteins or nucleic acids has been variously ascribed to the thermodynamic effect of 'water replacement' by sugar molecules or to the kinetic effect of slowed α relaxation associated with sugar matrix vitrification. While evidence for each of these hypotheses exists, we show that neither can adequately account for the observed stabilization of proteins embedded in sugar-glasses. Instead, we find firm evidence that protein stability in these glasses is directly linked to high frequency β relaxation processes of the sugar matrix. Specifically, we observe that when the β relaxation time, τ_β, of sugar-glasses is increased with antiplasticizing additives, protein stability increases in linear proportion to the increase in τ_β, even though these same additives simultaneously decrease the glass transition temperature, T_g, and the α relaxation time, τ_α, of the sugar matrix materials. Moreover, we find that while sugars 'replace' water by stabilizing protein native-like conformation in the dry state, the resulting enhanced protein conformational stability does not have a significant impact on the degradation rate of the proteins in sugar-glasses. We discuss implications of these findings for the fundamental physics of glass formation and for effective engineering of protein stabilizing glasses through the modification of τ_β.‡

Introduction

Many plants and animals possess the remarkable ability to survive long periods of time in extremely dry environments by producing high intracellular concentrations of sugars in response to dehydration stress. Delicate biological structures and labile macromolecules are protected by these sugars, even under complete desiccation, creating a suspended state of biological activity in the dry state that can be recovered almost miraculously upon hydration. The role of sugars in protecting dried biological structures and molecules was first recognized by Crowe *et al.*[1] for lipid membranes, and later for proteins by Carpenter *et al.*,[2] who subsequently demonstrated that sugar-based glasses could be used to stabilize labile analytical proteins for biotechnological applications.

Starting from these pioneering initial studies, the practice of preserving biological agents in carbohydrate glasses has become widespread. Indeed, the ability to store proteins in a dry state and the recovery of protein function after rehydration is essential in many fields: analytical enzymes, foods, and biopharmaceuticals all rely on sugar-based glassy matrices to stabilize proteins. The practical importance of this technology can be appreciated by considering that approximately one third of all therapeutic proteins in the $80 B biopharmaceutical industry are stabilized in sugar-based glasses. (*i.e.*, formulated as freeze-dried products).[3]

Despite the importance of this protein preservation method and decades of practical application, the mechanisms by which sugar-based preservative matrices ('sugar-glasses') impart dehydration stability to proteins are simply not well understood. As a consequence, many protein-based drugs cannot even be stabilized sufficiently for clinical trials, in spite of best efforts.[4] It is well documented that proteins sequestered in sugar-glasses can degrade through chemical pathways such as oxidation, hydrolysis and deamidation, or physical changes that may lead to aggregation upon rehydration,[5] but it is not clear what factors most strongly influence the rates of these processes, or how to best control or predict them. A better understanding of the fundamental mechanisms underlying protein degradation in glassy sugar-glasses will allow better control over degradation rates, and will have tremendous economic and social value.

Proteins degrade in glassy matrices by essentially the same modes as they do in aqueous solution. The major chemical degradation mechanisms are known for proteins in aqueous solution,[6] and typically involve protein conformational mobility as well as transport of small-molecule reactants, such as oxygen or water. There are likely to be differences in the details of these degradation mechanisms in the glass, but the requirement of protein local mobility and reactant transport is undoubtedly retained. Similarly, protein aggregation is likely to require local protein mobility while in the glass. It is thought that proteins may

Polymers Division, Materials Measurement Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, MD, USA. E-mail: cicerone@nist.gov; Jack.Douglas@nist.gov; Tel: +1 301 975 6779; +1 301 975 8104

† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c2sm06979b/

‡ Official contribution of the National Institute of Standards and Technology; not subject to copyright in the United States.

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

undergo small conformational changes while in the glass that expose hydrophobic groups, generating an "aggregation competent" state, and that aggregation occurs subsequently when the protein-sugar glass matrix is re-dissolved in buffer and the protein has significant translational mobility.

Although degradation mechanisms may be similar in glass and in aqueous solution, the glass presents an environment that is much more complex. Unlike most liquids above their melting points, glassy systems contain local dynamical fluctuations that can persist out to macroscopic timescales, and this can significantly impact transport properties.[7] Also, sugar-glasses containing proteins are generally dried from aqueous solution and typically contain some residual water. It is well established that water-protein enthalpic interactions are generally stronger than sugar-protein interactions,[8] and this results in residual water being preferentially found at the protein-sugar interface.[9] Proteins themselves are not homogeneous, and some regions of the protein surface are more hydrophilic than others.[10] Accordingly, residual water will be associated with some regions of proteins preferentially over others.[11] This complex glassy environment has made it difficult to definitively identify primary variables in protein stability.

Prevailing hypotheses for protein stabilization

Two classes of hypotheses for how proteins are stabilized in sugar-glasses have been widely discussed; one class focuses on dynamics, and the other on thermodynamics. Both classes include specific hypotheses that focus either on the sugar's role or on the role of residual interfacial water.

The thermodynamic hypotheses explicitly consider mechanisms for retaining near-native protein conformation and implicitly assume a connection between protein conformation and stability against degradation - ostensibly through thermodynamic suppression of conformational fluctuations thought to be required in degradation processes. In the 'water replacement' hypothesis, the sugar-glass matrix is proposed to substitute for water and thermodynamically stabilize the native conformation of protein by providing appropriate hydrogen bonding and polar interactions at the surface of the protein.[12] As mentioned previously, residual water in the glass will preferentially reside at the protein-sugar and a 'water entrapment' hypothesis would suggest that this interfacial water provides some thermodynamic stabilization of the native protein conformation.[13] On the other hand, it is clear that the sugars contribute to additional stability of the native secondary structure.[14] There is much evidence for a correlation between near-native conformation in the glass and stability against degradation,[15-17] but this relation has not been quantifiable, and, as we will show below, does not extend over the full range of sugar-glass compositions.

The alternative class of hypotheses focus on dynamics. These assume that degradation is inevitable (*i.e.*, thermodynamically favored), and that the rates of these chemical and physical processes are simply slowed down by the sluggish structural relaxation of the matrix.[22] The 'vitrification hypothesis' focuses on the dynamics of the sugar-glass itself; usually in terms of the α relaxation time, τ_α, where a larger τ_α is usually associated with slower degradation.[23-25] Tests of this hypothesis often indicate a rough correlation, but the dependence of degradation rate on τ_α is found to vary widely, even within a given study,[23,25] suggesting that some other significant factor is in play. Furthermore, other studies have specifically indicated no correlation between τ_α and protein stability.[26-28] An additional problem with the vitrification hypothesis is that it implies that the degradation rate should scale linearly with τ_α, leading to the conclusion that degradation should occur in the glass on unrealistically long timescales. In particular, since τ_α near T_g is often about 10 orders of magnitude slower than that its value in aqueous solution where degradation normally occurs over periods of days or weeks, protein degradation in a sugar-glass matrix should occur on geological timescales under this hypothesis. Since this is clearly not the case, the vitrification hypothesis of protein preservation in its naïve form certainly requires revision.

