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International Journal of Pharmaceutics 294 (2005) 73–87
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Aqueous two-phase systems as a formulation concept for spray-dried protein

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Received 30 July 2004; received in revised form 27 December 2004; accepted 4 January 2005

Abstract

This study investigates to what extent an aqueous two-phase system (ATPS) can encapsulate and protect the secondary structure of a protein during spray drying. The ATPSs contained polyvinyl alcohol (PVA) and dextran solutions, in different proportions. A model protein, bovine serum albumin (BSA) and, in some experiments, trehalose were added to the ATPS prior to spray drying. Electron spectroscopy for chemical analysis (ESCA), differential scanning calorimetry (DSC), UV spectrophotometry, size exclusion high-performance liquid chromatography (SEC-HPLC) and Fourier transform infrared spectroscopy (FTIR) were used for analysis of solid and reconstituted samples. The anticipated function of the ATPS was to improve the stability of the protein by preventing interactions with the air–liquid interface during drying and by improving the encapsulation of the protein in the dried powder. BSA was found to preferentially partition to the dextran phase and in the absence of PVA, BSA dominated the powder surface. In samples containing PVA, the polymer mainly covered the powder surface, even though the dextran-rich phase was continuous, thus preventing protein surface interactions and providing improved encapsulation. However, PVA was found to cause partial loss of the native structure of BSA although the protein was well encapsulated during spray drying.

Keywords: Aqueous two-phase system; Trehalose; Fourier transform infrared spectroscopy; Electron spectroscopy for surface analysis; Spray drying; Bovine serum albumin; Encapsulation; Protein formulation

1. Introduction

Freeze-drying, grinding and spray drying are used for producing pharmaceutical powders for inhalation, etc. The production method extensively influences the physical properties, such as flowability, hygroscopicity and dissolution, due to variation of the physical state (amorphous versus crystalline), particle size and composition at the particle surface. In addition, the biological activity of proteins, etc. included in the particles is influenced by the drying process.

In the particular case of spray drying, several processes impose stresses that can destabilise proteins and peptides, such as high pressure, high shear and im-

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doi:10.1016/j.ijpharm.2005.01.015

mense air–liquid interfaces during atomisation, heating and dehydration (Mumenthaler et al., 1994; Maa and Hsu, 1997). However, spray drying provides the possibility of formulating the active protein and creating particles suitable for inhalation in one step. Similar to protein stabilisation during freeze-drying, addition of disaccharides (such as sucrose and trehalose) or amino acids (such as arginine hydrochloride) to the liquid formulation increases the stability during spray drying and subsequent storage (Broadhead et al., 1994; Andya et al., 1999; Tzannis and Prestrelski, 1999).

The mechanisms behind disaccharide stabilisation are under constant debate in literature. First, the "Water Replacement Hypothesis" presented by Carpenter and Crowe (1988) describes the development of hydrogen bonds between the excipient and the protein as water is replaced during drying (Allison et al., 1999; Kreilgaard et al., 1999). Second, "Glassy State Stabilisation", first presented by Green and Angell (1989) and supported by Franks et al. (1991), describes a physical immobilization of proteins in an amorphous matrix formed by the excipients. Third, "Water Trapping" proposed by Belton and Gil (1994) suggests that water, essential for the conformation of the protein, is trapped in complexes formed by sugar and protein. Fourth, "Dilution" has been put forward by Costantino et al. (1994) as the stabilising mechanism, as the molecular interactions between protein molecules (aggregation) become less common upon adding sugars.

Alongside stabilisation of proteins by addition of disaccharides, surface-active additives can be utilised for prevention of protein adsorption to the air–liquid interface. Surfactants (such as polysorbate 20 (Maa et al., 1998), polysorbate 80 (Broadhead et al., 1994; Millqvist-Fureby et al., 1999; Adler et al., 2000), sodium lauryl sulphate (Adler et al., 2000) and Pluronic F88 (Maa et al., 1998)) reduce the adsorption of protein to the surface of the drying droplet thereby preventing or delaying the surface-induced denaturation of proteins.

A different, and yet unexplored, approach for the prevention of protein adsorption at surfaces and improvement of its stability in the dried state is encapsulation in an aqueous two-phase system (ATPS) formed by two immiscible polymers in solution (Albertsson, 1986). In addition to providing efficient encapsulation of the protein, the polymers can be selected to give the formulation desired release properties and dissolution profiles.

In an ATPS, formed by two non-ionic polymers, the phase separation is due to the small gain of entropy of mixing large polymer molecules. This is dominated by the large enthalpic contribution of interaction between the molecules (Flory, 1953; Albertsson, 1986). In other words, phase separation occurs at very low polymer concentrations because only few of these large molecules are needed to form many interactions between segments and water.

