Substantially Improved Stability of Biological Agents in Dried Form

The Role of Glassy Dynamics in Preservation of Biopharmaceuticals

Marcus T. Cicerone, Andrew Tellington, Landon Trost, and Alexei Sokolov

Recent advances in the field of biotechnology have led to products containing enzymes, antibodies, and many other biological agents suitable for use as pharmaceuticals, veterinarian preparations, and foods. Often, economic and ease-of-use considerations dictate that proteins be stabilized in a dry format. Well over a decade ago it became clear that carbohydrate glasses play a central role in anhydrous preservation of biological agents in nature (**1**, **2**). Currently, simple sugars and sugar alcohols are often used as the primary preservation medium for biological agents (**3–6**). Unfortunately, formulation of such dried preparations can be a very complex process. Consideration must be made for processing the aqueous formulation and for both the physical and biochemical stability of the final dried product.

Dried biopharmaceuticals are typically prepared in a glassy matrix by spray drying or lyophilization. When the glassformer is a simple sugar, physical properties of the dried products are often not robust. For example, even small amounts of residual moisture can cause the lyophilate to shrink and collapse or cause the spray-dried powder to fuse and cake up. In either case, the product does not redissolve readily. Polymer glasses have the advantage of yielding products with good physical characteristics, but when used alone, they seem to be less effective than small-molecule glassformers at preserving biological agents (**7–11**).

Formulations that combine small-molecule sugars with polymers (**12–15**) or amino acids (**16–19**) seem to yield products with both good physical characteristics and good biopreservation. Amino acids are used in freeze-drying for their propensity to crystallize and form a continuous crystalline network throughout the product, giving it structural support. The drawback to this approach is that proteins are not stabilized in the crystalline phase (**20**, **21**), and sometimes loss of product occurs in these formulations (**22**). Polymers also provide structural support to the product, but they do so by virtue of their ability to form physical networks, and crystallization is not necessarily involved. The advantage of product being nominally 100% amorphous must be weighed against the fact that such formulations often also have higher viscosity in the aqueous phase and may thus be more difficult to handle.

Table 1: A freeze-drying protocol

Temperature	Duration	Pressure
40 °C	≈ 1 h[a]	760 Torr
20 °C	≈ 6 h[b]	30 mTorr
8 °C	≈ 3 h	30 mTorr
25 °C	≈ 24 h[c]	30 mTorr

[a] Or until frozen
[b] Or until primary drying is done
[c] Final drying is complete before the 24-hour figure given here. We have not tried to determine the minimum time required for the final drying step.

Stability concerns. If liquid handling and physical properties of the dried product were the only concerns, formulation of biopharmaceuticals would be greatly simplified. Obviously, biochemical stability is also of utmost importance. As an added complication, the factors governing stability of biological structures or biomacromolecules in a dried format are only poorly understood. These factors may be broadly categorized as either related to dynamics or thermodynamics. Spectroscopic (**23**) and phenomenological (**24–26**)

PRODUCT FOCUS: PROTEINS, ANTIBODIES, POSSIBLY CELLS/VIRUSES

PROCESS FOCUS: FORMULATION, FILL AND FINISH

WHO SHOULD READ: MANUFACTURING, FORMULATIONS, QUALITY

KEYWORDS: STABILITY, DYNAMICS ANHYDROBIOSIS, LYOPHILIZATION, EXCIPIENTS, PLASTICIZATION

LEVEL: ADVANCED

evidence indicates that hydrogen bonding of the host glass with the guest species plays an important role in the stabilization of biological structures. Other components in the formulation, such as ionic species (**27–29**) and surfactants (**30**) also contribute to the thermodynamic stabilization of the protein in the glass.

The role of dynamics in anhydrobiosis is less well understood. The physical and chemical processes by which the biological agent degrades in the bioprotective glass may include reaction with small-molecule species, and partial denaturation (**31**). Such processes will be influenced by the dynamics of the host glass, and thus a clear understanding of the dynamics is central to understanding and manipulating the relative biochemical stability of the product.

Dynamics of bioprotective glass is often associated with the glass transition temperature (T_g), which is a measure of the structural relaxation (α process). Often the implicit assumption is that the higher the T_g, the slower the α process will be. It has been shown that in some cases high T_g is advantageous (**32–36**) to biopreservation, but other reports counter this (**37**, **38**). So no clear picture yet explains just how biopreservation is influenced by dynamics of the protective host.

There may be at least two reasons that bioprotective efficacy of a glass doesn't necessarily scale with the difference between the storage temperature and T_g. The first of these is that the α relaxation timescale of a glass is not a simple function of the storage temperature and T_g: Fragility and physical aging are both important factors (**39**), and thus α relaxation times should be measured directly if possible. The second potential reason for the lack of convergence in the literature is that glassformers in general exhibit dynamics over a broad range of timescales, and dynamics not associated with other than the α process will also have bearing on stability of biological agents stored in glass.

Figure 1 shows relaxation data for polybutadiene (**40**) obtained at temperatures near and above T_g. Polybutadiene is not used as a bioprotective glass, but the dynamic processes represented in this plot are more-or-less common to glassformers. In addition to the average timescale of the primary α relaxation process, there is a slightly faster secondary relaxation (sometimes denoted β process, γ process, or Johari-Goldstein process), which is typically brought about by small-amplitude local motions of the glass-former. At shorter times (or higher frequencies) still there is a fast (or fast β) process, which represents the timescale of intra- and intermolecular collisions. The timescale of this process is insensitive to temperature, but the amplitude is strongly temperature dependent. Even this figure does not fully describe the dynamics in glassy systems, because the α relaxation is typically not a single timescale phenomenon but occurs over a broad range of timescales, as much as three or four orders of magnitude in width.