The other prominent dynamics-based hypothesis, 'water anchoring', suggests that dynamics of the protein are coupled to dynamics of the sugar matrix *via* a water bridge that is hydrogen bonded to both. A number of experimental studies and simulation studies[18-20] have shown convincingly that interfacial water can play an important role in coupling dynamics of the glassy host with the protein. These studies have also shown that interfacial water anchors more effectively to trehalose glasses than to sucrose glasses,[18] and it appears that this is because trehalose is more likely than sucrose to form intermolecular rather than intramolecular hydrogen bonds.[21]

While it is fairly clear that each of the mechanisms in the four hypotheses mentioned above actually exist, it is not clear which is relevant to the issue of chemical and physical protein stability in sugar-glass. It is important to know which, if any, of these mechanisms are relevant since they suggest rather different approaches for engineering sugar-glass formulations to optimally suppress protein degradation. The water replacement and vitrification hypotheses have been considered and tested extensively in the context of protein stabilization, but neither has yielded a universal or quantitative metric for predicting protein stability in sugar-glass matrices. The lack of a reliable predictive model suggests to us that we should be looking elsewhere for the primary factors in protein stabilization.

Recent findings

We and others[29-33] have reported that high-frequency "β relaxation" processes in sugar-glasses correlate strongly with protein or other drug stability; *i.e.*, with rates of physical and chemical degradation of processes in the glass. From this we infer that these motions couple to the conformational flexibility of proteins and to small-molecule transport in these glasses. A large body of literature supports this inference. In general, glasses exhibit at least two distinct high-frequency relaxations, or β processes, the so-called "fast β" (β_{fast}) and the slow, or Johari-Goldstein β (β_{JG}) process.[34] The β_{fast} relaxation is associated with super-diffusive exploration of the molecular cage of neighbors, and occurs on a picosecond timescale,[35] while the β_{JG} relaxation is associated with small amplitude intermolecular motions and typically occurs on timescale of microseconds to milliseconds in sugar-glass.[36,37] Small amplitude protein motions appear to be "slaved" to the β_{fast} dynamics of the fluid matrix.[38-40] Small molecule transport appears to be coupled to both β_{fast} and β_{JG}; the former through a connection between free volume and the amplitude of

 This journal is © The Royal Society of Chemistry 2012

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

fast β relaxation[41–43] and the latter through small-scale matrix motions such as glucose ring flips.[44] Furthermore, it has long been held that high frequency motions in general can gate reactive and diffusive processes in highly viscous media.[45-46] The β_{fast} and β_{JG} relaxation seem to be linked, at least qualitatively[47] through similar response to antiplasticizing additives, and both have been associated with α relaxation through theory and phenomenological correlation.[48–53]

In the present work, we first critically examine the currently held dynamic and thermodynamic hypotheses, and show that, as commonly conceived, none can account for the data. We then provide evidence that protein degradation after freeze-drying is instead directly linked to β-relaxation of the glass as a whole. In particular, we show that stability trends are largely or entirely accounted for by trends in β-relaxation of the entire glassy system. We suggest a significant modification of the vitrification hypothesis where β-relaxations, rather than α-relaxation, are the gating motions governing the degradation of proteins in sugar glasses. We further suggest that the relationship of degradation rates to both α and β relaxation of the glass can be understood in terms of transport-limited reactions. In doing so we arrive at a firm physical foundation for engineering the stabilization of proteins and potentially many other biological materials.

Evidence for the central role of β relaxation

Below we consider the central role of fast relaxation processes in relation to protein stability in the glass. We first demonstrate a strong correlation between β_{JG} relaxation (μs to ms timescales) and degradation rates of model analytical proteins. Using data from the same formulations we show that α relaxation does not correlate at all with protein stability, even thought α relaxation and protein degradation occurs on comparable timescales. We then consider β_{fast} relaxation (ns timescale) in biopharmaceutical formulations, showing that it too correlates well with protein stability. Using the β_{fast} results we also show that the thermodynamic stabilization hypotheses are untenable as currently conceived. Evidently high frequency dynamics associated with very small molecular displacements of the matrix is the predominate factor in determining protein stability in glasses. The correlation between β relaxation and protein degradation is truly remarkable given that these processes can be separated by as much as 17 orders of magnitude in time.

β_{JG} relaxation

As mentioned above, there is reason to suspect that fast processes, β-relaxation rather than α-relaxation, may control protein stability in the glass. Here we use the antiplasticization phenomenon to independently control α and β relaxation so that we may resolve their relative roles in the stability of freeze-dried proteins. Antiplasticizers are molecular[47,54–56] or nanoparticulate additives[57,58] that speed up α relaxation (lower T_g) in the glass, while simultaneously *slowing down* the higher frequency β relaxation process. This is in contrast to ordinary plasticization where both α and β relaxation are accelerated with the addition of a diluent.

Fig. 1a provides an illustration of the antiplasticization effect, as exhibited by the behavior of the β_{JG} relaxation for a series of melt-quenched trehalose-glasses with varying amounts of glycerol.[36,47,54] We see that as glycerol is added to trehalose, the activation energy for the β_{JG} relaxation process, $E_{a,\beta}$, increases and the logarithm of the Arrhenius prefactor, $\log(a_{\infty,\beta})$, decreases proportionally in such a way that there is a crossing temperature, the antiplasticization temperature T_a, below which the β_{JG} relaxation time slows down with the additive, and above which it speeds up. This proportionality also leads to a linear relationship between $E_{a,\beta}$ and $\log(\tau_\beta)$ at all temperatures investigated, as is shown explicitly for glycerol mass fractions up to 0.2 at 23 °C in the inset to Fig. 1a. At high glycerol mass fractions (>0.2), the proportionality relation between $E_{a,\beta}$ and $\log(a_{\infty,\beta})$ breaks down, and τ_β decreases at all temperatures with further addition of glycerol. We have recently shown that this antiplasticization behavior can be understood based on simple analyticity arguments related to the activation free energy governing the β_{JG} relaxation process.[47] The effect, often termed "entropy-enthalpy compensation," arises when the activation entropy and enthalpy terms have opposite signs and the leading concentration term in a Taylor expansion of the activation free energy dominates so that the activation energy and entropy are proportional and extensive in the amount of diluent added to the glass.[47] Because the leading term in a Taylor series expansion is by definition linear in the perturbation (such as the addition of an antiplasticizer), entropy-enthalpy compensation is fulfilled over a sufficiently small range for almost any variable that significantly affects the reaction rate. Of note here is that, for the dielectric data of Fig. 1a (and protein stability data introduced below) this linear response persists over a 20% change in the formulation concentration and holds for over six orders of magnitude change in dynamic response! A model by Douglas *et al.*[59] predicts entropy-enthalpy compensation effects over a similarly large concentration range for associating proteins, and Freed[60] has recently shown that the same mathematical origin as described above can account for entropy-enthalpy compensation with concentration of "inert" additives in model reaction processes.