The ATPSs are generally used in the liquid state for separation of proteins, cell organelles and cells (Albertsson, 1986). The degree of partitioning is determined by the surface properties of the materials included in the ATPS. When applying ATPS for encapsulation purposes, the ATPS can be viewed as a "water-in-water" emulsion, in which one phase is dispersed in the other, continuous phase. It can be expected that the phase separation occurring in the ATPS in the liquid state is preserved during spray drying and that encapsulation of polymer droplets containing the protein will reduce the level of protein interaction with the potentially harmful air–liquid interface. During drying, the phase separation may even proceed further as the polymer concentration increases, although the extent of this may be limited in such a rapid drying process as spray drying. However, during freeze-drying, phase separation has been confirmed by multiple glass transitions for one-phase aqueous mixtures of polyethylene glycol (PEG)-dextran (Heller et al., 1997). Due to low interfacial tension (between 0.0001 and 0.1 mN/m compared with 1–20 mN/m for conventional water–organic interfaces) between the polymer phases adsorption of protein at the liquid–liquid interfaces is less likely (Forciniti et al., 1990). The low interfacial tension further implies that only a low level of energy is required to obtain and maintain an emulsion structure in the ATPS. Moreover, the polymers alone can stabilise particle structures and biomolecules (Albertsson, 1986). For example, polyvinyl pyrrolidone (PVP) and dextran were successfully used as a novel concept for the encapsulation and spray drying of the bacterium *Enterococcus faecium* (Millqvist-Fureby et al., 2000). However, when considering ATPS as a formulation approach for spray drying of a protein, care must be taken in combining the protein with polymers. The polymers must form an ATPS at reasonably low polymer concentra-

tions, the viscosity of the ATPS must be sufficiently low to allow spray drying, polymers that exhibit extensional viscosity may cause practical difficulties with respect to powder production and powder structure, and the protein should preferentially partition to one of the phases.

In the present study, in a two-phase system consisting of polyvinyl alcohol (PVA) and dextran, the partitioning and stabilisation of a model protein, BSA, during spray drying, was investigated. During spray drying, one of the polymer phases will be encapsulated by the other (depending on the phase volume ratio), and provided that the protein partitions preferentially to the encapsulated phase, improved protein stabilisation is anticipated.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), Cohn fraction V, >96% purity and potassium bromide (IR-grade) were obtained from Sigma Chemical Co. (St Louis, MO). Dextran T70 (M_w 67.2 kDa) was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden) and polyvinyl alcohol (PVA) was supplied as Airvol 205 by Air Products and Chemicals, Inc. (c/o Air Products Nederland B.V., Utrecht, The Netherlands). The degree of hydrolysis of PVA was 88.26% and the molecular weight was approximately 30 kDa. D(+)-Trehalose dihydrate, $HNa_2PO_4{\cdot}12H_2O$ and $NaH_2PO_4{\cdot}H_2O$, used for the buffer solution, were purchased from Fluka Chemie GmbH (Buchs, Germany) and Merck (Darmstadt, Germany), respectively. Milli-Q water was used throughout the experiments.

2.2. Preparation of ATPS

Particles were prepared by spray drying of ATPS solutions containing BSA in two types of matrices: PVA–dextran and PVA–dextran-trehalose. The ATPS solutions were prepared by mixing stock solutions (10%, w/w) of PVA and dextran in different proportions (Table 1). BSA solution (10%, w/w) was added to the ATPS system so that the protein:polymer ratio was 5:95 on a weight basis. In formulations containing trehalose, disaccharide corresponding to 20% of the total polymer dry weight was dissolved in the BSA-ATPS solutions prior to spray drying. All solutions were prepared with a 10-mM Na-phosphate buffer at pH 7.

2.3. Spray drying

Spray drying was performed in a laboratory spray dryer built at the YKI, Institute for Surface Chemistry. The dryer operates in a co-current mode, with a jacketed two-fluid nozzle and has a drying column of 750 mm in length and 150 mm in diameter. Compressed air from an in-house supply was used for atomisation of the feed solution. The emulsion was maintained by continuously stirring the feed solution. The spray-drying conditions used in all experiments are presented in Table 2. Particles were separated from the drying air by a membrane filter, as described earlier (Elversson et al., 2003).

Table 1
Compositions of ATPS

PVA content of total polymer (%)	PVA content of total composition (%)	Dextran content of total composition (%)	BSA content of total composition (%)	Trehalose content of total composition (%)
0	–	9.5	0.5	–
10	0.95	8.55	0.5	–
20	1.9	7.6	0.5	–
25	2.375	7.125	0.5	–
50	4.75	4.75	0.5	–
100	9.5	–	0.5	–
0	–	9.32	0.49	1.86
10	0.93	8.39	0.49	1.86
25	2.33	0.70	0.49	1.86
50	4.66	4.66	0.49	1.86
100	9.32	–	0.49	1.86

Table 2
Conditions for spray drying of BSA-ATPS solutions

Parameter	Setting
Inlet temperature (°C)	180
Outlet temperature (°C)	70–75
Liquid feed rate (ml/min)	5
Drying air flow (m^3/min)	0.8
Nozzle jacket temperature (°C)	25
Nozzle orifice diameter (mm)	1.5
Atomisation air flow (l/min)	29

2.4. Phase diagram and phase composition of PVA–dextran

Sample solutions containing 4–10% (w/w) polymer were prepared from the stock solution and buffer. The phase diagram of PVA–dextran was determined by letting equal volumes of each polymer solution at each concentration separate in graduated 10-ml test tubes. The phase volume and phase composition were analysed after 21–24 h of settling at room temperature (23–25 °C). One millilitre sample was withdrawn from each phase with a syringe. Absorbance was measured at 280 nm for the determination of PVA content in both bottom and top phases. The absorbance of dextran at this wavelength was negligible compared to the absorbance of PVA and the total solid content was used for the calculation of the dextran concentration of each phase. The total solid content was obtained from drying the samples in a drying cabinet (UT6, Heraeus Instruments GmbH, Hanau, Germany) at 105 °C for at least 10 h.

