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Research Paper

Drying-Induced Variations in Physico-Chemical Properties of Amorphous Pharmaceuticals and Their Impact on Stability II: Stability of a Vaccine

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Objectives. To investigate the impact of drying method on the storage stability of dried vaccine formulations.
Materials and Methods. A sucrose-based formulation of a live attenuated virus vaccine of a parainfluenza strain, with and without surfactant, was dried from by different methods; freeze drying, spray drying and foam drying. Dried powders were characterized by differential scanning calorimetry, specific surface area (SSA) analysis and by electron spectroscopy for chemical analysis (ESCA) to evaluate vaccine surface coverage in the dried formulations. Dried formulations were subjected to storage stability studies at 4, 25 and 37°C. The vaccine was assayed initially and at different time points to measure virus-cell infectivity, and the degradation rate constant of the vaccine in different dried preparations was determined.
Results. SSA was highest with the spray dried preparation without surfactant (~ 2.8 m^2/g) and lowest in the foam dried preparations (with or without surfactant) (~ 0.1 m^2/g). Vaccine surface coverage was estimated based on ESCA measurements of nitrogen content. It was predicted to be highest in the spray dried preparation without surfactant and lowest in the foam with surfactant. Stability studies conducted at 25°C and 37°C showed that the vaccine was most stable in the foam dried preparation with surfactant and least stable in spray dried preparations without surfactant and in all freeze dried preparations regardless of the presence of surfactant. Addition of surfactant did lower the SSA and vaccine surface coverage in freeze dried preparations but still did not improve storage stability.
Conclusions. In drying methods that did not involve a freezing step, good storage stability of Medi 534 vaccine in the dried form was found with low SSA and low vaccine surface accumulation, both of which integrate into low fraction of vaccine at the surface. Ice appears to be a major destabilizing influence.

KEY WORDS: drying vaccines; electron spectroscopy for chemical analysis (ESCA); glass transition temperature (T_g); parainfluenza virus; specific surface area; surface coverage; total surface accumulation; vaccine stability.

INTRODUCTION

Vaccines often are not sufficiently stable in aqueous solution to allow distribution and storage, particularly at room temperature. Therefore, solid dosage forms are produced by drying, typically freeze-drying. The focus of the present work is a study of the impact of variations in the drying process on storage stability of a representative solid vaccine formulation (sucrose-based), with and without surfactant.

Vaccine products are usually designed for mucosal (oral, skin or intranasal) or parenteral delivery (1). Vaccines remain a class of very unstable and vulnerable "active ingredients" in pharmaceutical preparations. The problem arises partly because of their complex structure. In a vaccine product, the "active ingredient" can be live attenuated, genetically modified live, inactivated, synthetic peptide-based, nucleic acid or subunit vaccine (2,3). Additionally, it is a common practice to combine two or more different vaccines in the same formulation.

Vaccines may be of bacterial or of viral origin. Viruses in viral vaccines consist of a nucleic acid genome (RNA or DNA). A viral particle can be as simple as a naked genome or a genome surrounded by a protein-based protective shell (capsid) to form a 'nucleocapsid.' The nucleocapsid may constitute the 'virion' (the complete viral particle) or may be surrounded by an envelope made of a lipid bilayer with enzymes and structural glycosylated or nonglycosylated proteins (2,3).

More than 60% of the vaccines in the market are in solution or suspension dosage form (4), and stabilizers are added to these formulations to improve storage stability (5). When dealing with a formulation of a simple naked genome vaccine in solution, one is usually concerned about chemical

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degradation via hydrolysis of glycosidic bonds (such as those linking DNA bases to deoxyribose backbone or carbohydrate monomers in polysaccharides). However when dealing with more complex vaccines, multiple routes of chemical degradation arise such as hydrolysis reactions involving both glycosidic and peptide bonds (4). Viral vaccines are also sensitive to and respond differently to physical stresses, and commonly viral-based vaccines can be affected differently by solvents, pH, ionic strength or extreme temperature (heat or cold) (4,6,7). These stresses can trigger chemical and/or physical instabilities. Because of the low stability of some vaccines in solution and the high cost of vaccines, drying of vaccine formulations is becoming a common practice. Additionally, the high cost of preservation of vaccine solutions and suspensions due to stringent cold storage requirements is reduced by drying. More than a dozen formulations of lyophilized vaccine products are in the U.S. market (4–6). Active research is also underway to develop alternate drying methods for alternate delivery systems such as spray drying (for inhalation technology) and spray freeze drying or spray coating (for dry powder needle-free injection) (8,9). Drying methods based on the principle of foaming and rapid evaporation of water (foam drying) were developed in the 1960s for preservation of vaccines and bacteria (10–12). Now, these methods or modifications of these methods such as Xerovac (13), foam freeze drying (14) or vacuum drying (15,16) are currently under re-investigation as alternate drying methods.

Depending on the structure of the vaccine, different stresses to which the vaccine is exposed during drying could cause significant in-process loss of activity and compromise storage stability. For example with freeze drying and spray freeze drying, some viral vaccines such as respiratory syncytial virus (RSV), influenza, measles and rubella are unstable during freezing apparently due to exposure to extreme low temperature and/or exposure to ice (6,17,18). Other important stresses include salt concentration, large pH shifts due to buffer crystallization, and possibly mechanical damage to membranes by growing ice crystals (19,20). The degree of damage is temperature and time dependent (6,17, 18), and although proper use of cryoprotectants can minimize freezing damage, significant degradation during freezing is often encountered. In addition, care should be taken with drying methods that generate a large air/water interface, high shear forces and high temperatures such as those characteristic of spray drying (21). Utilizing solvents during drying that may be harmful to vulnerable materials such as might occur with supercritical fluid technology should also be avoided (22). In short, selection of the drying method should be based on sensitivity of the material to any of the unit operations involved in the drying method (that may have a negative impact on the active ingredient), and on the intended mode of administration (i.e. the intended drug delivery system).

Powders produced by different drying methods have different physical properties. Differences in powder properties arise from differences in moisture content, particle size, particle morphology, powder density, specific surface area, surface composition, thermal properties, and other properties (8,9,23–25). Many drug delivery systems require a special drying method to achieve certain required powder characteristics to maximize delivery and bioavailability. For example, dried particles of a vaccine formulation for needle-free injections are required to have a particle size <70 μm to avoid tissue injury, narrow particle size distribution for uniform acceleration and penetration in the tissue, and high density for efficient acceleration by helium gas (9). All these attributes can be achieved by using spray coating or spray freeze drying (8,9). Achieving certain physical attributes for a powder can sometimes be at the expense of other physical properties important to long term storage stability of the vaccine in the dried state. For example, different drying methods result in large differences in residual moisture content, and many studies have shown that excessive moisture can be detrimental to long term storage stability of vaccines in the dried state (8,26,27). Drying also may cause changes in the structure of proteins, such changes may be method-dependent and impact storage stability (27–29). Freeze drying, for example, was shown to cause significant changes in the secondary structure of a tetanus toxoid (27). Although it is difficult to measure structural changes in dried formulations of more complex vaccine systems, one would expect that conformational changes in viral proteins or structural changes in the lipids that constitute the outer envelope may affect in-process and storage stability. Another potentially important factor is that different drying methods result in dried powders of varying specific surface area (SSA) (23,24). Additionally it is documented in several studies—by solid state surface analysis techniques such as Electron Spectroscopy for Chemical Analysis (ESCA)—that the drying method does influence the surface composition of the dried formulation (23–25,30). This probably occurs due to differences in surface activity or diffusion coefficient differences between the active ingredient and the stabilizer (31,32). A combination of high SSA and component separation may result in chemical or composition heterogeneity, that is, the separation of chemical components without phase separation. Since one normally finds that the stability of a vaccine improves as the weight ratio of stabilizer to vaccine increases (5,13,33), it follows that separation of components might have adverse stability consequences. That is, the vaccine near the surface would be much less stable than the overall composition would suggest, and the overall degradation rate could be higher for those samples that have a higher degree of component separation, at least if the relationship between degradation rate and composition is non-linear (34–36).

