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ScienceDirect

Vaccine 25 (2007) 8306–8317

Vaccine

www.elsevier.com/locate/vaccine

Spray drying of an attenuated live Newcastle disease vaccine virus intended for respiratory mass vaccination of poultry

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Received 23 March 2007; received in revised form 13 September 2007; accepted 20 September 2007
Available online 11 October 2007

Abstract

A powder vaccine intended for aerosol vaccination of poultry was formulated by spray drying a live attenuated Newcastle disease virus with potential stabilizers (mannitol, trehalose, polyvinylpyrrollidone (PVP), bovine serum albumin (BSA)). Thermodynamic properties, water sorption, particle size distribution, nebulization properties, density and morphology of the powders were evaluated and the virus survival during spray drying and storage was determined by incubation in embryonated eggs and subsequent haemagglutination assay. All powders had a narrow size distribution with a median volume diameter of ±30 μm (suitable for primary respiratory vaccination of chickens) and good aerosolization characteristics. Four amorphous, hygroscopic formulations were produced (trehalose, trehalose-PVP, trehalose-BSA, trehalose-PVP-BSA), where addition of BSA was beneficial for virus survival during production and storage at 6 and 25 °C. A crystalline, non-hygroscopic powder (mannitol) had a lower stabilizing capacity during production but maintained the remaining virus titre during storage. In conclusion, the study demonstrates that it is possible to produce a dry powder formulation of an attenuated live vaccine for mass vaccination of poultry in a one-step spray drying process.

Keywords: Spray drying; Live attenuated vaccine; Mass poultry vaccination

1. Introduction

The upscaling of the poultry industry can facilitate the rapid spread of infectious diseases over large geographical areas [1], increasing the significance of disease outbreaks. The use of efficient and economical vaccinations against important poultry and zoonotic pathogens are of great significance in limiting the number and extension of disease outbreaks [2]. For respiratory infections such as Newcastle disease (ND), mass spray and aerosol vaccination with a reconstituted freeze-dried live attenuated vaccine are often applied.

However, the efficiency of these vaccinations could be improved. A first problem is the broad droplet size distribution generated by the nebulizers currently used for liquid spray and aerosol vaccination: the presence of small, inhalable droplets in a coarse spray for primary vaccination often results in post-vaccination reactions [3–5] and a large fraction of non-inhalable droplets generated by fine aerosol nebulizers (e.g., 10–1000 μm [6]) reduces the efficiency of secondary vaccinations. Furthermore, the efficiency of vaccines administered by spray or aerosol might be jeopardized due to the use of tap water for reconstitution (often containing virucidal agents, such as chlorines) [7], by large shear forces which are applied to the liquid in order to transform it into droplets [8] and most importantly, due to inactivation of the vaccine virus by evaporation of droplets after generation [3,5,9].

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doi:10.1016/j.vaccine.2007.09.049

These harmful effects could be overcome by formulating the vaccine in dry powder form with defined particle size. Dry powder vaccines have been produced successfully by mechanical milling of a freeze-dried pellet of a live ND vaccine [10,11] and a live measles vaccine [12,13]. The former research never found large-scale application in poultry industry, probably related to difficulties in obtaining defined narrow particle size spectra. However, spray drying, where a liquid is transformed in dry powder particles by nebulization of droplets in hot drying air, has been recommended as an alternative to freeze drying for the preparation of inhalation products, as it allows to produce individual particles with a controlled size and shape in a one-step process [14–17]. As it is important in veterinary vaccinology to have inexpensive vaccines [18], especially in low-profit sectors such as poultry industry [19], spray drying has the additional advantage of being a faster and more cost-effective dehydration process than freeze drying [20].

The spray drying process can be divided in three major steps: nebulization of the liquid by means of a nozzle that provides the energy for droplet formation, drying of the droplets in the warm air of the drying chamber, and separation of the resulting powder particles from the drying air. Despite the application of higher than ambient temperatures during a spray drying process, co-current (i.e., where air and liquid flow through the drying chamber in the same direction) spray drying can be used to dry heat-sensitive materials as the materials are hardly exposed to the higher temperatures. During evaporation, the temperature of a droplet does not rise above the wet-bulb temperature (i.e., temperature of the saturated surface): heat is used for evaporation of liquid and not for heating of the droplet. As evaporation continues, the original temperature of the drying air (inlet temperature) decreases to lower values (outlet temperature). Only when all liquid has evaporated, the remaining dry particle becomes sensitive to heating. However, dried particles are immediately transported to a collection vessel where the temperature of the air further decreases [14–16].

Spray drying of heat-sensitive materials for inhalation such as proteins has been performed previously. In general, stabilizing excipients are added to the proteins before spray drying to prevent degradation during processing and storage, where immobilization of the labile materials in an amorphous glass is believed to be advantageous for maintaining the activity of the incorporated molecules [21–24]. Disaccharides are amongst the most used excipients, with trehalose being particularly successful [25,26]. However, polyvinylpyrrolidone (PVP) is sometimes added when formulating proteins as a dry powder to prevent re-crystallization of stabilizing sugars and the thereby induced inactivation of incorporated labile materials during storage [27–29]. Also proteins such as albumins have been shown to prevent crystallization of spray-dried and freeze-dried excipients [21,30]. The resistance to crystallization can be evaluated by measuring the glass transition temperature, which is the temperature at which the transition from the glassy to the rubbery state or from a low molecular mobility to a high molecular mobility (and therefore, higher risk of crystallization) occurs. PVP and albumins are known to increase the glass transition temperature, which means that the formulations can be exposed to higher ambient temperatures before the glass transition occurs. Equally important is a low hygroscopicity of the formulations as water molecules are known to increase the molecular mobility, thereby decreasing the glass transition temperature [31–34]. This low hygroscopicity is additionally important when formulating inhalation products as the absorption of moisture in a powder might induce agglomeration of aerosol particles [17,35].

In this study, the usefulness of spray drying to formulate an attenuated live ND dry powder vaccine was investigated. Firstly, placebo formulations were characterized for their protein stabilizing potential and for their particle size distribution. The assessment of the protein stabilizing potential was based on glass transition temperature, residual moisture content, water uptake and crystallization rate [36]. The particle size was compared with the optimal particle size required for respiratory vaccination of differently aged chickens, being >20 and >5 μm for primary vaccination of 1-day-old and 2-week-old chickens, respectively, and <5 and <10 μm for secondary vaccination of 2- and 4-week-old chickens, respectively [37]. Subsequently, an attenuated ND vaccine virus was spray-dried with the different formulations and the loss of titre upon drying and storage was evaluated.

