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# Addition of Monovalent Electrolytes to Improve Storage Stability of Freeze-Dried Protein Formulations

Hiroshika Goshima [1], Kelly M. Forney-Stevens [1], Ming Liu [2], Ken K. Qian [3], Madhusudan Tyagi [3], Marcus T. Cicerone [3], Michael J. Pikal [1,*]

[1] Department of Pharmaceutical Sciences, School of Pharmacy, University of Connecticut, Storrs, Connecticut 06269
[2] Department of Nuclear Engineering, North Carolina State University, Raleigh, North Carolina 27695
[3] National Institute of Standards and Technology, Gaithersburg, Maryland 20899

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ABSTRACT

This study investigates the effect of low levels of electrolytes on storage stability in freeze-dried sucrose-based protein formulations. Both bovine serum albumin and recombinant human serum albumin were freeze dried with sucrose and alkali halides (LiCl, NaCl, KCl, RbCl, and CsCl) at selected low levels. All formulations were stored at 50°C and 65°C up to 2 months and then assayed for protein aggregation. The data demonstrate that low levels of LiCl and NaCl enhance stability. No obvious correlations with either protein secondary structure or global dynamics (structural relaxation time) were found. However, good correlations were found between stability and both free-volume hole size via positron annihilation lifetime spectroscopy (PALS) and fast dynamics by neutron scattering. Volume changes on mixing and the partial molal volume of salt were also studied in an effort to detect decreases in free volume. These data did not support the hypothesis that reduction in free volume was the primary mechanism for salt-induced stabilization. Finally, a positive effect of postlyophilization annealing on stability was demonstrated. In summary, we find that small amounts of LiCl and NaCl significantly stabilize these proteins, which is a result at variance with conventional formulation wisdom.



## Introduction

Stability has historically been a challenge in protein formulation development, and refrigerated storage is a standard requirement. Freeze drying is commonly used to stabilize a protein product nominally because drying removes the reactant water and significantly immobilizes the protein system, but other stabilization mechanisms may also operate.[1] The first choice for the protein-stabilizing agent is frequently sucrose.[2-4] However, addition of other components to the disaccharide-based formulation has also been investigated, such as polyols, polymers, and amino acids.[5-19] Nominally, polymers would seem to be good stabilizer candidates because they increase the glass transition temperature ($T_g$).[1,6-8] However, whether $T_g$ is a reliable predictor of stability well below $T_g$ is doubtful.[6] Indeed, the observation that addition of low levels of several small molecules, such as glycerol[17] and sorbitol,[5] decreases $T_g$ but yet stabilizes disaccharide-based protein formulations suggests that it is perhaps local or "fast dynamics" that is a better predictor of stability than $T_g$ or global dynamics. The addition of such small molecules seems to antiplasticize "fast dynamics" and therefore stabilizes.[17]

Electrolytes are relatively common in freeze-dried formulations, as buffers and often as NaCl arising from prior purification steps.[9] However, because NaCl and buffer salts depress the collapse temperature,[11] use of salts in formulations is normally restricted to very low levels. In this study, we investigate addition of alkali chlorides, such as sodium chloride, as stability enhancers in sucrose-based formulations. Currently, the expected effect of an electrolyte on stabilization is unclear. Some studies suggest that NaCl destabilizes factor VIII[9] and human growth hormone.[10] However, another report recommended NaCl as a bulking agent for factor VIII, suggesting that at least crystalline NaCl did not significantly damage stability.[7]

The primary objective of this study was to investigate the effects of amorphous electrolytes on storage stability in sucrose-based protein formulations. Electrolytes studied were the series of alkali

* *Correspondence to:* Michael J. Pikal (Telephone: +1 860-486-3202).
*E-mail address:* michael.pikal@uconn.edu (M.J. Pikal).

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metal chlorides, that is, LiCl, NaCl, KCl, RbCl, and CsCl. To include divalent cations and anions was judged inappropriate for the purposes of this study because those ions often have protein-specific interactions that may significantly impact protein conformation and therefore impact stability. For example, it has been reported that the storage stability of phosphofructokinase has been improved by adding $Mg^{2+}$,[12] and coagulation factor VIII, somatotropin, and human serum albumin are affected by $Ca^{2+}$, $Zn^{2+}$, and $Cu^{2+}$, respectively.[13] Anions such as $I^-$ and $SCN^-$ tend to impact protein structure according to the Hofmeister series, at least in aqueous solution.[14,15] Hence, we used the chloride anion and monovalent cations, that is, $Li^+$, $Na^+$, $K^+$, $Rb^+$, and $Cs^+$, for the purpose of studying "general effects" of ions on protein stability in freeze-dried solids.

