Rapid communication

Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix

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ABSTRACT

Spray drying is a valuable technique in pharmaceutical dosage formulation, capable of producing amorphous, spherical powders, suitable for pulmonary deposition and further downstream processing. In this study, we show that spray drying bacteriophages together with trehalose results in an amorphous powder matrix with high glass transition temperature (between 116 and 118 °C), typical for amorphous trehalose. These powders are stable at low temperatures (4 °C) and relative humidity (5%). However, high humidity causes crystallization of the amorphous matrix, destroying the embedded phages. Furthermore, storage at higher temperature (25 °C) causes thermal instability of the embedded phages. The results show that storage conditions are important parameters to take into account in phage therapy development. The resulting particles are hollow spheres, with suitable aerodynamic diameters for deposition into the deep lungs. This opens possibilities to use these phage-containing powder formulations to tackle pulmonary infectious diseases, especially caused by antibiotic-resistant pathogens.

1. Rapid communication

(Bacterio)phage therapy recently gained scientific and medical attention. As most studies focus on liquid bacteriophage formulations, little is known about the possibilities of processing bacteriophages into different pharmaceutical formulations, with the few studies mainly using lyophilization to process liquid formulations into dry powders (Merabi-Shvili et al., 2013). Recently, the potential of spray drying bacteriophages into readily usable powder formulations for aerosolization was shown (Matinkhoo et al., 2011; Vandenheuvel et al., 2013). Trehalose was added because of its known capacity to protect biomaterials from desiccation and thermal stress (Crowe et al., 1998; Grasmeijer et al., 2013). This ability of thermal and dehydration protection is explained via the water-replacement hypothesis and the vitrification hypothesis. In these hypotheses, trehalose provides structural and conformational stability to the proteins by direct hydrogen bond formation or vitrifying the protein, hence limiting the protein’s mobility. The glassy state of trehalose shows a high glass transition temperature (Tg), between 79 and 115 °C (Willart et al., 2002), which promises a stable glassy matrix at room temperatures. This research focuses on the importance of storage conditions and the stability of the powder matrix as well as the negative effects of crystal formation on the viability of phages in spray-dried powders. These effects are important considerations in the development of phage-containing pharmaceuticals and the optimization of their mode of storage and packaging.

The powders in this study were manufactured as described previously (Vandenheuvel et al., 2013). In short, a 4% (w/v) trehalose solution (Acros Organics, Belgium), supplied with 1% (v/v) phage suspension of Pseudomonas aeruginosa phage LUZ19 or Staphylococcus aureus phage Romulus was spray dried using a Micro Spray laboratory-scale spray dryer (ProCepT, Belgium) with following process parameters: atomization was done using a bi-fluid nozzle with an orifice of 0.6 mm and atomizing airflow of 61/min, the liquid feed was supplied at 2 ml/min and dried in the drying chamber (300/min heated air of 85 °C). After spray drying, the powders were immediately stored at a temperature and humidity controlled atmosphere. Storage temperature was 4 or 25 °C. Using saturated salt solutions of phosphorus pentoxide and magnesium nitrate the relative humidity (RH) was adapted to 0 and 54%, respectively. The use of powders in respirable applications and metered dose inhalers (MDIs) is highly dependent on the particle size. Particles with an aerodynamic diameter ranging from 1 to 5 μm settle down in the deep lungs. We previously showed that particle size and size distribution was phage dependent, with phage Romulus resulting in a larger fine particle fraction compared to phage LUZ19 (Vandenheuvel et al., 2013). These results were confirmed with field emission gun scanning electron microscopy (FEG-SEM), performed with a Philips XL30 ESEM-FEG microscope.
Powder samples were fixed on aluminum stubs using double-sided carbon tape and gold coated by gold sputtering for 45 s at 20 mA and images were taken at a 10 kV electron acceleration voltage. Fig. 1 shows microscopic images of the powder samples, confirming that the powder particles were hollow spheres with relative smooth to golf ball-like surfaces. Although SEM cannot provide absolute numbers, the images clearly show that the powder sample containing LUZ19 results in more large particles compared to the Romulus-containing powder, supporting the previous light microscopic data. The average density of spray-dried non phage-containing and phage-containing trehalose powders was measured via helium pycnometry and found to be 1.47 g/cm³. For spherical particles, the geometric and aerodynamic diameter are related as follows: \( d_g = \frac{d_a \sqrt{\rho / \rho_0}}{\chi} \), with \( d_a \) the aerodynamic diameter, \( d_g \) the geometric diameter, \( \rho \) and \( \rho_0 \) the particle and unit density, respectively, and \( \chi \) the shape factor which is 1 for spherical particles (Pilcer and Amighi, 2010). This results in suitable geometric diameters for deep pulmonary deposition ranging from 0.82 to 4.12 \( \mu m \). The powders are confirmed to have a suitable fine particle fraction, with spherical, hollow particles which favors pulmonary delivery through aerosolization.