2. Materials and methods

2.1. Spray drying

An experimental spray dryer with a 30 μm piezo-actuated nozzle, kindly provided by Niro A/S (Soeborg, Denmark), was used to produce particles with an extremely narrow size distribution. The feed solutions were prepared in distilled water, with a total concentration of solids of 5% (w/w). Five different feed solutions were prepared: 5% mannitol (C*Mannidex 16700; Cerestar, Mechelen, Belgium), 5% trehalose dihydrate (C*Ascend 16400; kindly provided by Cerestar), 4% trehalose dihydrate with 1% polyvinylpyrrolidone (PVP) (Kollidon®30; BASF, Ludwigshaven, Germany), 4% trehalose dihydrate with 1% bovine serum albumin (BSA) (Sigma–Aldrich, Steinheim, Germany), and 3% trehalose dihydrate with 1% PVP and 1% BSA. The feed solutions were filtered (paper filter 595, Schleicher & Schuell, Dassel, Germany) to remove remaining particulates in order to avoid clogging of the nozzle. Then, a feed solution was pumped to the nozzle (feed rate: 30 ml/h) where a droplet generation frequency of 50 kHz was applied on the feed solution by the piezo-actuator. The inlet temperature was set at 90 °C and the drying gas flow rate at 25 kg/h, resulting in an outlet temperature of 70 °C. The powder was separated from the drying air using a cyclone. After production, the powders were stored at 25 °C in glass vials closed with silica-containing stoppers.

After drying and evaluating the placebo powders, the formulations were used to produce the powder vaccines. For this purpose, the attenuated Clone 30 strain of the ND virus, developed by Intervet International (Boxmeer, the Netherlands), was harvested from the allantoic fluid of embryonated eggs and added to the feed solution in a concentration of 10^7 50% egg infectious doses (EID_{50}) per ml of feed solution. As 100 ml of a feed solution with 5% solids was dried, a maximum titre of 10^9 EID_{50} was incorporated in 5 g powder. The collection vessel was regularly exchanged during production to prevent heating of the vaccine particles. The same drying conditions as described before were used and the powder vaccines were stored in sealed aluminium bags at 6 and 25 °C.

2.2. Solid state characterization

2.2.1. Dynamic vapour sorption (DVS)

Water sorption isotherms of the powder formulations were determined gravimetrically at 25 ± 0.1 °C using a DVS Advantage 1 with a Cahn D200 microbalance (Surface Measurement Systems, London, Great Britain). During a DVS experiment, materials are exposed to a changing relative humidity (RH), which is regulated by electronic mass flow controllers that mix saturated and dry carrier gas streams. The increase or decrease of RH induces a change of mass of the material due to moisture uptake or release, which is measured by comparing the mass of the sample cup with the mass of a reference cup, both attached to the microbalance [34].

Between 10 and 20 mg powder was weighed in the sample cup of the DVS instrument, subjected to a drying step in order to bring the sample to a constant weight and subsequently exposed to increasing RH (using 10% increments up to 90% RH). Following the sorption phase, the samples were exposed to a decreasing RH (in steps of 10% until 0% RH). Each step continued until equilibrium was reached (i.e., when the change of mass was smaller than 0.002% min^{-1} during at least 10 min) or until 6 h had passed. The mass change was recorded every minute with a resolution of ±0.1 μg.

2.2.2. Karl Fischer titration

The residual moisture content after spray drying and the water content after 1 year storage were evaluated by an automated Karl Fischer titration. The samples (±50 mg) were heated in a Mettler DO337 oven (Mettler-Toledo, Beersel, Belgium) at 130 °C during 10 min. During this period the water vapour from the samples was transferred by a dry nitrogen flow to the titration vessel of the Karl Fischer titrator (Mettler DL35; Mettler-Toledo), containing absolute, dry methanol (Biosolve, Valkenswaard, the Netherlands). After these 10 min, the titration with Hydranal® Composite 2 (Sigma–Aldrich) started automatically. During this titration, water molecules react stoichiometrically with the Hydranal® reagent (one-component reagent for Karl Fischer titration); subsequently, the volume of Hydranal® used to reach the endpoint of titration (potentiometrically determined) is used to calculate the percentage of water present in the sample (1 ml Hydranal® = ±2 mg water). All titrations were performed in triplicate.

2.2.3. Differential scanning calorimetry (DSC)

The thermodynamic behavior (i.e., exothermic or heat liberating and endothermic or heat consuming energy changes) of the powders was determined on a DSC 2920 calorimeter (TA Instruments, Ghent, Belgium), operated in the modulated mode. This means that the powders are linearly heated with an overlaying sinusoidal modulation of the temperature. The energy difference of processes occurring in a sample pan and an empty reference pan placed in the calorimeter are measured during a DSC experiment as function of time and temperature [38]. The DSC was calibrated with indium (temperature calibration) and with sapphire (heat capacity and cell constant calibration). The sample cell was purged with a nitrogen flow of 25 ml/min. The samples (5–10 mg) were loaded in sealed aluminium pans (TA Instruments), equilibrated at −20 °C and heated to 200 °C with a scanning rate of 2 °C/min and modulation amplitude of ±0.318 °C every 60 s. The glass transition temperature (T_g) was recognized on the reversing heat flow curve as an endothermic shift of the baseline and determined as the midpoint of this transition. Melting was seen as endothermic peaks, where the melting temperature T_m was determined after extrapolation on the baseline (onset melting temperature). Crystallization temperatures (T_c) were determined as the peak temperatures of the exothermic crystallization peaks.

2.3. Particle characterization

2.3.1. Scanning electron microscopy (SEM)

A thin layer of powder was dispersed on a sample holder and coated with platinum in an Auto Fine Coater (JFC-1300; Jeol, Zaventem, Belgium). A Jeol JSM 5600 LV scanning electron microscope (Jeol) was used to evaluate the particle morphology. Powders were evaluated immediately after production and after 1 year storage.

2.3.2. Particle size distribution

Particle size distributions were measured by laser diffraction (Mastersizer-S long bed; Malvern Instruments, Malvern, UK). Laser diffractometry is based on the fact that particles, passing through a laser beam, scatter light at an angle that increases logarithmically when the particle size decreases. The instrument consists of a laser source that generates monochromatic light of high intensity, a sample presentation unit that ensures that the material is passed through the laser beam, and a diode array detector that detects the intensity of the diffracted light beams.