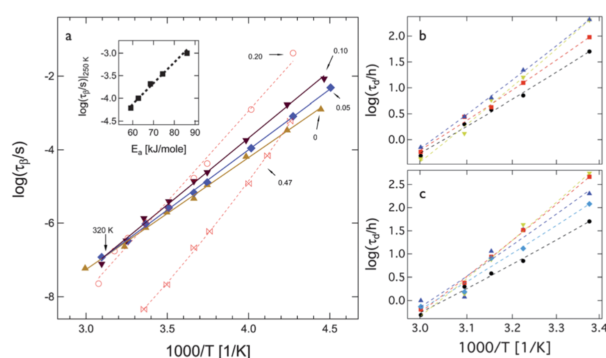

Fig. 1 Antiplasticization effects in sugar/diluent glasses. a: Dielectric relaxation times for melt-quenched trehalose glasses containing varying amounts of glycerol, indicated as mass fraction. Data taken from ref. 54. Relaxation times at 320 K are independent of glycerol content for glycerol mass fraction $\lesssim 0.2$ Inset: Linear dependence of $\log(\tau_\beta)$ on activation energy E_a for glycerol mass fraction $\lesssim 0.2$. b and c: Arrhenius plots of HRP degradation rates in antiplasticized maltitol glasses. b: maltitol with the following mass fractions of glycerol –0 (●), 0.05(▼), 0.1 (▲), 0.2 (■). c: maltitol with the following mass fractions of dimethyl sulfoxide –0 (●), 0.05(▲), 0.1 (▼), 0.15 (■), 0.2 (◆).

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

Taking advantage of entropy-enthalpy compensation for τ_β, we differentially vary α and β_{JG} relaxation rates in a series of antiplasticized glasses in order to clarify whether either of these dynamical properties has a dominant impact on protein stability. Panels b and c of Fig. 1 show degradation times (τ_d) of a model protein, horseradish peroxidase (HRP) in two such series of antiplasticized glasses. Values of τ_d were determined by measuring reconstituted HRP enzymatic activity after holding the freeze-dried protein-sugar samples under constant temperature for varying times. (See Supplementary Material for details.) Error bars represent intervals of one standard deviation derived from at least three replicate determinations of τ_d at each temperature. The behavior of the τ_d values precisely mimics that of the β relaxation seen in Fig. 1a and its inset: τ_d behaves in an Arrhenius manner, having E_a values that are tuned by varying the glycerol or dimethyl sulfoxide (DMSO) concentration. Also, the apparent antiplasticization temperature T_a for each of these plots is similar to the 320 K value observed in Fig. 1a, a common finding in systems exhibiting an entropy-enthalpy compensation relationship.[47,54,56,61–63] The trends are furthermore non-monotonic with antiplasticizer concentration, having a reversal in the sign of the effect on τ_d somewhere in the range of a (0.05 to 0.1) mass fraction DMSO or glycerol, which is a common pattern in entropy-enthalpy compensation with antiplasticizing additives. This precise coincidence of behavior between τ_d and β relaxation clearly shows that changing the β relaxation time of the glass significantly impacts HRP stability. We have gone on to measure the enzyme stability in more than 100 such antiplasticized glasses and find this same behavior in every case. The collected data are plotted in Fig. 2.

Fig. 2 shows log(τ_d) for HRP and equine alcohol dehydrogenase (ADH) at room temperature (T = 23 °C) for 22 antiplasticized sugar glass systems, comprising >100 glass formulations. (See Supplemental Material for full specification of these glasses.) The lower abscissa is the apparent activation energy, E_a, for the temperature dependence of τ_d in the various glasses. The solid lines are best fits to the data. The linear relationship here between log(τ_d) and E_a is a clear sign that β_{JG} relaxation plays an important role in the protein degradation processes for all of these sugar-glasses.

In addition to being linear, the relationship between log(τ_d) and E_a has the same slope as that for log(τ_β) and E_a in the inset to Fig. 1a. We demonstrate this by using the linear relationship shown in inset to Fig. 1a to obtain log(τ_β) values for a process with E_a values from the bottom axis of Fig. 2, and plot those values on the top axis of Fig. 2. Obtained in this way, the slope of the fits to log(τ_d) against log(τ_β) is 1 for both ADH and HRP. Thus, we find the degradation rates scale linearly with τ_β in all of these glasses, even though the timescales of τ_β and τ_d are separated by more than 12 decades in time. This strengthens our conclusion that β relaxation of the sugar matrix is a predominant kinetic parameter governing protein degradation in these systems.

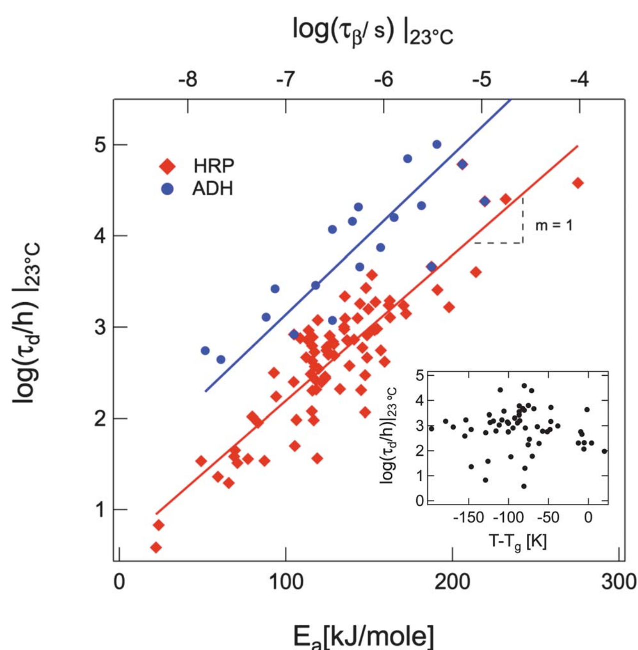

Fig. 2 Enzyme degradation rates at 23 °C in >100 plasticized and antiplasticized sugar-glasses. Top abscissa values are derived from activation energies for degradation and the relationship shown in the inset to Fig. 1. The solid lines are best fits to the data and yield a slope near 1 for log(τ_d) *vs.* log(τ_β) Inset: $T–T_g$ for most of the glasses shown in the main figure, plotted against enzyme degradation rate. No correlation is found between the abscissa value, a surrogate for τ_α, and enzyme stability.

α relaxation

We contrast this strong correlation between protein stability and β_{JG} relaxation with a consideration of the relationship between protein stability and α relaxation. The inset to Fig. 2 shows τ_d values of HRP plotted against values of ($T–T_g$) for the systems shown in the main figure. Here ($T–T_g$) values are expected to track with τ_α; a larger negative value of ($T–T_g$) generally indicates a slower α-relaxation. While the precise relationship between ($T–T_g$) and log(τ_α) depends on the relative heat capacity difference between the glass and supercooled liquid,[64] which varies somewhat from glass to glass, a strong correlation between ($T–T_g$) and log(τ_α) is expected for these glasses. In contrast to this expectation, there is no evident correlation between τ_d and ($T–T_g$) in the measurements. The lack of any discernable trend in the inset to Fig. 2 indicates that α relaxation does not have a direct and significant influence on protein stability, at least for formulations deep in the glassy regime. This observation is consistent with previous reports[26–28] and with the data of Fig. 1b and c where the protein stability in the glass state increases as we add DMSO or glycerol to maltitol, even though τ_α is reduced.

On one hand one might expect that the slow α relaxation dynamics would gate protein degradation processes in the glass since the two processes occur on approximately the same time scale. On the other hand, α relaxation is related to center-of-mass molecular displacements and large-scale protein conformational fluctuations.[40] It is not obvious that displacements on such a scale are really relevant to the problem at hand. These types of motions may be coupled to α relaxation in the glass, but would occur on vastly longer timescales, requiring many cumulative elemental α relaxation events.