Each motional process represented in the figure is likely to influence the preservation of biomolecules in hydrophilic glasses. The α process is linked most closely with viscosity and will strongly influence the rate of global motions leading to denaturation (**41**). The average value and width of the α relaxation will influence the diffusivity of reactive species that are intermediate in size (**42**). The β relaxation time will influence diffusion of small molecules such as gasses (**43**) and potentially small-amplitude protein motions that are precursors to denaturation.

Slowing any of these processes has potential to reduce the rate of biodegradation, as exemplified by the work of de Pablo et al. (**36**), where protein and bacterial preservation was improved by suppressing the α relaxation of disaccharides. In a similar spirit, it is possible to slow (antiplasticize) the β relaxations of a glass by adding a small amount of an appropriate plasticizer. Addition of such a species speeds up (plasticizes) the α relaxation, and this phenomenon is often referred to as plasticization/antiplasticization. This phenomenon has been observed in synthetic polymers (**44**), polysaccharides (**45**), sugars (**46**), and sugar alcohols. Although the presence of plasticizers is typically avoided in bioprotective glasses (**47**), judicious use of appropriate plasticizers can have a dramatic positive effect on stabilization of biological agents.

In the present study we modified disaccharide and polymeric

Figure 1: Many dynamic processes exist in glasses, covering a broad range of timescales. Polybutadiene serves here as an example. With the possible exception of the E-process, the various processes represented are more or less generic for glassformers. *Taken from reference 32 with permission.*

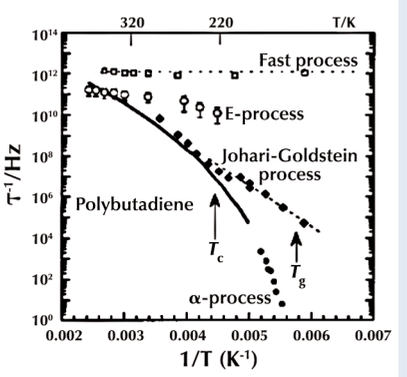


Figure 2: Enzyme degradation times in freeze-dried glasses, plotted in Arrhenius format; (□) ADH in trehalose, (○) HRP in trehalose, (△) HRP in a glass composed of dextran, inulin , and glycerol, mass ratio 80:10:10. Linear fits give R^2 values of 0.997 and .990 for the HRP data in trehalose and dextran glass, respectively, and 0.81 for the ADH data. The error bars represent standard uncertainties of ± 1 standard deviation.

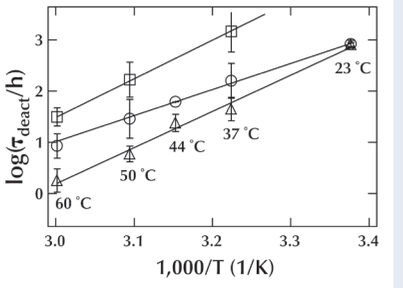
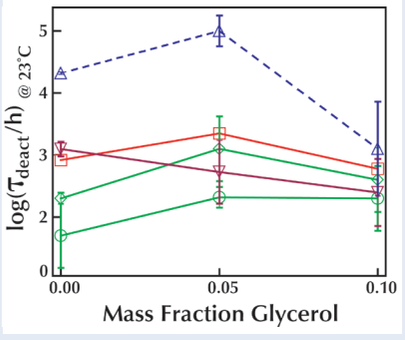


Figure 3: Enzyme deactivation times extrapolated to room temperature for bioprotective glasses plasticized with varying amounts of glycerol; (blue △) ADH in trehalose; (red □) HRP in trehalose; (purple ▽) HRP in raffinose; (green ◇) HRP in lactose, (green ○) HRP in maltitol. The lines between points are guides to the eye. The error bars represent standard uncertainties of ±1 standard deviation.



lyoprotective glasses by this plasticizing/antiplasticizing treatment, and in this way effected a substantial improvement in room-temperature storage lifetime of proteins lyophilized in sugar, sugar alcohol, and polymer glasses. We report protein stabilization in a plasticized polymeric glass that is improved by more than 1,000-fold over that in the unplasticized glass and that is approximately 100 times better than the stability we obtain in an unplasticized trehalose glass.

Experimental Information

Materials. All enzymes, reagents, glassforming materials, and plasticizers were obtained from Sigma (www.sigmaaldrich.com). All materials were used as received. The peroxidase (HRP) was type II from horseradish, and the alcohol dehydrogenase (ADH) was from bakers' yeast. The bovine serum albumin (BSA) was fraction V. Dextran of 70k and ficolls of 70k and 400k MW were used in these studies. These gave no discernible difference in results. Polyvinyl-pyrrolidone was K = 29–32.

Sample Preparation. All samples were made in aqueous solution in preparation for freeze-drying. Solutions contained 100 mM $CaCl_2$, 300 μg/mL Tween 20, 0.5% by mass BSA, and 60 nM enzyme for stabilization (HRP or ADH). All solutions contained a combined glassformer and plasticizer concentration of 20% by mass, except solutions containing polyvinylpyrrolidone(PVP), which were 13% by mass. Although lower excipient concentrations are often used in some freeze-drying applications, we chose a 20 wt% target based on the work of de Pablo et al. (**36**), who found this to give the most robust product. Salt and surfactant were first added to stock solutions of glassformer and plasticizer. After these were mixed well, the BSA and enzyme to be stabilized were added. HRP solutions were made up in 50 mM histadine buffer (pH 6.0), and ADH solutions were made up in 50 mM Tris buffer (pH 7.0). All solutions were made with milliQ water.

Each sample was divided into aliquots of approximately 150 μL previous to freeze-drying. The aliquots were dispensed into 1.7 mL microfuge tubes, then placed uncapped into the freeze-dryer for lyophilization.

Table 1 gives a typical freeze-drying protocol. After completion of the final drying step, the glassy samples are removed from the freeze-dryer and immediately capped to prevent excessive reabsorption of moisture from the ambient air. We determined that there was typically ⩽0.01 mass fraction residual water. Dried masses were consistent with plasticizer not being lost during the freeze-drying. This is noteworthy, because some plasticizers, such as DMSO, would in the pure state be completely lost to evaporation during the last stage of the freeze-drying protocol (24 h at 25 °C and 30 mTorr).