In this study, we investigated the relationship between drying method and both in-process and storage stability of a vaccine formulation, in the presence and absence of a nonionic surfactant. This study is the first of its kind. The drying methods employed were spray drying, foam drying, freeze drying with a "usual" freezing process and freeze drying with annealing above T_g' (the glass transition temperature of freeze concentrate) during the freezing stage. The base formulation chosen is a simple formulation consistent with good formulation practice (37,38). If one is to understand process effects, it is essential that all processes use the same formulation, and for ease of interpretation, it is also useful if the formulation is relatively free of phase chemistry complexity. The sucrose-based formulation satisfies these criteria. These processes result in powders with the same composition but large differences in thermal history and physical properties. We studied the changes in some physical properties caused by using different drying methods and their

impact on in-process and storage stability. For storage stability studies, bioassays were carried out on the vaccine formulations. Due to a multitude of degradation pathways in complex viral vaccine systems, it is difficult to measure their chemical and/or physical instability. Since the latter instabilities result in the loss of bioactivity, it is common to simply perform biological assays that measure the activity of the viral vaccine, although these assays are not high precision assays. The vaccine of choice in this study (Medi 534) is a genetically engineered bivalent live attenuated virus vaccine candidate for human parainfluenza virus 3 (hPIV 3) (a chimeric bovine/human PIV 3—b/h PIV3) which harbors the genes for F (fusion) and HN (hemagglutinin-neuraminidase) proteins of hPIV3 and expresses the surface glycoprotein of respiratory syncytial virus (RSV) (39,40). PIV 3 is an enveloped RNA virus that belongs to the family Paramyxoviridae, has a molecular weight of $6–8\times10^6$ Daltons and is 150–300 nm in diameter (2,39,40). Primary infection with PIV 3 in young infants and children of less than 2 years of age is a common cause of bronchiolitis. RSV is another paramyxovirus that causes acute lower respiratory tract infections in young children, the elderly, and in immuno-compromised individuals (39,40).

MATERIALS AND METHODS

Materials

Medi 534 (Lot 31) was obtained in 3.6% w/v sucrose and 1 mM phosphate buffer pH 7.0 from MedImmune Purification Development Group (Santa Clara, CA 95050) concentrated 20 fold by tangential flow filtration (TFF) using a 0.45 μm membrane (500 kDa MWCO). In this form, the "vaccine" concentration (i.e. total protein) was ~ 18.6 mg/mL (~0.8 ng viral protein per μg of total protein as determined by SDS gel electrophoresis). Chemicals and excipients were all used as supplied. Sucrose powder was purchased from Pfanstiehl Laboratories (Waukegan, IL). Pluronic F68 (a hydrophilic nonionic surfactant with an HLB value > 25 and a CMC of ~ 8 μM) was purchased from Sigma. 13 mm 2 ml and 20 mm 5 ml type I borosilicate clear tubing glass vials, as well as V2 F451 13 mm and V10 F451 20 mm single-vent Flurotec® (Daikyo Seiko) lyophilization stoppers were purchased from West Pharmaceuticals (Lititz, PA).

Preparation and Drying of Vaccine Solutions

Using a stock solution of 80% w/v sucrose, the ratio of vaccine-to-sucrose was adjusted to 1:100 by weight. Solutions were prepared with and without Pluronic F-68, and the concentration of surfactant was adjusted to 0.2% w/v in the final solution (well above critical micelle concentration). Solutions with different solids content were prepared to enable optimum drying by the various methods, each of which has its own requirements for processing (14,21,39,40). The total solids content in solution was adjusted for each drying method by diluting with de-ionized distilled water. Solids content for solutions to be spray dried was 10% w/v. Solids content for solutions to be freeze dried was 5% w/v. Solids content for solutions to be foam dried was ~ 30% w/v.

Spray Drying

Solutions were spray dried with a Buchi 190 Mini Spray Drier (Flawil, Switzerland) assembled inside an enclosure to maintain low humidity and temperature control. The spray dryer was equipped with a pump for the atomization gas, a pump to feed the solution to be spray dried, a line for liquid nitrogen to maintain temperature and a line for nitrogen gas to maintain humidity. The Buchi atomizer nozzle was replaced by a "custom-built" atomizer nozzle designed to produce high pressure effervescent atomization (19,41). The solution feed rate was 2 ml/minute, the inlet temperature was 40°C and the outlet temperature was 30°C. Relative humidity in the chamber was maintained below 5%, atomizing gas was nitrogen and atomizing pressure was 1,300 psi. Spray dried powders were subjected to a post drying time of 30 min in the collector tube at 34–35°C.

Moisture content in spray dried samples, as measured by Karl Fischer titration, ranged between 4 and 5% w/w (results not shown). Since the target moisture content was ~ 1–2% w/w in all dried preparations, spray dried powders were subjected to vacuum drying to reduce the moisture levels in the samples to the target 1–2% w/w. Vacuum drying was performed in a Virtis Genesis freeze dryer equipped with a condenser model 25EL (Gardiner, NY) at a shelf temperature of 35°C and a chamber pressure of 25 mTorr for 1 hour. Vials were sealed under nitrogen at the end of the drying cycle.

Freeze Drying

Solutions were freeze dried using a FTS Systems Lyostar II (Stone Ridge, NY). Solutions were lyophilized in 5 ml serum vials with a fill volume of 1 ml. Solutions were first frozen by placing on shelves pre–cooled at −45°C and holding at −45°C for 90 min. It is conventional when freeze drying vaccines to load on pre-chilled shelves. Furthermore, since the fill depth was very small (<0.5 cm), the degree of supercooling was relatively uniform throughout the vial. Shelf temperature was raised to −30°C at a heating rate of 1.3°C/minute for carrying out primary drying (below the collapse temperature). Shelf temperature was held at −30°C for 48 h during primary drying while maintaining a chamber pressure of 75 mTorr (for sublimation of ice). Average product temperature during primary drying was −37 to −38°C. The end of primary drying was marked by a rise in product temperature above the shelf temperature with a concurrent decrease in dew point to a constant value. For secondary drying (evaporation of water in the amorphous phase), shelf temperature was heated at 0.1°C/minute to 0°C. Temperature was maintained at 0°C for 30 min followed by another heating step at 0.1°C/minute to 10°C. Temperature was maintained at 10°C and 75 mTorr for 280 min. While 10°C is not a typical temperature to carry out secondary drying, selection of this temperature was designed to control moisture content in the final product at an intermediate value of ~ 1–2 % w/w.

Annealing Followed by Freeze Drying

Some vaccine solutions were annealed in the frozen state prior to primary drying to produce samples of lower specific

surface area than the regular freeze drying cycle and to investigate the impact of annealing on vaccine stability.

Annealing was done at −15°C (above T_g' of ~ −35°C) for 33 h. Shelf temperature was lowered again to −45°C and samples held to equilibrate at this temperature for 90 min again prior to primary drying. Conditions for primary and secondary are the same as under '*Freeze drying.*'

Foam Drying

Solutions were foam dried using a Virtis Genesis freeze dryer equipped with a condenser model 25EL (Gardiner, NY). Solutions were foam dried in 2 ml serum vials with a fill volume of 0.3 ml. Vials containing solutions were placed on shelves at 15°C and kept for 15 min to equilibrate. Foaming was initiated by reducing the chamber pressure below the vapor pressure of the solution to 50 mTorr at a shelf temperature of 15°C. Product temperatures initially decreased to ~ −20°C, due to evaporative cooling, and remained at this temperature during most of primary drying. None of the solutions froze, presumably due to very high solute content. The drying process is considered to be composed of two drying phases: primary drying and secondary drying. Primary drying was done at a shelf temperature of 15°C for 1 h and a chamber pressure of 50 mTorr. This step is a much shorter step than primary drying in a freeze drying cycle and water is removed by rapid evaporation. Secondary drying, a step to remove remaining moisture in the amorphous phase by evaporation, was done by heating shelf temperature to 33°C at a rate of 1°C/minute and maintained at that temperature for 48 h at 25 mTorr. The unusually longer secondary drying time in foam drying is probably attributable to the very low specific surface area of the foams.