The secondary objective was to better understand the role of electrolytes in stabilization based on two hypotheses of protein stabilization in disaccharide matrices; one commonly discussed mechanism is a thermodynamic stabilization mechanism, acting by stabilizing the native conformation by having the stabilizer hydrogen bond to the protein as does water and is termed the "water substitution mechanism." Here, stabilization results from maintaining "native-like" structure. In the present work, correlations between "structure" and stability were studied using infrared spectroscopy.[20,21] The other is a purely kinetic stabilization mechanism which acts by suppressing molecular mobility, often denoted the "glass dynamics mechanism."[3,5] Mobility was studied by evaluation of both "global mobility," as represented by the structural relaxation time measured by isothermal calorimetry[22,23] and by evaluation of "fast dynamics" by analysis of neutron scattering.[16,17] A number of protein systems have been studied using neutron scattering, by which one can characterize the local dynamics in the glassy solid. Generally, it has been found that this local dynamics or "fast dynamics" correlates well with storage stability of the protein in glassy systems.[16-19] In addition, recently, high-precision density measurement for glassy materials was used to characterize the system free volume, which is potentially a predictor of molecular mobility and therefore, stability.[24,25] Good correlations were found between free volume (i.e., density) and storage stability.[3] Thus, one might expect precision density measurements may be useful in understanding the role of alkali metal salts in stabilization of proteins in the glassy state. To interpret variations in density in terms of free volume, we applied specific volume-based approaches; (1) volume change on mixing and (2) partial molal volume of solute (i.e., salt) to evaluate free-volume changes in glassy formulations on addition of electrolyte. To directly measure size of the "holes" constituting free volume, a positron annihilation lifetime spectroscopy (PALS) study[26-35] was carried out. In addition, we investigated the effect of "post-lyophilization-annealing," which can be executed after the freeze-drying process by heating dried cakes to induce structural relaxation[36,37] or densification,[25] resulting in enhancement of storage stability. This thermal treatment has been found to be effective not only for stabilization of small molecules[36,38] but also for stabilization of an IgG protein,[39] so it was deemed of interest to determine if annealing had any impact on stability of formulations containing low levels of added salt.

As model proteins, bovine serum albumin (BSA) was selected because that is a well-investigated protein, and large amounts of protein were readily available for the experiments. Additionally, as a highly purified protein was desirable in the protein aggregation stability studies, recombinant human serum albumin (rHSA) was used for the purpose of "validation" of the BSA results. Monovalent electrolytes in various amounts were colyophilized with a 1:1 weight ratio of protein to sucrose. We found that small amounts of LiCl and NaCl, that remain amorphous, significantly stabilize these proteins, a result that is at variance with conventional formulation wisdom.

## Materials and Methods

### *Materials*

Bovine serum albumin, sucrose, potassium chloride, rubidium chloride, cesium chloride, potassium phosphate, and sodium sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Lithium chloride, sodium chloride, sodium phosphate monobasic anhydrous, sodium phosphate dibasic heptahydrate, and potassium phosphate monobasic were purchased from Fisher Scientific (Pittsburgh, PA). Recombinant HSA in sterile ultrahigh purity grade was a product of Albumin Bioscience (Huntsville, AL).

### *Preparation of Freeze-Dried Protein Formulations*

BSA and rHSA were dialyzed against 5-mmol/L sodium phosphate buffer at pH 7 using a membrane with MWCO: 6-8,000 (Spectrum Laboratories, Dominguez, CA). Protein concentrations were determined by UV-VIS spectroscopy (Cary 50 Bio, Varian) at 280 nm and then filtered by 0.22 μm polyether sulfone Millex-GP (Millipore, Billerica, MA) to prepare the protein-buffered solutions of BSA and rHSA at 50 mg/mL and 5 mg/mL, respectively. Separately, sucrose and the electrolyte, that is, LiCl, NaCl, KCl, RbCl, or CsCl, were dissolved and also filtered before mixing with the protein solution to prepare the protein-sucrose-electrolyte solutions at 1:1 in weight ratio for protein:sucrose with various weight fractions of electrolyte. All percentages described in this article were the weight fraction or mole fraction relative to total solute components. After dispensed into 5-mL tubing clear glass vials (Schott, Lebanon, PA) and semistoppered with Daikyo Fluoroteck stoppers (West Pharmaceutical, Lititz, PA), lyophilization was carried out using a laboratory freeze dryer (Dura-Stop, SP Scientific). Samples were frozen by cooling to −40°C and holding for 1 h, followed by setting the shelf temperature to −33°C and the chamber pressure to 60 mTorr (8 Pa) for primary drying. Although, given the collapse temperature ($T_c$) is usually about 2°C higher than the glass transition temperature of freeze concentrate ($T_g'$),[40] primary drying conditions for each formulation were designed based on each $T_g'$ in an attempt to maintain the product temperature below the assumed $T_c$, there were some cases where the product temperature did increase above the $T_g'$; yet no sign of collapse or loss of quality was observed even in the "worst case" of 9.0% LiCl-BSA and/or sucrose formulation with $T_g'$ of −47°C, where a maximum product temperature of −37°C was observed. Such unconventional phenomena have also been observed in recent works.[41,42] That is, collapse temperature and $T_g'$ can become high, and one may significantly exceed the collapse temperature measured by freeze-drying microscopy and still not observe collapse when freeze-drying in vials. Secondary drying was conducted at 40°C for 4 h before backfilling with nitrogen and sealing.

All samples freeze dried without visual collapse, and the water content was determined to be less than 1% by Karl Fischer titration (756 KF coulometer, Metrohm).

The glass transition temperatures ($T_g$) and the changes in heat capacity at $T_g$ ($\Delta C_p$) were measured by Modulated DSC (Q1000, TA Instrument) at 1°C/min with modulating ±0.5°C every 100 s. Samples were compressed into thin disks and placed in hermetically sealed aluminum pans. All sample handling was carried out in a dry bag continuously purged with dry nitrogen or air (relative humidity <3%). Glass transition temperatures were taken as the midpoint of the heat capacity shift. To confirm amorphous before and after storage, powder X-ray diffraction (PXRD; AXS D2 Phaser,