Enzymatic Assay. Assays of enzyme activity were carried out in 96-well plates on a Ceres UV 900 HDI plate reader (Bio-Tek Instruments Inc. www.biotek.com). Standard colorometric methods were used to assay enzymatic activity of both HRP (**48**) and ADH (**49**). Calibration curves were established with standard solutions of enzyme, obtained by serial dilutions of a known concentration of fresh enzyme. HRP concentrations for calibration curves ranged from 10 nM to 10 pM, and those for ADH calibration ranged from 25 nM to 2.5 pM. The nominal enzyme concentration of the rehydrated, stabilized-enzyme aliquots is 7.6 nM in all cases, giving us three orders of magnitude over which we could reliably measure enzyme activity. In all assays, initial rates of change of optical density are established by acquiring data for 7 min, with readings at 5–s intervals.

Evaluation of Formulations. The enzyme-stabilizing efficacy of a glass formulation was evaluated by measuring enzyme activity after separate sealed aliquots of the sequestered enzyme were placed at a controlled temperature for a series of time periods. Heat-stressed and frozen (control) aliquots were rehydrated in buffer and tested in adjacent rows on a 96-well plate for residual enzyme activity as described above. The characteristic time (τ_{deact}) for enzyme deactivation in a formulation was typically determined from one frozen control (unstressed) aliquot and three heat-stressed aliquots. Errors and data plots are estimated from multiple determinations of τ_{deact}.

We observed that enzyme activity (both HRP and ADH) showed an initial exponential decrease with time under heat-stress. We occasionally observe another, slower activity decay after extended heat stress. Similar bimodal activity decay has been seen before and has been linked to structural collapse of the freeze-dried cake (**50**); the values of τ_{deact} quoted here are all for the *faster* initial decay.

Figure 2 shows the deactivation time of HRP in several glasses at temperatures in the range wherein we performed our studies. The error bars represent intervals of 1 standard deviation derived from replicate determinations of τ_{deact} at each temperature. The main point we want to emphasize with respect to these data is the Arrhenius-like temperature dependence of the deactivation lifetimes over the measured temperature range. Such

Arrhenius-like behavior of τ_{deact} was observed for essentially all the enzyme-bearing glasses we evaluated. This was not surprising given that the measurements were made at temperatures well below the T_g of the protein-bearing glasses and over a relatively narrow temperature range. Under these conditions, Arrhenius-like behavior is expected (**51–53**).

The Arrhenius nature of τ_{deact} here can be exploited to predict the room-temperature enzyme stability by extrapolation from higher-temperature data (still within the glassy regime). We measured τ_{deact} directly at 23 °C for the two HRP-bearing glasses in Figure 2 (as well as several others). However, in most of what follows we plot enzyme activity lifetimes for HRP and ADH in several glasses, *extrapolated* to 23 °C, with error bars estimated from the extrapolation.

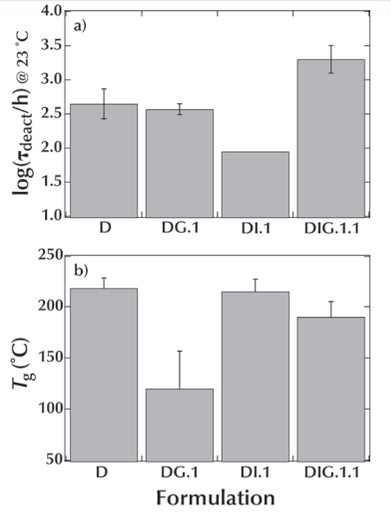
Figure 3 shows the estimated HRP and ADH deactivation times at 23 °C for several glasses plasticized with varying amounts of glycerol. The abscissa indicates the mass fraction of glycerol in the final product (the freeze-dried glass). The effect glycerol content has on τ_{deact} is nonmonotonic; the benefit of a small amount of plasticizer can be negated with too much of it. We observed this trend also for the other plasticizers we experimented with, namely dimethyl sulfoxide (DMSO), ethylene glycol, and propylene glycol. We also note that the stability increase in the glycerol-plasticized glasses seems to be quite robust, being neither protein-specific nor depending strongly on the choice of disaccharide glassformer.

The nonmonotonic relationship between added glycerol and enzyme stability is reminiscent of the suppressive effect of glycerol on the β relaxation seen in sugar glasses (**46**). We make this connection more solidly in a separate report (**54**).

Figure 3 shows that HRP in raffinose does not exhibit an increased stability on addition of glycerol. It may be that raffinose, a trisaccharide, responds to glycerol in a way similar to the polymeric glasses we studied (see below). The dynamics of small-molecule solutes will sometimes be essentially unconnected with that of the host glass (**55**). It seems clear to us that the dynamics of the glassformer and plasticizer must be coupled in some way for the antiplasticization of the fast dynamics that we wish to induce, although it is unclear whether the lack of effect we see with glycerol and raffinose is due to a complete decoupling of the dynamics.

Panel a) of Figure 4 demonstrates that a diluent alone may not improve enzyme stabilization when added to a polymeric host glass. In this case we see a 30% drop in τ_{deact} on the addition of 10–wt% glycerol to a dextran glass. On the other hand, although the addition of inulin alone also does not improve HRP stability in dextran, the addition of inulin along with glycerol produces a 4-fold increase in τ_{deact} over that of dextran alone.

Figure 4: HRP stability and plasticization of hosts. Panel a) displays HRP deactivation times, extrapolated to 23 °C for various glasses. The formulation names describe the glass composition as follows: D represents 70k dextran, I inulin, and G glycerol. The major component is listed first, and the fractional numbers following the letter designators refer to the mass fraction composed by the minor components, in order. The error bars represent standard uncertainties of ±1 standard deviation. Panel b) displays T_g of the glassy formulations listed on the abscissa. The error bars represent the width of the glass transition (see text).