Karl Fisher Moisture Determination:

Residual moisture content of all formulations was measured by direct injection using coulometric Karl Fischer titration (Denver Instrument Company). Powders were weighed and filled into vials in a glove bag where a low relative humidity (RH) was maintained (<2%) by flushing with dry nitrogen. Powders were dissolved in 2 ml of low moisture formamide and 0.5 ml of the solution was injected. Blank corrections were applied. Standard deviation from replicate measurements was not more than 0.1% H_2O.

Modulated Differential Scanning Calorimetry (MDSC) Studies

MDSC studies were performed to determine the glass transition temperature (T_g) of the different dried formulations to allow proper design of storage stability studies, and to detect any differences in thermal behavior that may exist between different formulations due to differences in drying method or surfactant addition. A Q-1000 Differential Scanning Calorimeter equipped with a DSC refrigerated cooling system, TA instruments, Inc. (New Castle, DE) was used. A mass of 5–15 mg of the powdered sample was filled and pressed into compact pellets in aluminum DSC pans and hermetically sealed using a sample encapsulation press. The entire process was performed in a glove box, with RH maintained under 2%. Nitrogen was used as a purge gas for MDSC at 30 cm^3/minute. All MDSC measurements were done at least in triplicate. The MDSC protocol was as follows:

- ➢ Equilibrate at 0°C.
- ➢ Modulate +/− 1°C every 120 s.
- ➢ Ramp 2°C/minute to 100°C.

Specific Surface Area (SSA) Measurement

Brunauer Emmett Teller (BET) Specific Surface Area (SSA) analysis was performed using a Micromeritics, Flow-Sorb II 2300 BET surface area analyzer (Norcross, GA). Sample size was at least 100 mg. Powder samples were degassed for at least 3 h in the Flow Prep oven at 25°C using krypton purge prior to measurement. Single point calibration was performed prior to taking surface area measurements using 0.1 mol krypton gas. Percent relative standard deviation (%RSD) for SSA measurements was not more than 2.5.

Electron Spectroscopy for Chemical Analysis (ESCA) studies

Each component in the powder is characterized by the specific ratio between its elements. ESCA, a widely used surface analysis technique, analyzes the elemental composition at the surface of solids with an analysis depth of ~ 50 Å (23). The area under the peaks is a measure of the relative amount of each atom. (42). From an analysis of the relative amount of the different elements in the pure components and in the powder using ESCA, one can estimate the percentage of powder surface covered with each chemical component (i.e., estimate vaccine surface coverage) (25,30).

ESCA was used to probe elemental composition of the surfaces of dried formulations. Survey spectra were collected with a VG ESCALAB MK II series Spectrometer (pass energy=60 eV). An $Al_{k\alpha}$ radiation (emission current of 11 kV anode voltage and 34 mA emission current) from Al Kα X-ray source was directed onto a circular region of approximate diameter of 3 mm. Pressure was maintained in analysis chamber at less than 10^{-8} Torr. Measurements were made on three samples. The peak areas from the spectra were converted to elemental atomic concentrations on the surface of each sample. Percent relative standard deviation (%RSD) for ESCA measurements ranged between 5 and 13.

Bioassay of Medi 534

Bioactivity assay of Medi 534 was performed in different formulations to (a) assess and compare vaccine recovery after drying by different methods, and (b) assess differences in storage stability of vaccine in different formulations.

The method involves primarily inoculation of 100 µl of a Vero cell suspension by different dilutions of the virus (10-fold serial dilutions from 10^{-1} to 10^{-8}) in cell growth medium using 96-well flat bottomed tissue culture grade microliter plates (ten wells for each dilution, 100 µl/well). This was followed by incubation of the plates at 33±2°C, 5±1% CO_2 for 4 days. At the end of incubation, virus-infected cell plates were fixed with cold 4% paraformaldehyde, washed, followed

by addition of a primary antibody (Numax, a humanized monoclonal antibody anti-PIV virus) to the plates and a second incubation at 37±2°C for 60 min. A secondary antibody was added (50 µl of Alexa Fluor 488-conjugated goat anti-human IgG) to each well and the plates incubated at 37±2°C for 60 min. The plates were read by a Wallac Victor ^{2} 1420 multilabel Counter to obtain total fluorescence intensity (FU) for each well, the data processed and converted to a $TCID_{50}$ (50% tissue culture infectious dose) titer by an immunofluorescence (IF)-TCID macro. $TCID_{50}$ is the dilution at which 50% of the test cell monolayers show evidence of infectivity by the viral vaccine (43). The macro determines the number of cytopathic effect (CPE) positive wells, and then converts CPE to $TCID_{50}$ titer using the Karber method (44). The titer is expressed as the 50% median effective dose $TCID_{50}$/ml. At least two samples of each formulation were assayed, and each sample was assayed in duplicate. Inter-vial variability (i.e. short term variation) for the bioassay method ranged between 2–6% (%RSD) while inter-day variability (i.e. long term variation for control samples) ranged between 3–8% (%RSD).

Storage Stability Studies

All vaccine formulations were subjected to stability studies at 4 and 25°C for up to 20 weeks. Bioassays were carried out on the formulations for up to 20 weeks. Additionally, some formulations were subjected to stability studies at 37°C for 1 and 2 weeks. Additional details are provided in the results and discussion section.

RESULTS

Moisture Content and Crystallinity

Initial moisture level in all dried preparations ranged between 0.8 and 2.3 % w/w, as measured by Karl Fisher titration (Table I). Additionally, all samples were non-birefringent when examined using polarized light microscopy, indicating complete absence of crystallinity.

Thermal Analysis

The thermal properties of the dried powders were analyzed by MDSC. Two major transitions were observed in the MDSC thermograms of preparations with and without surfactant; a glass transition event with an associated enthalpy recovery (endothermic), followed by a crystallization event. Additionally in the presence of surfactant, a small glass transition event preceding the main glass transition was observed with the freeze dried preparations (Fig. 1), indicating a possible phase separation with a surfactant-rich phase (MDSC scans of pure Pluronic-F68 showed an apparent T_g of ~ 40°C) and a sucrose-rich phase (higher T_g) (45,46). Similarly, a slight phase separation was observed with the foam dried preparation, but not with the spray dried preparation (Fig. 1).

Glass transition temperature (midpoint)—T_g—values as a function of moisture content for the 1:100 vaccine surfactant-free freeze dried formulation are shown in Fig. 2 as a straight line. To generate these samples, the freeze dried vaccine formulation was subjected to different secondary drying times, leading to a lower moisture content as drying time increased. T_g and ΔH at each moisture content were measured by MDSC from the reversing and non-reversing heat flow signals, respectively. As expected, T_g values decreased as moisture content increased. Over this limited range of moisture content, T_g varied linearly with residual moisture, and the straight line in Fig. 2 represents the linear fit. T_g values (midpoint) ranged between 47–60°C for surfactant-free vaccine preparations, and ranged between 50–62°C for vaccine preparations with surfactant. T_g was measured mainly to allow proper design of storage stability studies. The measured T_g values of the various dried vaccine powders were compared to the linear fit for T_g-moisture data in Fig. 2. Within reasonable experimental error, T_g data for all dried preparations were consistent with the linear fit, except possibly for one point (surfactant-free foam dried material) which seems to have a somewhat higher T_g than one would expect from its moisture content. Whether or not this observation is real or an artifact is unknown.