To obtain the size distribution of individual particles, the powders were suspended in Miglyol 812N (capric triglyceride; Sasol, Witten, Germany) with 0.2% Tween™ 80 (Federa, Brussels, Belgium) and subsequently circulated through the wet sample presentation cell of the laser diffrac-

tor. Additionally, the particle size distribution of dispersed powders was determined to verify if the powders could be de-agglomerated into individual particles during air-assisted dispersion. The experimental powder dispersion device was constructed from a vacuum flask, in which air (10 l/min; 2.5 bar) was blown with an Omron CX3 compressor (Omron, Hoofddorp, the Netherlands). The air flow suspended the particles in air and carried them through the outlet of the flask into the dry powder sample presentation unit of the particle sizing equipment. The environmental conditions during powder dispersion were 21 °C and 35% RH. An additional experiment was performed for the trehalose-PVP-BSA formulation using dry air (dried using silica gel) to disperse the powder. All measurements were performed in triplicate.

The results obtained from laser scattering experiments are volume-based and the obtained diameters are calculated as the diameters of spheres that have the same volume as the measured (non-spherical) particles. The volume-based size distribution is particularly useful when characterizing aerosols in human and veterinary medicine as it is more valuable to know the deposited mass than the deposited number of aerosol particles. For example, 100 particles of 1 μm (representing a total volume of 52.3 μm^3) will have a lower therapeutic effect than 1 particle of 10 μm (representing a volume of 523 μm^3), despite that the 1 μm particles are present in a higher number. The volume particle size distributions as measured, are characterized by the diameters $D[v, 0.1]$, $D[v, 0.5]$ and $D[v, 0.9]$. This means that 10, 50, and 90% of the total measured volume of particles, respectively, is represented by particles that have a volume that is smaller than the volume of a sphere with the corresponding diameter. $D[v, 0.5]$ is also known as the mass median diameter or MMD. The span or width of the distribution is defined as $(D[v, 0.9] - D[v, 0.1])/D[v, 0.5]$, and is zero when the distribution is perfectly monodisperse.

2.3.3. Particle density

A helium pycnometer (AccuPyc 1330; Micromeritics, Brussels, Belgium) was used to evaluate the true density of the spray-dried powders. The pycnometer determines the volume of a sample from the change of pressure of helium gas in a calibrated cell when the sample is introduced in the cell. The mass of the sample is accurately determined before starting the experiment and allows to calculate the density of the powder particles (density = mass/volume).

2.4. Evaluation of vaccine virus stability

ND virus titres in the original feed solutions and in the corresponding powders were determined by titration in 10–11-day-old embryonated chicken eggs. Ten-fold dilution series were prepared in phosphate buffered saline (PBS) with 1% penicillin-streptomycin. Subsequently, 100 μl of each dilution was inoculated in the allantoic cavity of five eggs. After 72 h incubation at 37 °C, allantoic fluids were collected and tested for haemagglutinating activity with chicken red blood cells (RBCs). Fifty microliters of the allantoic fluid of each egg was pipetted in a U-bottomed microtitre plate (containing 50 μl PBS per well) and two-fold dilutions were made across the plate. Afterwards, 50 μl of a 0.5% chicken RBC suspension was added to each well, the fluids in the well plate were gently mixed and RBCs were allowed to settle during 1 h at room temperature. In case ND virus was present, haemagglutination was detected. Virus titres were calculated using the method of Reed and Muench [39] and expressed as $\log_{10}$ EID_{50} per gram powder. The detection limit was $10^{2.7}$ EID_{50} per gram of powder. Comparison of the titre in the feed solution with the titre in the corresponding powder formulation allowed to evaluate the stability of the virus during the drying process. Virus titrations were also performed on powders stored for 1, 5 and 10 months at 6 and 25 °C in order to evaluate the ability of the different formulations to stabilize the vaccine during storage.

2.5. Statistical analysis

Water content, as determined by Karl Fischer titration, was evaluated by two-way ANOVA testing (SPSS version 12.0; SPSS, Chicago, IL, USA) for the influence of storage time and formulation. The particle size data were also analyzed using two-way ANOVA tests: a first analysis evaluated the influence of dispersion method and formulation, while in a second analysis the influence of storage time and formulation was assessed. Four particle size parameters were included in each analysis, namely $D[v, 0.1]$, $D[v, 0.5]$, $D[v, 0.9]$, and span. True densities were compared statistically for the influence of formulation type by a one-way ANOVA. Differences were considered significant at $P < 0.05$.

In all analyses, the normality of the data was evaluated with the Kolmogorov–Smirnov test. The homogeneity of variances was checked and the data were transformed where necessary. For the evaluation of water content, the main effect of the formulation ignoring the effect of storage time or the main effect of the storage time ignoring the formulation effect was investigated when no significant interaction was present. When the interaction between both factors was significant, the simple effects of the formulation within each storage time or the simple effects of the storage time within each formulation were investigated with a Bonferroni post hoc test. The same procedure was followed when analyzing the particle size data.

3. Results

3.1. Solid state characterization

3.1.1. Dynamic vapour sorption (DVS)

The water sorption and desorption profiles at 25 °C are shown in Fig. 1. Mannitol was the least hygroscopic powder, absorbing only 1% of water when exposed to 90% RH.