Panel b) shows the onset T_g values of formulations listed in panel a) for a DSC scan rate of 10 °C/min. The error bars indicate the width ($T_{endpoint} - T_{onset}$) of the transition. The dramatic drop in T_g of dextran is accompanied by a significant broadening of the glass transition. Unplasticized dextran shows a glass transition with a width of 10 °C, whereas the width of the transition is 35 °C in the presence of 10–wt% glycerol. In a report by Lourdin et al., a broadening in T_g upon addition of glycerol to a starch-water system was ascribed to poor mixing of glycerol with dextran in the glass, and we suspect that this might be occurring here (**46**). Note that the addition of a small amount of inulin to the dextran/glycerol mixture changes this behavior dramatically: The T_g of the resulting glass jumps back to within 20 °C of the value for pure dextran, and the width of the transition falls back to 15 °C, although another transition also occurs near the T_g of pure inulin (not shown). It is with this tertiary mixture that we obtain an increase in τ_{deact}, shown in panel a).

Inulin is only poorly soluble in water (<4 wt%), but with an equal mass of glycerol, an aqueous solution containing upwards of 25–wt% inulin can be prepared. It appears that the inulin plays a role in linking the plasticizer to the host glass, either making components of the mixture compatible or coupling the dynamics of the glycerol to the host.

Figure 4 indicates that an intermediate or "linker" species will sometimes be useful for increasing stabilization of proteins in plasticized polymeric glasses by addition of plasticizer. As Figure 4 shows, polymeric species showing apparently strong interactions with the plasticizer seem to be effective in this regard. A small-molecule species that is miscible in the plasticizer and host glass and with a T_g that is intermediate between the plasticizer and host also serves effectively as a linker.

Figure 5 displays τ_{deact}, extrapolated to 23 °C, for HRP in

dextran glasses plasticized with one of several diluents, in the presence of a linker species. In each case the final product contained 10 wt% each of the linker and plasticizer in the dextran glass. In almost all cases we observed an increase in stabilization of HRP, although we are not sure why the combination of propylene glycol and maltitol with dextran performed so poorly. So, to a first approximation, it doesn't seem to matter too much which plasticizer is used, as long as it has a low T_g (56) and is not prone to crystallization, although it appears that some linker/plasticizer pairs may be less effective than others.

Figure 6 shows τ_{deact} for HRP, extrapolated to 23 °C in a series of polymer glasses, unplasticized and plasticized with 20 wt% of a 1:1 mass blend of inulin and glycerol or maltitol and glycerol. We see that enzyme stability was improved in all the polymer glasses upon plasticization, in the presence of a linker species. We note that the difference between τ_{deact} for unplasticized ficoll and the values for plasticized ficoll shown in Figure 6 is largely made up for by adding 10–wt % glycerol only to the polymer. However, adding the intermediate species still appears to confer increased stability to the enzyme.

Not shown in Figure 6 are results of HRP stabilization in polyvinyl alcohol glasses. The results were qualitatively similar to those shown for ficoll and PVP, but a significant loss of enzyme activity occurred upon freeze-drying (so that even the frozen controls had low activity). We did not therefore include these results.

Figure 7 shows τ_{deact} extrapolated to 23 °C for HRP in dextran glass, plasticized with a 1:1 mass blend of maltitol and glycerol. We note that τ_{deact} seems to be increasing with content of the plasticizer/linker blend, even at the point where this is the major component in the glass. This is particularly striking when compared with Figure 3 because the best

Figure 5: HRP deactivation times, extrapolated to 23 °C for dextran glasses plasticized with mixtures of either inulin or maltitol and the plasticizer listed in the abscissa. The error bars represent standard uncertainties of ± 1 standard deviation.

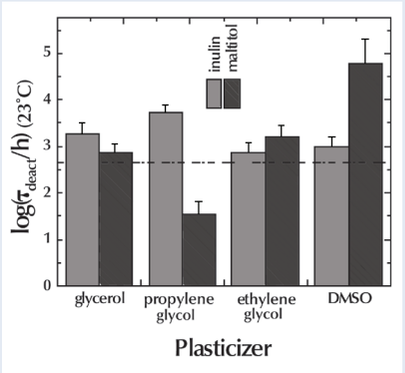
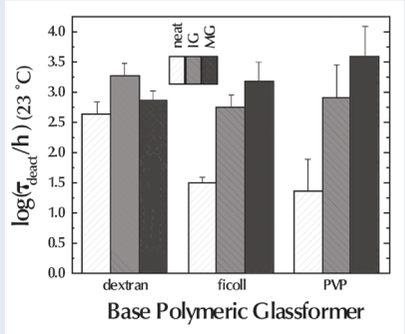


Figure 6: HRP deactivation times, extrapolated to 23 °C for polymeric glasses, unplasticized (neat), plasticized with 10 wt% each of inulin and glycerol (IG), or with 10 wt% each of maltitol and glycerol (MG). The polymers on which the glasses are based are indicated in the abscissa. The error bars represent standard uncertainties of ± 1 standard deviation.



stability we obtained in a maltitol/glycerol glass was when glycerol was only 10 wt% of the glass. In that case the best τ_{deact} we obtained was $10^{2.3}$ h, whereas in this case we obtain $\tau_{deact} = 10^{3.4}$ h.

We suggest that the significantly increased value of T_g (undoubtedly suppressed α relaxation) in the polymeric system as compared with the maltitol/glycerol system of Figure 3 accounts for the marked increase in τ_{deact} of the former. We also point out that T_g will decrease across the series displayed in Figure 7, so that while T_g drops, enzyme stability increases. We propose that suppression of the faster dynamics is responsible for this trend, and that the α relaxation in this system is probably sufficiently suppressed so that the current plasticization will not materially affect τ_{deact}. We are not aware of any dynamics measurements made on analogous tertiary systems to which we can compare these data.