ΔH values ranged between 7.5–9 J/g for all foam dried preparations, and ranged between 1.6–3 J/g for the surfactant-free spray dried and lyophilized preparations. For the spray dried preparation with surfactant, ΔH was ~5 J/g. We were not able to effectively measure a ΔH for the lyophilized preparations with surfactant due to overlap of the enthalpy recovery endotherm with the crystallization peak (not shown). Clearly, the enthalpy recovery values do vary with the preparation technique, but the meaning of this observa-

Table I. Moisture Content, Specific Surface Area (SSA) Measurements and Atomic % of N Atom on the Surface of Dried Vaccine Formulations (The Raw Signal from ESCA Measurements for the N Atom)

Formulation	Treatment	Moisture Content (% w/w)	SSA (m^2/g)±SD[a]	% Surface N as Measured by ESCA (from ESCA N Raw Signal)±SD
1:100 (Vaccine: Sucrose)	Freeze dried	1.0	1.42±0.03	2.05±0.27
	Annealed/ Freeze dried	2.2	0.71±0.03	0.70±0.03
	Spray dried	1.0	2.80±0.07	2.18±0.22
	Foam dried	2.2	0.13±0.02	0.88±0.05
1:100 (Vaccine: Sucrose) with Pluronic F-68	Freeze dried	0.8	1.15±0.02	0.77±0.04
	Annealed/ Freeze dried	1.9	0.64±0.03	1.08±0.15
	Spray dried	1.9	1.26±0.03	1.17±0.10
	Foam dried	2.3	0.10±0.01	No N signal detected

[a] SD: standard deviation

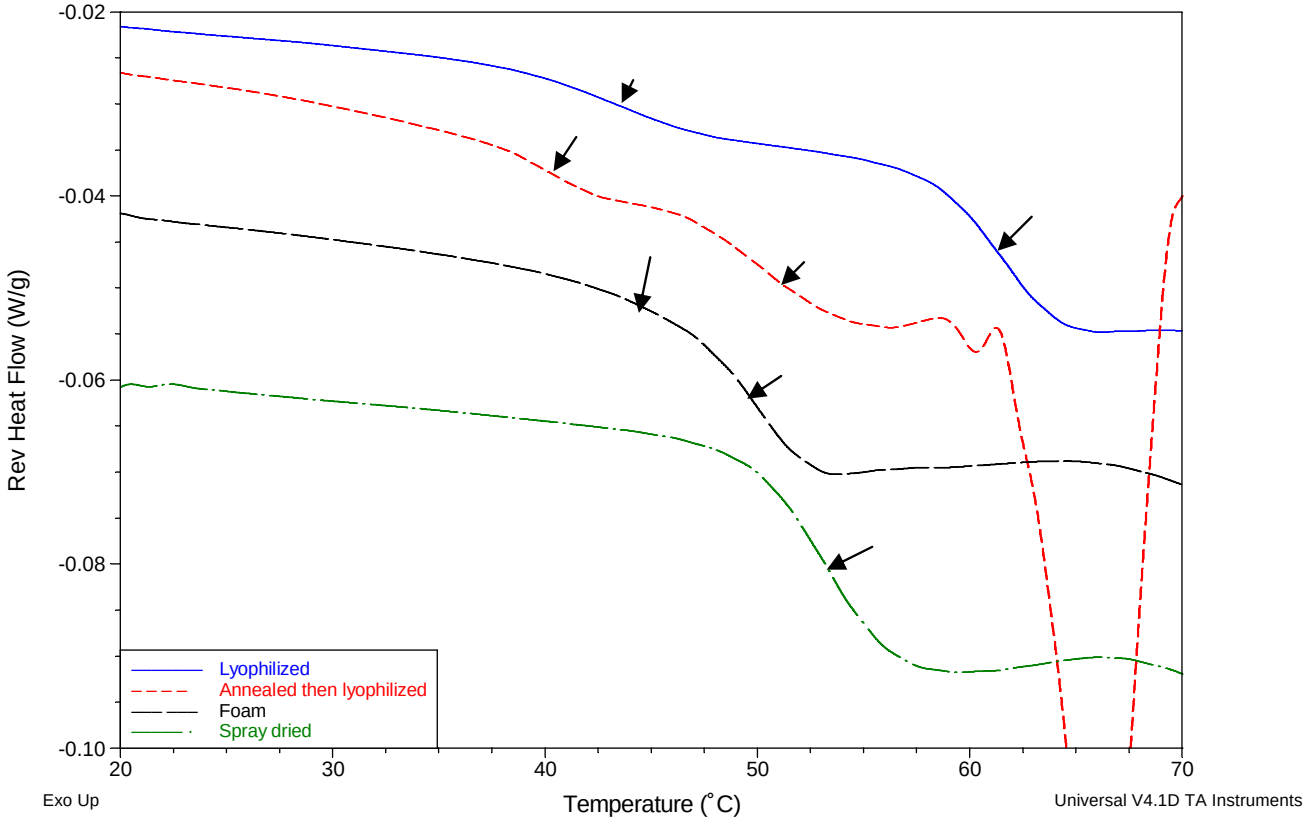

Fig. 1. Reversing heat flow signals from MDSC thermal traces of preparations containing surfactant showing the main glass transition event and the differences between their glass transition temperatures (*arrows pointing to midpoint of minor and major transitions*).

tion is not completely clear. Greater enthalpy recovery means greater enthalpy relaxation during processing, and nominally, for materials identical in composition, greater enthalpy recovery for a sample would mean that sample has a longer structural relaxation time (i.e., lower mobility) than the comparator. However, the moisture contents are not all identical, so the simple interpretation offered may not be completely correct. No consistent trends in other glass transition parameters (width of the glass transition temperature, ΔT_g, and the heat capacity change at T_g , ΔC_P) between the drying methods were evident.

SSA and Surface Analysis

Results for SSA measurements on all preparations are reported in Table I. In surfactant-free preparations, SSA was highest in spray dried preparation and extremely low with the foam dried samples. Intermediate values for SSA were observed with the freeze dried preparations. Annealing for 33 h at −15°C prior to primary drying caused roughly a 2 fold reduction in the SSA of the freeze dried formulation (Freeze dried vs. Annealed/Freeze dried). Annealing is a process by which samples are held at a temperature between the ice melt temperature and the glass transition temperature of the freeze concentrate (T_g') for a period of time. Annealing allows larger ice crystals to grow at the expense of small ones in a process called Ostwald ripening (47). Therefore annealing should have dramatic effects on SSA of the ice crystals, and therefore the dried solid (48,49). Upon addition of the surfactant, SSA's of spray dried and freeze dried preparations decreased and were comparable. Again, SSA was less with the preparation annealed prior to freeze-drying and was extremely low with the foam dried formulation.