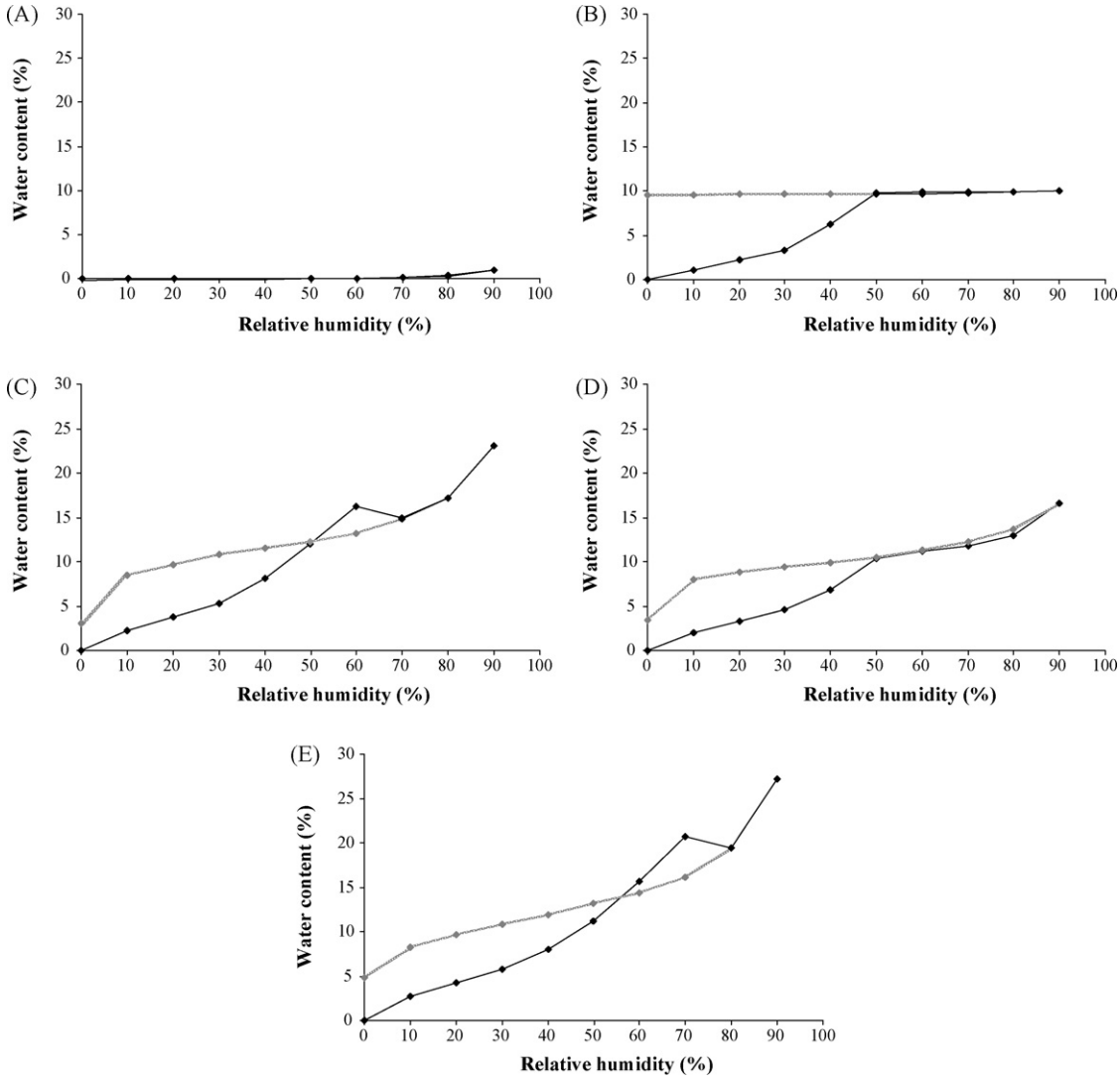

Fig. 1. Water sorption (black) and desorption curves (grey) obtained from dynamic vapour sorption experiments. (A) Mannitol; (B) trehalose; (C) trehalose-PVP; (D) trehalose-BSA and (E) trehalose-PVP-BSA.

The other formulations had a similar water sorption up to 50% RH and absorbed about 5 and 11% water at 30 and 50% RH, respectively. Below 30% RH the water sorption rate was lower in comparison to the sorption rate above 30% RH, resulting in an inflection point in the absorption curve. Above 50% RH, the influence of the composition of the formulation was evident. Pure trehalose did not sorb additional water when exposed to higher RH and did not release water during the desorption phase. When PVP and/or BSA were added to trehalose, no plateau in water sorption was observed and the water content increased up to maximum values of 23, 17 and 27% for trehalose-PVP, trehalose-BSA and trehalose-PVP-BSA, respectively. At a specific RH (correlated with a specific water content), the raw DVS data (not shown) of these combined powders showed a decrease in mass during the sorption phase: for trehalose-PVP this occurred at 70% RH (18% water content decreased to 15%), for trehalose-BSA at 60% RH (15% water content decreased to 11%), and for trehalose-PVP-BSA at 80% RH (22% water content decreased to 19%). The desorption phase of these powders was similar when water evaporated from the samples. However, hysteresis occurred and 3–5% water was still present in the samples at the end of the sorption/desorption cycle.

3.1.2. Residual moisture content and moisture uptake during storage

Table 1 shows the residual moisture content (Karl Fischer titration) after spray drying and after 1 year storage of the powders in glass vials with silica-containing stoppers. Mannitol retained significantly less water during production (<1.0%) compared to the other formulations (3.0–3.5%). No significant differences were detected between trehalose-PVP, trehalose-BSA and trehalose-PVP-BSA. Storage during

Table 1
Average moisture content (±S.D.) ($n = 3$) and glass transition temperature ($n = 1$) immediately after production and after 1 year storage at 25 °C in glass vials closed with silica-containing stoppers

	Immediately after production		1 Year after production	
Formulation	Moisture content (%)	Glass transition temperature (°C)	Moisture content (%)	Glass transition temperature (°C)
Mannitol	0.7 ± 0.1	–	1.4 ± 0.1[a]	–
Trehalose	3.1 ± 0.1	71.6	7.9 ± 0.0[a]	38.3
Trehalose-PVP	3.4 ± 0.1[b]	75.8	8.9 ± 0.1[a,c]	41.1
Trehalose-BSA	3.4 ± 0.1[b]	76.8	7.2 ± 0.1[a]	48.0
Trehalose-PVP-BSA	3.3 ± 0.1[b]	82.1	8.8 ± 0.1[a,c]	47.4

The moisture content immediately after production represents the residual moisture content after spray drying.
Water content of all formulations increased significantly (superscript letter 'a') after 1 year storage in glass vials.
Formulations with the same superscript letters 'b' and 'c' have similar water contents (no significant difference).

1 year significantly increased the moisture content of the mannitol formulation to 1.4%, while the other formulations contained 7–9% water. The PVP-containing formulations showed similar (±9%), but significantly higher results than those of the other formulations.