Figure 8 shows the temperature dependence of τ_{deact} for HRP in several polymeric glasses. The polymers dextran, ficoll, and PVP are represented here, reinforcing the idea that the choice of a particular hydrophilic polymer may not be crucial or that there may be a range of polymers sufficient for a given protein. In each case the polymeric glass was plasticized with 10 wt% of maltitol and 10 wt% of DMSO or glycerol. Maltitol seems to serve as the best linker species that we have experience with; in addition to the polymeric intermediates discussed above, we have evaluated the effectiveness of lactose, trehalose, and sorbitol in this role. This apparent superiority of maltitol over other linkers holds for stability measurements of HRP and ADH and may be related to the unusually small "void volumes" detected in amorphous maltitol by positron annihilation spectroscopy (57).

The formulations represented in Figure 8 have not been optimized for their ability to stabilize HRP. They were "first guess" based on information we had at the time of the experiments. The results displayed in Figure 7 suggest that potentially significant improvement can be made to each of these formulations, because they correspond to a composition of 10 wt% each of the plasticizer and linker. Even without optimization, the improvement in HRP stability over that of the unplasticized polymeric glass is quite remarkable. Figure 6 shows estimated τ_{deact} values for HRP in both ficoll and PVP to be about $10^{1.5}$ h at 23 °C, whereas a similar extrapolation of the data in Figure 8 yields values on the order of $10^{4.5}$, a 1,000-fold improvement.

Discussion

In a separate publication we establish the relationship between increased protein stability and the

suppression of fast dynamics in the glass upon addition of plasticizer (**54**). The suppression of dynamics associated with the τ_{deact} process that occurs with addition of an appropriate plasticizer is well documented experimentally. It has been shown to be accompanied by several changes in physical and dynamic material properties, particularly reduction in small-molecule diffusivity (**43**). These changes in material properties of the bioprotective glass are expected to affect protein stability, because common degradation pathways include processes that rely on diffusion of small-molecule species, such as oxidation and deamidation.

General Formulation Considerations. We believe that the phenomenon we have documented is closely related to the plasticization/antiplasticization effect reported in the polymer and carbohydrate literature. Thus, formulation questions become questions about what combinations of materials will best effect an antiplasticization of fast dynamics in a bioprotective glass, while allowing the slow dynamics (α relaxation) to occur on as long a timescale as possible. To ensure the latter, one might add a cross-linking agent such as borate salts (**36**) or a gel-forming agent, or simply start with a glassformer that has an intrinsically high T_g, such as a polymeric system. Indeed, we observed that the plasticized polymeric glasses were more effective in protein stabilization than the plasticized small-molecule glasses we explored.

Finding the conditions under which an antiplasticization of the fast dynamics will occur is fairly straightforward, because the phenomenon is well documented, and one can take advantage of this literature. The concept of "free volume" is a framework commonly used for thinking about the plasticization/antiplasticization phenomenon (**58**). The general picture is that molecular mobility in amorphous materials is related to the amount of the free volume, which free volume comes about by the suboptimal efficiency of molecular packing in these disordered solids. Although no way yet exists to apply this concept in such a way that it is quantitatively in agreement with a broad range of observed phenomena, the concept may still be useful for thinking about general trends. Using the conceptual framework of free volume, we can rationalize many of the trends that are observed with the antiplasticization phenomenon, and hopefully derive some qualitative predictive power from the concept.

Within a conceptual free volume framework, the plasticizers fill small volumes left open by the larger (or stiffer) host glassformer, restricting motion and thereby slowing the fast dynamics. As more of the low T_g material is added, there is excess over what is needed to occupy the original free volume, and the plasticizer becomes a lubricant, speeding up the dynamics on all timescales. In literature reports wherein antiplasticization is observed, the optimal range of plasticizer is commonly between 5 wt% and 25 wt%, with only plasticization occurring at higher concentrations. In reconciling this statement with the data of Figure 7, we reiterate that we know of no dynamics measurements on systems that are analogous to those presented in that figure, and thus we believe it is possible that a higher plasticizer/linker concentration will effect an optimal antiplasticization in these glasses, as our data suggest.

The need for phase compatibility is another qualitative feature that can be rationalized by the free volume picture. For single diluent molecules to occupy the free volume associated with the host glass, they must interact intimately with the host molecule. Vilics et al. suggest that miscibility is the most important factor for inducing antiplasticization, as they observed significant diminution of the antiplasticization in cases of poor mixing (**56**). Their observations are consistent with our experience. Berquist et al. proposed that diluent molecules serve as lubricants to dynamics when they begin to pair up (**58**). Fortunately, poor miscibility could be detected by differential scanning calorimetry (DSC) (**46**).

Compatibility of the biological agent with the various components in the glass is also a very important issue. In a separate report, we showed that diluents that interact strongly with the protein of interest (e.g. serve as substrate analogs) seem to confer an added degree of stabilization to the protein (**54**).

The factor considered by Vilics et al. to be second in importance for inducing the plasticization/antiplasticization effect is the difference in T_g between the plasticizer and host — a low T_g material being a stronger antiplasticizer (**56**). Although the literature supports this second conclusion up to a point, it has been shown that a plasticizer with a T_g value too far below that of the host

Figure 7: HRP deactivation times, extrapolated to 23 °C for dextran glasses, plasticized with varying amounts of a 1:1 mass blend of maltitol and glycerol. The error bars represent standard uncertainties of ± 1 standard deviation.

