

Stability of sugar glass stabilized biology essay

[Science](#), [Biology](#)



Abstract:

Stabilized influenza vaccines are more advantageous over conventional influenza vaccines as they can be used as alternative influenza vaccinations. However, the major concern is the stability of the stabilized influenza vaccine and the stability has to be maintained for storing the vaccine through a wide range of temperatures. Lyophilization is an excellent technique employed for stabilizing the proteins and vaccines. Here, we used inulin, dextran and mixture of dextran and trehalose as lyoprotectants and studied the storage stability of whole inactivated influenza virus vaccine through a range of temperatures (-20 - 40 °C) for three months. The physical and biochemical characteristics were found to maintained till 30 °C. The in vivo data suggest that immunogenicity was maintained after storage at 30 °C. In conclusion, this study suggests that stabilized influenza vaccines can be stored through a range of temperature with the preservation of in vivo antigenicity and powder characteristics of the stabilized influenza vaccine, and this could be helpful in stockpiling of influenza vaccines for future pandemics.

Introduction

Influenza vaccination is the main tool for the containment of virus in influenza outbreaks. Unfortunately, every year many doses of influenza vaccines are discarded as the potency of the vaccine is lost due the failure of the cold chain {{44 Bordon, Yvonne 2010}}. The main disadvantage of current influenza vaccines is that they remain stable only within a narrow temperature range, and this is the main parameter that limits the widespread use of vaccination during pandemic outbreaks. Moreover, liquid

vaccines are mainly administered via conventional routes like intramuscular (i. m.) and subcutaneous (s. c.) injection. The drawback of these vaccination routes is the lack of compliance due to pain at the site of injection, needle stick injuries and needle fear. In addition, help from trained health care workers is required for conventional vaccination. Finally, the liquid influenza vaccines predominantly induce a systemic immune response, which only provides protection at the systemic level and is considered to be less effective in protecting the most vulnerable population {{24 Cox, R. J. 2004}}. Although live attenuated intra nasal (i. n.) influenza vaccine provides both local and systemic immune responses{{59 Greenbaum, E. 2004; 58 Atmar, R. L. 2007}}, it shares other drawbacks with conventional vaccines such as the requirement of a cold chain. An attractive alternative to these dosage forms could be a dry powder for pulmonary administration. Already more than 40 years ago several studies have been performed to identify the potential of pulmonary immunization using liquid influenza vaccine formulations{{51 Waldman, R. H 1969; 56 Haigh, W. 1973; 52 Waldman, RH 1969}}. The outcomes of these studies indicate that pulmonary immunization can induce the same level of systemic immune response as that induced by conventional vaccines. In addition, they also induce a local immune response, which provides the population with additional protection against the influenza virus at the site of entry and might also provide cross-protection against heterologous viruses{{60 Holmgren, Jan 1992; 31 Amorij, J-P. 2007}}. Another major advantage of pulmonary immunization is the improvement in patient compliance (no needle pain). In addition, these vaccines do not require the involvement of

trained personnel as they can be self-administered. This procedural simplicity would be an advantage in particular during a pandemic. However, the disadvantage of these liquid vaccine formulations of being unstable still remains. Furthermore, in contrast to dry powder formulations, administration of liquid formulations to the lungs often leads to poor and irreproducible deposition of the pharmaceutical in the lung{{61 Frijlink, Henderik W. 2005}}, which might have been the reason for discontinuation of this research in the seventies of the previous century. The pulmonary administration of influenza vaccines recently has regained interest because it became apparent that biopharmaceuticals can be brought in the dry and stable state by incorporating them in a sugar glass matrix by freeze drying, spray drying or spray freeze drying (SFD){{31 Amorij, J-P. 2007; 53 Audouy, S. A. 2011; 20 Amorij, J. P. 2007}}. Previous studies from our group have shown that influenza vaccines can be stabilized with the oligosaccharide inulin by freeze drying technique{{20 Amorij, J. P. 2007}}. But for pulmonary delivery of influenza vaccine the WIV would be the better option, since it can be more immunogenic than subunit influenza vaccine when administered through pulmonary route {{62 Geeraedts, Felix 2008}}. However, for pulmonary delivery the WIV has to be formulated into a powder with aerodynamic particle size ranging from 1-5 μm {{63 Zanen, Pieter 1994; 61 Frijlink, Henderik W. 2005; 64 Westerman, E. M. 2007}}, and this can be achieved by spray freeze drying (SFD){{31 Amorij, J-P. 2007; 63}}. SFD is a well-known technique to stabilize the vaccines with appropriate lyoprotectants like sugars. Previously WIV influenza vaccine was SFD in the presence of inulin, and the biochemical integrity of WIV influenza vaccine