β_{fast} relaxation

We now turn to β relaxation on very short timescales. We have used incoherent inelastic neutron scattering[65] intensity to obtain

 This journal is © The Royal Society of Chemistry 2012

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

the mean-squared displacement ($\langle u^2 \rangle$) of hydrogen atoms[66] in sugar-glasses on a nanosecond timescale. The value of $\langle u^2 \rangle$ reflects the competition between thermally induced particle motion and elastic effects associated with interparticle interactions that impede motion. We have previously shown that $\langle u^2 \rangle^{-1}$, as measured by neutron scattering, strongly correlates with protein stability in sugar glasses.[33,67] In those studies, as in the present work, $\langle u^2 \rangle$ measurements are made on a timescale slightly longer than that on which the β_{fast} relaxation occurs. However, in the glassy state there is very little relaxation after β_{fast} relaxation on the ps – ns timescale,[68] so $\langle u^2 \rangle$ measured at a nanosecond is a good measure of the amplitude of the β_{fast} relaxation. We emphasize that the values of $\langle u^2 \rangle$ we obtained in these studies were influenced both by the protein and the glassy host in which the protein was encapsulated.

Fig. 3a shows the relationship between $\langle u^2 \rangle$ and sugar mass fraction (Φ) in freeze-dried glasses for three globular proteins, an IgG (125 kD), lysozyme (14 kD), and a cytokine (19 kD). We observe that $\langle u^2 \rangle$ changes in an essentially linear manner with Φ for all these proteins. Under the assumption that this linear relationship between Φ and $\langle u^2 \rangle$ is general for globular proteins, we can estimate relative changes in the amplitude of β_{fast} relaxation when Φ is systematically changed in reported stability studies of pharmaceutically important proteins.

Fig. 3b shows protein degradation rates as a function of $\langle u^2 \rangle^{-1}$ for all published stability studies that we could find where Φ is systematically changed and stability of a globular protein is measured.[17,33,67,69,71] These data include both aggregation and chemical degradation rates of proteins in sucrose and trehalose-based sugar-glass formulations. Here we focus on the enhancement of protein stability upon the addition of sugar by plotting relative degradation rates of the proteins in sugar-glasses; the absolute protein degradation rates have been arbitrarily shifted on the log axis. For three of these proteins,[25,33,67] we have neutron backscattering data from which we obtain $\langle u^2 \rangle^{-1}$. For the remainder, we estimate $\langle u^2 \rangle^{-1}$ as a function of Φ and based on the data of Fig. 3a.

Here we observe a linear relationship between $\langle u^2 \rangle^{-1}$ and degradation rate for chemical as well as physical degradation processes of eleven proteins in sucrose and trehalose glasses at various temperatures. The linear relationship is remarkable given that measured $\langle u^2 \rangle^{-1}$ and the degradation rates are measured on timescales differing by 15 orders of magnitude.

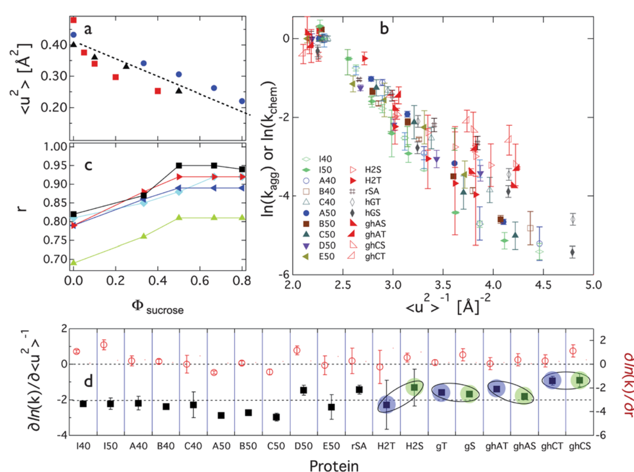

Fig. 3 Relative effects of fast β relaxation and protein conformation on stability. a: Amplitude of mean-squared displacements in protein/sucrose glasses for three proteins of MW = □ 19 kD cytokine, ▲lysozyme, ● 150 kD IgG. b: Aggregation and chemical degradation rates of proteins freeze-dried in sucrose or trehalose-based glasses plotted as a function of $\langle u^2 \rangle^{-1}$. Abscissa values are estimated from the relationship in panel a. c: Spectroscopic correlation coefficient for protein secondary structure as a function of sucrose mass fraction in freeze-dried glasses for proteins of MW = ◀185, ▲150, ◆125, ▶79, and □ 19 kD, data from ref. 22. d: Relative impact of protein conformation and of $\langle u^2 \rangle$ on protein stability. Left ordinate – (□) values of $\partial \ln k/\partial \langle u^2 \rangle^{-1}$ obtained from $\langle u^2 \rangle^{-1} > 3.0$ ($\Phi > 0.5$) of panel c. Right ordinate – (□) values of $\partial \ln k/\partial r$ obtained from $\langle u^2 \rangle^{-1} < 3.0$ region of panel c, using $\partial \ln(k)/\partial \langle u^2 \rangle^{-1}$ values obtained from $\langle u^2 \rangle^{-1} > 3.0$. Abbreviations for protein identities in c and d: I40, I50 – IgG aggregation in sucrose[71] at 40 and 50 °C, respectively. A40, A50, B40, B50, C40, C50, D50, E50 – Aggregation of proteins A–E in sucrose[33] at (40 and 50) °C. H2S, H2T – Aggregation of her2 antibody in sucrose or trehalose respectively[17] at 40 °C. rSA – chemical degradation of serum albumin in sucrose[71] at 50 °C. gT40, gS40 – Aggregation of rhuMAB in sucrose or trehalose.[69] ghAT, ghCT – Aggregation or chemical degradation respectively, of hGH in trehalose.[25] ghAS, ghCS – Aggregation or chemical degradation respectively, of hGH in surose.[25]

Thermodynamic stabilization

We now turn to evaluation of the thermodynamic stabilization hypotheses. We discuss these primarily in terms of secondary rather than tertiary protein structure because the trends in secondary structure with sugar: protein mass ratio are well established, whereas there is very little, if any, analogous data on trends in tertiary structure. Furthermore, secondary structure is the context in which these ideas are applied practically; protein formulators will often use FTIR spectra of proteins in glass in an attempt to predict stability.

Regarding 'water replacement', it appears that sugars "replace" water to some extent as protein solutions are dried, stabilizing the protein secondary structure. Fig. 3c shows how a measure of protein secondary structure changes with Φ in freeze-dried glasses for five globular proteins, ranging in molecular mass from 19 to 180 kD.[33] The spectral correlation coefficient, r, is plotted on the ordinate. Here $r = \Sigma_i x_i y_i/(\Sigma_i x_i^2 y_i^2)^{1/2}$ is obtained through a comparison between IR spectra of a reference native protein in an aqueous phase to the protein in the glass where x_i and y_i are the second derivatives of spectral absorbance values of the reference and glass-sequestered protein in the amide I region (1600 to 1700) cm^{-1}. The trend observed here is observed quite generally; globular proteins become increasingly native-like with increasing sugar mass fraction (Φ) for $\Phi < 0.5$, but r plateaus at $\Phi \approx 0.5$, becoming independent of Φ for $\Phi > 0.5$.[33,70,71] The plateau in secondary structure appears to occur at Φ where there are just enough hydroxyl groups from the sugar to titrate polar and hydrogen-bonding groups on the protein surface.[15] The sugar to protein mass ratio needed to meet this criterion is typically in the vicinity of $\Phi = 0.5$, and changes only weakly with protein molecular mass (M), scaling as $M^{1/3}$. Previous work[70] has supported the view that the sugars stabilize the secondary structure of the protein by demonstrating that increased hydrogen bonding between the sugar and protein leads to increasingly native secondary protein structure in the glass. That

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

sugars stabilize the secondary structure is incontrovertible, but the significance of this effect for chemical and aggregation stability of the protein in the sugar glass is not clear. Given the correspondence of $\langle u^2 \rangle$ to degradation rate observed in Fig. 3b, we are in a position to address the question of whether a more native-like secondary structure leads intrinsically to an increased stability for proteins embedded in the glass.

