Stability in Freeze Dried Proteins

Can Physical Characterizations Predict with Useful Accuracy?

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Basic Objective

- **Do physical measurement in a few hours/days…**
- **Predict impact of formulation change or processing change…**
  - **Qualitatively**
  - **Trends quantitatively valid at temperature of interest**

Pharmaceutical Stability in Solids is Not Driven by Thermodynamics

- **Thermal Denaturation of proteins in solids is very high, ≈ 130°C-190°C**
  - **addition of disaccharides stabilizes against degradation, but lowers thermal denaturation temperature.**
- **Molecular mobility in glasses is very slow---> system is Not in equilibrium!**
  - **thermodynamics does not apply?**
- **Some limited correlation between secondary structure and pharmaceutical stability**
  - **thermodynamics "could" be critical in determining structure formed in the freeze drying process (where mobility is high),… or not!**

Physical Parameters Predictive of Stability?

- **Dynamic**
  - Tg (simplest!)
  - Enthalpy Relaxation
  - "Fast Dynamics"
  - "Free Volume" (density)
  - "Coupling" of protein to matrix (how measure?)
  - What else?
- **Structure**
  - Protein Conformation
    - FTIR, what else?
- **Surface Effects**
  - Surface "high reactivity"?
  - Migration to surface (ESCA)
  - Specific Surface Area

- **Formulation Effects and Processing (thermal history) Effects**

Classes of Dynamics in Glasses

- **Global Dynamics (α relaxation)**
  - **Directly related to viscosity**
    - **Enthalpy relaxation**
    - **Dielectric relaxation**
    - **Thermally stimulated Current (dipole re-orientation)**
  - **Long time scale, long length scale**
  - **Tg marks division between "solid" and "liquid" behavior**
- **"Fast" Dynamics (example: β relaxation)**
  - **"local" motion,**
  - **small length scale, short time scale**
  - **Various measures**
    - **β-relaxation via Dielectric,**
    - **amplitude of nanosec time scale motion via neutron scattering.**
    - **NMR Relaxation Times**

Tg as a Predictor of Stability

Glasses and Stability

Temperature -------->

glass transition

T_g

Solid

low molecular mobility

more stable

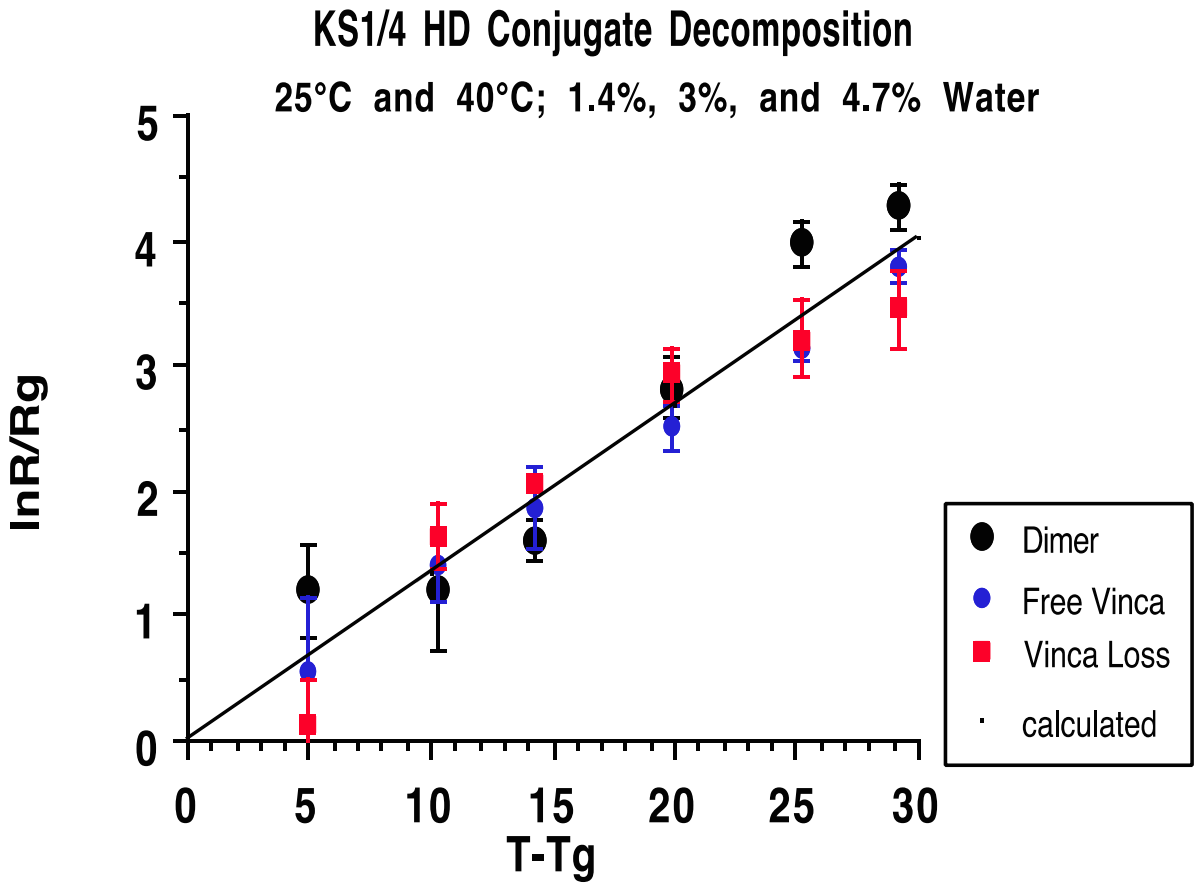
Liquid

high molecular mobility

more reactive

Stability and "T-Tg" for KS1/4 MoAb:Vinca Conjugate

Roy, et. al., Develop. biol. Standard., 74, 323-340 (1991)

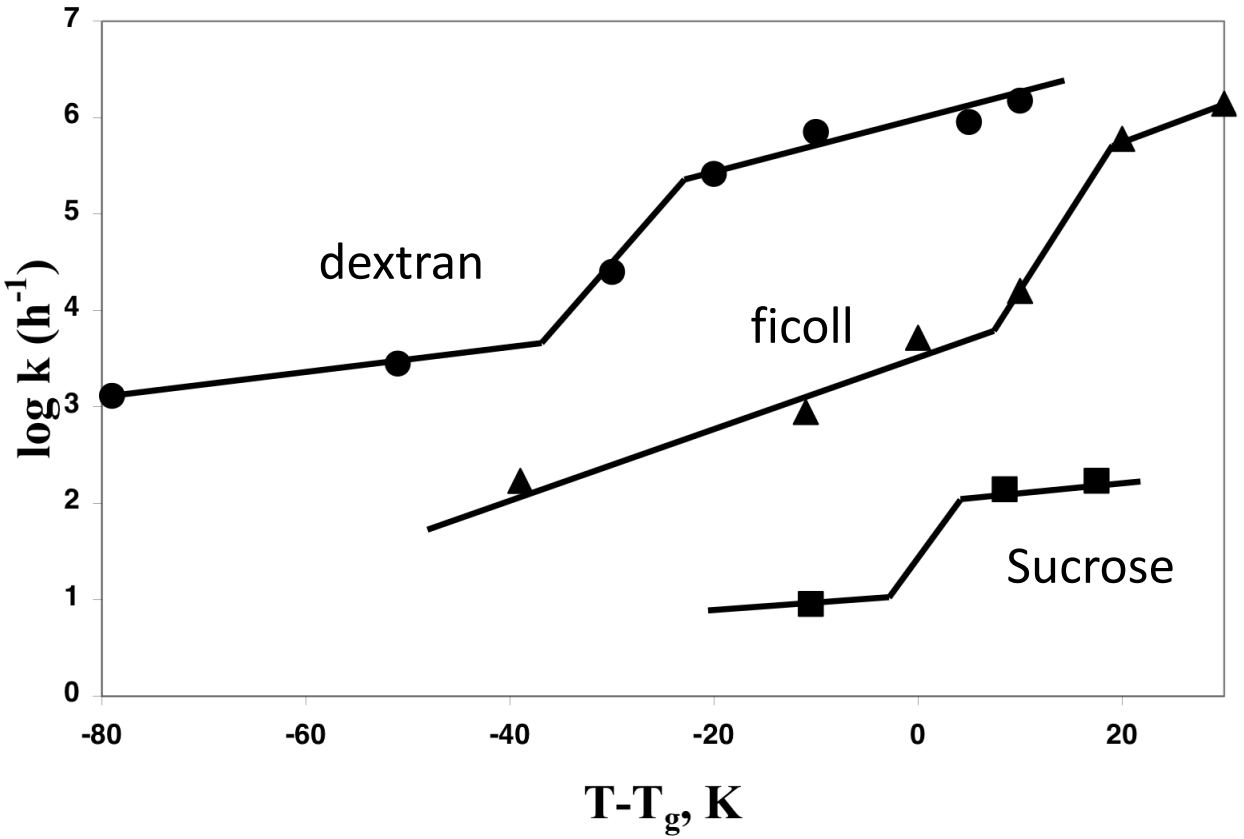


- **Good Correlation of Stability with T-Tg (above Tg), as in WLF equation!**

Chemical Stability and Tg

Hydrolysis of 2-(4-nitrophenoxy) tetrahydropyran

- Streefland, L., Auffret, A. D., and Franks, Felix, Pharm. Res., 1998. 15(6): p. 843-849.