was found to be maintained during the drying process. In addition, the dry powder was found to be suitable for pulmonary administration {{31 Amorij, J-P. 2007; 32 Saluja, V. 2010}}. Later, it has been shown that the biochemical integrity of WIV influenza vaccine is maintained during freeze-drying in the presence of either inulin or trehalose, and that the dried product exhibited an excellent storage stability {{38 Geeraedts, F. 2010}}. However, it was not investigated whether the biochemical integrity of WIV influenza vaccine in the formulation was maintained along with the physical powder characteristics suitable for pulmonary delivery during storage. Hence, in the present study we investigated the storage stability of SFD WIV influenza vaccine powders for a period of three months at various temperatures by evaluating the following parameters, (i) biochemical integrity of WIV influenza vaccine, (ii) physical powder characteristics, and (iii) in vivo antigenicity after pulmonary administration in mice. Additionally, we studied the possibilities of replacing inulin by dextran or a mixture of dextran and trehalose as a stabilizer. The dextran has the excellent amorphous bulking property when processed into SFD powder. However, dextran may not stabilize the antigen to the same extent as inulin. Whereas, trehalose a disaccharide can preserve the antigen in powdered vaccines{{66 Allison, S. Dean 2000; 68 Carpenter, JohnF. 1997}}. Hence, the dextran and the combination of the dextran and trehalose was investigated in this study.

1. Material and Methods1. 1 VirusLive influenza virus A/Hiroshima/52/2005 (A/Hir/H3N2) was kindly provided by Solvay Biologicals (Weesp, The Netherlands). Water filtered through the miller filter (pH 7, 18. 2 MΩ) was used throughout this study. 1. 2. Vaccine preparationWIV was produced by

inactivating the live A/Hir/H3N2 virus by overnight incubation with 0.1% β -propiolacton (Acros Organics, Geel, Belgium) at 4 °C in citrate buffer (125 mM Na₃C₆H₅O₇, 150 mM NaCl, pH 8.2) under continuous rotation. The inactivated virus was then dialyzed overnight at 4 °C against hepes buffer saline (HBS, 2 mM Hepes, 125 mM NaCl, 0.9 mM CaCl₂ · 2H₂O and 0.5 mM MgCl₂, pH 7.4). WIV protein content was determined by micro-Lowry assay and purity was analyzed by SDS gel followed by silver staining.

1.3. Spray freeze-drying WIV was SFD together with stabilizers i. e. inulin (4 kD; Sensus, Roosendal, The Netherlands) or dextran (6 kD; Sigma-Aldrich, Zwijndrecht, The Netherlands) or a mixture of dextran and trehalose (Cargill, Kerfeld, Germany) at a weight ratio of 1/1. A dispersion of WIV in HBS buffer containing 5 % w/v stabilizer was prepared at a HA: sugar weight of 1: 200, and for placebo powder dispersion containing 5 % w/v stabilizer in HBS buffer was used. The dispersion was pumped at a flow rate of 5 ml/min through a two-fluid nozzle (diameter 0.5 mm) of a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) and sprayed using an atomizing air flow of 600 l/hour in liquid nitrogen. The liquid nitrogen was allowed to evaporate after which the frozen vaccine dispersions were placed on the shelf (pre-cooled to a temperature of -55 °C) of a Christ Epsilon 2-4 freeze dryer. Drying was performed at a pressure of 0.220 mBar with a condenser temperature of -85 °C. The shelf temperature was gradually increased from -55 °C to 4 °C over 32 hours. Thereafter, the pressure was decreased to 0.055 mBar and the shelf temperature was gradually increased to 20 °C over 11 hours. The powder vaccine was then collected in a hood at a relative humidity of 10% or less and was stored at 4 °C in hermetically sealed