The data in Fig. 3b, along with the existence of two distinct regions of secondary structure dependency on Φ (*i.e.*, regimes above and below $\Phi = 0.5$) allows us to determine whether secondary structure is *causally* related to protein stability in the glass. Assuming protein degradation is influenced only by β relaxation (specifically, $\langle u^2 \rangle$) and protein conformation, we can write

$$\frac{d \ln k}{d\langle u^2\rangle^{-1}} = \frac{\partial \ln k}{\partial\langle u^2\rangle^{-1}} + \frac{\partial \ln k}{\partial r}\frac{dr}{d\Phi}\frac{d\Phi}{d\langle u^2\rangle^{-1}} \quad (1)$$

From Fig. 3c, we know that $dr/d\Phi = 0$ for $\Phi > 0.5$, so any change in degradation rate should be entirely due to β-relaxation in this regime. Thus, the slope of *ln* k *vs.* $\langle u^2 \rangle^{-1}$ for $\Phi > 0.5$ should give us an accurate estimation of $\partial ln\ k/\partial\langle u^2 \rangle^{-1}$.

In Fig. 3d, we plot values of $\partial ln\ k/\partial\langle u^2 \rangle^{-1}$ for each of the protein systems of Fig. 3b. These values are determined from the region of Fig. 3b where $\langle u^2 \rangle^{-1} > 3.0$, for which also $\Phi > 0.5$, and thus where $dr/d\Phi = 0$. If we assume that the values of $\partial ln\ k/\partial\langle u^2 \rangle^{-1}$ obtained for $\Phi > 0.5$ hold also in the regime $\Phi < 0.5$, we can solve for $\partial ln\ k/\partial r$ *via* eqn (1), given that we have known, nonzero values of $dr/d\Phi$ for $\Phi < 0.5$. Values of $\partial ln\ k/\partial r$ obtained in this way are plotted for each protein as open circles in Fig. 3d, against the right ordinate. With a few exceptions, these values are equal to zero within measurement uncertainty. In other words, although there are significant changes in the secondary structure of the proteins, *these changes typically have no effect on stability* since values of $\partial ln\ k/\partial\langle u^2 \rangle^{-1}$ obtained at $\Phi > 0.5$ accounts entirely for the stability trends in Φ.

A few of the protein systems appear to present an exception to this general trend. One is represented by I40 and I50, an IgG in sucrose at 40 °C and 50 °C[60], which gives positive values of $\partial ln\ k/\partial r$. In Fig. 3b we see that all but the last data point for this system is consistent with a uniform linear relationship between *ln* k and $\langle u^2 \rangle^{-1}$ over all Φ, and therefore consistent with $\partial ln\ k/\partial r = 0$. Thus, the apparent positive values of $\partial ln\ k/\partial r$ may be an artefact of errors in a single data point for this system. Two of the other three systems (I40 & gS40) that show positive values of $\partial ln\ k/\partial r$ behave in this same way, but we cannot rule out the possibility that changes in conformation significantly impact stability in the glass for these three proteins. It is conceivable that some proteins will be more susceptible to degradation when they lose native secondary structure if reactive peptides, such as cysteines are exposed that would be otherwise buried in the native conformation.

While it is clear that sugars help to stabilize a native-like secondary structure, we conclude from the data of Fig. 3d that the state of the protein secondary structure in a freeze-dried glass has no general, significant impact on protein stability, and that the water replacement hypothesis in its present form is thus not able to account in an important way for the stabilization effect of the sugar matrix.

Why have protein secondary structure and α-relaxation in the glass persisted so long as working metrics for predicting protein stabilizing efficacy glassy formulations, even though neither is entirely reliable or quantitative? The answer is two-fold. Firstly, until now, there has been no hypothesis that better explains experimental results. Secondly, both the water replacement and the traditional vitrification pictures of protein stabilization have associated plausible physical models and supporting, albeit circumstantial, evidence. It is a highly attractive idea that chemical and aggregation stability should be enhanced for native-like proteins and, indeed, correlation is often observed between protein stability and protein conformation. It is a similarly attractive idea that vitrification, or slowing of α relaxation, should slow chemical reactions that underlie degradation processes in the glass. Degradation rates often correlate with α-relaxation times in *a given glass*, but the extent of this correlation is highly variable from glass to glass.[23,25]

In contrast to secondary protein structure and α relaxation, β relaxation quantitatively tracks degradation rates from aggregation or chemical degradation for all the protein/glass systems we have investigated. This relationship provides a robust metric for rapidly estimating protein stability and evaluating glassy formulations, a process which currently requires months of laborious testing. The drawbacks of this metric are that convenient measurement methods have not been available. High-frequency dielectric relaxation such as used to obtain data of Fig. 1 cannot be used reliably for freeze-dried powers due to presence of spurious signals[72] in systems of high surface area, and neutron scattering facilities are not generally accessible for routine formulation testing. To address this practical issue, we have recently shown that one can obtain information equivalent to neutron backscattering using a spectroscopic method involving fluorescent Stokes shifts.[70]

Potential mechanisms

Why do these degradation processes track β relaxation which occurs many orders of magnitude faster, and why do they track changes in both β_{JG} and β_{fast} relaxation? Timescale of β_{JG} relaxation and the amplitude of β_{fast} relaxation both respond in similar ways to changes local fluctuations accompanying changes in stiffness and packing of the material in the glass[29,47] brought about by antiplasticization.[56] It also appears that these local fluctuations associated with β relaxation either directly gate the degradation processes, or share a causal link with other factors that do govern protein degradation processes.

How could these local fluctuations gate protein degradation? One potential mechanism could operate through local protein conformations. Simmons *et al.*[73] have shown through simulation that antiplasticization in polymer materials leads to a decrease in $\langle u^2 \rangle$, corresponding to an increase in material stiffness. The local stiffness of the glass will directly impact local conformational flexibility of the protein,[40] and this connection is particularly important in the glass, since the elastic constant, or local stiffness, varies significantly on a spatial scale comparable to that of the protein, as illustrated in Fig. 4.