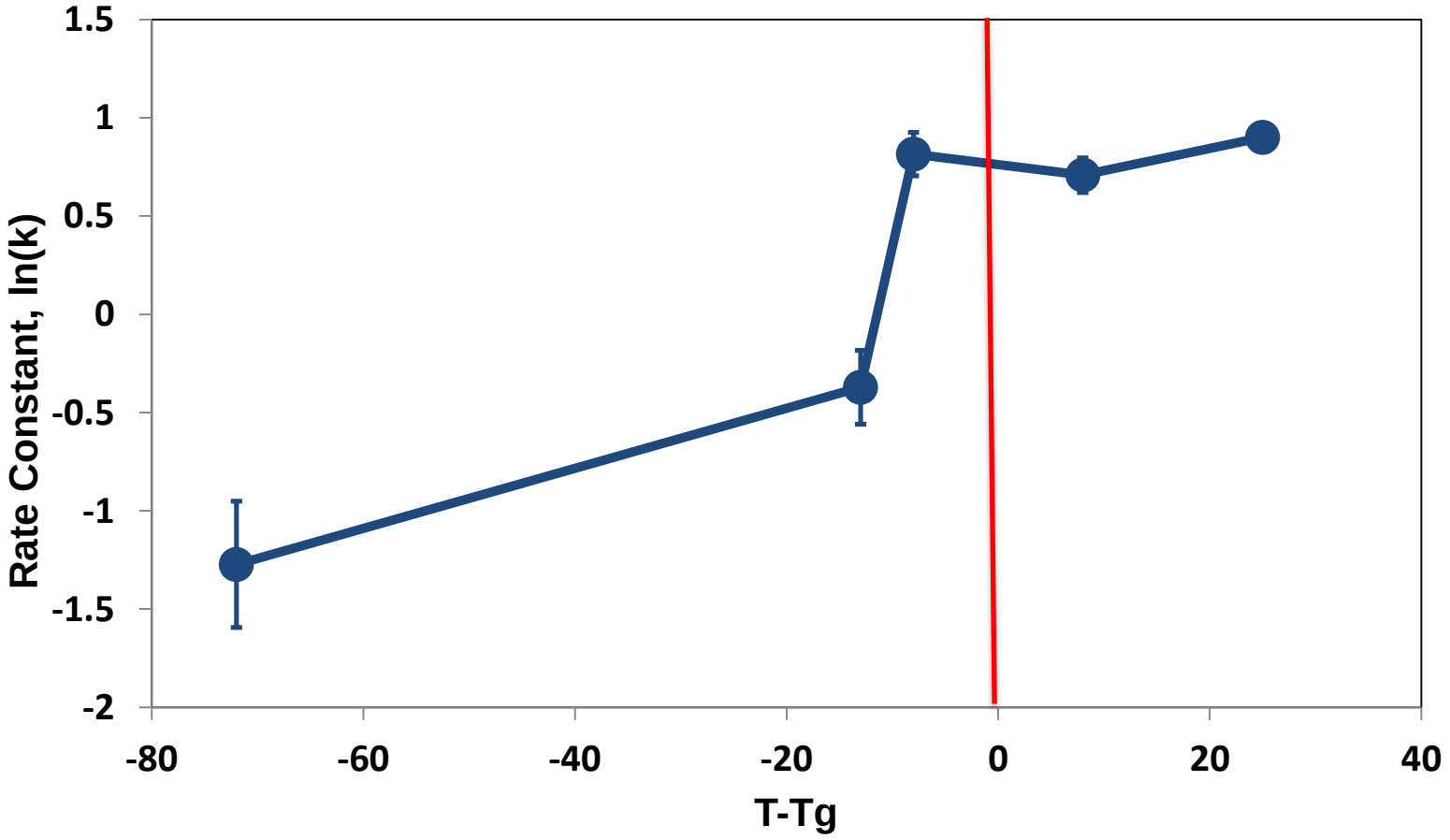


Stability Correlates with Tg Qualitatively

- **differences in coupling with matrix?**

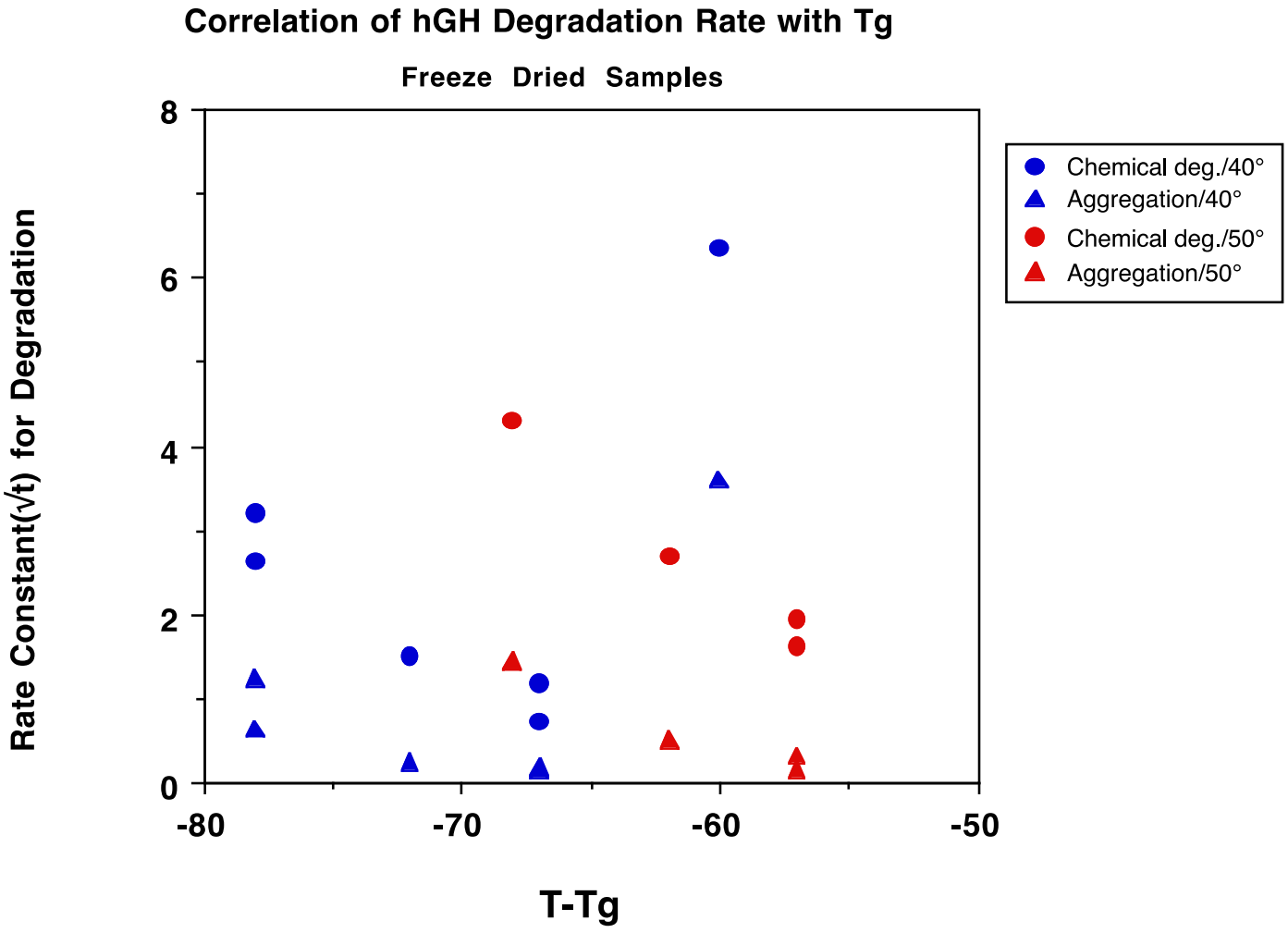
Stability and Tg

IgG1:Trehalose (0.5:1) with Glycerol



Correlation of Stability and "T - Tg"

"Dry Solids" Stored Well Below Tg

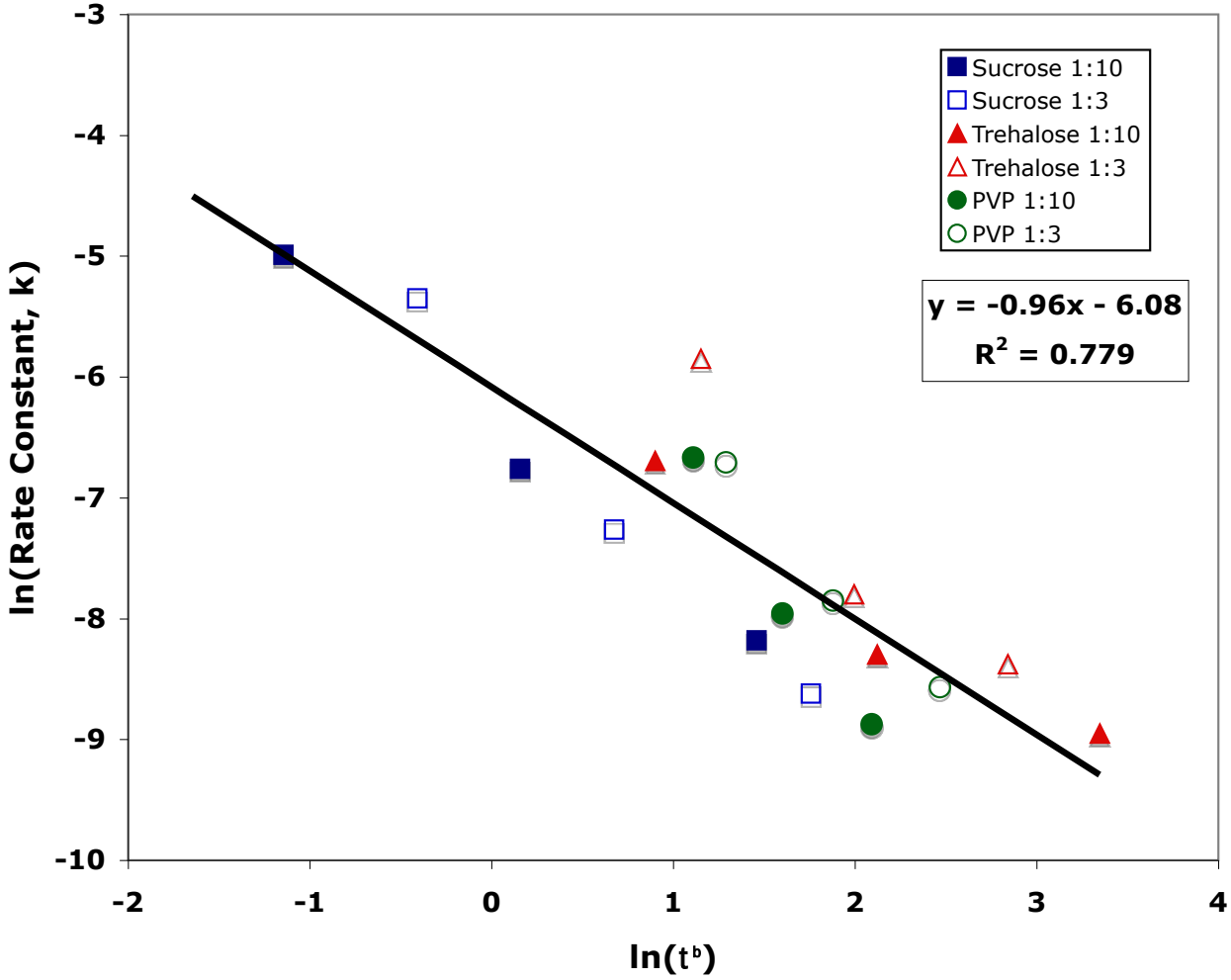


- No Obvious Sensible Correlation!

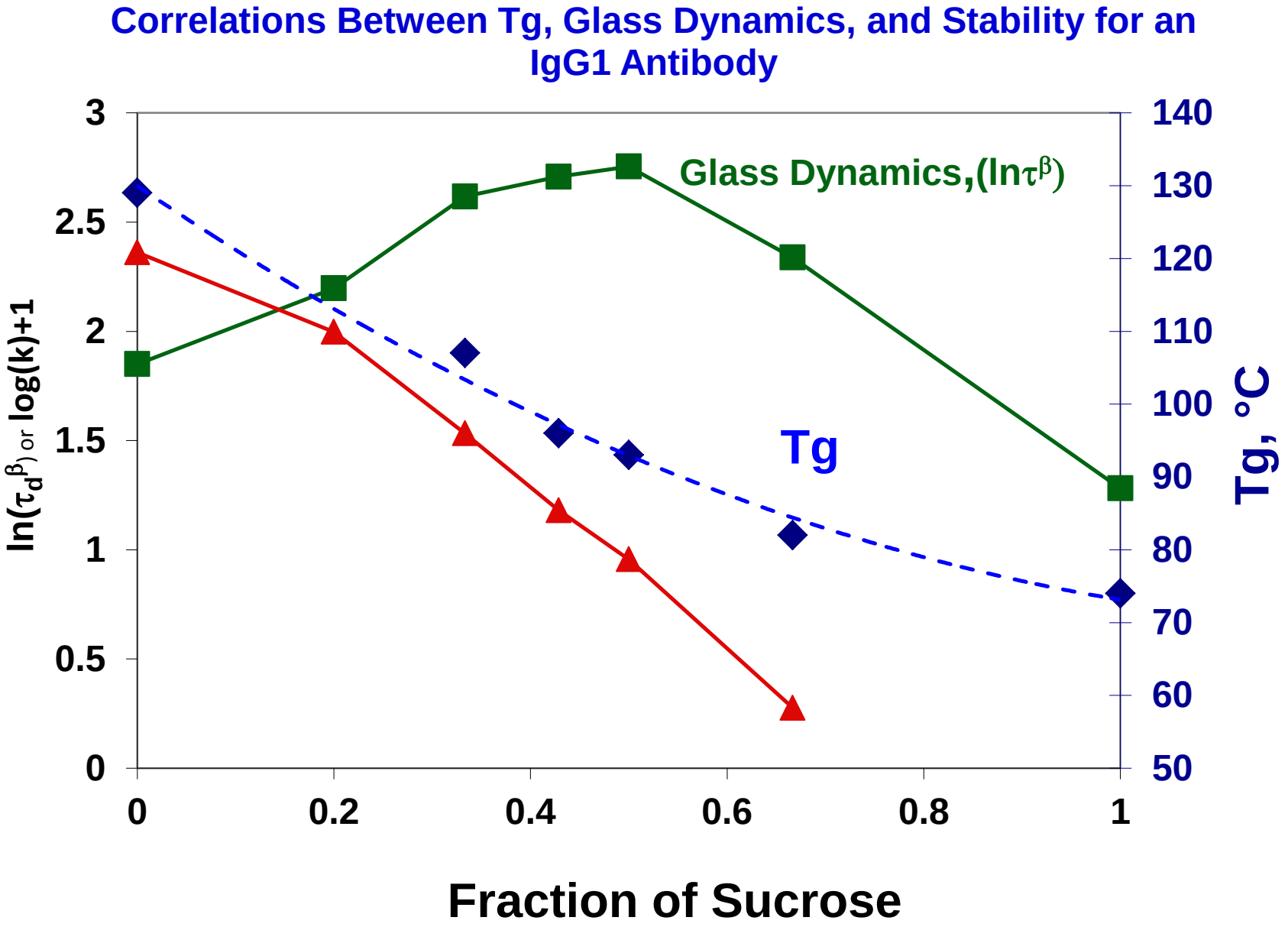
Stabilization and Molecular Mobility

- **Mobility in a glass depends on more than "T-Tg" particularly well below Tg**
- **Does molecular mobility determine pharmaceutical degradation in the solid state?**
  - **Or, at least is it a critical factor?**
    - **Degradation Rate = $(\text{Mobility})^{C}$, C = coupling coefficient, = 1 for diffusion controlled Rx with Stokes-Einstein where Diffusion $\propto 1/\eta$**
- **What kind of molecular mobility is most relevant?**
  - **All?**
  - **Enthalpy Relaxation Time?**
    - **Structural relaxation time via calorimetry**