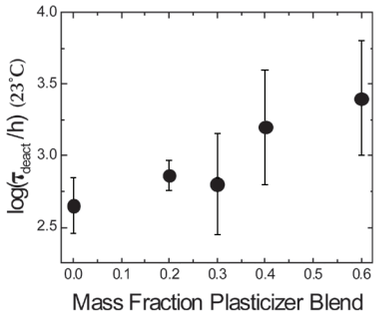
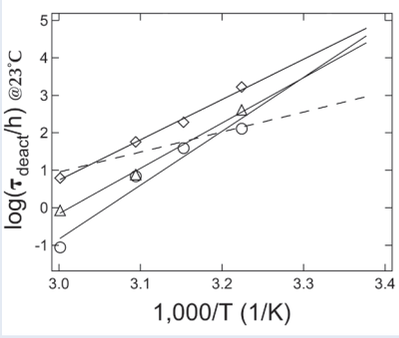


Figure 8: HRP deactivation times in Arrhenius format in glasses made up of (◇) 80 wt% dextran, 10 wt% maltitol and 10 wt% DMSO; (△) 80 wt% ficoll, 10 wt% maltitol and 10 wt% glycerol; (○) 80 wt% PVP, 10 wt% maltitol and 10 wt% glycerol.



will not induce an antiplasticization (**58**). A model by Ngai, based on dynamics rather than free volume, predicts a lack of antiplasticization when timescales of motion of the diluent and host are excessively separated (**59**). We note that the positive impact of the presence of a linker molecule on τ_{deact} in a plasticized polymeric glass is consistent with this idea; the linker having dynamic timescales that are intermediate between that of the polymer and the plasticizer.

The free volume picture suggests that the plasticizer molecule should be of relatively low molecular weight to elicit an antiplasticization of the fast dynamics. Although we don't have convincing data that this is the case, our experience with oligomeric ethylene glycol in the role of plasticizer in a dextran glass suggests some validity to the idea; lower molecular weight oligomers gave better stabilization. If the free volume picture leads us in the right direction in this case, then large surfactant molecules may be ineffective in producing an antiplasticizing effect, even though they might be miscible and have low T_g.

Excessive residual water (amounts of more than just a few mass percent) may have a profound effect on the stabilizing ability of a glass/diluent formulation and can seriously degrade the beneficial properties associated with antiplasticization. Work by Noel et al. shows that water will antiplasticize glucose and maltose (**60**). Their data show, however, that maltose is antiplasticized by water only at temperatures below –45 °C, and a small extrapolation of their data shows that glucose is antiplasticized only at temperatures below 23 °C. Above those temperatures, water has only a plasticizing effect on α and β relaxations in both of these materials.

The presence of some small amount of residual water is almost unavoidable in pharmaceutical preparations, and it may be beneficial. One might speculate that just as glycerol was an effective antiplasticizer for polymers only in the presence of an intermediate companion molecule, a small amount of residual water actually may be beneficial in the presence of the plasticizer, as an agent for "filling" the free volume that was too small to be filled by the plasticizing additive. The possibility of this effect is suggested by the results of Noel et al. (**61**).

Finally, we suggest that the antiplasticizing of a formulation by addition of plasticizer be reserved until the last formulation step, and that measurements of material dynamics or rheological properties be used to guide the formulation with respect to amounts of diluent required for an optimal antiplasticization. Once a range of plasticizer/linker concentrations are determined by these relatively fast methods, biostabilization experiments can be performed on the narrowed set of candidate formulations.

Acknowledgments

We thank Chris Soles for pointing out the antiplasticization literature. Alexei Sokolov acknowledges partial financial support from NSF (DMR-0080035).

References

1 Mouradian R; Womerseley, C; Crowe, LM; et al. Preservation of Functional Integrity During Long Term Storage of a Biological Membrane. *Biochem. Biophys. Acta* 1984, 778: 615–617.

2 Carpenter, JF; Crowe, LM;. Crowe, JH. Stabilization of Phosphofructokinase with Sugars During Freeze-Drying: Characterization of Enhanced Protection in the Presence of Divalent Cations. *Biochem. Biophys. Acta* 1987, 923: 109–115.

3 Carpenter, JF; Martin, B; Loomis, SH; et al. Long-Term Preservation of Dried Phosphofructokinase by Sugars and Sugar/Zinc Mixtures. *Cryobiology* 1988, 25: 327–376.

4 Franks, et al. US Patent 6,071,428; 6 June 6 2000.

5 Magneson, et al. US Patent 5,547,873; 20 August 1996.

6 Roser, US Patent 5,149,653; 22 September 1992.

7 Hinrichs, WL; Prinsen, MG; Frijlink, HW. Inulin Glasses for the Stabilization of Therapeutic Proteins. *Int. J. Pharm.* 2001, 215 (1–2): 163–174.

8 Sun, WQ; Davidson, P. Effect of Dextran Molecular Weight on Protein Stabilization During Freeze-drying and Storage. *CryoLett.* 2001, 22: 258–292.

9 Anchordoquy, TJ; Izutsu, KI; Randolph, TW; et al. Maintenance of Quaternary Structure in the Frozen State Stabilizes Lactate Dehydrogenase During Freeze-drying. *Arch. Biochem. Biophys.* 2001, 390 (1): 35–41.

10 Walker, et al. US Patent 5,565,318; 15 October 1996.

11 Yoshioka, S; Aso, Y; Kojima, S; et al. Effect of Polymer Excipients on the Enzyme Activity of Lyophilized Bilirubin Oxidase and Beta-galactosidase Formulations. *Chem. Pharm. Bull.* (Tokyo) 2000, 48 (2): 83–85.

12 Saleeb, et al. US Patent 5,972,395; 26 October 1999.

13 Allison SD; Manning MC; Randolph TW; et al. Optimization of Storage Stability of Lyophilized Actin Using Combinations of Disaccharides and Dextran. *J. Pharm. Sci.* 2000, 89 (2): 199–214.

14 Kreilgaard L; Frokjaer S; Flink JM; et al. Effects of Additives on The Stability of Recombinant Human Factor XIII During Freeze-Drying and Storage in the Dried Solid. *Arch. Biochem. Biophys.* 1998, 360 (1):121–134.