The background in Fig. 4 shows spatial fluctuations in the elastic constant of a glass formed from a coarse-grained polymer melt simulation exhibiting fragile glass formation.[56] A ribbon

 This journal is © The Royal Society of Chemistry 2012

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

model of RNAse-A has been superimposed on this background with its scale matched to that of the glass matrix. Although this image is only schematic, it qualitatively illustrates fluctuations in local elastic constants and associated mobility that have sufficient spatial extent to facilitate conformational changes in structural motifs of the protein, making it possible for these to occur in the glassy state, provided the *local* mobility in one of these domains is sufficiently high. The high local mobility domains dictate the overall protein conformational mobility in the glass. Stiffening of the glass upon antiplasticization suppresses the fastest of these high-mobility domains,[56] reducing both $<u^2>$ and the possibility of local conformational fluctuations that could lead to degradation by partial unfolding or by transiently exposing highly reactive, and otherwise buried moieties to reactive species diffusing in the host material.

Another potential mechanism could operate through diffusion rates of small reactive species in the host matrix. The amplitude of fast β relaxations measured by $<u^2>$ serve as a read-out for the nanoscopic pore-space, or "free volume,"[42] and facilitate diffusion of small molecules such as gasses.[43] Furthermore, β_{JG} relaxations, are known to facilitate diffusion of slightly larger species such as water in saccharide glasses.[44] Under conditions that prevail in these glasses[74] one expects reactions involving multiple species to be diffusion rate limited, so that k_d would be directly proportional to D_T, the diffusion coefficient of the relevant species. This prediction is made by both the Smoluchowski theory of diffusion-limited reactions[75] and the classical Kramers theory under diffusion-limited reaction conditions.[76]

These two mechanisms are not mutually exclusive, and either or both could be important, depending on the protein and the degradation modes. One expects that small molecule diffusion would be particularly important for chemical degradation pathways.

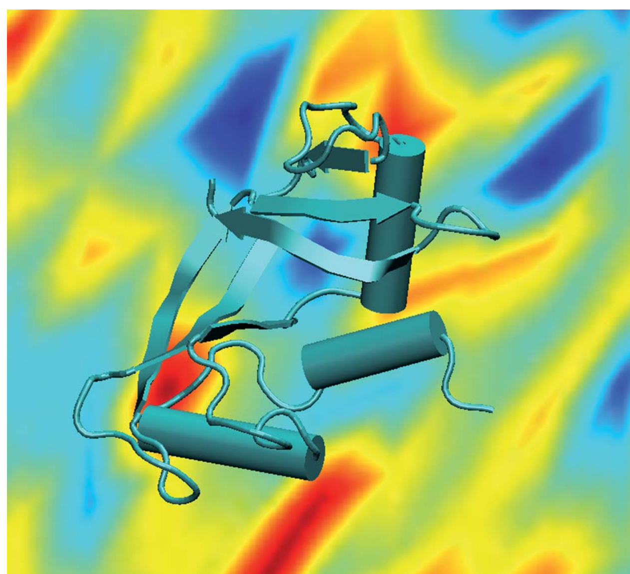

Fig. 4 Ribbon model of RNAse-A, drawn to scale against a background with a color map indicating the magnitude of the local shear elastic constant of the background fragile glass. Red green and blue indicate relatively high, moderate and low values of the shear modulus and thus amplitudes of the local atomic motions. The elastic constant fluctuations correspond to a model glass forming polymer melt,[56] but this phenomenon is expected to be general for glasses, including our sugar-based glass protein preservation matrices.

With the relevance of reactant diffusion rates in mind, we can now readily understand the highly variable behavior of the relationship between α relaxation and degradation rate. To appreciate this point, we briefly review what is known of the relationship between D_T and τ_α. In a dynamically homogeneous system one expects the Stokes–Einstein (SE) relation, $D_T \sim \tau_\alpha^{-1}$, to hold. On the other hand, spatial heterogeneity in dynamics, as observed in the glass, is sufficient to cause dramatic violation of the classical SE behaviour.[77,78] Here, rather than the classical behavior, a 'fractional Stokes–Einstein' relationship ($D_T \sim \tau_\alpha^{-\delta}$) is observed, where $0 < \delta < 1$.[79] This altered scaling relationship between D_T and τ_α has significant implications for rates of reaction in glass-forming and other complex liquids in that it implies a corresponding fractional power law relationship between the rate of reaction k_d and τ_α, *i.e.*, $k_d \sim \tau_\alpha^{-\delta}$. Such a relationship is commonly observed in water[80] and numerous other complex liquids[79,81-85] under conditions where dynamic heterogeneity is prevalent. In such systems, D_T can be enhanced by as much as five orders of magnitude at T_g[78] over the value expected in the classical Stokes–Einstein picture. The magnitude of this enhancement in D_T is strongly influenced by the properties of the most mobile domains in the glass, and thus the breadth of the distribution of dynamics, or, correspondingly, the fragility of the glass.[78,86,87]

The fact that D_T (and thus k_d) decouples from τ_α in glasses generally, and that this decoupling varies with glass composition provides rationale for results that were previously difficult to understand. Pikal *et al.*[25] observed a monotonic relationship between k_d and τ_α for chemical degradation and aggregation of hGH, although this relationship varied significantly between sucrose and trehalose formulations. As it turns out, their results are consistent with $k_d \sim \tau_\alpha^{-\delta}$ in sucrose with $\delta = 0.75$, and in trehalose with $\delta = 0.25$. Duddu *et al.*[23] obtained similar results for aggregation of an IgG in trehalose and sucrose. The monotonic change in k_d with τ_α have been interpreted as supporting the traditional vitrification hypothesis. However, until now the considerable variation in this relationship between formulations had not been explained.

Unlike temperature-dependent studies on a single formulation, no clear pattern emerges between k_d and τ_α when multiple formulations are compared at fixed temperature,[26-28] and these observations have been viewed as evidence against a relationship between τ_α and k_d. A clear example of variability in the relationship between k_d and τ_α is shown in the inset to Fig. 2; we see from this figure that among a number of formulations with $(T\text{-}T_g) = 75\ ^\circ C$ at room temperature, where one expects values of τ_α to be nominally equivalent, the variation in k_d can be on the order of 10^5. This variation is perfectly understandable for a fractional SE relationship between D_T and τ_α in which the coupling exponent δ varies over the observed range (0.25 to 1), depending on the particular sugar-glass matrix.

The mere existence of the decoupling also addresses a basic concern that we expressed above regarding the adequacy of the traditional vitrification hypothesis for describing the rate of protein degradation. The simple proportionality assumption $k_d \sim \tau_\alpha$ between the rate of protein degradation rate and the

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

structural relaxation underlying the vitrification hypothesis simply ignores SE violation and thus it is no surprise that this model can lead to wildly underestimated degradation rates upon extrapolating from the liquid state. A modification of the vitrification hypothesis based on an extrapolation of the fractional power relation $k_d \sim \tau_\alpha^{-\delta}$ might well lead to more reasonable estimates of protein degradation rates, and this possibility remains to be checked.

Conclusions

We have shown that stability against aggregation, chemical degradation, and enzymatic activity loss for proteins sequestered in a sugar-glass is directly related to relatively high frequency processes in the glass, both β_{JG} and β_{fast} relaxation. We observe a linear relationship between degradation rates and β relaxation that persists over at least five orders of magnitude in rate. Quantitative tracking of any variable with protein stability in sugar-glass over this dynamic range is unprecedented.