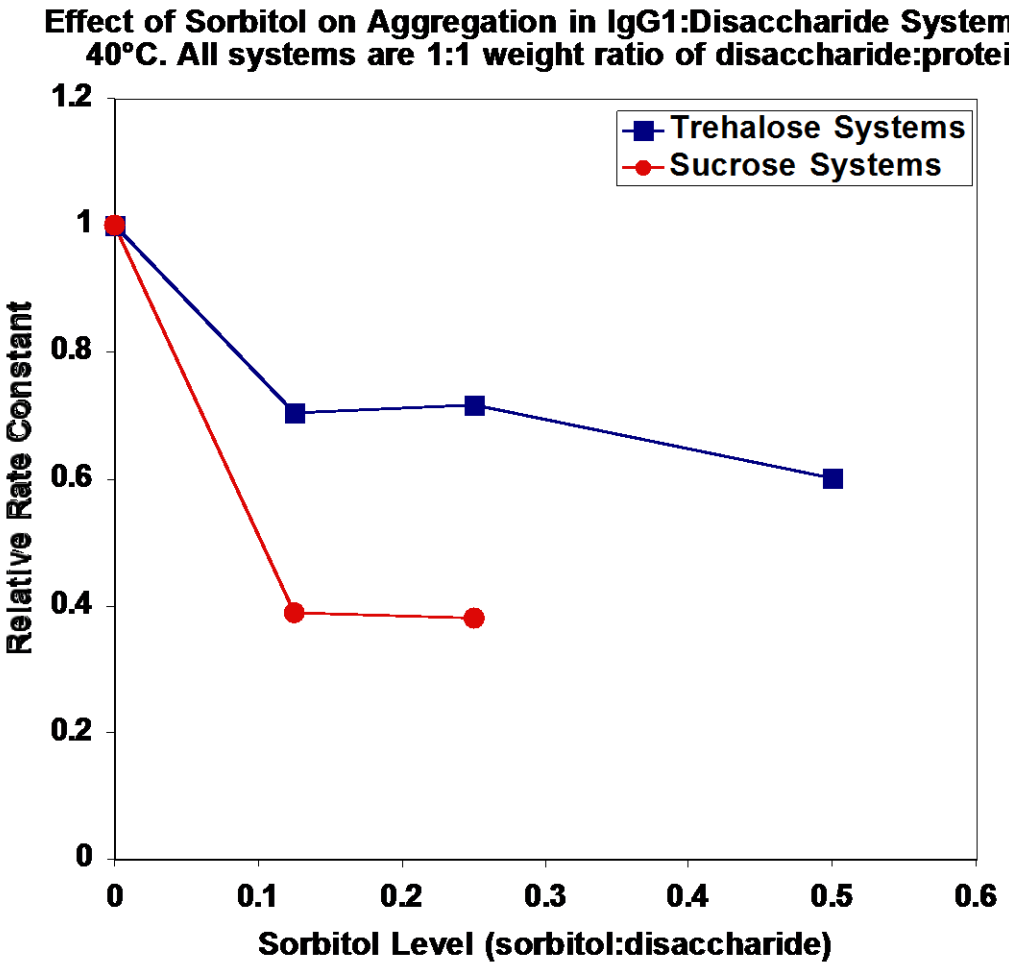
Stability of Sodium Ethacrynate: Correlation of Dimerization Rate with Relaxation Time



- Good Correlation; coupling coefficient ≈ 0.45


Correlations Between Tg, Glass Dynamics, and Stability for an IgG1 Antibody
Glass Dynamics,(lnτβ)
Tg
ln(τdβ) or log(k)+1
Tg, °C
Fraction of Sucrose
0
0.5
1
1.5
2
2.5
3
50
60
70
80
90
100
110
120
130
140
0
0.2
0.4
0.6
0.8
1

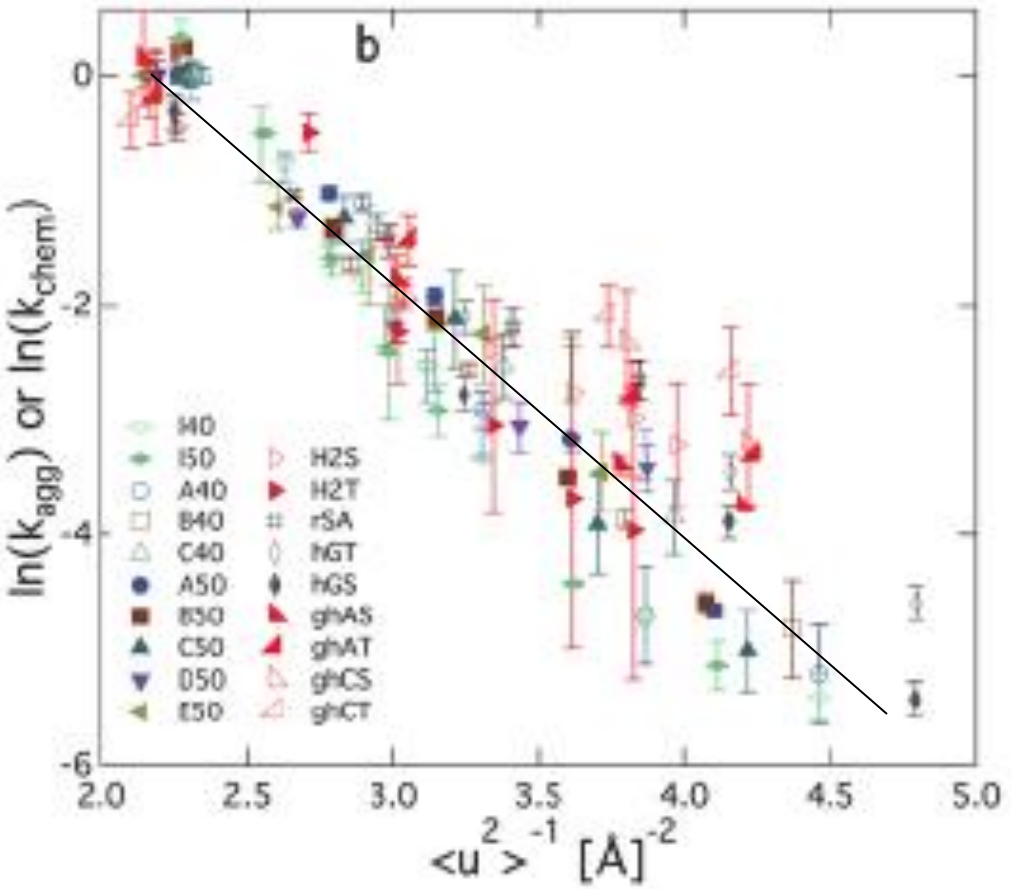
Aggregation in IgG1:Disaccharide Systems
Effect of Small Additions of Sorbitol



- **Small amounts of sorbitol stabilize but lowers Tg! WHY?**

Stability and Fast Dynamics

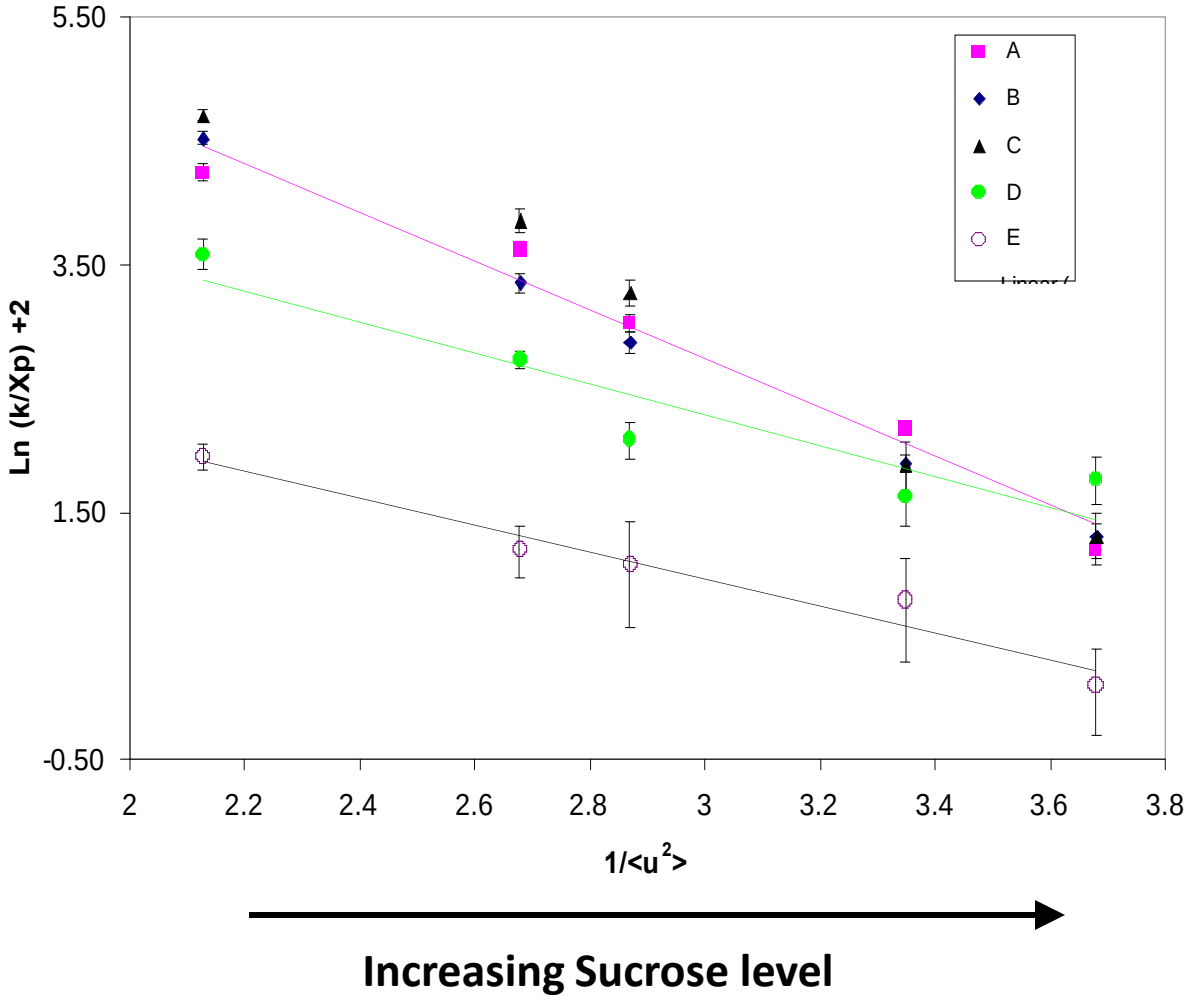
- **Reciprocal of mean amplitude of fast motion by neutron scattering, $1/\langle u^2 \rangle$; relates to diffusional motion**



M. Cicerone et al. *Soft Matter,* 8, 2012

Stability and "Fast Dynamics"

Relationship between the normalized aggregation rate constant and fast local mobility ($1/\langle u^2 \rangle$) at 50 °C for five different proteins



- **Excellent correlation between stability & "Fast Dynamics".**

Mobility and Stability: Hypothesis

- **Seems like:**
  - **Mobility directly relevant to instability is "diffusional motion"—Marc Cicerone**
  - **Fast dynamics (diffusional motion) decouples from "viscous like" motion (structural relaxation time), at least well below Tg**

Annealing Impacts Stability!