injection vials. 1. 4. Transmission electron microscopy Spray freeze-dried vaccines were reconstituted with sterile water. Liquid and SFD formulations were dialyzed against ammonium acetate buffer (75 mM ammonium acetate, 2. 5 mM hepes, pH 7. 4) overnight at 4 °C. Dialyzed samples were placed on a glow discharge 200 mesh copper grid covered with Formvar film. Samples were stained with 3% ammonium molybdate, pH 7. 2 and analyzed on Philips CM 12 transmission electron microscope (TEM). 1. 5. Hemagglutination assay WIV containing 0. 1 µg/µl of hemagglutinin (HA) was diluted 1: 10 (w/v), in PBS (154 mM NaCl, 12 mM Na₂HPO₄ 0. 9 mM KH₂PO₄, pH 7. 4) and 50 µl was added in 96-well V-bottom plates containing 50 µl of PBS and serially diluted twofold in PBS. Subsequently, 50 µl of 0. 1% guinea pig red blood cells (RBC; Harlan, Zeist, The Netherlands) in PBS was added.

Hemagglutination was determined two hours after incubation.

Hemagglutination titers were expressed as log₂ of the highest dilution showing agglutination of RBC. 1. 6. Physical characterization of the powders Scanning electron microscopy (SEM) was performed with a JEOL JSM 6301-F microscope (JEOL Ltd., Tokyo, Japan). Samples were prepared by placing the powders on double-sided sticky carbon tape on a metal disk. Then the particles were coated with 30 nm of gold using a Balzers 120B sputtering device (Balzer UNION, Liechtenstein). Images were captured at a magnification of 1000x. The geometric particle size of the SFD powders was measured using a HELOS compact model KA laser diffraction apparatus (Sympatec GmbH, Clausthal-Zellerfeld, Germany). The powders were dispersed at a pressure of 1 bar using a RODOS dispersing system. The specific surface area of the SFD powders was determined using a Tristar

surface analyzer (Micrometrics Instrument Corp., USA). The samples were loaded on the surface area analyzer, and the surface area was determined using the multipoint BET method from the nitrogen adsorption isotherm at 77 K.

1. 7. Immunization of mice Animal experiment handling and work protocols were approved by the local animal welfare and use committee of the University of Groningen, The Netherlands. An in vivo study was carried out in 6-8 weeks old female BALB/c mice (Harlan, Zeist, The Netherlands). Mice were immunized twice at an interval of three weeks with WIV formulations containing 5 µg HA. For pulmonary vaccination, mice were anesthetized by inhalation of Isoflurane/O₂. Then the mice were brought to a vertical position and intubated with a modified Autograde catheter (Becton Dickinson) into the trachea. Powder vaccine was delivered using a dry powder insufflator (Penn-Century Inc., Wyndmoor, USA). Approximately 1 mg of vaccine powder (also containing 5 µg HA) was delivered to the lungs by delivering three puffs. 50 µl of liquid aerosol vaccine was administered using an IA-1C Micro-sprayer aerosolizer for mice attached to the FMJ-250 high-pressure syringe (Penn-Century Inc., Wyndmoor, USA), and 50 µl of liquid aerosol HBS was administered to control group. Mice were placed in a recovery incubator with the 25 °C for two hours, and then placed back in the housing facility. One week after the second dose was administered, mice were sacrificed for evaluation of the immune response. After sacrifice, blood was withdrawn by heart puncture. Serum was stored at -20 °C until used. Nose wash and broncho-alveolar lavages (BAL) were obtained using 1 ml PBS, pH 7.4, containing complete protease inhibitor cocktail tablets (Roche, Almere, The Netherlands).