2.5. BSA partitioning in ATPS

Partitioning of BSA was measured in samples of the same compositions as those used for spray drying (duplicate analysis). The concentration of PVA in each phase and the concentration of BSA in the dextran-rich phase were recorded from absorbance at 320 and 280 nm, respectively, using a corresponding ATPS without protein as a blank. The concentration of BSA in the PVA-rich phase was calculated from the total load of BSA, BSA in the bottom phase and the phase volumes of the settled system. Test tubes, settling parameters and sampling were as described above. When necessary, samples were diluted with buffer for the analysis.

The partition coefficient K of BSA was calculated according to Eq. (1), where C is the BSA concentration (%, w/w) in top and bottom phases:

$$K = \frac{C_{\text{top}}}{C_{\text{bottom}}} \tag{1}$$

2.6. Electron spectroscopy for chemical analysis (ESCA)

Electron spectroscopy for chemical analysis (AXIS HS photoelectron spectrometer, Kratos Analytical, UK) was used to determine the elemental composition of the powder surface. The instrument used a monochromatic Al Kα X-ray light source. The circular analysis area was approximately 1 mm^2 and the analysis depth was less than 100 Å. Three measurements were made for each sample, each at different spots in a total sample area of approximately 20 mm^2.

The surface composition of the powder is estimated by analysing the relative amounts of different elements (carbon, oxygen, nitrogen) in the pure components (excipients and BSA) and in the spray-dried samples (Fäldt and Bergenståhl, 1993). Assuming the elemental composition of the surface is a linear combination of the elemental compositions of the different components in the sample, the data of the elemental composition of the pure components can be used to calculate the composition of the surface by solving a matrix equation, by using Eq. (2):

$$A\gamma = f \tag{2}$$

where A is the matrix containing the elemental composition of the pure components, f the vector containing the elemental composition of the sample surface and γ the relative coverage of the different components. Applying the least-squares method for solving Eq. (2) overdetermined systems could be solved using Eq. (3):

$$\gamma = (A^{\mathrm{T}}A)^{-1}A^{\mathrm{T}}f \tag{3}$$

2.7. Fourier transform infrared spectroscopy (FTIR)

The biological activity in terms of secondary structure of BSA was analysed with Fourier transform infrared spectroscopy. FTIR spectra were obtained using a Bomem HB 104 instrument (ABB, Inc., Norwalk,

CT) equipped with a TGS detector operating in a nitrogen atmosphere. A spectral resolution of 4 cm^{-1} was used and the spectra were obtained from 128 scans. Spectra were acquired using the direct mode where the air background was recorded without any sample in the spectrometer. Spray-dried samples were analysed both as solids and after rehydration in water.

Solid samples were added to approximately 0.4 g of dry KBr to obtain a protein–KBr ratio of 1:1000. The KBr–sample mixture was ground by hand in an agate mortar to mix and reduce the particle size of the components and a thin tablet was pressed under vacuum at a load of approximately 100 MPa. This procedure does not alter the protein structure (Prestrelski et al., 1993; Forato et al., 1998). The absorbance spectra of pure protein were obtained by subtraction of absorbance spectra of placebo samples containing similar amounts of excipients but without BSA. Second-derivative spectra were obtained from Savitsky-Golay derivation with a seven-point smoothing. The spectra were baseline corrected and area-normalised in the amide I region, 1720–1580 cm^{-1}, using the GRAMS32 software (Galactic Industries Corp.).

Liquid samples were applied to a cell with CaF_2 windows, separated by a 6-μm spacer (Mylar X-Ray Film, Chemplex, Palm City, FL). Absorbance of pure protein was obtained by first subtracting the air background from both buffer and protein sample and then subtracting the buffer absorbance from the protein sample absorbance to obtain a straight baseline from 2200 to 1800 cm^{-1}.

2.8. Size exclusion high-performance liquid chromatography (SEC-HPLC)

Size exclusion high-performance liquid chromatography was performed on a Hewlett-Packard (series II, 1090) HPLC (Palo Alto, CA) equipped with a diode array detector, using a detection wavelength of 280 nm. A few samples were analysed at 5 mg/ml of BSA, but the level of aggregates was higher in these samples compared to those analysed at a lower concentration, therefore 1 mg/ml of BSA was selected for the further SEC-HPLC analyses. The amount of native protein and non-native aggregates in hydrated samples (1 mg/ml of BSA) was quantified using a Tosoh Bioscience TSK-gel G2000SWXL column (Tokyo, Japan) eluted with Na-phosphate buffer, pH 7.0, at 0.6 ml/min flow rate.

The amounts of native BSA monomers and aggregates were calculated from peaks eluting at 12.0 and 10.8 min, respectively. Results were expressed as peak percentage area of the total area.

2.9. Scanning electron microscopy (SEM)

A scanning electron microscope, model XL 30 TMP (W) (FEI Company, Hillsboro, ON), in high vacuum mode was utilised for evaluation of surface morphology of the particles. Powder was applied to a carbon tape covered Al-stub and coated with Au of 520 Å thickness (Sputter Coater SCD 050, Balzers Union AG, Balzers, Lichtenstein) prior to analysis.