15 Izutsu K; Yoshioka S; Kojima S.. Increased Stabilizing Effects of Amphiphilic Excipients on Freeze-Drying of Lactate Dehydrogenase (LDH) By Dispersion into Sugar Matrices. *Pharm. Res.* 1995, 12 (6): 838–843.

16 Kim, AI; Akers, MJ; Nail, SL. The Physical State of Mannitol After Freeze-Drying: Effects of Mannitol Concentration, Freezing Rate, and a Noncrystallizing Cosolute. *J. Pharm. Sci.* 1998, 87 (8): 931–935.

17 Lueckel, B; Bodmer, D; Helk, B; et al. Formulations of Sugars with Amino Acids or Mannitol: Influence of Concentration Ratio on the Properties of the Freeze-Concentrate and the Lyophilizate. *Pharm. Dev. Technol.* 1998, 3 (3): 325–336.

18 Kasraian, K; Spitznagel, TM; Juneau, JA; et al. Characterization of the Sucrose/Glycine/Water System By Differential Scanning Calorimetry and Freeze-Drying Microscopy. *Pharm. Dev. Technol.* 1998, 3 (2): 233–239.

19 Heller, MC; Carpenter, JF; Randolph, TW. Protein Formulation and Lyophilization Cycle Design: Prevention of Damage Due to Freeze-Concentration Induced Phase Separation. *Biotechnol. Bioeng.* 1999, 63 (2): 166–174.

20 Pikal, MJ; Rigsbee, DR. The Stability of Insulin in Crystalline and Amorphous Solids: Observation of Greater Stability for the Amorphous Form. *Pharm. Res.* 1997, 14 (10): 1379–1387.

21 Izutsu, K-I; Yoshioka, S; Terao, T. Effect of Mannitol Crystallinity on the Stabilization of Enzymes During Freeze-Drying. *Pharm. Soc. Jap.* 1994, 424 (1): 5–8.

22 Lueckel B; Helk B; Bodmer D; et al. Effects of Formulation and Process Variables on the Aggregation of Freeze-Dried Interleukin-6 (IL-6) After Lyophilization and

on Storage. *Pharm. Dev. Technol.* 1998, 3 (3): 337–346.

23 See, for example, Allison, SD; Chang, B; Randolph, TW; et al. Hydrogen Bonding Between Sugar and Protein Is Responsible for Inhibition of Dehydration-Induced Protein Unfolding. *Arch. Biochem. Biophys.* 1999, 365 (2): 289–298.

24 Tanaka, K; Takeda, T; Miyajima, K. Cryoprotective Effect of Saccharides on Denaturation of Catalase By Freeze-Drying. *Chem. Pharm. Bull.* 1991, 39 (5): 1091–1094.

25 Constantino, HR; Carrasquillo, KG; Cordero, RA; et al. Effect of Excipients on the Stability and Structure of Lyophilized Recombinant Human Growth Hormone. *J. Pharm. Sci.*, 1998, 87: 1412–1420.

26 Cleland, JL; Lam, X; Kendrick, B; et al. A Specific Molar Ratio of Stabilizer to Protein Is Required for Storage Stability of a Lyophilized Monoclonal Antibody. *J. Pharm. Sci.* 2001, 90 (3): 310–21.

27 Carpenter, JF; Crowe, LM; Crowe, JH. Stabilization of Phosphofructokinase with Sugars During Freeze-Drying: Characterization of Enhanced Protection in the Presence of Divalent Cations. *Biochim. Biophys. Acta* 1987, 923: 109–115.

28 Carpenter, JF; Martin, B; Crowe, LM; et al. Stabilization of Phosphofructokinase During Air-Drying with Sugars and Sugar/Transition Metal Mixtures. *Cryobiology* 1987, 24: 445.

29 Mazzobre MF; Del Pilar Buera, M. Combined Effects of Trehalose and Cations on the Thermal Resistance of Beta-Galactosidase in Freeze-Dried Systems. *Biochim. Biophys. Acta* 1999, 1473 (2–3): 337–344.

30 Chang, BS; Kendrick, BS; Carpenter, JF. Surface-Induced Denaturation of Proteins During Freezing and Its Inhibition By Surfactants. *J. Pharm. Sci.* 1996, 85 (12): 1325–1330.

31 Prestrellski, SJ; Tedeschi, N; Arakawa, T; et al. Dehydration-Induced Conformational Transitions in Proteins and Their Inhibition By Stabilizers. *Biophys. J.* 1993, 65: 661–671.

32 Buitink, J; van den Dries, IJ; Hoekstra, FA; et al. High Critical Temperature Above T_g May Contribute to the Stability of Biological Systems. *Biophys. J.* 2000, 79 (2): 1119–1128.

33 Dudu, SP; Dal Monte, PR. Effect of Glass Transition Temperature on the Stability of Lyophilized Formulations Containing a Chimeric Therapeutic Monoclonal Antibody. *Pharm. Res.* 1997, 14: 591.

34 Bell, LN; Haeman, MJ; and Muraoka, LM. Thermally Induced Denaturation of Lyophilized Bovine Somatotropin and Lysozyme As Impacted By Moisture and Excipients. *J. Pharm. Sci.* 1995, 84 (6): 707–712.

35 Wang, W. Lyophilization and Development of Solid Protein Pharmaceuticals. *Int. J. Pharm.* 2000, 203: 1–60.

36 Miller, DP; Anderson, RE; de Pablo, JJ. Stabilization of Lactate Dehydrogenase Following Freeze-Thawing and Vacuum Drying in the Presence of Trehalose and Borate. *Pharm. Res.* 1998, 15 (8): 1215–1221. See also, Conrad, PB; Miller, DP; Cielenski, PR; et al. Stabilization and Preservation of Lactobacillus Acidophilus in Saccharide Matrices. *Cryobiology* 2000, 41: 17–24.