3.1.3. Thermodynamic properties

Glass transition temperatures (T_g) of the amorphous powders, determined immediately after production and after 1 year storage, are shown in Table 1. The T_g of pure spray-dried trehalose (72 °C) increased to values between 76 and 82 °C

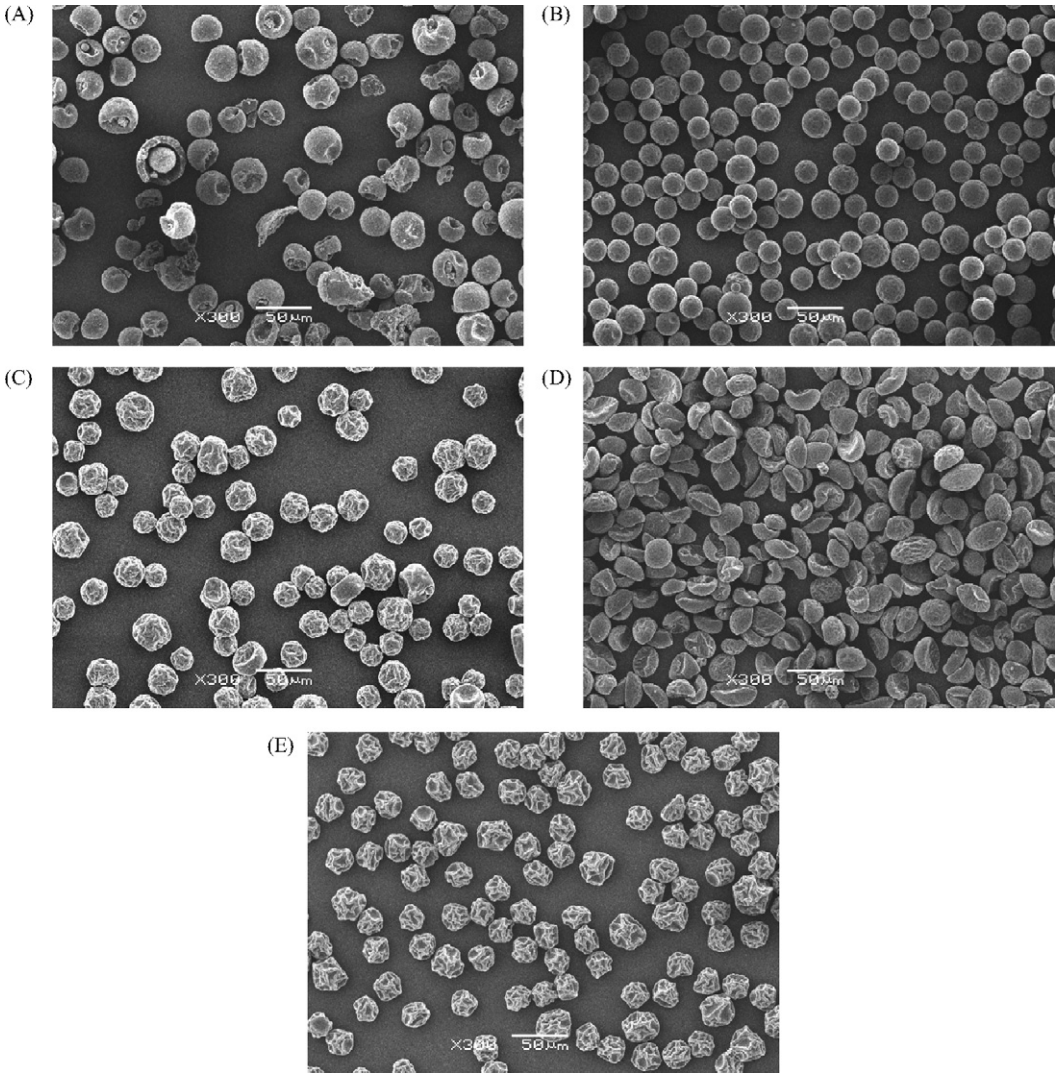

Fig. 2. Surface morphology of the different formulations immediately after production. (A) Mannitol; (B) trehalose; (C) trehalose-PVP; (D) trehalose-BSA and (E) trehalose-PVP-BSA.

Table 2
Average particle size distribution parameters (±S.D.) ($n = 3$) of powders measured after suspension in Miglyol + 0.2% Tween™ 80, compared to the particle size distribution after air-assisted dispersion with the experimental powder dispersion device

Formulation	Dispersion method[a]	$D[v, 0.1]$ (μm)	$D[v, 0.5]$ (μm)	$D[v, 0.9]$ (μm)	Span
Mannitol	Susp.	19.5 ± 0.8	28.2 ± 0.2	37.8 ± 0.4	0.7 ± 0.0
	Aer.	22.8 ± 0.3[b]	29.3 ± 1.0[b]	43.2 ± 3.7[b]	0.7 ± 0.1
Trehalose	Susp.	4.1 ± 0.7	24.8 ± 0.6	37.0 ± 1.0	1.3 ± 0.4
	Aer.	20.4 ± 0.2[b]	24.1 ± 0.4	29.4 ± 1.3[b]	0.4 ± 0.0[b]
Trehalose-PVP	Susp.	3.2 ± 0.1	25.8 ± 0.1	30.3 ± 0.0	1.1 ± 0.0
	Aer.	21.8 ± 0.5[b]	26.5 ± 1.1	32.6 ± 1.6[b]	0.4 ± 0.0[b]
Trehalose-BSA	Susp.	16.1 ± 0.3	28.2 ± 0.0	36.2 ± 0.2	0.7 ± 0.0
	Aer.	20.6 ± 0.6[b]	27.9 ± 0.1	38.3 ± 0.7	0.6 ± 0.0
Trehalose-PVP-BSA	Susp.	19.5 ± 0.9	29.0 ± 0.0	36.1 ± 0.5	0.6 ± 0.0
	Aer.	25.3 ± 0.2[b]	34.1 ± 1.3[b]	305.3 ± 258.5[b]	8.1 ± 7.5[b]

[a] Dispersion method: Susp. = suspended in wet dispersion cell of Mastersizer with Miglyol + 0.2% Tween™ 80 as medium; Aer. = aerosolized with experimental powder dispersion device into dry powder cell of Mastersizer.
[b] Significant difference ($P < 0.05$) between the primary and aerosolized particle size.

with the addition of other excipients. After 1 year storage at 25 °C, T_g values decreased, the PVP-containing powders having the largest reduction (35 °C). At both time points, the BSA-containing powders had the highest T_g values. The spray-dried mannitol powder was crystalline with a melting temperature of 163 °C.

Upon heating of pure trehalose, a crystallization peak was observed at 55 °C above T_g. This crystallization peak was delayed to 70 and 90 °C above T_g for trehalose-PVP and trehalose-BSA, respectively. The trehalose-PVP-BSA powder did not show non-isothermal crystallization within the scanned temperature range.