1. 8. ELISA HA specific IgG, IgG1, IgG2a and IgA

antibodies in nose wash, BAL and serum were detected using ELISA. The ELISA plates (Greiner bio-one, Alphen a/d Rijn, The Netherlands) were coated overnight at 37 °C with 500 ng/well of A/Hir/H3N2 WIV. Coated plates were washed once with coating buffer (17.8 mM Na₂CO₃, 22.5 mM NaHCO₃, pH 9.6) and blocked with 2.5 % milk powder solution. After washing once with coating buffer and twice with PBS containing 0.05 % Tween 20 (PBST, pH 7.2), serial dilutions of the samples were done in plates. Plates were then incubated at 37 °C for 90 minutes and were subsequently washed with PBST. Then, 100 µl of appropriate horse radish peroxidase (HRP) conjugated anti mouse IgG, anti-mouse IgG1, anti-mouse IgG2a or anti-mouse IgA (Southern Biotech, Birmingham, USA) diluted 1: 5000 in PBST was added followed by incubation for at 37 °C for 60 minutes for the detection of IgG, IgG1, IgG2a and IgA, respectively. After extensive washing, 100 µl citrate phosphate buffer (1 M NaH₂PO₄, 0.1 M Na₂HPO₄, pH 5, containing 0.04 % o-phenylenediamine and 0.012 % H₂O₂) was added. The enzymatic reaction was allowed to proceed at room temperature for 30 minutes and stopped by adding 50 µl of 2 M H₂SO₄. The absorbance was measured at 492 nm using a Synergy HT reader (BioTek, Winooski, USA). Average IgG titers were determined as log₁₀ of the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at a wavelength of 492 nm. IgA titers are presented as average of maximum absorbance of 1: 1 diluted nose and lung washes. IgG1 and IgG2a concentrations were determined using calibration curve made by overnight coating 0.1 µg anti mouse IgG at 37°C. Following extensive washing, increasing concentrations of 100 µl of IgG1 or IgG2a (Southern Biotech, Birmingham, USA) was added to the plates. Average influenza HA-

specific IgG1 or IgG2a responses are presented as concentrations $\mu\text{g/ml}$. 1. 9
Statistical analysisThe antibody titers are mentioned as geometric mean \pm standard error mean. The difference in antibody titers was analyzed by student-t test at a confidence interval of 95 % ($P \leq 0.05$). The increase / decrease in significance was denoted by an increase in the number of symbols: one symbol ($P \leq 0.05$); two symbols ($P \leq 0.01$).

Results

Physical characterization of powders

The sugar solutions were sprayed into liquid nitrogen to produce fine frozen-droplets. These frozen droplets were then freeze dried to yield SFD powders. The SFD vaccines and placebo powders were evaluated for physical characteristics like powder morphology, specific surface area and particle size.

1. Particle size of vaccine and placebo powders

The geometric particle size of the spray freeze-dried inulin, dextran and mixture of dextran and trehalose vaccine powders was compared with the respective placebo powders on $t=0$ and found to be maintained with the incorporation of WIV in sugar matrix. Hence, the geometric particle size of the placebo powders can be directly correlated with the geometric size of the spray freeze-dried vaccine powders (table 1). Since there is no significant difference in geometric particle size between SFD vaccines and placebo powders, the placebo powders were used for studying the powder characteristics for 3 months. For a powdered particle to be inhaled it should be in an optimum size range. The bigger size particle may get stuck in the

upper respiratory tract and whereas smaller size particles can be exhaled without getting deposited in the lung. Hence the particles for pulmonary delivery should be formulated within the optimum size range of 1-5 μm . The laser diffraction measurements revealed that SFD powders yielded particle of volume average size of about 8 μm , and the size large compared to requirements for pulmonary delivery. However, with laser diffraction the geometric diameter of powder particles can be measured and not the aerodynamic diameter of the particles. The aerodynamic diameter of the particles can be calculated by the equation mentioned below {65 Zijlstra, Gerrit S. 2007}. d_{ae} is the aerodynamic diameter, d_g the geometric particle size, ρ_p the density of the particles (g/cm^3), ρ_0 the unit density ($1 \text{ g}/\text{cm}^3$), χ the dynamic shape factor. The particle size of the spray freeze-dried sugars was evaluated at $t = 0$. The weight of the buffer and vaccine is negligible compared to sugar in the SFD powder, so the density of the particles is: $\rho_p = 0.05 \text{ g}/\text{cm}^3$, and the dynamic shape factor of spherical particles is 1. Hence, it can be calculated that volume average aerodynamic diameter of the SFD vaccine and placebo powders as ~ 1.8 . Since, the aerodynamic particle size was found to be within optimum range it can be concluded that the SFD vaccine and placebo powders are suitable for inhalation. To evaluate whether the particle size of the SFD powders maintained during storage for 3 months at different temperatures, the powders were analyzed by laser diffraction at various time intervals. The measurements (fig. 3) revealed that the particle size of SFD inulin, dextran and mixture of dextran and trehalose stored at -20 , 4 or 30 $^\circ\text{C}$ did not change. However, the particle size of SFD inulin stored at 40 $^\circ\text{C}$ was reduced 15 day after storage. The particle size of

the SFD dextran was found to remain the same for 3 months at 40 °C. In case of the dextran-trehalose mixture, a trend towards decreased particle size of the sample stored at 40 °C was observed 15 day after storage, however, noticeable reduced size was observed after 3 months of storage.