2.10. Differential scanning calorimetry (DSC)

The physical state of solid samples was measured by differential scanning calorimetry. Samples of 1.5–3.8 mg were placed in 40 μl Al-pans with a perforated lid. Samples were heated from 20 to 250 °C at a rate of 10 °C/min with a nitrogen flow of 50 ml/min. Thermograms of all samples were obtained in triplicate.

2.11. Pycnometry

A gas pycnometer (AccuPyc 1330, Micromeritics) was used to determine the apparent particle density (i.e. mass of the particle divided by the volume of the particle including closed pores, but excluding open pores) of spray-dried material. Two-thirds of a 1-cm^3 insert was filled with powder, weighed and flushed with N_2 for volume analysis. Each sample was run five times and the relative standard deviation of the average apparent density was less than 0.05%.

3. Results

3.1. Phase diagram and phase composition of PVA–dextran

The phase diagram and phase composition of the PVA–dextran system were determined at four tie lines at ambient temperature, 21–23 °C (Fig. 1). The mean value of the tie-line slope (STL) was -1.006 ± 0.035,

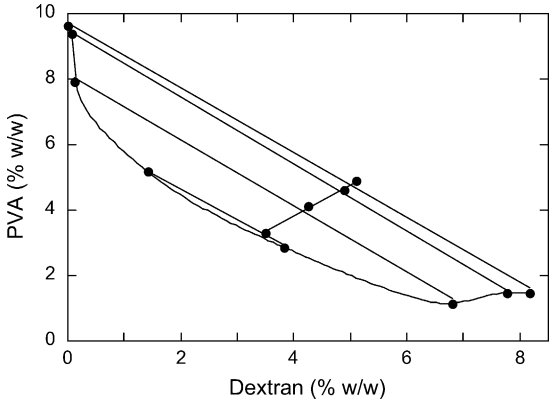

Fig. 1. Phase diagram of PVA and dextran (STL = −1.006 ± 0.035).

using Eq. (4):

$$\mathrm{STL} = \frac{\Delta C(P_1)}{\Delta C(P_2)} \quad (4)$$

where C is the polymer concentration (%, w/w), and P_1 and P_2 are the two polymers. Phase inversion occurred at approximately equal phase volumes, in this case at 50:50 PVA–dextran ratio.

3.2. BSA partitioning in ATPS

The phase composition and BSA partitioning of the formulations produced for spray drying was determined at ambient conditions (Table 3). BSA strongly partitioned to the dextran-rich phase. The partition coefficient (K) ranged from 0.24 to 0.66, demonstrating a strong favouring of the dextran-rich phase even though volumes of the PVA-rich phase were small (Table 3). In the case of equal concentration of the two polymers, 84% of the BSA was detected in the dextran-rich phase.

Table 3
Partitioning of BSA in PVA–dextran ATPSs, with various polymer compositions

PVA content of total polymer (%)	V_{top}/V_{bottom}	K	Percentage BSA in bottom phase (%)
0	–	–	–
10	0.04 ± 0.01	0.66 ± 0.05	98 ± 1.0
20	0.14 ± 0.03	0.48 ± 0.14	94 ± 0.6
25	0.20 ± 0.03	0.39 ± 0.05	93 ± 0.2
50	0.80 ± 0.04	0.24 ± 0.02	84 ± 0.3
100	–	–	–

The total polymer concentration was 9.5% (w/w) and the BSA concentration was 5% (w/w). The BSA content was measured in both top and bottom phases.

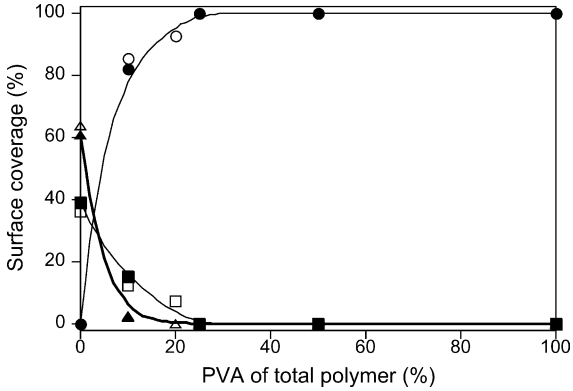

Fig. 2. Surface composition estimated by ESCA of PVA (◯), dextran (□) and BSA (△) in powders without trehalose (unfilled symbols) and with trehalose (filled symbols). The BSA content was 5% of the dry weight.

3.3. Electron spectroscopy for chemical analysis

Even at low PVA concentrations, the particle surface were dominated by PVA, e.g. 86% surface coverage at 10% PVA content of total polymer (Fig. 2). Complete surface coverage was achieved at a PVA content of 25%. At 10% PVA, the BSA surface coverage was only 2% while a 64% surface coverage was observed in a pure dextran matrix. Increasing the BSA content of the formulations from 5 to 10% on a weight basis increased the surface coverage of BSA to slightly more than 70% (in pure dextran/trehalose). The surface composition seemed unaffected by the addition of trehalose to the ATPS.