3.2. Particle characterization

3.2.1. Scanning electron microscopy (SEM)

The different powder particles are visualized in Fig. 2. Most of the mannitol particles were regular shaped, but some of the particles seemed to be fragmented. In contrast, trehalose particles were nearly perfect spheres. The addition of PVP to trehalose induced the formation of raisin-like, wrinkled spherical particles, with the indentations becoming more pronounced when additionally BSA was included in the formulation. Combining trehalose with BSA (but without PVP) resulted in completely collapsed particles. In general, all powders showed a very homogeneous size distribution.

3.2.2. Dispersability of spray-dried powders

Table 2 compares the particle size distribution after suspending the powders in Miglyol (primary particle size) and after air-assisted dispersion with the experimental powder dispersion device. Most parameters were similar after air-assisted dispersion (although the small differences were in some cases statistically different related to the good reproducibility of the measurements). Only for mannitol and trehalose-PVP-BSA, all three size parameters increased significantly. These differences were small, except for $D[v, 0.9]$ of trehalose-PVP-BSA which increased from 36 to 305 μm upon dispersion, thereby increasing the span value significantly. However, when the powder was dispersed with a dry air stream, $D[v, 0.1]$, $D[v, 0.5]$ and $D[v, 0.9]$ were 21, 29 and 37 μm, respectively.

3.2.3. Influence of storage time on particle size distribution

The particle size distribution (determined in Miglyol) immediately after production is compared in Table 3 with the distribution after storing the powders for 1 year in glass vials with silica-containing stoppers. Similar to the evalua-

Table 3
Average particle size distribution parameters (±S.D.) ($n = 3$) of powders measured after suspension in Miglyol + 0.2% Tween™ 80 immediately after production and after 1 year storage at 25 °C in glass vials with silica-containing stoppers

Formulation	Storage time	$D[v, 0.1]$ (μm)	$D[v, 0.5]$ (μm)	$D[v, 0.9]$ (μm)	Span
Mannitol	–	19.5 ± 0.8	28.2 ± 0.2	37.8 ± 0.4	0.7 ± 0.0
	1 year	19.9 ± 1.4	28.7 ± 0.3	39.9 ± 0.5[a]	0.7 ± 0.0
Trehalose	–	4.1 ± 0.7	24.8 ± 0.6	37.0 ± 1.0	1.3 ± 0.4
	1 year	14.9 ± 0.4[a]	38.1 ± 2.6[a]	97.4 ± 3.4[a]	2.2 ± 0.1[a]
Trehalose-PVP	–	3.2 ± 0.1	25.8 ± 0.1	30.3 ± 0.0	1.1 ± 0.0
	1 year	4.1 ± 0.0[a]	26.9 ± 0.2[a]	37.4 ± 1.7[a]	1.2 ± 0.0[a]
Trehalose-BSA	–	16.1 ± 0.3	28.2 ± 0.0	36.2 ± 0.2	0.7 ± 0.0
	1 year	12.9 ± 5.5	29.7 ± 0.0[a]	40.9 ± 0.3[a]	0.9 ± 0.2[a]
Trehalose-PVP-BSA	–	19.5 ± 0.9	29.0 ± 0.0	36.1 ± 0.5	0.6 ± 0.0
	1 year	6.1 ± 2.1[a]	29.3 ± 0.0	37.9 ± 0.0[a]	1.1 ± 0.0[a]

[a] Significant differences ($P < 0.05$) between the particle size immediately after production and after 1 year storage at 25 °C.

Table 4
Inactivation of Clone 30 live attenuated ND vaccine virus during the spray drying process

Formulation	EID_{50} in total amount of feed	EID_{50} in total amount of powder	EID_{50} per gram powder
Mannitol	$10^{9.0}$	$10^{4.9}$	$10^{4.3}$
Trehalose	$10^{8.5}$	$10^{8.3}$	$10^{7.6}$
Trehalose-PVP	$10^{8.0}$	$10^{7.2}$	$10^{6.5}$
Trehalose-BSA	$10^{8.5}$	$10^{8.3}$	$10^{7.7}$
Trehalose-PVP-BSA	$10^{8.5}$	$10^{8.7}$	$10^{8.0}$

tion of the dispersability, most differences were small and not relevant from a practical point-of-view (even if statistically different). Only pure trehalose showed a clear shift towards larger particle sizes after 1 year storage. This was confirmed by SEM pictures as the trehalose particles agglomerated due to the formation of solid bridges between the primary particles (Fig. 3). No particle agglomeration was observed on SEM pictures of the other powders.

3.2.4. Particle density

The true density of the formulations, with significant differences between all formulations, was influenced by the incorporated excipients. The pure components mannitol and trehalose had a density of 1.42 and 1.52 g/cm^3, respectively. The addition of PVP or BSA to trehalose lowered the density to 1.45 and 1.47 g/cm^3, respectively, and the additive effect of PVP and BSA resulted in the lowest density for the trehalose-PVP-BSA formulation (1.40 g/cm^3).

3.3. Evaluation of vaccine virus stability

The effect of spray drying on the infectivity of the Clone 30 ND vaccine virus can be derived from Table 4, where the titre before drying (i.e., in the total volume of feed solution) is compared with the titre after drying (i.e., in the total amount of spray-dried solids). Additionally, the latter titre was expressed per gram powder. Three formulations retained the vaccine activity completely: trehalose, trehalose-BSA and trehalose-PVP-BSA. The addition of PVP to trehalose decreased the vaccine titre with 0.8 $\log_{10}$ EID_{50}, but the largest loss of activity was seen in the mannitol formulation, as the titre was reduced with 4.1 $\log_{10}$ EID_{50}.

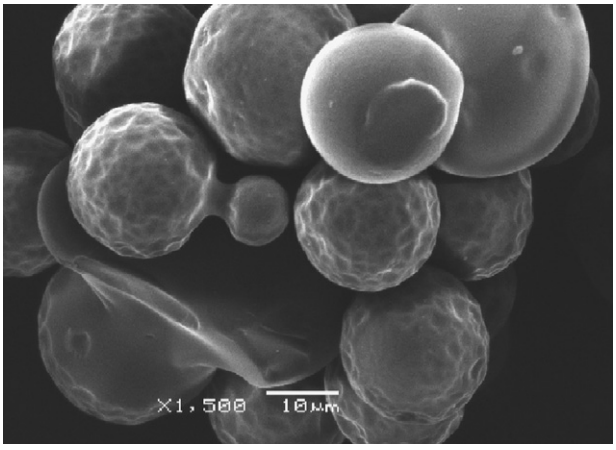

Fig. 3. Agglomerate of trehalose particles, formed after 1 year storage at 25 °C in glass vials with silica-containing stoppers.