2. Evaluation of dry powder insufflator

The powder dispersing efficiency of the dry powder inhaler was evaluated with the SFD inulin, dextran, and mixture of dextran, and we found that the powder dispersions were highly inconsistent between the puff, which was mainly due to the inefficiency of the dry powder insufflator in dispersing the powders. Moreover, we also found that the dry powder insufflator was dispersing both primary particles and agglomerates of particles from the SFD powders when compared to powders dispersed by RODOS disperser. The particle size distribution of SFD powders dispersed by dry powder insufflator and RODOS are shown in Table 2. A bimodal particle size distribution was seen (data not shown) indicating that both primary and agglomerate particles were dispersed by the powder insufflator.

3. SEM of SFD powders

The morphology of SFD inulin, dextran and mixture of dextran and trehalose as analyzed by SEM revealed that in all three cases highly porous spherical particles with interconnected pores having a particle size ranging from 1 μm to 10 μm were formed. The high porosity and interconnectivity of the pores can be contributed to the removal of the ice crystals, which are formed during freezing, by sublimation. Hence, mirror images of ice crystals are seen in the SEM images.

4. Specific surface area of placebo SFD powders

The specific surface area of placebo SFD powders before and after storage at different temperatures up to three months was evaluated by BET analysis.

Analysis of specific surface area of the powders (fig. 4) indicated that, immediately after preparation, SFD inulin and SFD dextran both had a higher specific surface area (100-120 m²/g) than SFD dextran-trehalose mixture (around 75 m²/g). The specific surface area of all SFD powders remained unchanged during storage for 3 months at -20, 4 or 30 °C. However, the specific surface area of the SFD inulin stored at 40°C was significantly reduced after 15 days of storage. The specific surface area of the SFD dextran sample was maintained during storing at 40 °C for 3 months.

However, the specific surface area of SFD dextran- trehalose mixture was not stable during storage at 40 °C as it was reduced to 40 m²/g after 2 months and to 5 m²/g after 3 months. In conclusion all three SFD powders could be stored at temperatures up to 30°C for at least 3 months without substantial change of their physical powder characteristics. However, exposures to higher temperatures lead to changes in the physical properties of SFD inulin and SFD dextran-trehalose.

Analysis of whole inactivated influenza virus after SFD

5. TEM of WIV

To evaluate whether the particulate nature of WIV was preserved during the drying process, the powders were analyzed by TEM. TEM images of liquid WIV (fig. a), WIV SFD in the presence of inulin (fig. 5b), dextran (fig. 5c) and mixture of dextran and trehalose (fig. 5d) and then reconstituted revealed that stress related to SFD did not affect the particulate nature of WIV.

Presence of WIV of 100 nm to 150 nm in TEM images indicate that inulin, dextran and dextran-trehalose are suitable stabilizers for SFD of WIV

6. Hemagglutination titers

To evaluate the biological activity of HA upon SFD with inulin, dextran and dextran-trehalose mixture and subsequent storage at -20, 4, 30 or 40 °C up to three months, hemagglutination assay was performed. The activity of HA in unprocessed WIV (fig a) was reduced to 4. 7 (log₂) s when stored at 30 °C or 40 °C for 1 month. In addition, there was a gradual loss in hemagglutination titers for samples stored at -20 °C and refrigerated conditions. The hemagglutination titers of the WIV after SFD with inulin, dextran or mixture of dextran and trehalose was slightly decreased but remained more or less constant upon subsequent storage for a period of 3 months at a temperature up to 40 °C. To determine the molecular stability of the proteins present in WIV, SDS-PAGE analysis was performed using freshly prepared and vaccine formulations stored for three months at 30 °C. The gel showed (fig. 6e) band pattern consistent with the structural proteins present in freshly prepared WIV. Four bands associated with surface proteins of WIV were observed on the gel, viz., HA dimer: 130 kD, HA1: 70 kD, NA: 40 kD and HA2: 20kD. No difference was found in the band pattern between SFD formulations stored at 30 °C and freshly prepared liquid or reconstituted SFD formulations. Minimal difference in the band color intensity can be observed between SFD formulations stored at 30 °C and freshly prepared SFD formulations. The WIV proteins present in stored SFD vaccine were stained less intense than the WIV proteins present in freshly prepared SFD formulations. However, a significant difference can be observed in the

banding pattern between liquid WIV stored at 30 °C and freshly prepared WIV. Clear bands of HA1 and HA2 were observed in freshly prepared WIV which were almost absent in WIV stored at 30 °C. These band patterns on the SDS PAGE gel demonstrate that stress of SFD and exposure of the SFD formulations to 30 °C for three months preserved the WIV proteins.