small molecules and proteins

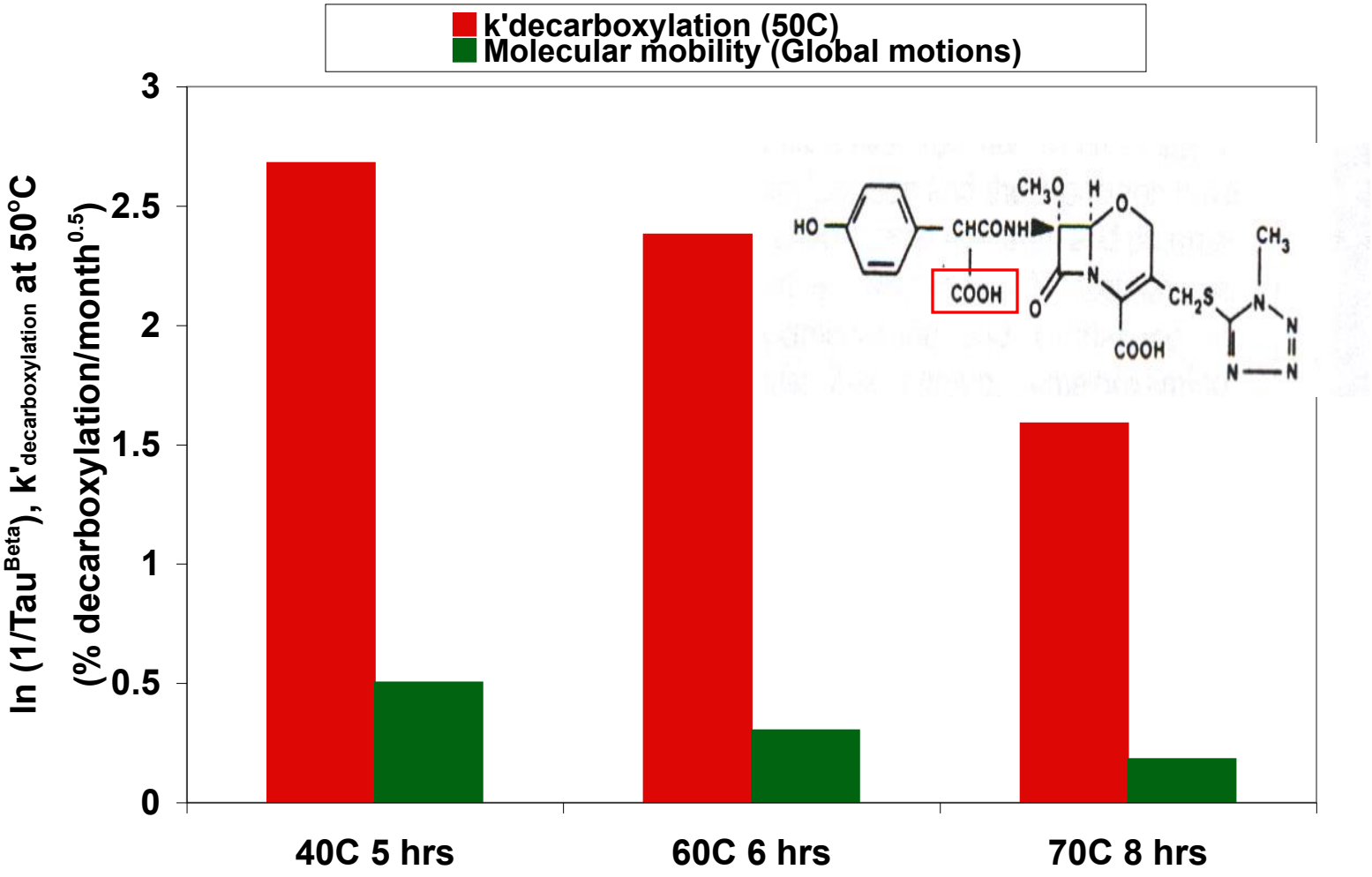
"Amorphous" is not a complete description of the state for an amorphous solid

-because such systems are not in thermodynamic equilibrium!

Annealing a Glass

- **Hold sample at T<Tg for given time(s)**
- **Energy decreases,**
- **Structure Increases,**
- **Free volume decreases,**
- **Relaxation time increases,**
- **If relaxation dynamics is a predictor of pharmaceutical stability,**
  - Much evidence suggest it is, so…
- **Stability improves!**

The Annealing Effect for Moxalactam Disodium

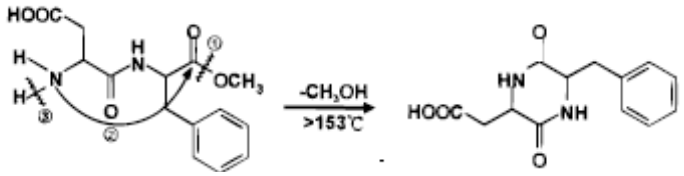


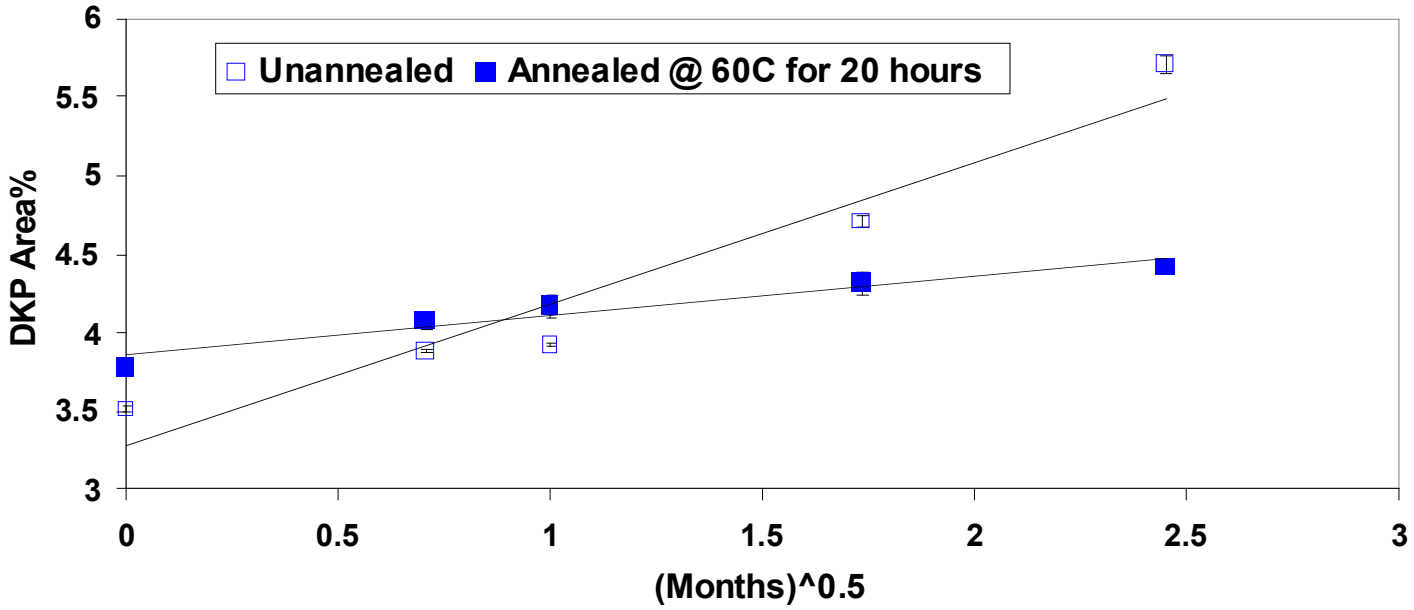
Annealing decreases mobility (enthalpy relaxation) and decreases Degradation rate: "Cooking Stabilizes"

Annealing Can Improve Purity at end of Storage

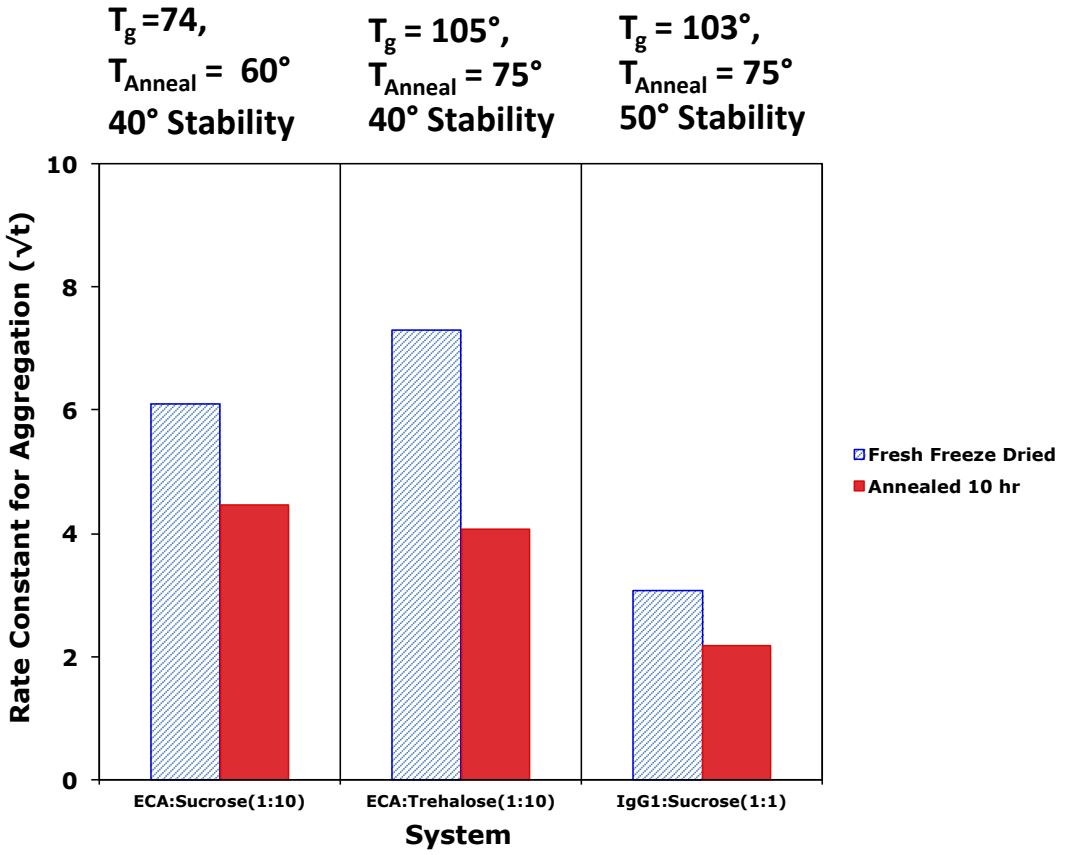
Appearance of DKP Degradation Product as a function of time at 50°C storage temperature.

Aspartame: sucrose (1:10) formulation





Effect of Annealing on Aggregation in Small Molecule (NaECA) and Protein (IgG1*) Systems

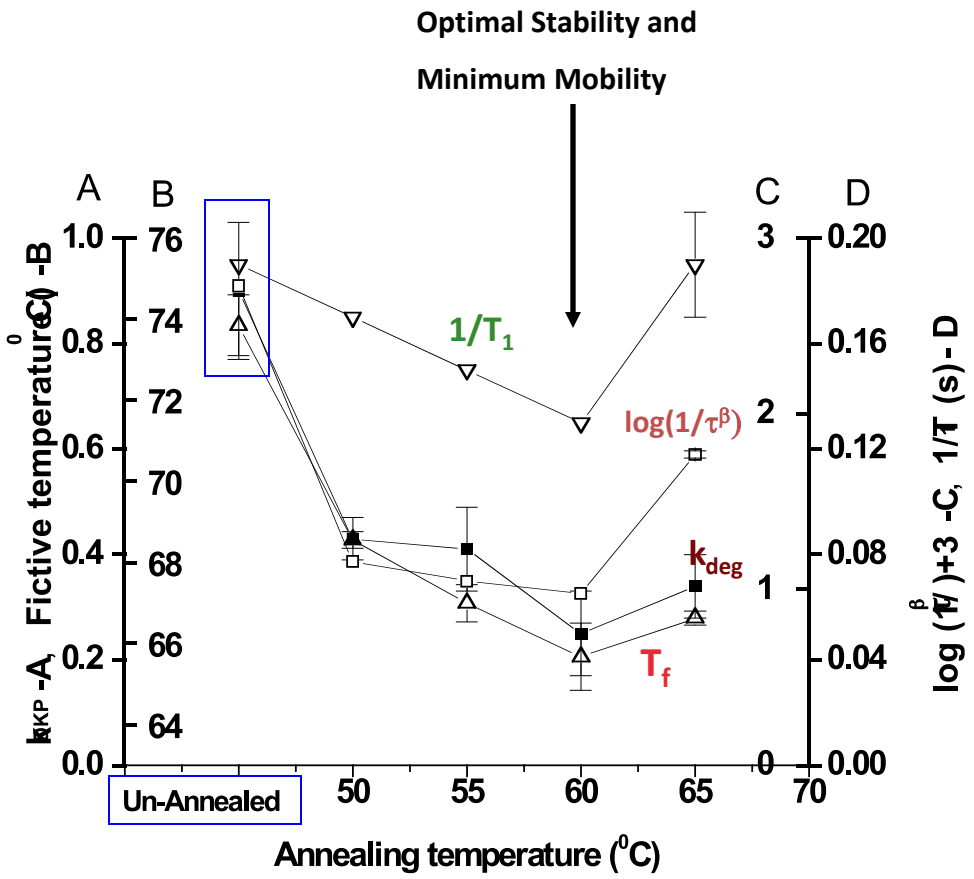


- Effect of Annealing on Stability appears to be general!