Results for ESCA analysis of vaccine powders are also summarized in Table I. In a two-component system of A and B, it is possible to determine the surface concentration % (w/w) of the powder with regard to each component A and B (i.e., surface coverage) (31,32,50). Even though we did not have knowledge of the exact elemental composition of the vaccine, the relative abundance of N atom in the vaccine (the source of which is component proteins of the vaccine) allowed the use of N peak as the marker peak for the presence of the vaccine on the surface of the dried formulations (Fig. 3). Other excipients (Pluronic F-68 and sucrose) lack N atom. From a knowledge of the SSA and %N on the surface of the dried formulations (% w/w), one can qualitatively estimate how much of the total vaccine in the formulation has accumulated at the surface of the dried powder (i.e., total surface accumulation) (34,35). Assuming the vaccine composition is identical to a typical protein (~14–15% N), then one would expect the % surface N in a homogeneous 1:100 vaccine formulation to be ~ 0.2–0.3%. Therefore, ESCA results in Table I suggest that none of the dried formulations were homogeneous. In fact, almost all dried formulations showed a surface excess of the vaccine. In surfactant-free preparations, spray dried and freeze dried preparations showed higher vaccine surface coverage while foam dried and freeze dried preparations annealed prior to primary drying showed lower vaccine surface coverage. Based on SSA and ESCA results, therefore, the spray dried

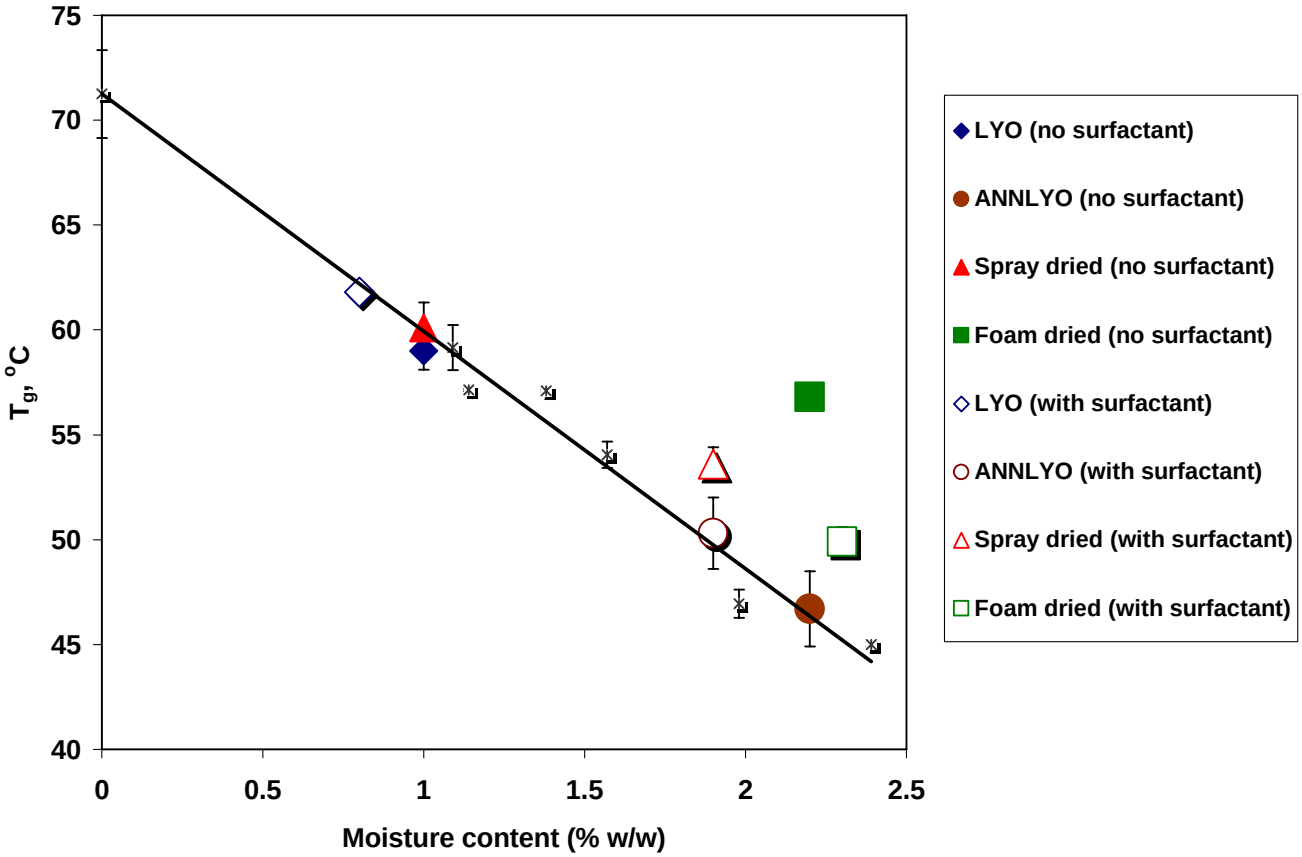

Fig. 2. T_g of dried powders of the vaccine as a function of moisture content, compared to the straight line fit from a regression analysis of T_g—moisture data for a lyophilized vaccine formulation with different moisture levels.

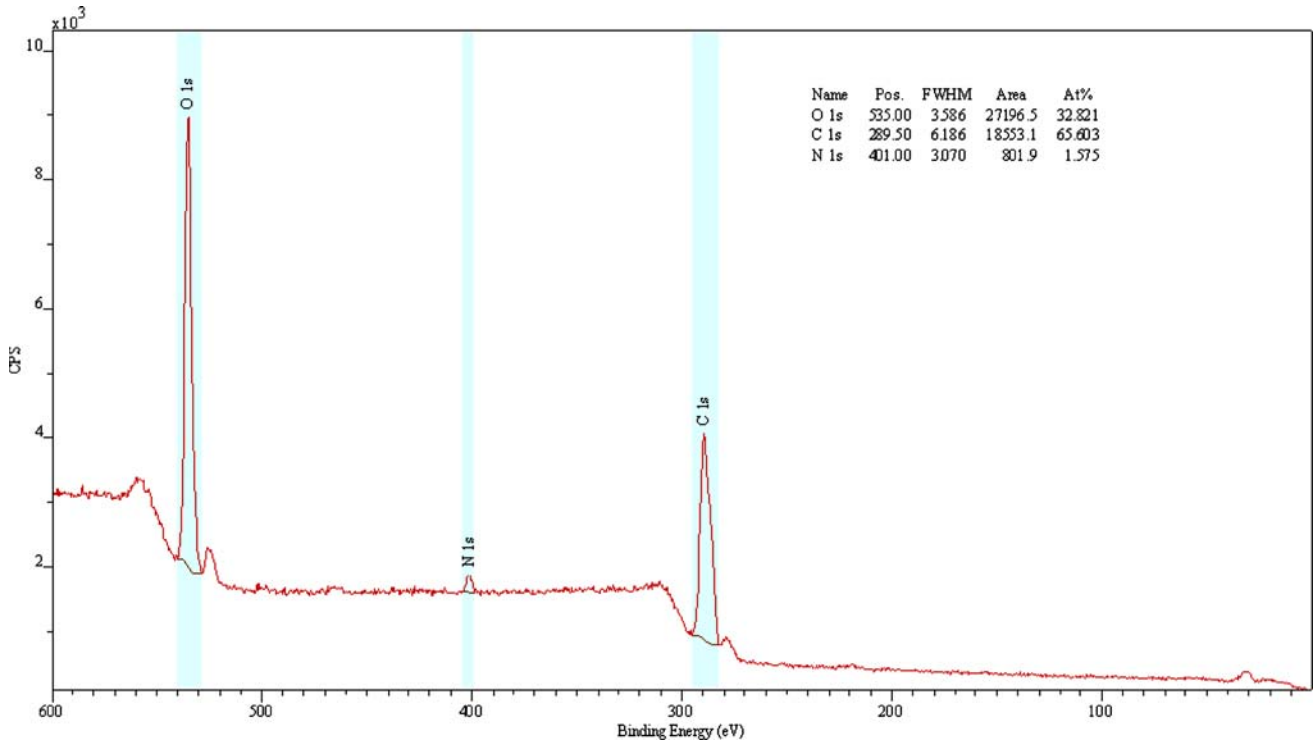

Fig. 3. ESCA scan for a freeze dried vaccine formulation without surfactant showing the count per second (CPS) as a function of kinetic (binding) energy for ejected electrons from each characteristic atom. The sensitivity of the instrument for any particular element (At%) is $\leq 0.5\%$.

preparation has the highest total vaccine surface accumulation while the foam dried preparation has the least. Upon addition of the surfactant, a decrease in vaccine surface coverage was observed in freeze dried, spray dried and foam dried preparations as indicated by a significant decrease in % of atomic surface N. While our results are limited to the study of one non-ionic surfactant, our observations are consistent with observations made by other researchers on the effect of nonionic surfactants on surface coverage of proteins in spray dried and freeze dried formulations (23,25,30,51). Furthermore, no N peak was detected by ESCA on the surface of the foam dried preparation with the surfactant. Therefore, along with a reduction in SSA in the presence of the surfactant, one would predict extremely low (if any) total vaccine surface accumulation in the latter foam. Based upon SSA and ESCA results, vaccine surface accumulation in preparations containing the surfactant would be in the following order:

Spray dried, Freeze dried > Annealed/Freeze dried ≫ Foam dried.