3.4. Fourier transform infrared spectroscopy

For native BSA (dissolved in buffer), the predominant secondary structure was the α-helix (approximately 55%), which resulted in a strong band at 1657 cm^{-1} (Fig. 3). Upon spray drying all of the formulations showed deviations from the native BSA spectrum, particularly in the dry samples. In all samples, the α-helix band was both broader and lower in intensity; in some cases it was shifted towards a higher band-

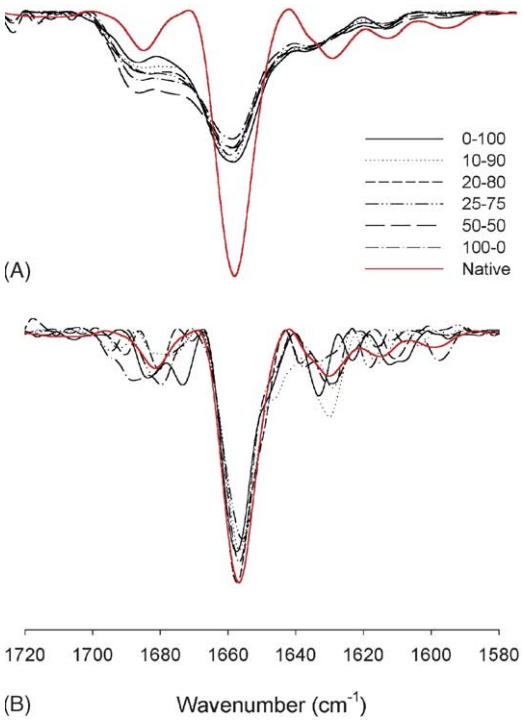

Fig. 3. Second derivative amide I spectra of BSA spray-dried in ATPS with varying composition (PVA:dextran) compared to native BSA, before (A) and after (B) reconstitution of the powders in water. Arrows indicate the direction of band amplitude changes with increasing PVA content of total polymer.

width (1659 cm^{-1}); and new features appeared, especially in the region around 1680 cm^{-1} (corresponding to unordered structures). Minor changes were also observed in the 1600–1640 cm^{-1} region (corresponding to intra- and intermolecular β-sheet).

In dried samples, the content of PVA in the ATPS clearly affected the α-helix content of BSA. The band intensity at 1657 cm^{-1} strongly decreased as PVA was added and the loss of native structure further increased as the content of PVA increased. The depth of the α-helix band relative to native BSA in solution was used as a measure of native structure (Heller et al., 1997), and this is illustrated in Fig. 4. In the solid state, between 43 and 52% of the native helix structure was lost, with increasing concentration of PVA in the formulation (Fig. 4). Conformational loss occurred to a lower degree in samples with trehalose (32–46%). However, after reconstitution in water 81–99% of the helix structure was recovered (Fig. 3).

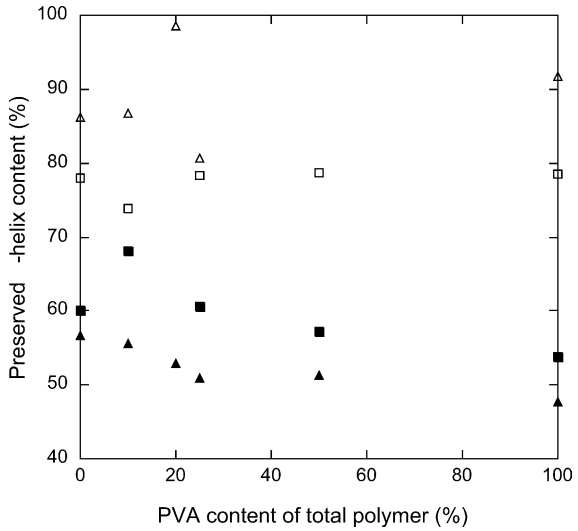

Fig. 4. Percentage preserved α-helix in spray-dried (filled symbols) and reconstituted BSA in ATPS (open symbols), with (■) and without (▲) addition of trehalose.

3.5. Size exclusion high-performance liquid chromatography

The monomer content of spray-dried and reconstituted BSA in ATPS was surprisingly low: between 30 and 40% of the protein was found to be aggregated in all formulations studied (Fig. 5). In addition, considerable fragmentation occurred. Formulations without dextran were more prone to aggregation and consequently the amount of native protein decreased. However, formulations with more extensive aggregation demonstrated less fragmentation. When trehalose was added to the formulations the level of native BSA increased in most cases, due to less fragmentation and less soluble aggregates. The level of insoluble aggregates was similar at low PVA content, whereas high PVA content in the presence of trehalose increased the level of insoluble aggregates.