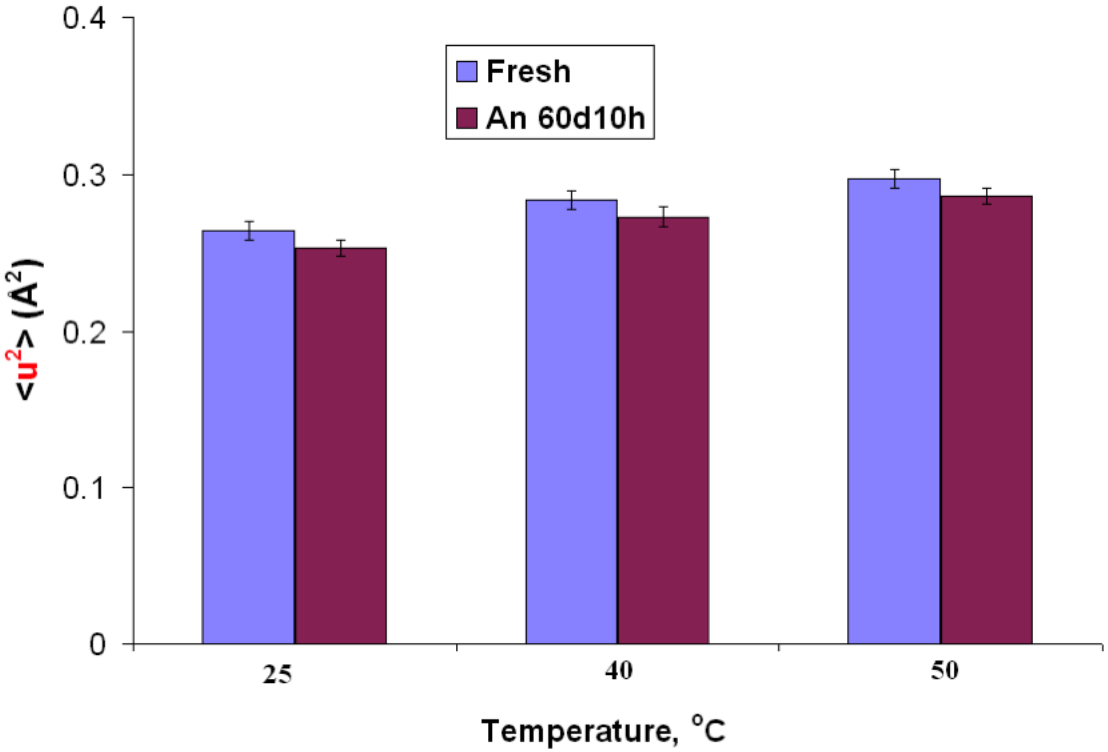
Effects of Annealing on Mobility:

$1/\tau^{\beta}$, Fictive Temperature (T_f), T_1(NMR) and Degradation Rate

Aspartame:Sucrose (1:10) Annealed for 20 hr



Fast local mobility of IgG1/Sucrose= 1:1 studied by neutron backscattering at 25, 40 and 50 °C



- No large change in the fast local mobility upon annealing, but no large change expected
  - From correlation of ln(k) with $1/\langle u^2 \rangle$ and differences in $\langle u^2 \rangle$ above, we predict:

k_{anneal}/k_{fresh} = 0.78, where direct annealing stability experiment gives 0.71

Good Agreement!

Complications

- **Specific Effects do seem to be present**
  - **Different proteins behave differently**
    - **Sucrose stabilizes hGH better than trehalose, but with KGF, no real difference**
- **Factors other than fast dynamics may control trends when fast dynamics variations are small**
  - **Stability in protein/disaccharide/amino acid systems do not correlate well with fast dynamics (data not shown)**
  - **Likely that "coupling" between matrix and protein varies between stabilizers**
    - **How to measure?**
  - **Surface Effects (fraction protein at surface)-NEXT!**

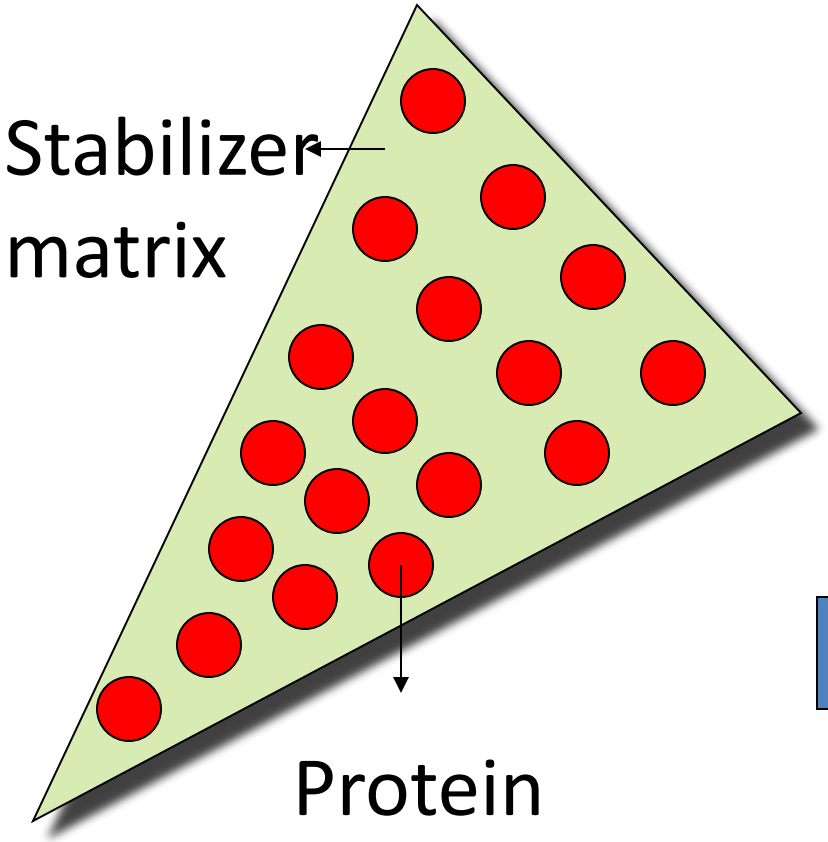
Maybe Protein at Surface is “Reactive”

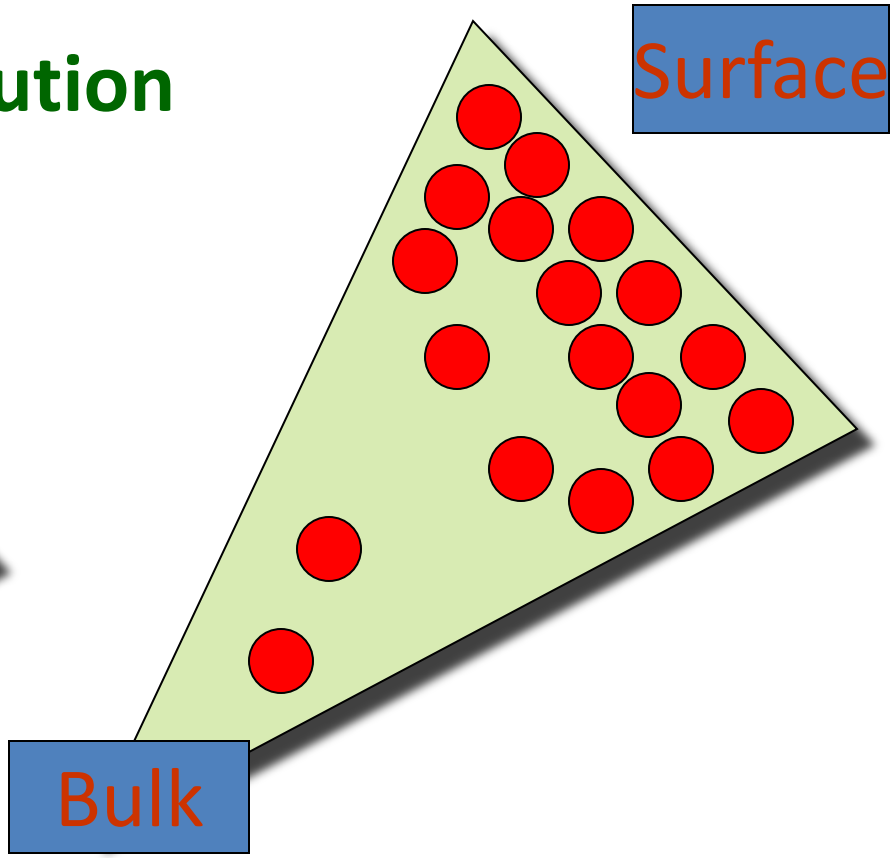
- Interaction with ice during freezing at aqueous:ice interface
  - unfolding
- Protein at surface is “concentrated”
  - Partial separation from stabilizer
- Protein at surface is in “reactive environment”
  - Surface phase has “air” on one side

Composition Heterogeneity

Uneven distribution of protein and stabilizer throughout the dried particle

Homogeneous distribution

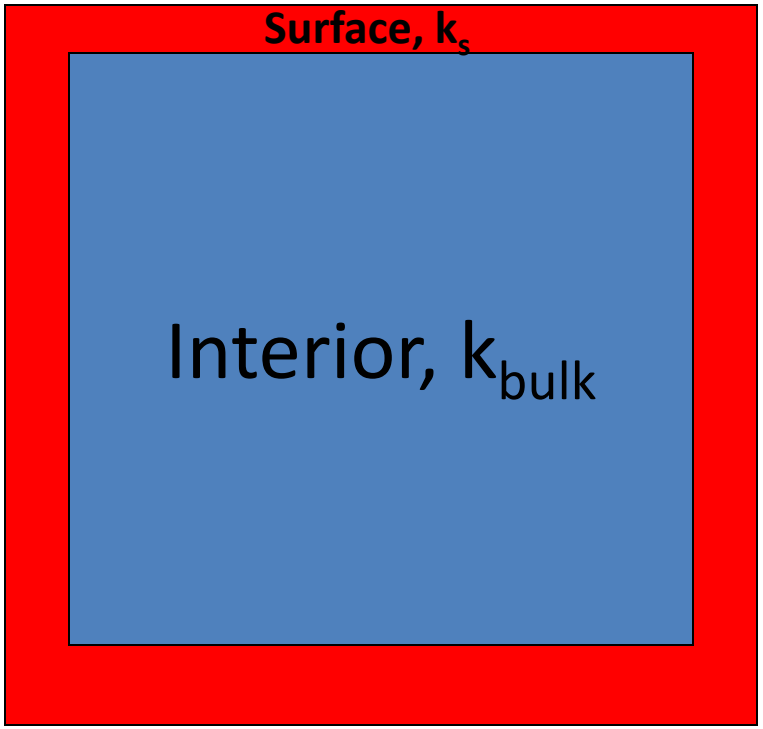




Heterogeneous distribution

Chemical Heterogeneity

Surface is Richer in Protein than is "bulk"