To study the viability of powder-embedded phages during long-term storage, the surviving infectious phages were titrated immediately after powder production and subsequently on a monthly basis for one year. Titers were determined by plating a proper dilution of a dissolved powder sample in presence of a host bacterium using the standard double agar overlay method as described earlier (Vandenheuvel et al., 2013) (Fig. 2). Freshly spray-dried powder samples were divided in four groups and stored at

Fig. 1. SEM images of bacteriophage-containing trehalose powders. (A) LUZ19-containing powder particles. (B) Romulus-containing powder particles. (C) Close-up on a Romulus-containing particle, showing the hollow inside.

Fig. 2. Stability of bacteriophage titer. (A) Loss of viable LUZ19 phages during one year of storage in controlled conditions. (B) Loss of viable Romulus phages during one year of storage in controlled conditions \((n=3)\). Standard deviations were calculated but not shown to improve the readability of the graphics. For data points marked with an asterisk (*) \( n=2 \) due to detection limitations.
Fig. 3. MDSC curves. (A) Trehalose dihydrate. (B) Spray-dried amorphous trehalose. (C) Romulus-containing spray-dried trehalose. (D) LUZ19-containing spray-dried trehalose. Arrows mark the \( T_g \).

Fig. 4. X-ray diffraction profiles of stored samples. X-ray diffraction patterns of unprocessed trehalose dihydrate and samples stored at different atmospheres.
different atmospheric conditions: 4 °C and 0% RH, 4 °C and 54% RH, 25 °C and 0% RH, and 25 °C and 54% RH. Each powder sample was made and analyzed in triplicate. Both phases were the most stable at 4 °C and 0% RH. LUZ19 showed no significant loss of phage titer. However, the logarithmic loss of phage titer for Romulus at these conditions was about 2 log units over the course of one year, but seemed to stabilize after the initial loss. Remarkably, after one year of storage the most damaging conditions were found to be 25 °C and 0% RH.

