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Research Paper

Shrinkage of spray-freeze-dried microparticles of pure protein for ballistic injection by manipulation of freeze-drying cycle

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ABSTRACT

Spray-freeze-drying was used to produce shrivelled, partially-collapsed microparticles of pure proteins that may be suitable for use in a ballistic injector. Various modifications of the freeze drying cycle were examined for their effects on collapse of the pure protein microparticles. The use of annealing at a shelf temperature of up to +10 °C resulted in no visible particle shrinkage. This was because of the high T_g' of the pure protein. Inclusion of trehalose or sucrose led to particle shrinkage because of the plasticizing effects of the disaccharides on the protein. Only by extending the duration of primary drying from 240 to 2745 min at shelf temperatures in the range −12 to −8 °C were shrivelled, wrinkled particles of bSA and bCA of reduced porosity obtained. Manipulation of the freeze-drying cycle used for SFD can therefore be used to modify particle morphology and increase particle density.

1. Introduction

The Powderject ballistic injector propels a dose of up to 3 mg of a powder at supersonic speed into the epidermal layer of the skin (Kendall et al., 2004). It has therefore potential applications for delivery of high-potency drugs and also vaccines (Weissmueller et al., 2013). To penetrate into the epidermis, the powder particles require sufficient momentum. This can be achieved by a particle diameter in the range of 30–70 μm combined with as high a density as is possible (Kendall et al., 2000). The production of such microparticles has centred on the techniques of spray freeze drying (Maa and Prestrelski, 2000) and lyophilization/milling (Etzl et al., 2014) of carbohydrate-based formulations. These processes produce, however, highly porous carbohydrate particles that have low density and are mechanically fragile (Ziegler et al., 2010). This can be, at least in part, avoided by inducing partial collapse of the system to decrease porosity and hence increase both density and mechanical strength. This has been achieved by adopting two techniques: inclusion of a dextran in the formulation (Maa et al., 2004; Schiffter, 2008; Schiffter et al., 2010) and running primary drying close to (Rochelle and Lee, 2006) or above (Anamur et al., 2015) the glass transition temperature, T_g. The result is wrinkled, shrunken particle morphology of higher density than that the carbohydrates alone (Maa et al., 2004; Etzl et al., 2014). The concentration of the active ingredient should be high, as this results in a low powder mass per dose that has to penetrate into the dermis.

The technique of spray freeze drying (SFD) involves ultrasonic nozzle-atomisation of a liquid feed into a bath of a cryogen, usually liquid nitrogen, to produce a dispersion of frozen droplets. This is then lyophilized to yield a dry powder of, for example, a biomolecule in a carbohydrate-carrier (Maa and Prestrelski, 2000). In this paper we address the question whether a pure active ingredient, for example a protein without additional carbohydrate or dextran, could be prepared by SFD as shrunken, partially-collapsed particles suitable for use in the powder injector. This shrinkage should result in higher particle density and hence better skin penetration and lower mechanical fragility. The protein will be amorphous after SFD (Sonner et al., 2002; Yu et al., 2002) and should therefore show plastic flow at a temperature in the region of the glass transition (Rambhatla et al., 2005). This should be possible in the frozen solution state when the glass transition temperature of the maximally freeze-concentrated solution, T_g', is relevant, or after starting primary drying when the glass transition temperature of the drying solid phase, T_g, is relevant. We selected two enzymes as model proteins: bovine serum albumin (bSA) and bovine carbonic anhydrase (bCA). bSA is a readily-available model protein which has been used in numerous studies of drying processes; bCA was selected to determine the effects of particle shrinkage on aggregation status which can be measured for this model protein by size exclusion chromatography. Because the formulation is fixed, i.e, the pure protein dissolved in water, the only way to achieve partial collapse and shrinkage of particles is to vary the lyophilization process cycle (Rochelle and Lee, 2006; Etzl et al., 2014). Aqueous solutions of the proteins were therefore put through the SFD process using progressive modifications of the

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lyophilization cycle that could result in partial collapse. Our rationale was to use the following three cycle modifications. First, an annealing procedure of the frozen microparticles was introduced to promote viscous flow by raising product temperature to the vicinity of T_g' (Rochelle and Lee, 2006). Secondly, the shelf temperature during primary drying was increased to push product temperature closer to T_g and hence also promote plastic flow (Rambhatla et al., 2005). Thirdly, the duration of primary drying was greatly extended at different shelf temperatures to give more time for plastic flow to occur (Rambhatla et al., 2005). The results of this work illustrate that shrunken, high-density SFD particles of the pure proteins can be made. They also throw some light on the effects of protein content on the plastic flow of materials during freeze drying of frozen microparticles.

2. Materials and methods

2.1. Materials

Both bovine erythrocyte serum albumin (bSA) (66.5 kDa) and bovine erythrocyte carbonic anhydrase (bCA) (molecular mass ca. 30 kDa) were obtained from Sigma-Aldrich (Munich, Germany). The base and hydrochloride salt of tris(hydroxymethyl)aminomethane (Trizima) were also obtained from Sigma-Aldrich and used to prepare the tris buffer of pH 7.5 at 50 mM. Trehalose dihydrate and sucrose also came from Sigma-Aldrich. Water was double-distilled from an all-glass apparatus.