3.6. Scanning electron microscopy

The shape and surface morphology of particles were strongly affected by the composition of the two-phase system. As the PVA content increased, the particles turned from highly wrinkled to nearly smooth spheres (Fig. 6). Thread-like structures and small doughnut-shaped particles were also observed as well as a slight

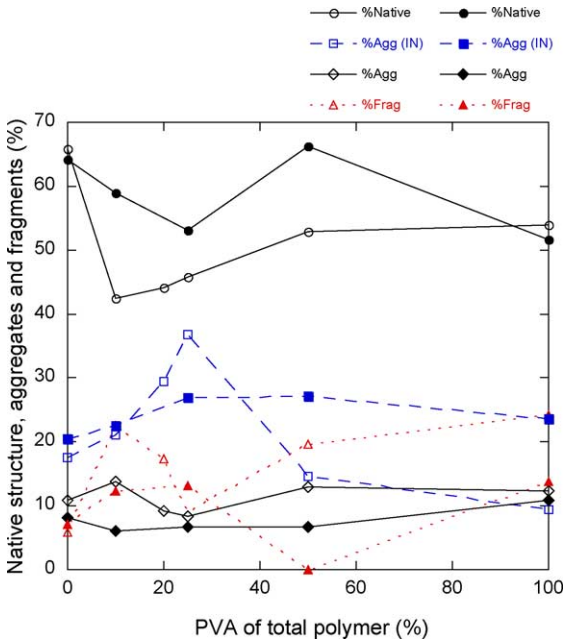

Fig. 5. Percentage of native structure (●), insoluble (■) and soluble (♦) aggregates and fragments (▲) of reconstituted BSA from PVA–dextran (dotted line) and PVA–dextran–trehalose (solid line) powders.

increase of the particle size when the PVA content increased. No visible change of shape, morphology or size of the particles was observed after addition of trehalose to the various compositions of polymers. Neither did an increase of the BSA content from 5 to 10 mg/ml give any notable difference in appearance.

3.7. Differential scanning calorimetry

Up to three thermal transitions were observed on the DSC curves of each spray-dried sample: two-phase systems had two separate glass transitions, at 44–50 °C (T_g^1) and at approximately 230 °C (T_g^2), as well as a melting peak at 196–199 °C (Table 4). In samples without PVA, both the lower glass transition and the melting endotherm were absent. In addition, the lower glass transition was not detected in samples containing the lowest amount of PVA (10%). However, all samples containing PVA showed recrystallisation between 123 and 132 °C upon cooling (data not included in Table 4).

Temperatures corresponding to the glass transition, melting and recrystallisation of pure spray-dried PVA were according to the DSC measurements: 49, 196 and 110 °C, respectively. A transition, assumed to be the glass transition of dried dextran occurred around 224 °C but neither melting nor crystallisation was observed within the scanning range (20–300 °C).

Samples supplemented with trehalose had T_g^1's similar to that of powders without trehalose. T_g^2's were difficult to analyse as the melting transitions (attributed to PVA) were shifted upwards, however, both T_g^2 and T_m were shifted to slightly lower temperatures in the presence of trehalose. Recrystallisation in PVA and trehalose-containing powders occurred between 114 and 116 °C. For pure spray-dried trehalose the thermal transitions according to the literature are glass transition at 118–119 °C (Naini et al., 1998; Mosén, 2003) and melting at 236 °C (Mosén, 2003).

3.8. Pycnometry

The apparent density of spray-dried ATPS particles was considerably affected by the composition of the powder (Fig. 7). Above all, an increasing PVA–dextran ratio resulted in a linear reduction of the apparent density from 1.3 to 0.4 g/cm^3, while addition of trehalose increased the density (the increase

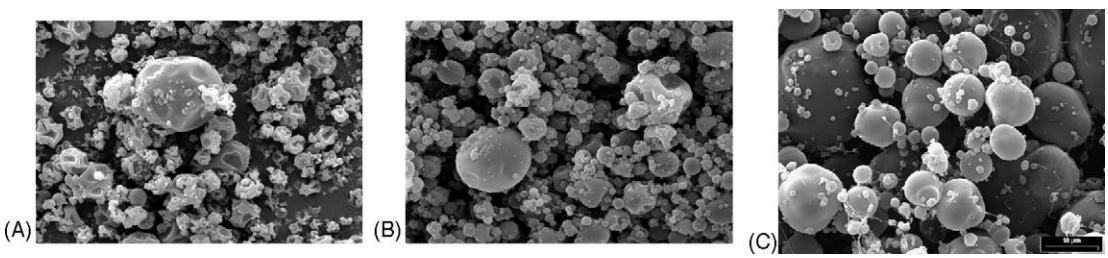

Fig. 6. SEM images of spray-dried of ATPS particles with BSA. The PVA content of the total polymer content was 0% (A), 10% (B) and 100% (C). Magnification was 2000×.

Table 4
Thermal transitions at heating in ATPS containing PVA, dextran and BSA, with and without trehalose

PVA content of total polymer (%)	PVA–dextran			PVA–dextran–trehalose		
	T_g^1 (°C)	T_m (°C)	T_g^2 (°C)	T_g^1 (°C)	T_m (°C)	T_g^2 (°C)
0	nd[a]	nd	229.7 ± 0.5	nd	nd	210.1 ± 0.2
10	nd	197.0 ± 0	229.5 ± 0.3	nd	nd	208.1 ± 0.6
20	44.4 ± 0.3	196.5 ± 0.5	229.3 ± 1.2	–	–	–
25	45.3 ± 0.7	196.2 ± 0.3	nd	47.8 ± 0.8	191.2 ± 0.1	nd
50	47.1 ± 1.1	196.2 ± 0.3	nd	47.3 ± 1.0	189.7 ± 1.7	nd
100	49.8 ± 0.7	198.3 ± 0.3	nd	45.3 ± 1.7	189.2 ± 0.6	nd

Pure excipients	T_g (°C)	T_m (°C)
PVA	49.3 ± 0.3	195.6 ± 0.5
Dextran	224.5 ± 1.0	nd
Trehalose	118.5[b]	236[c]

[a] nd = transition not detected.
[b] Naini et al. (1998) and Mosén (2003).
[c] Mosén (2003).

ranged from approximately 0.02 to 0.14 g/cm^3). A slight reduction of the density was also observed in the PVA–dextran–trehalose system upon doubling of the protein content while the density of ATPSs without protein were similar to the powders containing 5% (w/w) protein.