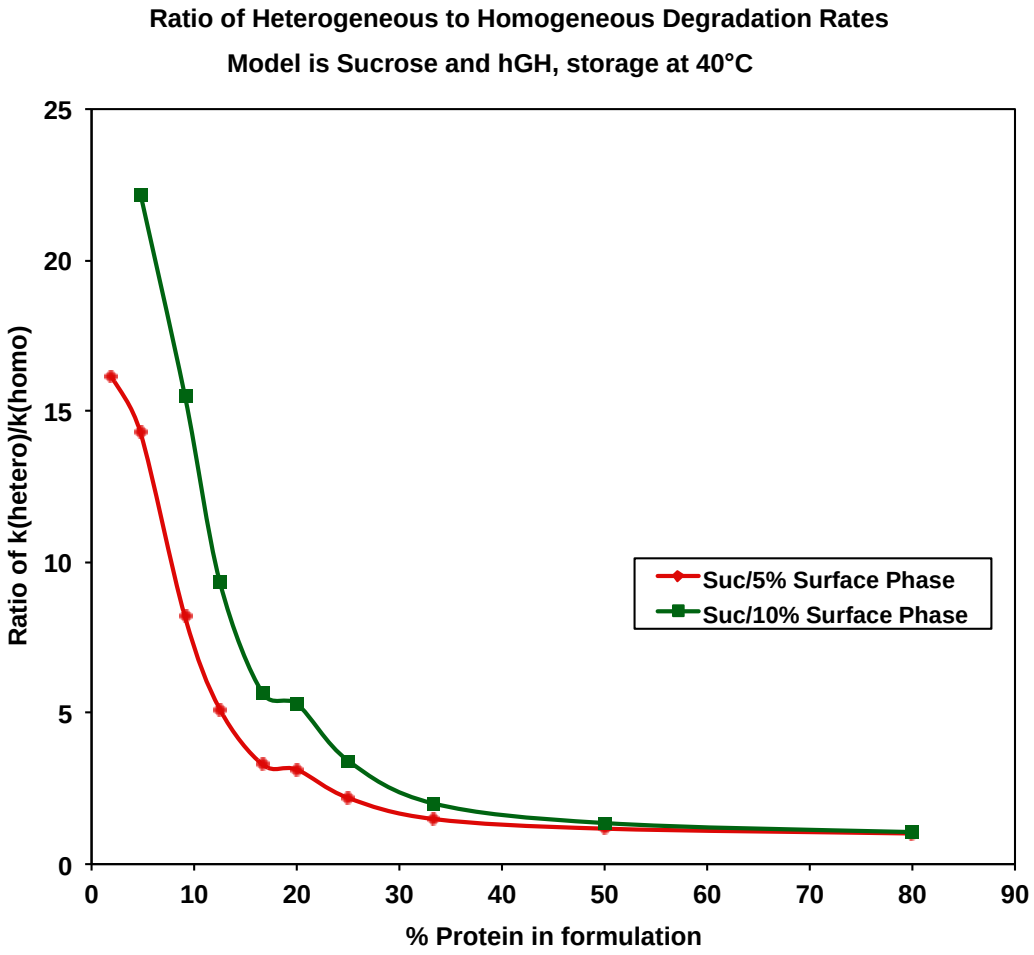


- Stability is weighted average of the two regions
- May Estimate the Effect
- Model for Calculations:
  - hGH:sucrose
    - ln(k) linear in % sucrose
      - "literature" data
  - SSA and ESCA used for
    - fraction of total protein on "surface"
    - composition of surface and bulk
  - Calculate overall (average) rate constant for degradation

- Calculations: extensive heterogeneity leads to inferior stability!

Heterogeneous Composition and Instability

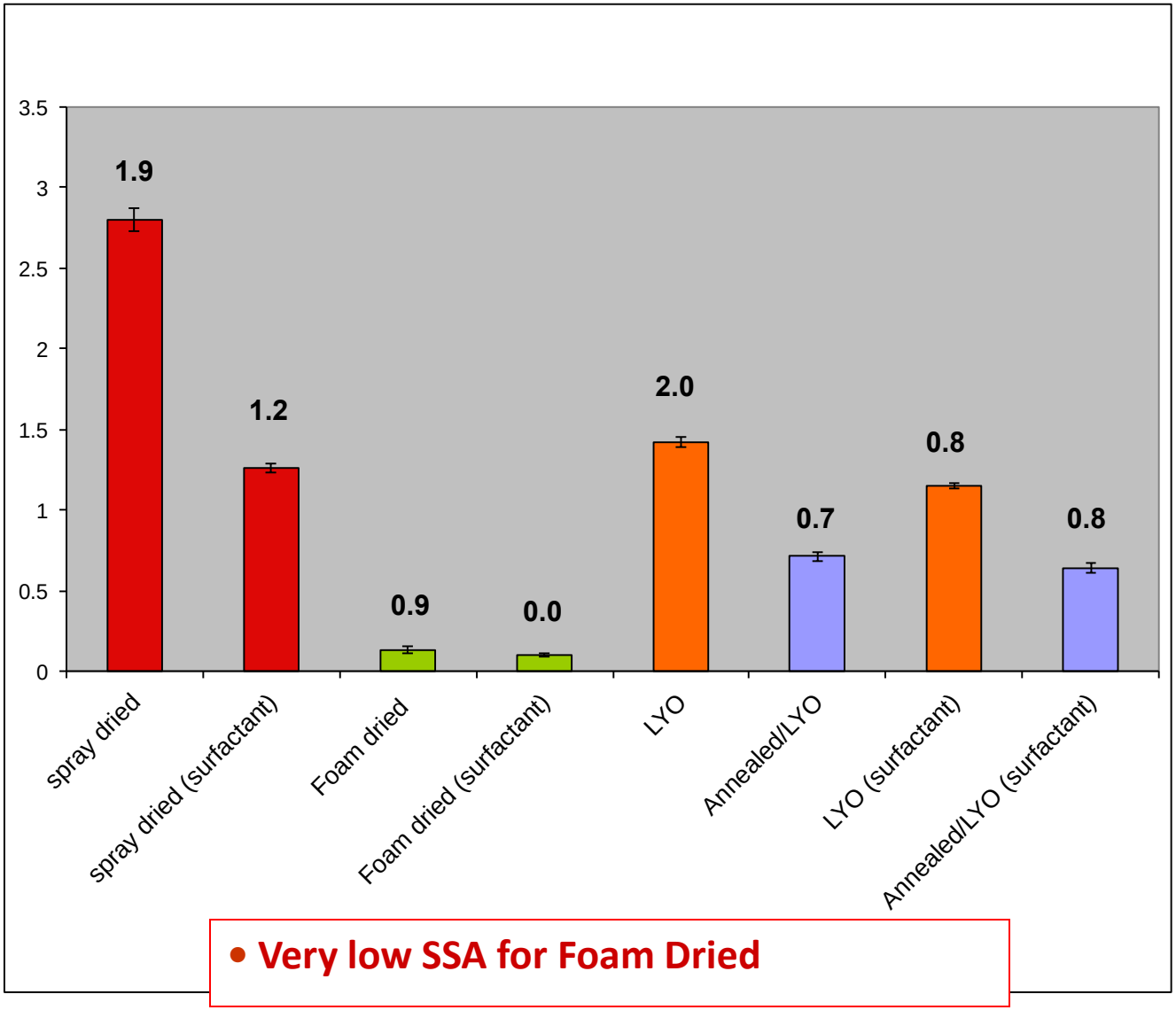
Ratio of k(heterogeneous) to k(homogeneous)



- **Effect is large only for stabilizer rich systems**

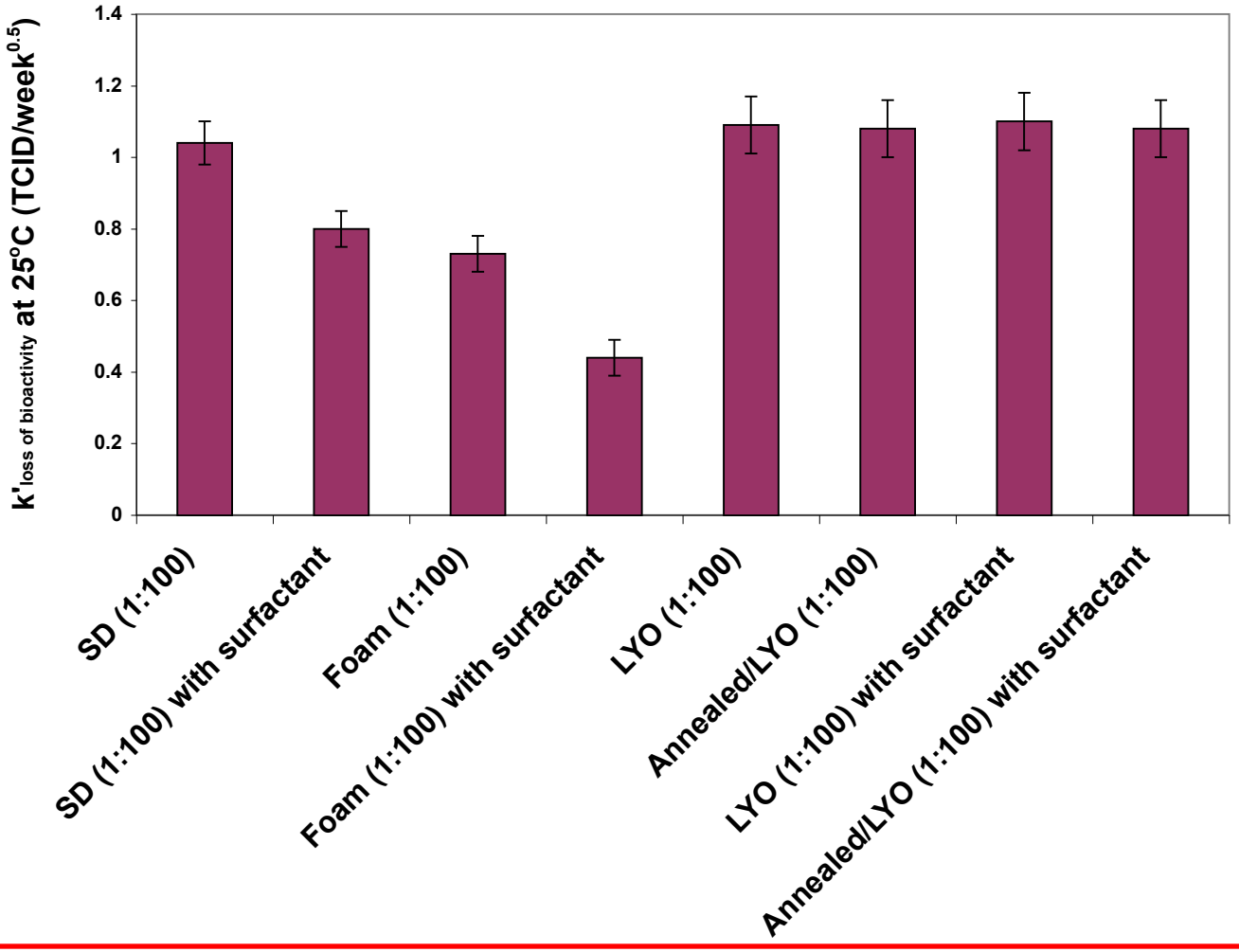
Surface composition and Specific Surface Area (SSA): Vaccines