2.2. Spray-freeze-drying (SFD)

Spray solutions were prepared by dissolving 15% w/w of either bSA or bCA in the tris buffer followed by filtration through a 0.2 µm pore-diameter membrane filter (Sartorius Stedim Biotech, Göttingen, Germany). In some cases trehalose dihydrate or sucrose was also added to the spray solution. 10 mL of each spray solution was then spray-freeze-dried using the laboratory-scale SFD rig fully described before (Sonner et al., 2002). A 60 kHz ultrasonic nozzle (Sono-Tek, Milton, NY-USA) was used to atomize the spray solutions at a rate of 1 mL/min into liquid nitrogen (LN2) contained in stainless-steel bowls. The bowls were then placed onto the pre-cooled shelf (−45 °C) of a VirTis Advantage Plus (SP Industries, Warminster, PA-USA) freeze dryer. The LN2 was allowed to evaporate before starting the freeze dry cycle. The different cycles examined are given in the Results & Discussion. Unless otherwise given, the primary drying step lasted 240 min and was followed by a ramp over 45 min up to a shelf temperature of +25 °C during secondary drying held for 600 min. This starting-point lyophilization cycle was selected because it has already been shown to be suitable for SFD of carbohydrate-based formulations that do not require partial collapse (Gieseler and Lee, 2009). After completion of secondary drying the VirTis was purged with dry nitrogen gas. The bowls were removed from the drying chamber to a glove box at 0.1% RH where the powders were transferred into glass tubes and sealed ready examination.

2.3. Scanning electron microscopy

An SFD-powder sample was fixed to an Al stub (Model G301; Plano, Wetzlar, Germany) using a self-adhesive film. The sample was then Au sputtered for 1.5 min at 20 mA/5 kV (Hummer JR Technics) and examined on an Amray 1810T Scanning Electron Microscope at 20 kV.

2.4. Mercury porosimetry

The pore size distribution of a sample was determined using a Porosimeter 200 (Carlo Erba, Renningen, Germany) combined with a Pascal 140 (Porotech, Hofheim, Germany). A powder sample of 50 mg was placed in the device's sample holder and examined up to a maximum intrusion pressure of Hg of 2000 bar.

2.5. BET surface area measurement

A powder sample of 300–800 mg was first degassed under vacuum for 12 h at 30 °C. The BET measurement was then performed using an Area-Meter II (Ströhlein Instruments, Berlin, Germany) using LN2 and nitrogen gas. The specific surface area, S_v [m^2/g], of the sample was calculated by the device software using the BET equation.

2.6. Differential scanning calorimetry

The glass transition temperatures of the maximally freeze-concentrated spray solutions were determined using a Mettler Toledo DSC822e (Columbus, OH-USA). 30 µL of sample were sealed in a 40 µL Al pan, cooled down to −60 °C and then reheated at 10 °C/min back to room temperature.

2.7. Size exclusion chromatography

The aggregation status of the bCA was determined in rehydrated samples of the SFD powders (2–10 mg/mL in 50 mM tris buffer pH 7.5). A Superdex 200 column (GE Healthcare, Munich, Germany) was connected to a Perkin Elmer HPLC system comprising 200 LC pumps and an ISS 200 auto-sampler (Perkin Elmer, Waltham, MA-USA). The column was kept at 30 °C in a column heater (CO30, EchoTherm, Hartkirchen, Austria). Detection was done using a UV diode-array (235C, Perkin Elmer, Waltham, MA-USA), a refractive index detector (RI-101, Shodex, Munich, Germany) and a miniDawn Treos multi-angle light-scattering detector (Wyatt Technology, Dernbach, Germany). The mobile phase was isocratic tris buffer pH 7.5 50 mM also containing 150 mM sodium chloride. The flow rate was 0.5 mL/min. All chromatograms were evaluated from the static light scattering (SLS) signal using the Astra Software (Wyatt Technology, Dernbach, Germany).

3. Results and discussion

3.1. Introduction of an annealing step

The annealing procedure was done not to enhance crystallization (Tang and Pikal, 2004), but rather to promote viscous flow of the amorphous components of the highly porous SFD particles (Rochelle and Lee, 2006). After the initial holding step at shelf temperature, T_{shelf}, = −45 °C, the primary drying (1°) was started by annealing at T_{shelf} = + 5 °C for either 2 h or 6 h (cycles # 1 and # 2 in Fig. 1a). The upwards temperature-ramp was started simultaneously with turn-on of the vacuum pump. This T_{shelf} was selected to be well above the temperature range of −15 to −10 °C where quenched aqueous proteins show their T_g' (Pikal et al., 2007) and should therefore bring product temperature closer to T_g' than without annealing. Yet despite this high T_{shelf}, the resulting microparticles of pure bSA show a spherical morphology with smooth porous surfaces and no visible signs of shrinkage or wrinkling caused by annealing (Fig. 2a for the example of cycle #1; SEM from cycle # 2 was identical). Even raising T_{shelf} to +10 °C for 2 h of annealing (cycle # 3 in Fig. 1a) gave the same picture (SEM not shown). This complete lack of visible shrinkage on annealing at a T_{shelf} of up to +10 °C differs greatly from the behaviour of SFD microparticles of pure trehalose which showed a strongly shrivelled appearance after just 1 h annealing at T_{shelf} = + 5 °C (Sonnner et al., 2002). Viscous flow and shrinkage can be expected when the amorphous product's temperature approaches and exceeds the critical temperature of the frozen solid and its viscosity decreases sharply (Rambhatla et al., 2005). This is taken to be T_g' for the annealing cycles used here (Rochelle and Lee, 2006). For quenched trehalose the value is −31 °C (Pyne et al., 2003) whereas quenched aqueous proteins show T_g' at higher temperatures of −15 to −10 °C (Pikal et al., 2007). A dialysed