In vivo Immune response

7. Evaluation of humoral immune response – SFD vaccine

To evaluate the influenza virus specific humoral response induced after pulmonary vaccination of liquid or SFD formulations stored at 30 °C for three months in comparison to freshly prepared formulations, samples were collected one week after the second dose and analyzed by ELISA. Analysis of IgA antibody in the nose and BAL washes showed that there was a trend towards decreased IgA antibody production in the mice vaccinated with SFD formulations stored for three months at 30°C compared to mice vaccinated with freshly prepared SFD formulations. The IgA levels induced by liquid WIV that was stored at 30 °C was also lower compared to IgA levels induced by the stored powdered formulation. On the similar lines, evaluation of IgG antibody in serum revealed that mice vaccinated with liquid or SFD formulations stored at 30 °C induced significantly lower IgG antibody than mice vaccinated with freshly prepared formulations.

8. Evaluation of humoral immune response - reconstituted SFD vaccine

To test the effects of storage at 30 °C for three months of storage on the in vivo antigenicity, the SFD vaccine powders were reconstituted with water

immediately after preparation or after storage and then administered to mice on day 0 and 21. The serum IgG antibody response was determined using ELISA (fig 8a). The evaluation of IgG antibody in serum shows that, in mice, liquid WIV stored induced significantly lower levels of IgG antibody compared to freshly prepared WIV or stored SFD vaccines, and it indicates the degradation of WIV when stored in liquid form. Also, mice vaccinated with stored powder vaccine induced similar level of IgG antibody in mice vaccinated with freshly prepared vaccines. HI titers are also in line with the IgG antibody titers whereas the stored liquid WIV showed significantly decreased HI titers compared to other stored or freshly prepared formulations (fig 8b).

9. Phenotype of immune response:

To evaluate the subtypes of antibody induced after pulmonary administration of stored (three months at 30 °C) or freshly prepared, liquid WIV or reconstituted SFD formulations, IgG2a or IgG1 antibody ELISA was performed. It was found that stored liquid WIV induced significantly lower amount of IgG2a antibodies (fig. 9a) and IgG1 antibodies than freshly prepared or stored SFD vaccines or freshly prepared WIV. Mice vaccinated with either stored or freshly prepared SFD vaccines induced similar levels of IgG2a and IgG2a antibodies. Analysis of IgG2a/IgG1 ratio (fig. 9c) shows that pulmonary vaccination induced highly skewed IgG1 antibody response. Overall, IgG2a and IgG1 ELISA results demonstrated that SFD vaccines preserved the antigenicity of WIV than the liquid vaccine upon storage at 30 °C for three months. However, pulmonary vaccination fails to induce balanced IgG2a and IgG1 antibody response.

Discussion

In this study we prepared a dry powder WIV formulation by SFD using inulin, dextran, and mixture of dextran and trehalose as stabilizing excipients. We showed that powdered vaccines can be stored at different temperatures ranging from of -20 °C to 30 °C for a time span of three months with the maintenance of antigenicity of the WIV. Additionally, the powder characteristics of the vaccine powders were suitable for inhalation (~ 1.8 µm) after storage. The powder characteristics of SFD vaccine were evaluated with the help of placebo powders. Since the physical powder properties of the placebo SFD powders were found to be similar to that of the stabilized vaccines and are confirmed by the particle sizes. Hence, we consider that physical powder properties of the vaccines are not affected by incorporation of WIV containing 5 µg of HA in SFD powders. The ratio of HA / sugar is 1:200, and the incorporation of WIV in sugar matrix and there will be no effect on powder properties. However, the particle sizes and surface of the SFD inulin and mixture of dextran + trehalose stored at 40 °C were reduced. The decrease in particle sizes and surface area are due to the glass transition temperature (T_g) of these sugars. The T_g of inulin and mixture of dextran + trehalose was found to be 154 °C and lower compared to dextran 220 °C (data not shown). Hence, the SFD vaccine samples stored at 30 °C were used study the particulate and biochemical integrity of WIV vaccine. Firstly, the particulate integrity of WIV after formulation was evaluated by TEM and, the intactness of the viral membrane was found to be preserved even after exposing them to stresses like spraying and freeze-drying. Secondly, the biochemical integrity of the SFD vaccine was assessed by the SDS-PAGE