Numbers = % Surface N as measured by ESCA



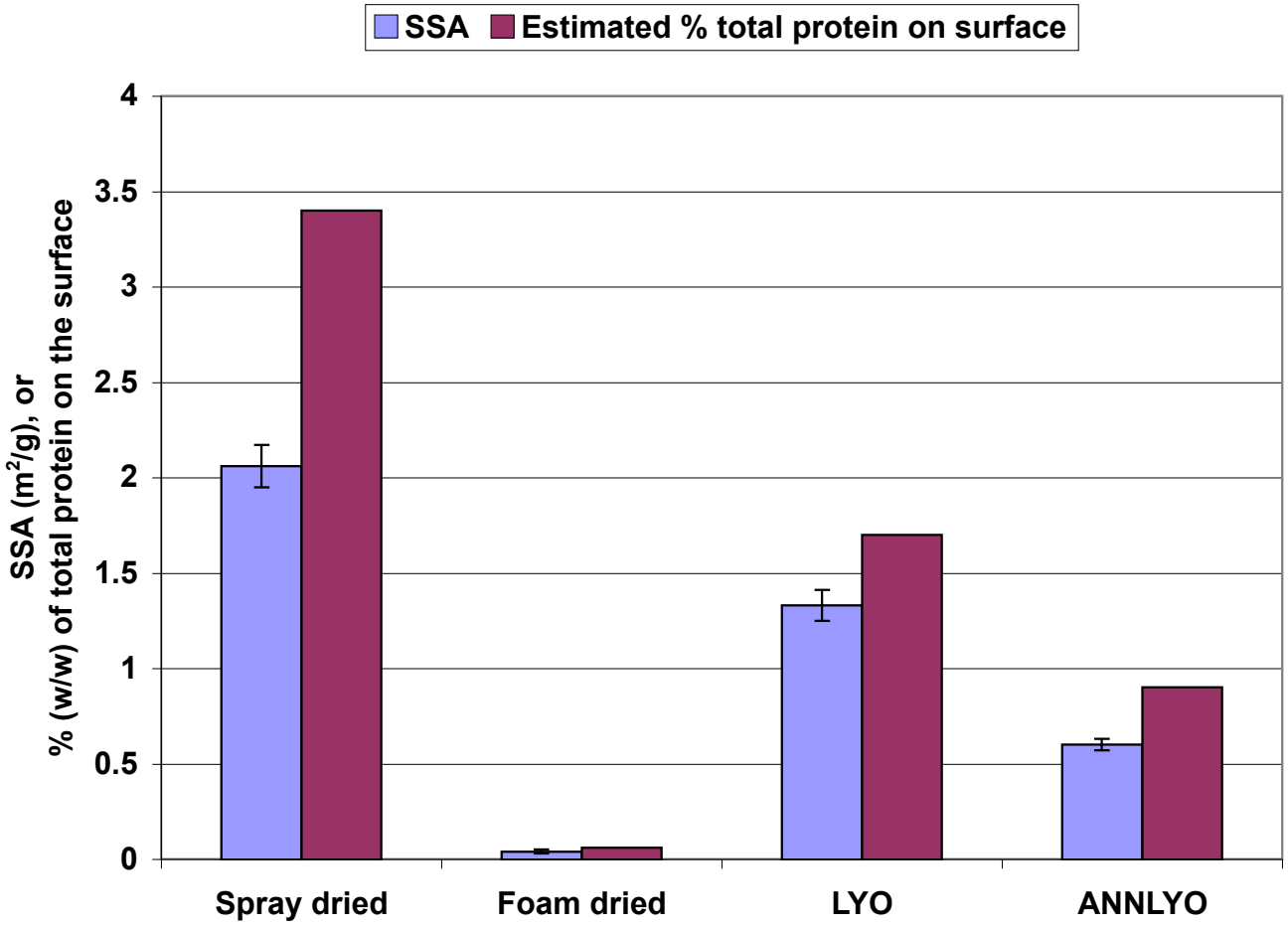
- **Very low SSA for Foam Dried**

Vaccine Stability: Rate of loss of activity at 25°C.



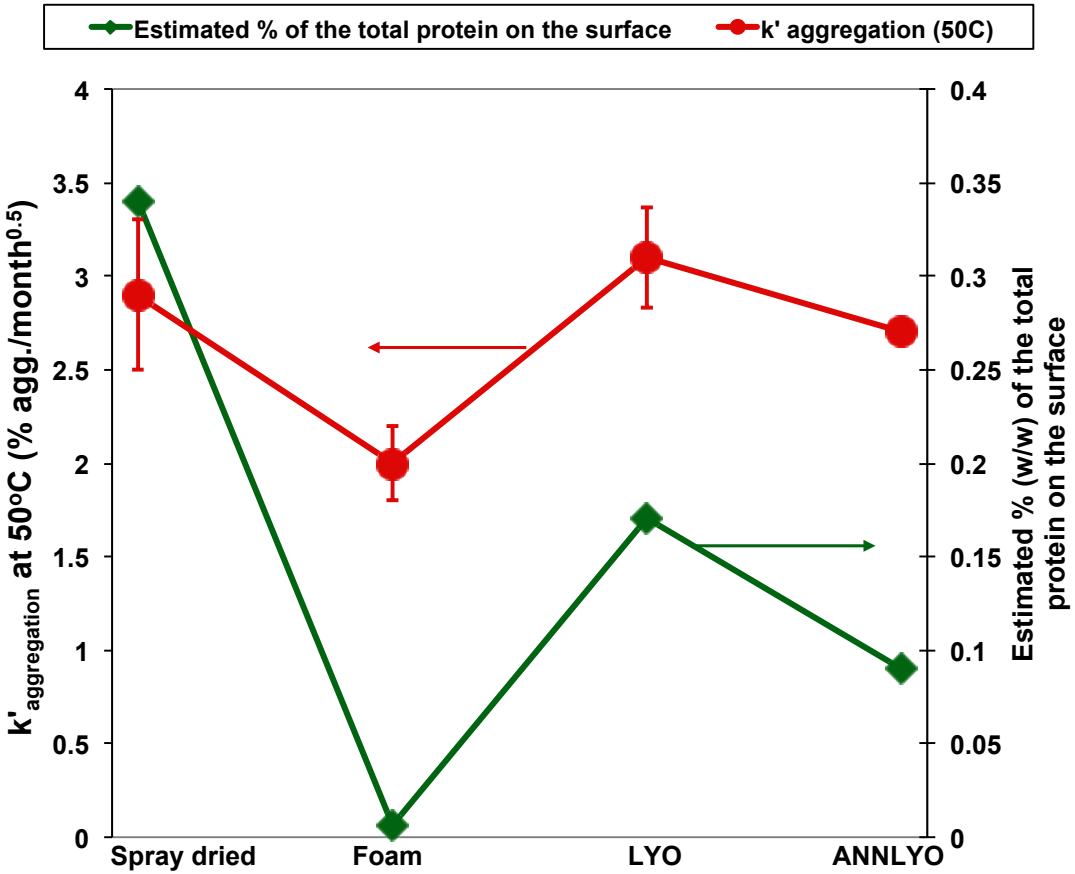
- **Foam Dried with surfactant <u>much</u> more stable**

Specific surface area (SSA) and surface composition: IgG1:Sucrose (1:4)



- **Foam Dried has <u>very</u> low SSA and " %surface protein"**
  -Spray dried has highest "% surface protein"

Stability (50°C) Correlations in IgG1:Sucrose (1:4)



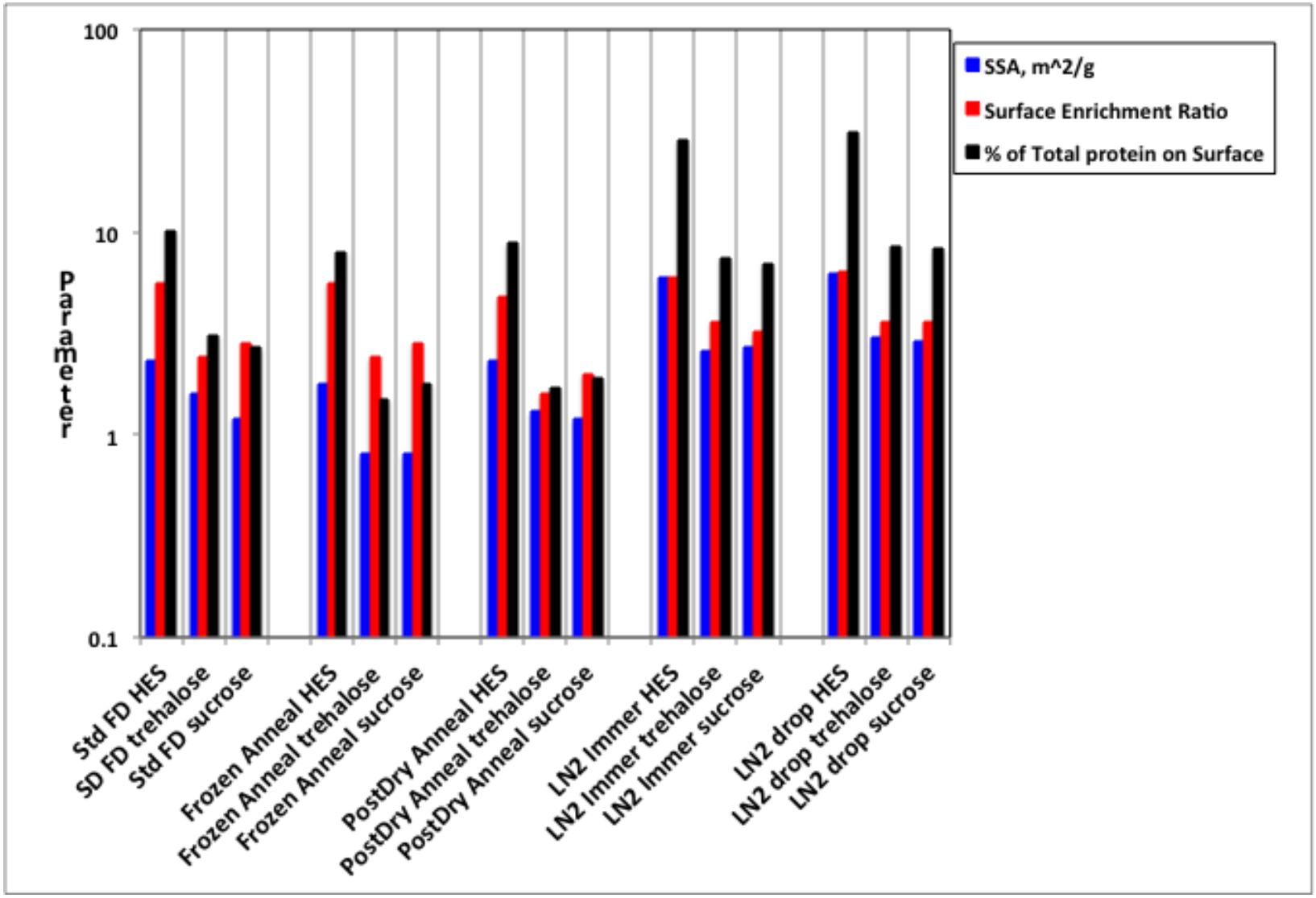
- **Fair correlation of stability with % of protein on surface**

-additional variable (not discussed) is thermal history variation giving mobility variation

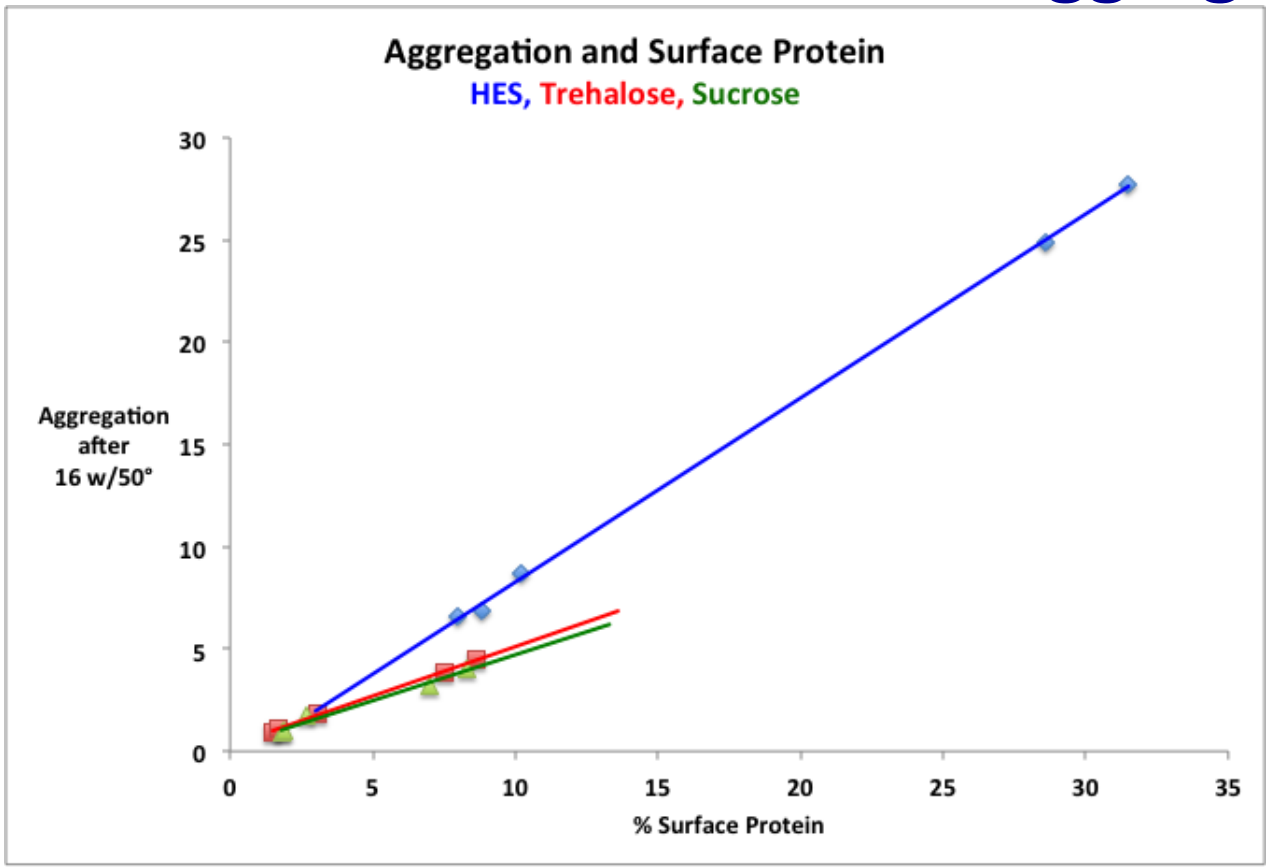
Recent Data for hGH

- Xu, Grobelny, van Allmen, Knudson, Pikal, Carpenter, and Randolph. "Protein Quantity on the Air-Solid Interface Determines Degradation Rates of hGH in Lyophilized Samples", J. Pharm. Sci., 103, 1356 (2014)
- A collaborative study between Univ. Colorado and Univ. Connecticut
  - Formulation varied and method of preparation varied to give large variation in SSA
  - Stability correlates with fraction protein at the surface

hGH: SSA, Surface Enrichment Ratio, and % Total Protein on Surface



Good Correlation Between % Surface Protein and Aggregation



Analysis shows:

Most degradation occurs in the surface region!!