To investigate the relationship between this loss of viable phages and crystallization of the amorphous trehalose, the powder matrix was studied by powder X-ray diffraction (PXRD) and modulated differential scanning calorimetry (MDSC). To confirm the amorphous character of the spray-dried trehalose powders, PXRD was used to analyze spray-dried trehalose powders. New powder samples were produced and kept at 4 °C and 0% RH until measurement, and analyzed in reflection mode using zero background sample holders, with an X’pert PRO diffractometer (PANalytical, The Netherlands), equipped with a Cu X-ray source ($\lambda_{Cu} = 1.5405\ \text{Å}$) from 4 to 40°2θ. Data acquisition and analysis were done using the X’pert Data Collector and the X’pert Data Viewer, respectively. The resulting diffractograms were compared to a diffractogram of unprocessed trehalose dihydrate. The absence of characteristic trehalose dihydrate crystal peaks (8.8° and 23.9°) and the presence of an amorphous halo showed that the powder samples were X-ray amorphous after spray drying (data not shown). Furthermore, the $T_g$ was determined using MDSC (Fig. 3), performed on a DSC Q2000 (TA Instruments, UK). Therefore, samples with a mass between 3.00 and 4.50 mg were placed in sealed aluminum pans and scanned from 70 to 150 °C, with a heating rate of 2 °C/min and a nitrogen purge of 50.0 ml/min. The modulation period was 40 s, with a 0.64 °C amplitude. The obtained data were analyzed using the Universal Analysis 2000 software (version 4.5A) (TA Instruments). Temperature calibration was done using indium and octadecane. Enthalpic and heat capacity calibration were done using indium and sulphur, respectively. Melting enthalpies and $T_P$ were studied using heat flow curves and reversing heat flow curves, respectively. Being fully crystalline, trehalose dihydrate did not show a glass transition. Instead, the heat flow curve showed an endothermic peak (93.1 °C), representing the melting of the crystals. Spray-dried trehalose without phages showed a glass transition temperature of 118.7 °C, for Romulus and LUZ19-containing powders this was 116.4 °C and 117.2 °C, respectively. The presence of a $T_g$ and the absence of the endothermic peak, indicate the fully amorphous state of the powders. Powder samples which were stored for at least eight months at controlled atmosphere showed different heat flow profiles (data not shown). Samples stored at the highest humidity agglomerated, regardless of the storage temperature. The samples did not show a $T_g$, but an endothermic peak with set off temperature between 86 and 91 °C appeared. This peak concurs with the melting transition of pure non-processed trehalose dihydrate. The X-ray diffractograms of these samples showed Bragg peaks typical for crystalline trehalose dihydrate (Fig. 4). This indicates that at 54% RH amorphous trehalose is able to form stable trehalose dihydrate crystals, regardless of the storage temperature. This leads to the conclusion that loss of the amorphous structure and the formation of crystals is causing the high drop in phage titer observed in these samples. Samples that were stored at a dry atmosphere did not show any change in DSC or PXRD patterns. The amorphous trehalose powder matrix was stable and no recrystallization took place, possibly explained by the absence of water in the atmosphere. Two Romulus-containing samples showed an unexpected high drop in viable phage titer (data not shown). These samples were stored at 0% RH and different temperatures. The heat flow curves revealed the presence of crystalline trehalose, which was also confirmed by X-ray diffraction analysis (data not shown). Since other samples stored at the same atmospheric conditions were amorphous, these samples were possibly subjected to an unstable atmospheric during storage (e.g., desiccator was not tightly sealed, drying capacity of the salt was exceeded) and crystallization occurred. Although it was impossible to link the exact moment of crystallization with the time of phage titer drop, these results indicate the importance of the amorphous glassy matrix and its protective effect on the embedded biologics.

In conclusion, we demonstrate that spray-dried phase-containing trehalose particles require specific storage conditions which limit crystal formation. A storage temperature below the $T_g$ of trehalose was insufficient to prevent crystallization and protect the embedded phases. Crystallization could occur at temperatures far below the glass transition temperature of the spray-dried trehalose powders when the relative humidity was high. Thus, to prevent crystallization, controlling the relative humidity seems to be the more important factor over temperature. This transformation of amorphous trehalose to crystalline trehalose dihydrate in high humidity atmospheres is consistent with previous studies (Iglesias et al., 1997; Nagase et al., 2002; Surana et al., 2004). Furthermore, storage temperature had a pronounced effect on phage viability. Over time, the phage titer declined at 25 °C even when no crystallization of the amorphous trehalose matrix was observed, but stayed more stable at 4 °C. As expected, the effects of crystal formation on phage viability appears phage specific. Larger phage viirions are more prone to inactivation upon crystal formation than smaller phage viirions. Although the observations above show that storage of spray-dried phase-containing trehalose powders is not straightforward, one should be mindful of the possibilities for further downstream processing, handling and aerosolizing these powders. The potential of these dry powders in aerosol therapy to tackle pulmonary bacterial infection diseases is only one example of the possibilities yet to be studied.

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References