We have proposed that the high frequency β relaxation processes in the glass affect protein degradation through coupling of the β relaxations to local protein motions and to diffusion of small molecule reactive species in the glass. In connection with the latter, we show that consideration of a material-dependent fractional Stokes Einstein relation between α relaxation times and transport properties of reactive species can easily explain the enigmatic behavior observed when comparing α relaxation to protein degradation rates in the glass. The behavior now explained includes variations in $\log(\tau_\alpha)$ *vs.* $\log(k_d)$ from glass to glass, and differences in k_d covering five orders of magnitude between glasses at nominally the same value of τ_α.

We have used this correspondence in k_d and β relaxation to demonstrate that the water-replacement hypothesis of protein stabilization is not correct. Sugars may partially substitute for water in stabilizing the secondary structure of proteins in the dry glass, but protein secondary structure is not, in general, directly related to degradation rate of the protein in the glass.

In this work we have used dielectric spectroscopy to measure β_{JG} relaxation in melt quenched glasses, and neutron scattering to quantify changes in β_{fast} in freeze-dried sugar-glasses. We are currently developing a bench top method for measuring β_{fast} relaxation that will be amenable to freeze-dried glasses, and suggest that other properties connected with β relaxation, such as compressibility, density, or modulus may also provide measures indicative of protein stability. As a practical matter, we expect additives and processes that allow for a tuning of β relaxation will have a marked impact on protein stability in glass matrices, allowing for a more rational design of protein preservation formulations. Tuning the fragility of glass-formation with molecular and nanoparticle antiplasticizer additives has many other applications in the design of materials where specific values of hardness and toughness are required.[88,89] β relaxation measurements may provide a useful and convenient metrology for quantifying the effect of such additives on the elastic properties of these composite materials as well.

Acknowledgements

We thank David S. Simmons and Hans Frauenfelder for simulating discussion and useful comments. This work was funded in part under NIH/NIBIB Grant R01 EB006398-01A1.

Notes and references

1 L. M. Crowe, R. Mouradian, J. H. Crowe, S. A. Jackson and C. Womersley, *Biochim. Biophys. Acta, Biomembr.*, 1984, **769**, 141–50.
2 J. F. Carpenter, L. M. Crowe and J. H. Crowe, *Biochim. Biophys. Acta, Gen. Subj.*, 1987, **923**, 109–15.
3 R. Nayar, M. C. Manning, in *Rational design of stable protein formulations: theory and practice* (Plenum Pub Corp, 2002), pp. 177.
4 H. GLENN, *C&E. News.*, 2007, **85**, 8.
5 M. C. Lai and E. M. Topp, *J. Pharm. Sci.*, 1999, **88**, 489–500.
6 M. C. Manning, D. K. Chou, B. M. Murphy, R. W. Payne and D. S. Katayama, *Pharm. Res.*, 2010, **27**, 544–75.
7 M. T. Cicerone and M. D. Ediger, *J. Chem. Phys.*, 1996, **104**, 7210–7218.
8 T. Arakawa and S. N. Timasheff, *Biochemistry*, 1982, **21**, 6536–6544.
9 P. S. Belton and A. M. Gil, *Biopolymers*, 1994, **34**, 957–961.
10 J. A. Rupley and G. Careri, in *Protein Hydration and Function*, ed. M. M. R. J. T. E Frederic, D. S. Eisenberg, Academic Press, 1991, pp. 37–172.
11 M. V. Fedorov, J. M. Goodman, D. Nerukh and S. Schumm, *Phys. Chem. Chem. Phys.*, 2011, **13**, 2294–2299.
12 S. J. Prestrelski, N. Tedeschi, T. Arakawa and J. F. Carpenter, *Biophys. J.*, 1993, **65**, 661–71.
13 L. Cordone, G. Cottone and S. Giuffrida, *J. Phys.: Condens. Matter*, 2007, **19**, 205110.
14 S. D. Allison, B. Chang, T. W. Randolph and J. F. Carpenter, *Arch. Biochem. Biophys.*, 1999, **365**, 289–298.
15 K. Tanaka, T. Takeda and K. Miyajima, *Chem. Pharm. Bull.*, 1991, **39**, 1091–4.
16 H. R. Costantino, K. G. Carrasquillo, R. A. Cordero and M. Mumenthaler, *et al.*, *J. Pharm. Sci.*, 1998, **87**, 1412–20.
17 J. L. Cleland, X. Lam, B. Kendrick and J. Yang, *et al.*, *J. Pharm. Sci.*, 2001, **90**, 310–21.
18 F. Francia, M. Dezi, A. Mallardi and G. Palazzo, *J. Am. Chem. Soc.*, 2008, **130**, 10240–10246.
19 G. Cottone, G. Ciccotti and L. Cordone, *J. Chem. Phys.*, 2002, **117**, 9862.
20 A. M. Massari, I. J. Finkelstein, B. L. McClain and A. Goj, *J. Am. Chem. Soc.*, 2005, **127**, 14279–14289.
21 A. Lerbret, P. Bordat, F. Affouard, M. Descampes and F. Migliardo, *J. Phys. Chem. B*, 2005, **109**, 11046–11057.
22 F. Franks, R. H. M. Hatley and S. F. Mathias, *Biopharm. – Tech. & Bus. Biopharm.*, 1991, **4**, 38–55.
23 S. P. Duddu, G. Z. Zhang and P. R. DalMonte, *Pharm. Res.*, 1997, **14**, 596–600.
24 Y. S. Guo, S. R. Bryn and G. Zografi, *J. Pharm. Sci.*, 2000, **89**, 128–43.
25 M. J. Pikal, D. Rigsbee, M. L. Roy and D. Galreath, *et al.*, *J. Pharm. Sci.*, 2008, **97**, 5106–21.
26 S. Rossi, M. P. Buera, S. Moreno and J. Chirife, *Biotechnol. Prog.*, 1997, **13**, 609–16.
27 M. P. Buera, S. Rossi, S. Moreno and J. Chirife, *Biotechnol. Prog.*, 1999, **15**, 577–9.
28 P. Davidson and W. Q. Sun, *Pharm. Res.*, 2001, **18**, 474–9.
29 M. T. Cicerone and C. L. Soles, *Biophys. J.*, 2004, **86**, 3836–45.
30 R. E. Moznine, G. Smith, E. Polygalov, P. M. Suherman and J. Broadhead, *J. Phys. D: Appl. Phys.*, 2003, **36**, 330.
31 S. Yoshioka, T. Miyazaki and Y. Aso, *Pharm. Res.*, 2006, **23**, 961–6.
32 S. Yoshioka, T. Miyazaki, Y. Aso and T. Kawanishi, *Pharm. Res.*, 2007, **24**, 1660–7.
33 B. Q. Wang, S. Tchessalov, M. T. Cicerone, N. W. Warne and M. J. Pikal, *J. Pharm. Sci.*, 2009, **98**, 3145–66.
34 G. P. Johari and M. Goldstein, *J. Chem. Phys.*, 1971, **55**, 4245–52.
35 P. Allegrini, J. F. Douglas and S. C. Glotzer, *Phys. Rev. E.*, 1999, **60**, 5714–24.
36 J. Obrzut, A. Anopchenko, J. F. Douglas and B. W. Rust, *JNCS.*, 2010, **356**, 777–81.

 This journal is © The Royal Society of Chemistry 2012

View Article Online

Published on 01 February 2012. Downloaded by National Institutes of Standards & Technology on 11/06/2018 16:53:19.