In-process Stability

Recovery of the vaccine after drying was dependent on the drying method as evidenced by a greater recovery in spray and foam dried preparations versus all freeze dried preparations, regardless of the presence of the surfactant (Table III). It seems clear that ice is a major destabilizing "stress" for the vaccine.

Storage Stability

Kinetics describing the loss of activity of virus showed at least biphasic behavior and appeared to approach a nearly constant value at longer times during storage stability studies (Fig. 4). Degradation kinetics in amorphous pharmaceuticals are frequently described by "square root of time" or "stretched exponential" kinetics (52–54). Multiple linear regression was carried out using a general linear model (GLM) to plot and fit the assay data with various models linear in a fractional power of time. F-values for each GLM model with different time exponent showed that the model with square-root of time kinetics fits the data most appropriately, confirming square root of time dependence (see Table II and Figs. 5 and 6). Viral potency (infectivity) measured with time, log P, thus followed the linear equation (52):

$$\log P = \log P_o + k\sqrt{t} \quad (1)$$

where log P_o is the initial potency and k is the apparent rate constant for degradation. Therefore from a plot of log activity versus square root of time, k at each storage temperature was determined from the slope of the straight line for each preparation (units of degradation rate are log activity/week$^{0.5}$). Even though samples stored at 4°C were consistent with either exponential kinetics or square root of time kinetics during the short 12 week stability study period, to maintain consistency in data analysis, the 4°C data were also analyzed in terms of Eq. 1.

Storage at 4°C for 12 weeks was not sufficient to distinguish between stability of the different preparations. However, storage at 25°C showed significant differences between the stability of the vaccine in the different preparations (Table III, Fig. 6). In the surfactant-free preparations, the foam dried preparation showed superior stability relative to the other preparations, and no significant differences were observed between the other preparations. Upon addition of the surfactant, there was a significant improvement in the stability of the vaccine in the spray dried and the foam dried preparations but no corresponding improvement in stability of any of the freeze dried preparations (Table III, Fig. 6). The order of stability at 25°C for spray dried and foam dried preparations in the presence and absence of the surfactant was:

Foam dried (with surfactant) > Spray dried (with surfactant), Foam dried (without surfactant) > Spray dried without surfactant.

Results from the 37°C storage stability studies were similar to 25°C results.

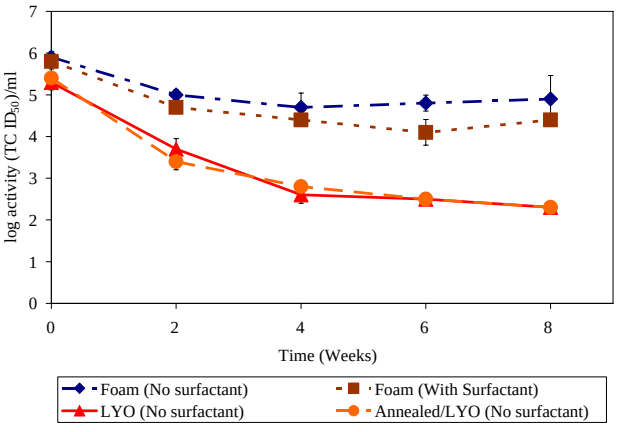

Fig. 4. Plots showing progress of stability studies for some representative preparations at 25°C (Viral potency vs. time) for 8 weeks.

Table II. F-values[a] for Fitting log $P = \log P_o + k.t^n$ to Storage Stability Data of Vaccine Formulations Using Different Exponents for Time (n)

n	F-value of Model at 25°C	F-value of Model at 37°C
1	995.9	455.2
0.25	1461.1	572.6
0.5	1734.4	638.2
0.6	1572.8	623.2
0.7	1391.9	

[a] F value is a statistical value calculated by SAS software when all data are collectively fitted to a general linear model. It indicates the goodness of fit for all data to the general model used. The higher the F-value, the more appropriate the model is. In our case, within a confidence limit of 95%, F-value increased as the data became more linear with the fraction of time. F-value was highest with square root of time at 50°C. F-value at 40°C data was high at both $t^{0.4}$ and $t^{0.5}$. However, to be consistent in treatment of data, we selected $t^{0.5}$. F-stat is computed using the following ratio:
Mean sum of square of an effect / Mean Square of Errors

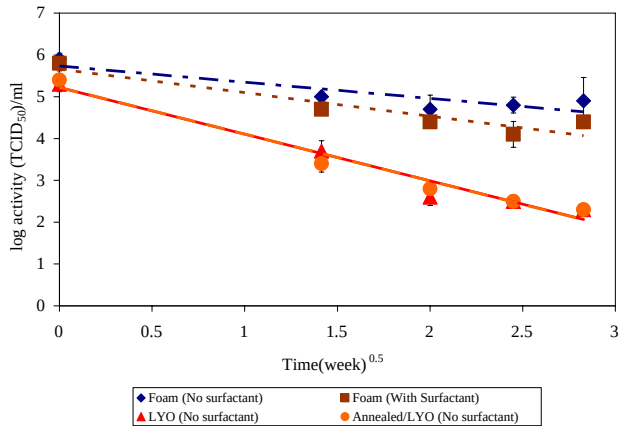

Fig. 5. Plots showing progress of stability for the same representative preparations (in Fig. 4) at 25°C (viral potency vs. square-root of time) for 8 weeks.

DISCUSSION

Role of Ice and Air-water Interface in Vaccine Stability

Vaccine in lyophilized preparations suffered a greater loss in initial activity (in-process damage) than vaccine in either spray dried or foam dried preparations. It seems clear that the vaccine undergoes damage during freezing, and that the vaccine was not as vulnerable to the air–water interface (encountered during foam drying but particularly during spray drying). With preliminary freeze–thaw studies (results not shown), a greater loss in activity was observed with a slow thaw, as opposed to a fast thaw procedure. We are not certain of the mechanisms operating here, but note that similar observations were made in freeze–thaw studies with different enzymes such as catalase (55) and lactate dehydrogenase (56).

Unlike with spray dried and foam dried preparations, no improvements were observed in the storage stability of the vaccine in lyophilized formulations upon addition of the surfactant. This suggests that while this surfactant was effective in preventing structural damage at the air–water interface (in spray drying and foam drying), it was not effective at an aqueous-ice interface (in freezing).