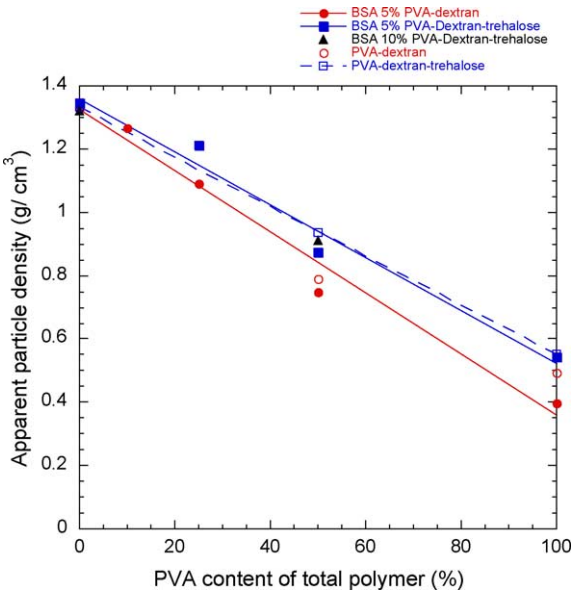

Fig. 7. The apparent particle density of various spray-dried ATPS particles: with (solid line) and without BSA (dotted line), and with (●) and without (■) trehalose. Linear fit: $R \geq 0.99$.

4. Discussion

In the present study the feasibility of using the ATPS concept to encapsulate a model protein, BSA, has been evaluated to investigate whether this would improve the protein stability during spray drying. The criteria for selecting the system was:

- The selected polymers must form an ATPS at such concentration that the viscosity of the solution is low enough to allow spray drying.
- The polymers should preferably not exhibit extensive extensional viscosity, as this imparts particle formation during spraying.
- The T_g of the respective polymers must be high enough to allow preparation of particles.
- The protein should preferably partition strongly to one of the phases.
- The protein should not interact negatively with any of the polymers.

Thus, we chose to formulate the ATPS using PVA and dextran, since PVA is easy to spray dry at molecular weights around 30 kDa, forms an ATPS with dextran at a relatively low polymer concentration and has a presumed ability of high surface coverage due to its surface activity. Any specific interactions between BSA and PVA were not known when initiating the study. The most frequently used system for partitioning of proteins in liquid systems, PEG–dextran, cannot be

used for spray drying at high levels of PEG since this polymer has a low T_g and particles were not obtained when spray drying such a system (data not shown). The PVP–dextran system, successfully used for preservation and drying of bacteria (Millqvist-Fureby et al., 2000), was however, not suitable since it displays extensive extensional viscosity causing thread spinning effects during spraying. Further, PVP is less suitable as a model system for analytical reasons, since the elemental composition of the polymer is similar to that of BSA. Thereby the ESCA evaluation becomes complicated and uncertain.

The most important parts of the present investigation was thus if a strong partitioning of BSA to one of the phases in the ATPS could be obtained and to determine whether any specific interactions between BSA and any of the components caused negative stability effects.

4.1. Distribution of components in the ATPS and surface composition of spray-dried powder (phase diagram, BSA partitioning, ESCA)

To allow spray drying, an ATPS has to be formed at low concentrations to avoid particle defects related to the viscosity of the polymers, as mentioned before. The critical point (i.e., the lowest polymer concentration where phase separation is observed) of a PVA (55 kDa)–dextran (57.2 kDa) system reported by Zaslavsky (1995) was 2.85% (w/w) of PVA and 2.65% (w/w) of dextran and the STL was −1.079. Addition of 0.11 M sodium phosphate buffer at pH 7.4 lowered the critical point to 2.57% (w/w) of PVA and 2.00% (w/w) of dextran and the STL increased to −1.771 (Zaslavsky, 1995). Our system was buffered with a 10 mM sodium phosphate buffer of pH 7.0 and had an STL −1.006. In addition, the molecular weights of the polymers used here were different, approximately 33 kDa for the PVA and 67.2 kDa for the dextran as well as the degree of hydrolysis. Such facts may account for differences in the STL values for the different systems (Fig. 1). The critical point was not possible to detect, due to low difference in refractive index between the phases.

Trehalose was presumed to distribute evenly between the phases according to the common assumption of distribution of small solute molecules, such as saccharides (Albertsson, 1986). Large molecules such as proteins are generally more unevenly distributed but chemical structure rather than size determines the partitioning in an aqueous two-phase system. The partition coefficient for BSA in the present PVA–dextran system (equal polymer concentrations) was 0.24, meaning that 84% of the BSA was found in the dextran-rich phase (Table 3). A similar system, PEG–dextran, would not be as efficient in encapsulating BSA because the reported value of K is higher, 0.45 at a pH close to the isoelectric point of BSA (Zaslavsky, 1995).