37 Davidson, P; Sun, WQ. Effect of Sucrose/Raffinose Mass Ratios on the Stability of Co-Lyophilized Protein During Storage Above the T_g. *Pharm. Res.* 2001, 18: 474–479.

38 Buera, MP; Rossi, S; Moreno, S; et al. DSC Confirmation That Vitrification Is Not Necessary for Stabilization of the Restriction Enzyme EcoRI Dried with Saccharides. *Biotechnol. Prog.* 1999, 15: 577–579.

39 Shamblin, SL; Tang, X; Chang, L; et al. Characterization of the Time Scales of Molecular Motion in Pharmaceutically Important Glasses. *J. Phys. Chem.* B 1999, 103: 4113–4121..

40 Kanaya, T; Kawaguchi, T; Kanji, K. Local Dynamics of Some Bulk Polymers Above T_g As Seen By Quasielastic Neutron Scattering. *Macromolecules* 1999, 32: 1672–2678.

41 Ansari, A; Jones, CM; Henry, ER; et al. The Role of Solvent Viscosity in the Dynamics of Protein Conformational Changes. *Science* 1992, 256: 1796–1798.

42 Cicerone, MT; Ediger, ME. Enhanced Translation of Probe Molecules in Supercooled o-Terphenyl: Signature of Spatially Heterogeneous Dynamics. *J. Chem. Phys.* 1996, 104: 7210–7218.

43 Sefcik, MD; Schaefer, J; May, FL; et al. Diffusivity of Gases and Main-Chain Cooperative Motions in Plasticized Poly(Vinyl Chloride). *J. Polym. Sci., Polym. Phys. Ed.* 1983, 21: 1041–1054.

44 Xiao, C; Wu, J; Yang, L; et al. Positron Annihilation Lifetime and Dynamic Mechanical Studies of g-Relaxation in PBA-PC and TMBPA-PC Plasticized By TOP. *Macromolecules* 1999, 32: 7913–7920.

45 Gaudin, S; Lourdin, D; Forssell, PM; et al. Antiplasticization and Oxygen Permeability of Starch-Sorbitol Films. *Carbohydr. Polym.* 2000, 43: 33–37.

46 Lourdin, D; Ring, SG; Colonna, P. Study of Plasticizer-Oligomer and Plasticizer-Polymer Interactions By Dielectric Analysis: Maltose-Glycerol and Amylose-Glycerol-Water Systems. *Carbohydr. Res.* 1998, 306: 551–558.

47 Carpenter, JF; Pikal, MJ; Chang, BS; et al. Rational Design of Stable Lyophilized Protein Formulations: Some Practical Advice. *Pharm. Res.* 1997, 14 (8): 969–975.

48 Bovaird, JH; Ngo, TT; Lenhoff, HM. Optimizing the o-Phenylenediamine Assay for Horseradish Peroxidase: Effects of Phosphate and pH, Substrate and Enzyme Concentrations, and Stopping Reagents. *Clin. Chem.* 1982, 28: 2423–2426.

49 Vallee, BL; Hoch, FL. *Proc. Nat. Acad. Sci. USA* 1955, 41: 327.

50 Sun, WQ; Davidson, P. Effect of Dextran Molecular Weight on Protein Stabilization During Freeze-Drying and Storage. *CryoLetters* 2001, 22: 258–292.

51 O'Connell, PA; McKenna, GB. Arrhenius-Type Temperature Dependence of the Segmental Relaxation Below T_g. *J. Chem. Phys.* 1999, 110: 11054.

52 Mazzobre, MF; Buera, MP; Chirife, H. Glass Transition and Thermal Stability of Lactase in Low-Moisture Amorphous Polymeric Matrices. *Biotechnol. Prog.* 1997, 13: 195–199.

53 Yoshioka, S; Aso, Y; Izutsu, K.-I; et al. Application of Accelerated Testing for Shelf-Life Prediction of Commercial Protein Preparations. *J. Pharm. Sci.* 1994, 83: 454–456.

54 Cicerone, et al. In preparation.

55 Vogel, M; Medick, P; Rossler, E. Slow Molecular Dynamics in Binary Organic Glass Formers. *J. Mol. Liquids* 2000, 86: 103–108. Blochowicz, Th; Karle, C; Kudlik, A; et al. Molecular Dynamics in Binary Organic Glass Formers. *J. Phys. Chem. B* 1999, 103: 4032–4044.

56 Vilics et al. DMA Study of the Suppression of The β Transition in Slightly Plasticized PVC Blends. *J. Thermal Anal.* 1996, 47: 1141–1153.

57 Unpublished data, private communication from Annelise Faivre.

58 Berquist, P; Zhu, Y; Jones, AA; et al. Plasticization and Antiplasticization in Polycarbonates: The Role of Diluent Motion. *Macromolecules* 1999, 32: 7925–7931.

59 Rizos, AK; Petihakis, L; Ngai, KL; et al. A Dielectric Relaxation Study of the γ-Relaxation in Tetramethylbisphenol A Polycarbonate Plasticized By Tris(2-Ethylhexyl) Phosphate. *Macromolecules* 1999, 32: 7921–7924.

60 Noel, TR; Parker, R; Ring, SR. A Comparative Study of the Dielectric Relaxation Behaviour of Glucose, Maltose, and Their Mixtures with Water in the Liquid and Glassy States. *Carb. Res.* 1996, 282: 193–206.

Corresponding author ***Marcus T. Cicerone*** *is a staff scientist at the National Institute of Standards and Technology, Gaithersburg, MD 20899-8542, cicerone@nist.gov. He developed this article while working as a visiting assistant professor at Brigham Young University in Provo, UT, where* ***Andrew Tellington*** *and* ***Landon Trost*** *were students.* ***Alexei Sokolov*** *is an assistant professor in the Polymer Sciences department at the University of Akron, OH, USA.*