Fig. 4 shows the loss of titre (calculated per gram of powder) in function of storage time, taking the titre immediately after production as a reference. In general, the vaccine titre decreased at higher storage temperature and longer storage time. However, the degree of inactivation depended on the excipients of the formulation. Although mannitol was not able to stabilize the vaccine during the drying process, the reduction of titre was only 0.3 $\log_{10}$ EID_{50} when stored at 6 °C for 10 months. In contrast, the reduction of titre in the other formulations ranged between 1.2 (trehalose-BSA) and 3.1 (trehalose-PVP) $\log_{10}$ EID_{50} under the same storage conditions. The titre of trehalose-BSA and trehalose-PVP-BSA vaccines stabilized during the last 5 months of storage at 6 °C. Storage at 25 °C resulted in a considerable decrease of titre in the trehalose and trehalose-PVP formulations, while the loss of titre in trehalose-BSA and trehalose-PVP-BSA formulations was lower. Additionally, the resulting titres after 10 months storage at 25 °C of the trehalose-BSA and trehalose-PVP-BSA formulation were only approximately 2 and 1 $\log_{10}$ EID_{50} lower in comparison to storage at 6 °C. The incorporation of the Clone 30 vaccine in trehalose-BSA and trehalose-PVP-BSA thus resulted in the highest retention of viral activity, with residual virus titres of $10^{6.5}$ and $10^{6.0}$ EID_{50}, respectively, when stored for 10 months at 6 °C and $10^{4.3}$ and $10^{4.7}$ EID_{50}, respectively, after 10 months storage at 25 °C.

4. Discussion

When developing an inhalable dry powder vaccine, the interaction between powder properties such as water uptake, glass transition temperature and particle characteristics largely influences the potential application of a formulation. The water sorption profiles obtained from the DVS experiments (Fig. 1) indicated clear differences between the formulations, which are mainly related to different thermodynamic properties. The mannitol powder, which was crystalline after spray drying, hardly absorbed water, while the amorphous trehalose-containing formulations were hygroscopic even at low RH. The larger water uptake of amorphous formulations is due to bulk absorption of water, while crystalline material only adsorbs water on readily accessible water binding sites at the surface [40]. This also explains

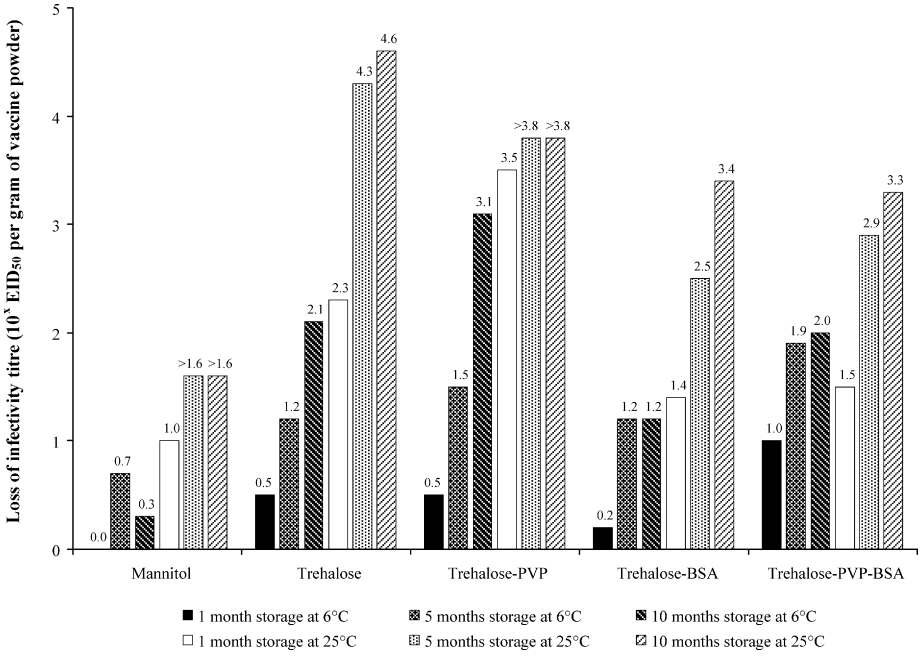

Fig. 4. Loss of infectivity titre of Clone 30 live attenuated ND vaccine virus incorporated in powder formulations in function of storage temperature and time in comparison to the titres obtained immediately after production. Results are expressed as $\log_{10}$ EID_{50} per gram powder with the darker bars representing results for storage at 6 °C, and the lighter bars showing results for storage at 25 °C. Some vaccines did not contain a virus concentration above detection limit (i.e., $10^{2.7}$ EID_{50} per gram of powder) and the loss of titre should then be considered as a minimal loss.

why mannitol had a much lower residual moisture content after spray drying compared to the other formulations. The amount of water absorbed by amorphous materials depended on the excipients used: the addition of the hydrophilic high molecular weight PVP increased the amount of water incorporated in the trehalose matrix after exposure to 90% RH (DVS experiments) and after 1 year storage (Karl Fischer titration).

The non-isothermal T_g (Table 1), as determined from the DSC thermograms, showed that the high-molecular weight additives PVP and BSA were able to maintain the glass transition temperature at a higher level after 1 year storage, even when larger amounts of plasticizing water were absorbed. Additionally, crystallization of the formulation was delayed to higher temperatures when PVP and/or BSA were added to trehalose. This delay of crystallization was also detected in the water sorption profiles (Fig. 1): although all components passed their isothermal glass transition (i.e., RH at which the glass transition temperature was reduced to 25 °C) at 30% RH (detected as an inflection point in the water sorption isotherm [34,40,41]), the moisture-induced crystallization (identified by a reduction of water content [34,40,41]) occurred at higher RH for the formulations containing PVP and/or BSA. Hence, these formulations can be exposed to higher temperature and RH, and can absorb more water without their molecules reaching the mobility necessary for crystallization. Several authors have used PVP to stabilize the amorphous state and explained this effect by limiting the molecular mobility in the system due to the addition of high molecular weight molecules [27–29]. Proteins have been described to limit the molecular mobility of sugars and prevent crystal nucleation and growth due to strong hydrogen bonds between protein and sugar [24,42,43].