37 K. Kaminski, E. Kaminska, K. L. Ngai and M. Paluch, *et al.*, *J. Phys. Chem. B*, 2009, **113**, 10088–96.
38 G. Caliskan, D. Mechtani, J. H. Roh and A. Kisliuk, *et al.*, *J. Chem. Phys.*, 2004, **121**, 1978–83.
39 T. E. Dirama, G. A. Carri and A. P. Sokolov, *J. Chem. Phys.*, 2005, **122**, 244910.
40 H. Frauenfelder, G. Chen, J. Berendzen and P. W. Fenimore, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 5129–34.
41 F. W. Starr, S. Sastry, J. F. Douglas and S. C. Glotzer, *Phys. Rev. Lett.*, 2002, 89.
42 K. L. Ngai, L. R. Bao, A. F. Yee and C. L. Soles, *Phys. Rev. Lett.*, 2001, 8721.
43 A. A. Gusev, S. Arizzi, U. W. Suter and D. J. Moll, *J. Chem. Phys.*, 1993, **99**, 2221.
44 V. Molinero and W. A. Goddard, *Phys. Rev. Lett.*, 2005, **95**, 045701.
45 R. F. Grote, G. Van der Zwan and J. T. Hynes, *J. Phys. Chem.*, 1984, **88**, 4676–84.
46 H. Sumi, *J. Phys. Chem.*, 1991, **95**, 3334–50.
47 T. Psurek, C. L. Soles, K. A. Page, M. T. Cicerone and J. F. Douglas, *J. Phys. Chem. B*, 2008, **112**, 15980–90.
48 R. W. Hall and P. G. Wolynes, *J. Chem. Phys.*, 1987, **86**, 2943–8.
49 J. C. Dyre, N. B. Olsen and T. Christensen, *Phys. Rev. B.*, 1996, **53**, 2171–4.
50 K. L. Ngai and S. Capaccioli, *J. Phys. Chem. B*, 2004, **108**, 11118–23.
51 K. Kessairi, S. Capaccioli, D. Prevosto and M. Lucchesi, *et al.*, *J. Phys.Chem. B*, 2008, **112**, 4470–3.
52 U. Buchenau and R. Zorn, *Europhys. Lett.*, 1992, **18**, 523–8.
53 L. Larini, A. Ottochian, C. De Michele and D. Leporini, *Nat. Phys.*, 2008, **4**, 42–5.
54 A. Anopchenko, T. Psurek, D. VanderHart, J. F. Douglas and J. Obrzut, *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.*, 2006, **74**, 031501.
55 E. B. Stukalin, J. F. Douglas and K. F. Freed, *J. Chem. Phys.*, 2010, **132**, 084504.
56 R. A. Riggleman, J. F. Douglas and J. J. de Pablo, *Soft Matter*, 2010, **6**, 292–304.
57 H. C. Wong, A. Sanz, J. F. Douglas and J. T. Cabral, *J. Mol. Liq.*, 2010, **153**, 79–87.
58 F. W. Starr and J. F. Douglas, *Phys. Rev. Lett.*, 2011, **106**, 115702.
59 J. F. Douglas, J. Dudowicz and K. F. Freed, *Phys. Rev. Lett.*, 2009, **103**, 135701.
60 K. F. Freed, *J. Phys. Chem. B*, 2011, **115**, 1689–92.
61 L. Liu and Q.-X. Guo, *Chem. Rev.*, 2001, **101**, 673–96.
62 R. A. Riggleman, K. Yoshimoto, J. F. Douglas and J. J. de Pablo, *Phys. Rev. Lett.*, 2006, **97**, 045502.
63 R. A. Riggleman, J. F. Douglas and J. J. de Pablo, *J. Chem. Phys.*, 2007, **126**, 234903.
64 S. L. Shamblin, X. Tang, L. Chang, B. C. Hancock and M. J. Pikal, *J. Phys. Chem. B*, 1999, **103**, 4113–21.
65 P. M. Gehring and D. A. Neumann, *Phys. B*, 1997, **241–243**, 64–70.
66 G. Zaccai, *Science*, 2000, **288**, 1604–7.
67 L. L. Chang, D. Shepherd, J. Sun and D. Ouellette, *et al.*, *J. Pharm. Sci.*, 2005, **94**, 1427–44.
68 M. T. Cicerone, Q. Zhong, J. Johnson, K. A. Aamer and M. Tyagi, *J. Phys. Chem. Lett.*, 2011, **2**, 1464–8.
69 S. U. Sane, R. Wong and C. C. Hsu, *J. Pharm. Sci.*, 2004, **93**, 1005–18.
70 S. D. Allison, B. Chang, T. W. Randolph and J. F. Carpenter, *Arch. Biochem. Biophys.*, 1999, **365**, 289–98.
71 L. L. Chang, D. Shepherd, J. Sun, X. C. Tang and M. J. Pikal, *J. Pharm. Sci.*, 2005, **94**, 1445–55.
72 R. W. Sillars, *Wireless Section, Institution of Electrical Engineers-Proceedings*, 1937, **12**, 139–55.
73 D. S. Simmons and J. F. Douglas, *Soft Matter*, 2011, **7**, 11010–11020.
74 S. M. Lievonen and Y. H. Roos, *J. Food Sci.*, 2002, **67**, 2100–6.
75 F. C. Collins and G. E. Kimball, *J. Colloid Sci.*, 1949, **4**, 425–37.
76 H. A. Kramers, *Physica*, 1940, **7**, 284–304.
77 J. F. Douglas and D. Leporini, *JNCS.*, 1998, **235**, 137–41.
78 G. L. Pollack, *Phys. Rev. A: At., Mol., Opt. Phys.*, 1981, **23**, 2660–3.
79 M. T. Cicerone, P. A. Wagner and M. D. Ediger, *J. Phys. Chem. B*, 1997, **101**, 8727–34.
80 L. M. Xu, F. Mallamace, Z. Y. Yan and F. W. Starr, *et al.*, *Nat. Phys.*, 2009, **5**, 565–9.
81 A. Voronel, E. Veliyulin, V. S. Machavariani, A. Kisliuk and D. Quitmann, *Phys. Rev. Lett.*, 1998, **80**, 2630–3.
82 G. Chirico, M. Placidi and S. Cannistraro, *J. Phys. Chem. B*, 1999, **103**, 1746–51.
83 S. H. Bielowka, T. Psurek, J. Ziolo and M. Paluch, *Phys. Rev. E.*, 2001, **63**, 062301.
84 S. R. Becker, P. H. Poole and F. W. Starr, *Phys. Rev. Lett.*, 2006, **97**, 55901.
85 F. Fernandez-Alonso, F. J. Bermejo, S. E. McLain and J. F. C. Turner, *et al.*, *Phys. Rev. Lett.*, 2007, **98**, 77801.
86 R. Zwanzig, *Chem. Phys. Lett.*, 1989, **164**, 639–642.
87 S. K. Kumar, G. Szamel and J. F. Douglas, *J. Chem. Phys.*, 2006, **124**, 214501.
88 D. P. Bentz, M. A. Peltz, K. A. Snyder and J. M. Davis, *Conc. Int.*, 2009, **31**, 31–6.
89 S. P. Delcambre, R. A. Riggleman, J. J. de Pablo and P. F. Nealey, *Soft Matter*, 2010, **6**, 2475–83.