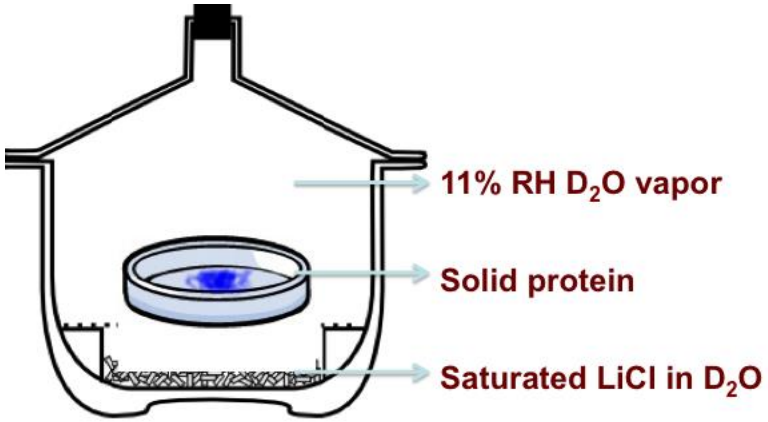
- very low protein (0.1%) & 5% saccharide = large heterogeneity effect (>20x)
- Some difference with stabilizer (HES poor stabilizer)

Conclusions on Surface Effects

- **Stability Depends on Specific Surface Area**
- **Degradation due mostly to degradation of protein at the surface**
  - **Increases with product of surface concentration and specific surface area**
  - **Consistent with chemical heterogeneity effect**
- **Impact of process due largely to effect of process on total protein at the surface.**
- **Surface effects can be critical!**

Another measure of Structure and Dynamics: Hydrogen/Deuterium Exchange Kinetics

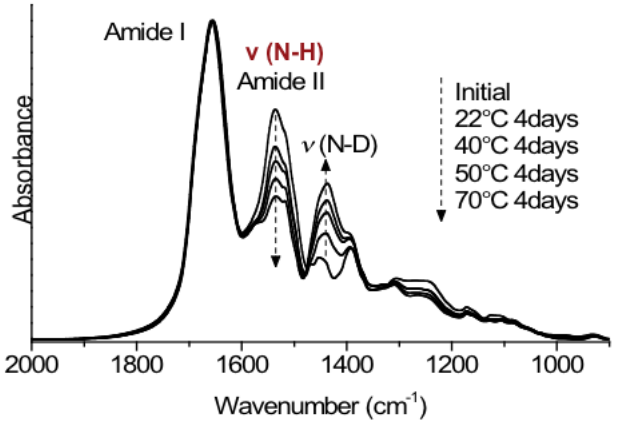
Equilibration



Detection

Purdue (Liz Topp)
-Mass Spec

UConn (Pikal/Bogner)
-FTIR



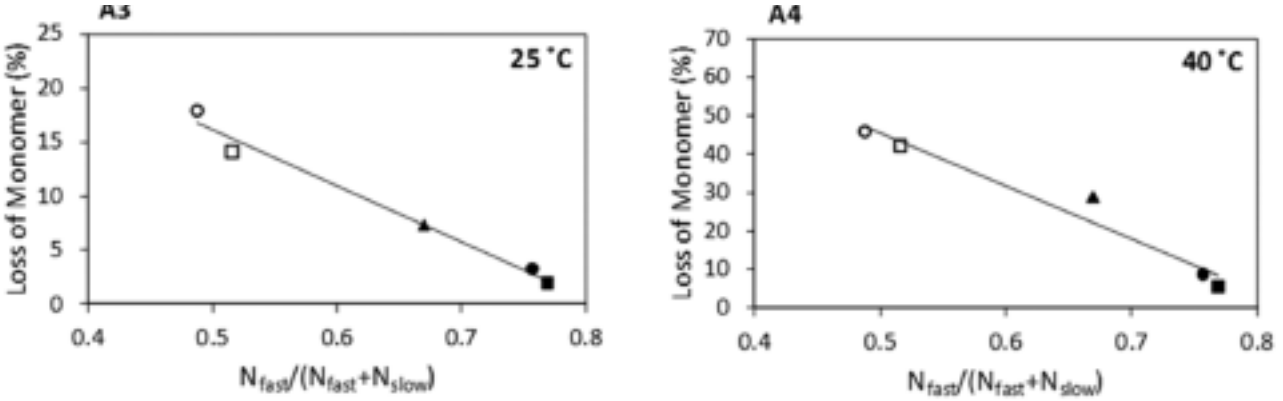
Example of BSA[1]
Follow time course of Amide II (ratio to Amide I, not impacted by H/D exchange)

Correlation of Myoglobin Aggregation after 1 yr at 25° and 40° with H/D Exchange (A) and FTIR Analysis (B)-PURDUE

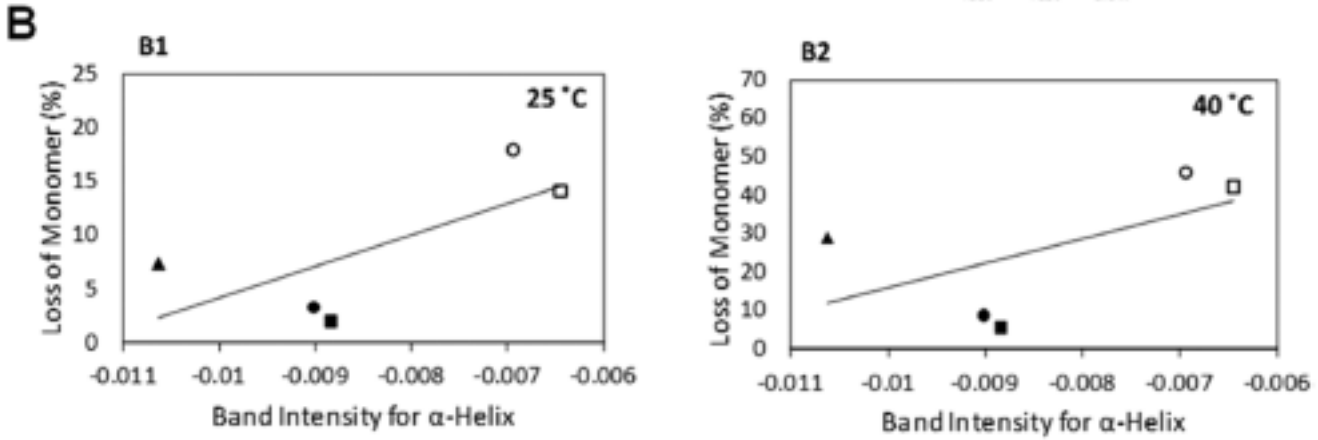
Data from: Moorthy BS, Schultz S, Kim S, and Topp EM. (2014). Predicting protein aggregation during storage in lyophilized solids using solid state amide hydrogen/deuterium exchange with mass spectrometric analysis (ssHDX-MS). *Molecular Pharmaceutics*, 11/6: 1869-1879, 2014

A. Good correlation H/D Exchange

$$\text{deuterium uptake} = N_{fast}(1 - e^{-k_{fast}t}) + N_{slow}(1 - e^{-k_{slow}t})$$



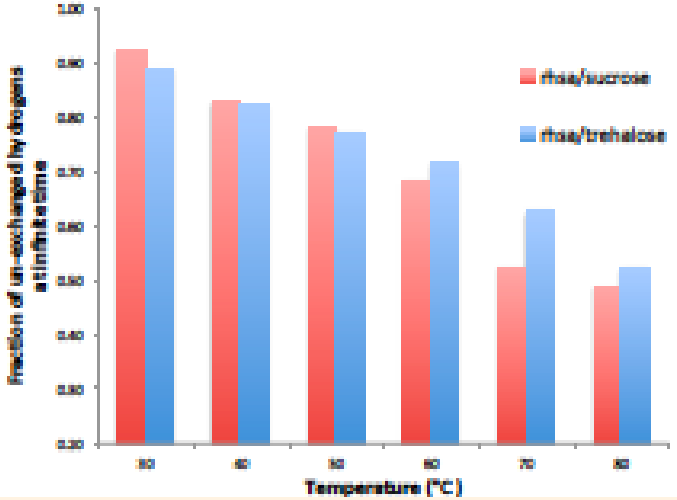
B. Poor correlation FTIR



Fraction Non-Exchangable Protons in 1:1 Disaccharide Formulations of rHSA-UCONN

v. Comparison of the extent of un-exchanged hydrogens at "infinite time" (X∞)

Temp (°C)	rHSA/sucrose	rHSA/trehalose
30	0.925	0.890
40	0.826	0.821
50	0.782	0.768
60	0.683	0.715
70	0.525	0.626
80	0.488	0.520

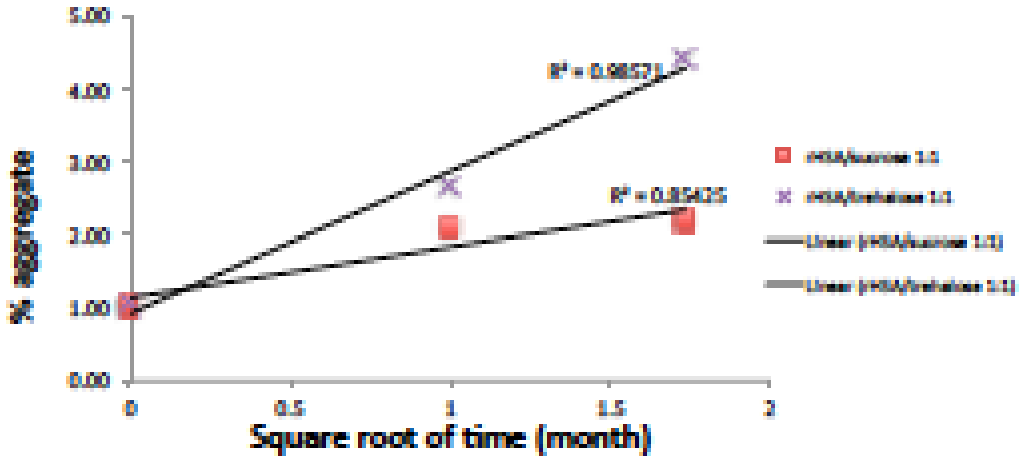


❖ The rHSA/trehalose formulation showed less H/D exchange than sucrose formulation at 60 °C, 70 °C, and 80 °C, implying less mobility and perhaps better stabilization of internal protein dynamics.

- **Trehalose retards exchange better than sucrose**
- **Means trehalose formulation more stable?**

Stability Data at 40°C (Dry samples)

vi. Comparison of physical stability during storage at 40 °C, 0% RH.



❖ The sucrose formulation showed better stability at 40 °C 0% RH.

- **Contrary to expectations, sucrose system more stable**
  - **however, stability is at 40°C, trehalose better at dampening motion at 60° and higher**
  - **Maybe because of 11% RH H/D data do not predict stability of dry product??**
    - **Stability studies on samples equilibration at 11% RH in progress**

Conclusion on Value of H/D EX

- **Need more data, more examples to test correlations with stability**