Pure trehalose crystallized with the formation of trehalose dihydrate [36,42], as can be derived from the constant water content during the desorption phase (Fig. 1). This hydrate formation was largely prevented by the addition of PVP and/or BSA. However, not all absorbed water could be removed after the final drying step of these formulations, which was previously related to the formation of small quantities of hydrate molecules during the sorption phase [34]. Therefore, the additives apparently could not completely prevent the hydrate formation of trehalose upon exposure to increasing humidity.

The evaluation of water sorption is essential in the formulation of dry powder aerosols due to the influence of water on the aerosolization properties. Moisture sorption can induce cohesion by liquid bridging during dispersion, and agglomeration via crystallization and formation of solid bridges during storage [17,35]. The first phenomenon can explain the increase in particle size of the trehalose-PVP-BSA powder upon air-assisted dispersion (Table 2) since vapour sorption from the air used to disperse the powder during the measuring period most probably caused cohesion of the powder. This was confirmed by repeating the experiment with dried air (using silica gel): the particle size distribution of the dispersed powder was similar to its primary particle size distribution. The other formulations were not as sensitive to this short-term moisture effect as their moisture sorption was slower compared to trehalose-PVP-BSA. For

example, after exposing the samples for 5 min to 30% RH (comparable to the ambient RH (35%) during the powder dispersion tests), the trehalose-PVP-BSA and trehalose powder absorbed 0.4 and 0.1% water, respectively (derived from the raw DVS data).

However, the median particle size of trehalose-PVP-BSA did not increase significantly upon storage, while trehalose showed largely increased particle sizes (Table 3). This was explained by the long-term effect of water on the particle size, i.e., agglomeration by induction of crystallization or dissolving of the outer layer of the particles and subsequent formation of solid bridges (as confirmed for trehalose in Fig. 3). A surface enrichment with PVP and BSA is suggested by the morphology of the particles: the raisin-like and collapsed appearance of the formulations containing PVP and BSA, respectively, have both been described previously and related to highly viscous forces that hinder particle smoothening due to surface enrichment of these components when combined with sugars [27,44]. As crystallization has been described to start at the surface [27], and as PVP and BSA have a higher T_g and lower solubility than trehalose, the particles were better protected against crystallization or dissolution and subsequent agglomeration compared to pure trehalose.

Although only minor deagglomeration forces were applied, the powders were easily dispersed, as only minor differences were seen between the particle size distribution in miglyol and the particle size distribution after air-assisted dispersion (Table 2). This can be explained by the homogeneous particle size distribution and the sphericity of particles (Fig. 2). Therefore, a monodisperse particle size distribution and shielding the powders from environmental humidity during storage and dispersion are the main requirements to ensure easy dispersion of these dry powders.

The powders manufactured with the experimental spray dryer had a median particle size of about 30 μm, depending on the design of the ultrasonic nozzle. These particles are well suited for primary respiratory vaccination of poultry where deposition in the lower airways should be avoided to prevent post-vaccination reactions in the more vulnerable lungs [3–5]. To obtain this, vaccination of 1-day-old chickens should be performed with particles larger than 20 μm, as determined in a previous study [37]. When designing powders for secondary vaccination (where the lower airways are targeted), the particle size should be reduced below 5 and 10 μm for 2- and 4-week-old chickens, respectively [37]. However, hygroscopic formulations, such as the amorphous powders, can be subject to hygroscopic growth in the airways [45]. This could decrease the chance that particles reach the lower airways, even if they are theoretically small enough. Hence, a non-hygroscopic powder, such as mannitol, could be beneficial for a secondary powder vaccination by avoiding particle growth due to moisture sorption.

Spray drying of the Clone 30 vaccine virus with the above-evaluated excipients did not result in loss of vaccine activity, except when the virus was incorporated in crystalline mannitol (Table 4). In contrast, during storage at 6 °C, the loss of infectivity titre was lower in the mannitol matrix than in the amorphous formulations (Fig. 4). These findings suggest that the ND virus did not behave like single proteins when incorporated in dry powder formulations. Water replacement with hydrogen bonding between stabilizing sugars and proteins is considered to be responsible for stabilization during dehydration [46,47]. However, enveloped viruses such as the ND virus contain a large amount of lipoproteins in their envelope [48], so that water replacement is an unlikely mechanism of stabilization. Immobilization in an amorphous matrix [47,49] does stabilize the virus better during the drying process, but less during storage. This may be due to the different structure of single proteins versus viruses. Virus particles, being a complex organization of proteins, might already have an intrinsic stability as is also described for enzymes consisting of several monomers [50]. As in freeze drying, a possible inactivation mechanism for the viruses during spray drying and storage might be aggregation [51]; however, additional research, which was out of the scope of the current project, should be performed to support this.

It was possible to prepare powders containing approximately 10^8 EID_{50} of the ND virus per gram powder. Knowing that a dose of approximately 10^3 EID_{50} of a Clone 30 vaccine should be inhaled by a chicken to induce a good immune response [4], it can be calculated that each chicken should inhale approximately 10 μg of the here developed powder vaccines.

In summary, a stable dry powder formulation of an attenuated live Newcastle disease vaccine for respiratory mass vaccination of poultry was developed by means of a one-step spray drying process. The addition of PVP and/or BSA to trehalose formulations revealed several advantages in solid state and particle characteristics. When formulating the vaccine in these excipients, the combination of trehalose with BSA (both in the binary trehalose-BSA combination or in the ternary trehalose-PVP-BSA combination) was beneficial for the maintenance of vaccine titres during 10 months storage at 6 and 25 °C. Although mannitol crystallized during spray drying and did not have a good process stability, it was able to stabilize the vaccine virus particles during storage at 6 °C. Combining the latter with its low hygroscopicity, mannitol might overcome the disadvantage of loss of titre during production.

In vivo experiments will be performed in the near future to evaluate the applicability of the dry powder vaccination concept. Chickens will be exposed in isolators to the here developed powder formulations and, for comparison, to classical liquid vaccines. Virus survival in the isolator air will be measured in order to evaluate whether the virus survives nebulization better when incorporated in a powder compared to a liquid. Further, the humoral immune response induced by the powder and liquid vaccines will be determined to evaluate the feasibility of mass vaccination of poultry with powder vaccines.

Acknowledgements

This study was supported by a grant of the Special Research Fund (BOF) from Ghent University, Belgium. Bart De Pauw is acknowledged for the SEM pictures, Lieve Sys and Nele Dennequin for the evaluation of the vaccine titres, and Els Adriaens for helpful suggestions regarding the statistical analysis.

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