While it is clear that exposure to ice is damaging, prolonging the exposure to ice by annealing prior to primary drying did not seem to add an additional stress to the vaccine. Both in-process stability and storage stability of the vaccine in the lyophilized formulations annealed during freezing were not significantly different from stability in the same lyophilized formulations without an annealing treatment. Therefore, residence time in a freeze concentrate above T_g' with ice present does not seem to be either a critical in-process or storage stability factor. Hence, the environment during exposure to ice is also a critical factor. For example, while it is surprising and very important that "annealing" during the freezing process indeed does no damage beyond that done by a normal freezing process, the environment during annealing is quite different than the environment during the first portion of the freezing process. Immediately after the ice first forms, the system is a mixture of ice and moderately concentrated solution at a temperature just below 0°C, with a reasonably low viscosity. However during the annealing process, the concentration of solutes is quite high, with a very high viscosity. In other studies, annealing did not impact either the in-process stability or the storage stability of freeze dried formulations of Medi 522 (an IgG_1) (57) and methionyl-human growth hormone (Met-hGH) (34). We also note that protein secondary structure in Met-hGH and IgG_1 lyophilized formulations was not impacted by annealing. Although ice may be thermodynamically destabilizing, perhaps the mobility during annealing was not sufficiently high to allow major structural alterations (i.e., protein unfolding). In this regard, we note that studies by Tang et al. (58) showed that even well above T_g', protein unfolding may be very slow on the time scale of freeze drying (calculations showed that protein unfolding may require a timescale of years in disaccharide freeze concentrates!). Furthermore, as the concentration of sucrose increased in the freeze concentrate, the cold denaturation temperature was dramatically lowered. The point is that not only time but also the dynamics and perhaps even the thermodynamic stability of the system that is controlling stability.

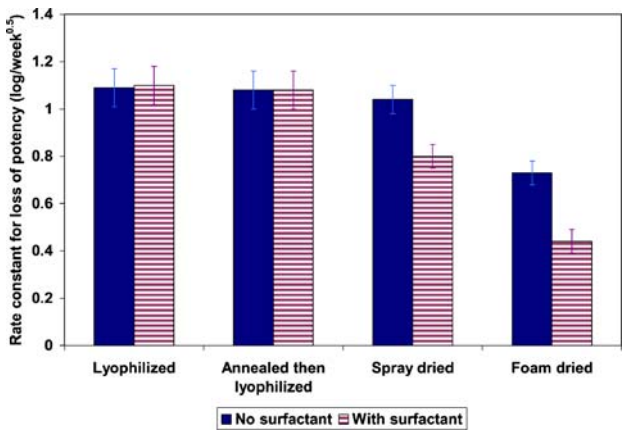

Fig. 6. Rate constant for loss of potency of vaccine in different preparations at 25°C from square-root-of-time kinetics.

Role of SSA and Vaccine Surface Coverage in Vaccine Stability

The product of SSA and % surface N usually gives a qualitative estimate of the total vaccine surface accumulation. As can be seen from Fig. 7, improvement in storage stability of the vaccine at both 25°C and 37°C correlates reasonably well with total vaccine surface accumulation, provided that the drying method did not involve prolonged exposure to ice (i.e., lyophilization). The rank order for storage stability at 25°C and 37°C was consistent with the rank order of the product of both % surface N and SSA, with low surface coverage and low SSA being associated with a small rate constant. Similarly, Sane et al. (59) argued that the storage stability of rhuMAb formulations were related to SSA, as evidenced by greater storage stability of lyophilized formulations, as compared to spray dried formulations. Also, Webb et al. (23) data suggest a correlation between storage stability of recombinant human interferon-γ (rhIFN-γ) in trehalose and total protein surface accumulation. Different drying methods used in both these studies did not result in significant differences in protein secondary structure among the drying methods, and none of the proteins studied were vulnerable to the ice-water interface.

Table III. Recovery of Vaccine after Drying and Rate of Loss of Viral Potency for Storage Stability at 4, 25 and 37°C for Dried Formulations of Medi 534

Formulation	Drying Process	Initial Loss in Viral Potency (log $TCID_{50}$/ml)[a]	Rate of Loss of Potency (k) (log/week$^{0.5}$)±SE[b]		
			4°C	25°C[c]	37°C
1:100 (Vaccine: Sucrose)	Freeze dried	1.4	0.25±0.07	1.09±0.08	2.65±0.30[d]
	Annealed/ Freeze dried	1.3	0.36±0.07	1.08±0.08	2.78±0.30[d]
	Spray dried	0.8	0.20±0.06	1.04±0.06	2.76±0.19
	Foam dried	0.8	0.18±0.06	0.73±0.05	2.31±0.19
1:100 (Vaccine: Sucrose) with Pluronic F-68	Freeze dried	1.3	0.26±0.07	1.10±0.08	
	Annealed/ Freeze dried	1.3	0.41±0.07	1.08±0.08	
	Spray dried	0.4	0.24±0.06	0.77±0.05	2.25±0.19
	Foam dried	0.9	0.24±0.06	0.44±0.05	1.20±0.19

[a] Initial loss in viral potency is the difference between recovered viral potency after drying and initial viral titer before drying (~ 6.7 log $TCID_{50}$/ml).
[b] SE: Standard Error
[c] Storage stability studies at 25°C were conducted for 20 weeks (for Spray dried and foam dried preparations) and for 8 weeks (for Freeze dried and Annealed/Freeze dried preparations). At 8 weeks, viral potency in all freeze dried preparations dropped below detection limits of the assay.
[d] k value was determined from only two time points: initial and 1 week. Beyond 1 week, viral potency in freeze dried preparations dropped below detection limits of the assay.
Energy of activation—E_a—(from three temperatures studied) for foam dried preparation with surfactant was calculated to be ~ 8 Kcal/mole. Other preparations (spray dried with and without surfactant, foam dried without surfactant) had higher E_a (~13 Kcal/mole).