under reducing conditions. The banding patterns of the stored samples were compared with the freshly prepared SFD vaccine powders and freshly inactivated WIV and, this reveals us that WIV proteins are still preserved even after storing them at 30 °C for three months. Finally, the conformational integrity of HA was assessed for a period of three months at different intervals by HA assay and, it revealed that HA was preserved by inulin, dextran and mixture of dextran and trehalose after SFD. Hence the SFD vaccine stored at 30 °C for three months were selected for the immunization in mice. The pulmonary vaccination induced SIgA in the nose and BAL washes and, serum IgG in mice immunized with vaccine powders. However, the antibody titers of the stored WIV powder vaccines were found to significantly lower than the freshly stabilized vaccines. The pulmonary vaccination induces highly skewed IgG1 immune response in mice and in line with previous studies ({{53 Audouy, S. A. 2011}}). In addition, these stored vaccines also induce the HI titers similar to that of the freshly formulated vaccines powders. The lower mucosal and systemic antibody induction in mice after pulmonary vaccination of SFD formulations stored at 30 °C can be due to loss of antigenicity, change in powder characteristics after storage or both. In contrast, the powder characteristics of the formulated powders were found to be preserved and the particle size measurement was performed by RODOS, which is considered to be good in dispersing the powders, and the biochemical integrity of SFD WIV stored at 30 °C was evaluated by the HA titers and SDS-PAGE analysis and found to maintained. Since the antigenicity of the formulated vaccines and powder characteristics of the SFD powders found be preserved, we reconstituted the powdered vaccines with water and

used for pulmonary delivery with micro-sprayer. The immunogenicity of the reconstituted vaccines was found to be comparable with freshly formulated vaccine powders and freshly inactivated WIV. The inconsistency was clearly seen between the animal groups immunized with stored vaccines and, animal groups vaccinated with the freshly formulated vaccine. The reason for the difference in antibody titers between the groups is due to the inefficiency of the powder dispersing device in dispersing the powder particles that are bound to each other by attractive forces on storage. However, the same SFD powders after storage were efficiently dispersed by RODOS dispenser. Hence, on storage the primary particles could be bound to each other by attractive force, and will require powder disperser as efficient as RODOS. The powder characteristics of SFD vaccines upon storage are not affected. However, subtle changes to the powder during storage which were not significant enough to be measured by BET and laser diffraction using the RODOS, but caused the poor delivery of SFD vaccine in mice by the dry powder insufflator. In previous studies, it has been demonstrated that various influenza vaccines can be stabilized by incorporating them in inulin matrices and the results of the present study are in agreement with these studies {{53 Audouy, S. A. 2011; 38 Geeraedts, F. 2010}}. Additionally, our results demonstrate that dextran and a mixture of dextran and trehalose can also stabilize the WIV for a period of three months through a range of temperatures i. e. $-20\text{ }^{\circ}\text{C}$ - $40\text{ }^{\circ}\text{C}$. Previously, it has been studied by Amorij et al, that freezing can be detrimental for the subunit influenza vaccine{{20 Amorij, J. P. 2007}}. However, in this study it has been shown that WIV can be stable at $-20\text{ }^{\circ}\text{C}$ for three months, and confirmed by HA titers. We

reconstituted the stabilized vaccine in water and vaccinated the mice to evaluate the immunogenicity of the vaccine. However, the powder delivery can be improved by using efficient powder dispensers like the Novalizer® or Twincer®, which are proved to be better dispensers than the dry powder insufflator used in this study. We showed that dextran and combination of dextran and trehalose are capable of stabilizing the WIV as effective as the inulin after SFD. In addition, these sugars preserve the powder characteristics of the SFD sugars on storage for three months at 30 °C. Consequently, the formulated vaccines can be safely stored at room temperature under controlled RH. The powdered influenza vaccines can be self-administered by which an improved compliance in a high-risk population during pandemics can be expected. In this study we studied the possibilities of stabilizing the WIV with different sugars for pulmonary delivery. Our data confirm that WIV has been stable for at least three months at 30 °C after incorporation in a matrix of inulin, dextran, and mixture of dextran and trehalose.