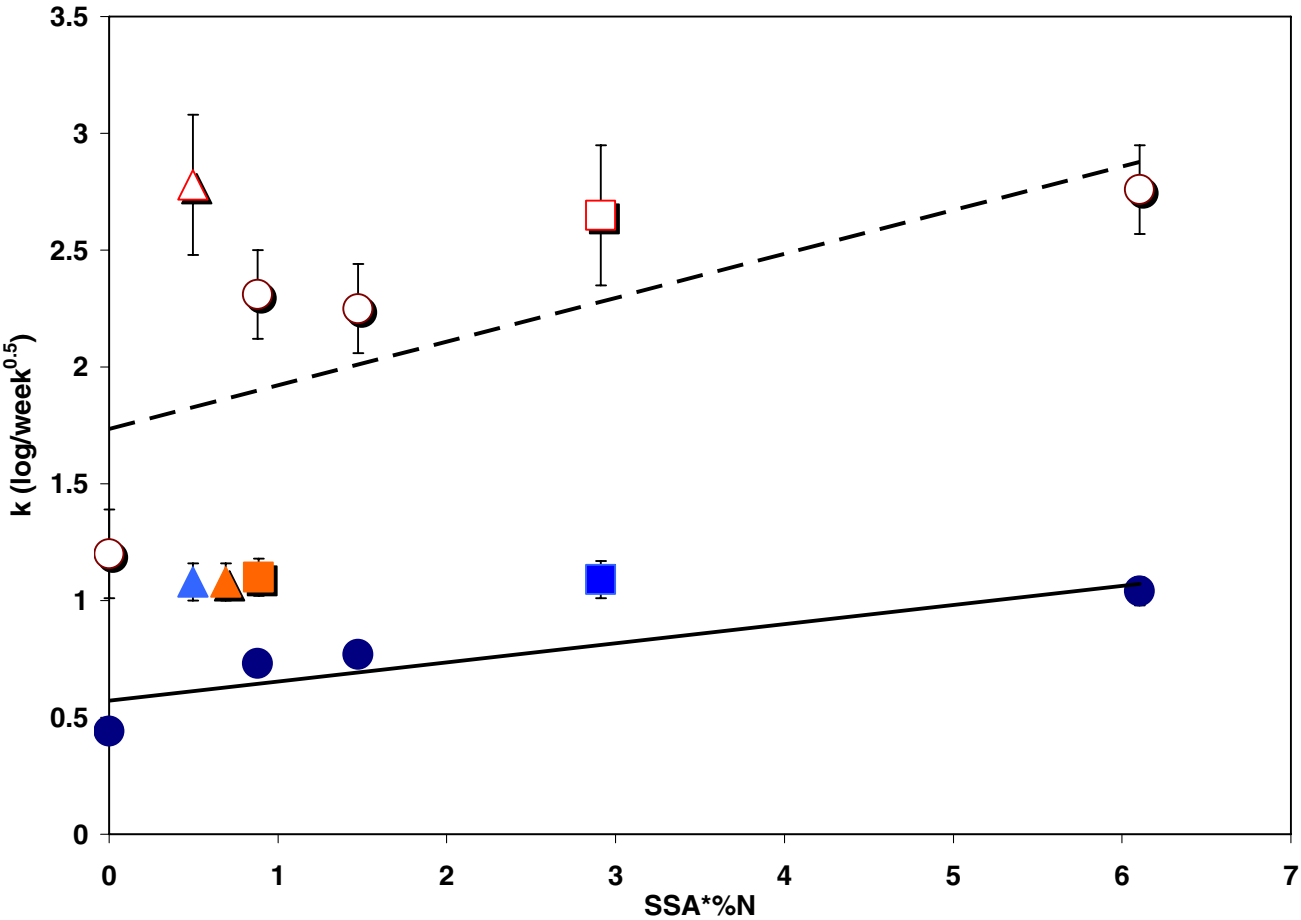

Fig. 7. Rate of loss of activity of vaccine (k) at both 25 and 37°C as a function of a qualitative estimate of total vaccine surface accumulation (estimated from the product of SSA and % surface N). *Closed symbols* are for 25°C stability studies (*closed squares and triangles*=freeze dried preparations; *closed circles*=non-freeze dried preparations), *open symbols* are for 37°C stability studies (*open triangle and square*=freeze dried preparations; *open circles*=non-freeze dried preparations).

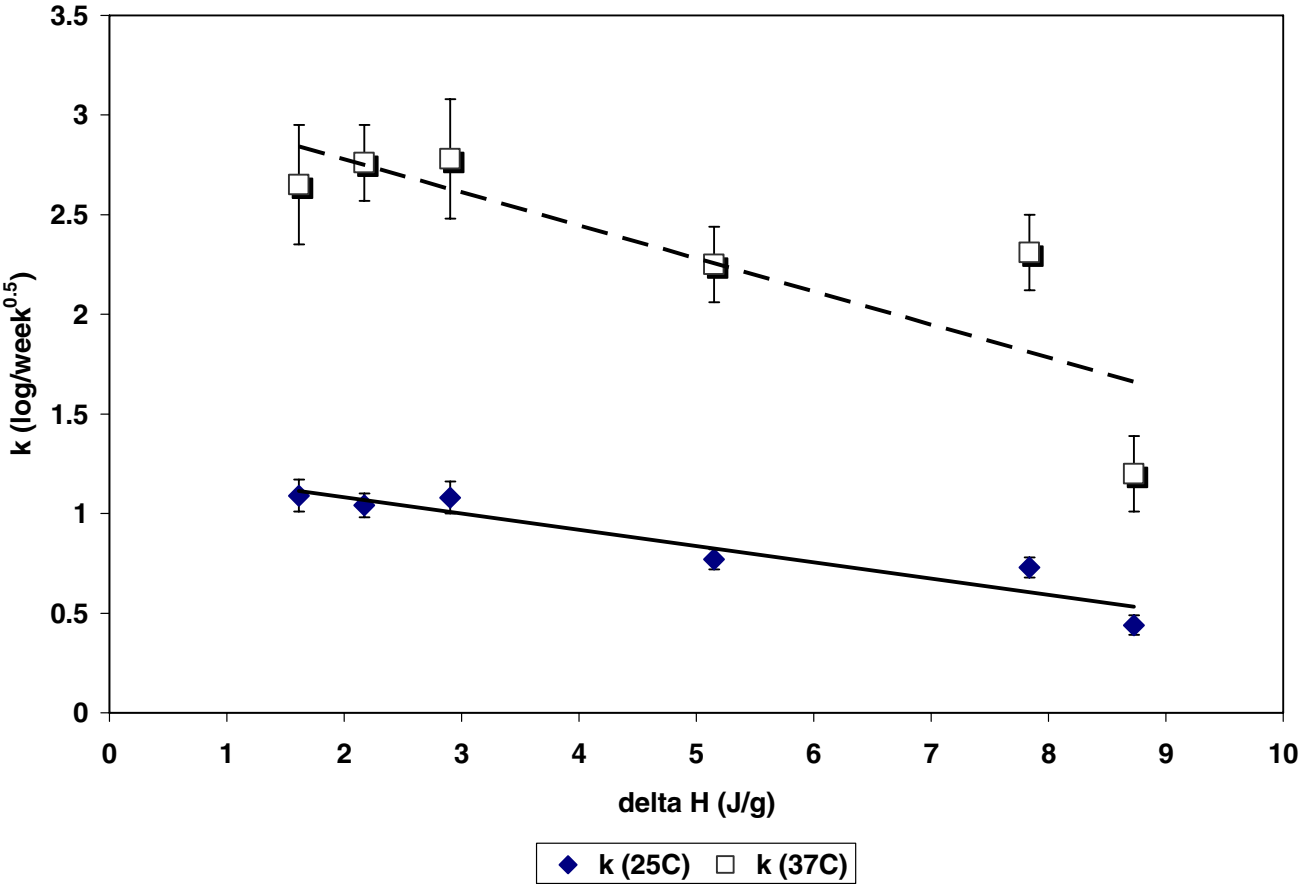

Fig. 8. Rate of loss of activity of vaccine (k) at both 25 and 37°C as a function of enthalpy recovery (ΔH).

Role of Enthalpy Recovery in Vaccine Stability

Due to differences in thermal history for each drying method, the drying method is expected to affect the energy state and free volume of the material, and consequently affect molecular mobility in the resulting glass. We therefore expect that drying methods that utilize a high temperature during drying and/or long drying times close to T_g would result in pronounced physical aging and hence produce a significant increase in the structural relaxation time. While we did not measure structural relaxation times in our studies, we did determine enthalpy recovery upon a DSC scan. As discussed earlier, enthalpy recovery is a result of "global" molecular mobility (motions that result from translational and rotational motions, and therefore strongly influence the diffusion of reactive molecular species), and at least in systems of constant chemical composition, would be expected to correlate directly with structural relaxation time of the sample. Thus, a high enthalpy recovery may correspond to a system which has been exposed to high temperature and has therefore annealed to a lower energy state and is therefore characterized by a long structural relaxation time (60).

The measured enthalpy recovery differed from one drying method to the other. Moreover, storage stability of the vaccine is well correlated with enthalpy recovery at both 25°C (R^2=0.90) and 37°C (R^2=0.71) (Fig. 8), regardless of exposure to ice (i.e. lyophilization). Foam dried preparations showed significantly higher enthalpy recoveries than other drying methods, regardless of the presence of surfactant, indicative of lower molecular mobility, and these samples were the most stable. It is also significant to note that a poor correlation was observed between moisture content and storage stability for the same formulations (i.e., those plotted in Fig. 7) at both 25°C (R^2=0.42) and 37°C (R^2=0.31) (results not shown), suggesting that the correlation between stability and enthalpy recovery is a result of the correlation between stability and molecular mobility in the glass (i.e., enthalpy recovery is a suitable surrogate for structural relaxation time in this case).

CONCLUSIONS

Freezing damaged both in-process and storage stability of the vaccine in the different formulations, regardless of the presence of the surfactant. However, annealing above T_g' during freezing did not cause any further damage to in-process or storage stability of the vaccine. Foam drying resulted in superior storage stability, both in presence and absence of surfactant. Improvement in stability with foams was associated with very low SSA, low vaccine surface coverage and high enthalpy recovery (i.e., a low molecular mobility).

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