As pointed out earlier, the phase separation in the droplets during spraying is likely to be preserved during drying. In addition, the composition at the air–liquid interface of the droplets during spray drying will be maintained in the solid state (Fäldt and Bergenståhl, 1994). Hence, analysis of the surface composition of the powder by ESCA enables the encapsulation of BSA to be monitored.

4.1.1. Surface composition of powders without PVA

From ESCA, 64% of the particle surface was found covered by BSA, at a protein–dextran ratio of 5:95 (Fig. 2). Landström et al. (1999) reported similar surface coverage, 65 and 77% for BSA–dextran ratios of 3:97 and 10:90, respectively. Similar surface coverage was also reported by Fäldt and Bergenståhl (1994) for BSA–lactose (65%) with a protein:sugar ratio of 5:95. However, in BSA–trehalose the same protein sugar ratio resulted in only 40% of surface coverage (Adler et al., 2000).

In addition, not more than 70% of surface coverage was reached even with a BSA concentration increased to 40 wt.% (Adler et al., 2000), which is in accordance with our findings of 70% surface coverage with a BSA–dextran ratio of 10:90 (Fig. 2). The reason for the high surface coverage of protein in relation to the overall composition is that the surface-active component adsorbs to the air–liquid interface during atomisation. Usually a threshold is found, above which the surface coverage of protein does not increase (Fäldt and Bergenståhl, 1994; Landström et al., 1999; Millqvist-Fureby et al., 1999). Addition of trehalose did not change the surface composition of the spray-dried powders (Fig. 2).

4.1.2. Surface composition of powders with PVA

In all two-phase systems containing more than 5% PVA (of total polymer), the powder surface was found

to be dominated by PVA (Fig. 2), even though the dextran-rich phase was continuous, i.e. it can be assumed that the PVA at the powder surface originated from the dextran-rich phase. PVA and BSA possess similar equilibrium surface activities (approximately 50 mN/m) (Bhattacharya and Ray, 2004; Absolom et al., 1981), but it appeared as though PVA adsorbed more "efficiently" than BSA to the air–liquid interface of the drying droplet. Since mass transport in the drying droplet is mainly controlled by diffusion and convection, the sizes of the polymer and protein molecules become a factor of consideration. PVA has about half of the mass of BSA (30 and 66 kDa, respectively) which result in a diffusion coefficient of approximately half that of BSA (6.7×10^{-11} m^2/s) (Shen and Probstein, 1977) and hence, diffusion is faster for PVA than for BSA (according to Stokes–Einstein's diffusion equation). However, diffusion alone is not enough to explain the high accumulation of PVA at the surface. In a water solution, approximately 30 ms is needed for the polymer to reach the surface of a 10-μm droplet by diffusion-controlled adsorption, which is much longer than the estimated drying time for such a droplet to reach the critical concentration where precipitation at the surface starts (about 4 ms). If the effects of viscosity on the diffusion during the gradual enrichment and drying of the system were also considered the diffusion time would increase further. However, droplets need to be transported from the centre of the spray cone to the edge in order to raise their temperature and start to dry (King, 1990), which also adds to the total drying time (up to 25 ms assuming a radial velocity that is 1% of the axial velocity of the spray at the orifice). The droplets in the spray will represent a range of droplet life times, but the droplet lifetime will be similar or shorter than the required time for diffusion of the polymers to the droplet surface. Therefore, competitive interfacial adsorption of polymers and proteins during spray drying is not explained by differences in diffusion alone but convection must be considered as well. Furthermore, the random coil configuration of PVA in solution will be beneficial for the kinetics of adsorption as compared to the ordered configuration of BSA which has to be partially unfolded for adsorption to occur. It can thus be assumed that the adsorption kinetics is of importance for the outcome of the competition between PVA and BSA for the interface. In the case of 10% PVA, the dextran-rich phase contained approximately 2.5% PVA, 7.8% dextran and 0.5% BSA (Fig. 1). PVA showed 3.7 times surface excess whilst BSA showed 0.5 times surface excess (Fig. 2). This corroborates the contention that PVA adsorbs more efficiently than BSA, and thus acts as an encapsulant, even when the PVA-rich phase is the dispersed phase.

4.2. Encapsulation effects on the physical particle properties (DSC, SEM, pycnometry)

In a solid solution, containing two components, T_g and T_m of the mixture will depend on the transitions temperatures of the individual components. However, in our spray-dried ATPS two separate glass transitions were found in samples with a PVA content equal to or higher than 20% (w/w) (Table 4). The first glass transition (T_g^1) was observed between 44–50 °C and the second glass transition (T_g^2) was detected around 230 °C, but only in samples containing 80% or more of dextran. T_g^1 corresponded to the glass transition of pure spray-dried PVA (T_g = 49 °C) and T_g^2 was likely to originate from amorphous dextran. The DSC curve of unprocessed dextran is rather uneventful but showed a baseline shift around 220 °C. The observation of two different glass transitions indicates that the two-phase system persisted in the solid state (Heller et al., 1997). In samples without PVA no melting or recrystallisation were detected during temperature cycling confirming that these two transitions originated from the PVA-rich phase.

If trehalose distributes to both polymers in the ATPS both the T_g^1 and T_m would increase as these transitions of amorphous trehalose occurs at substantially higher temperatures (Naini et al., 1998; Mosén, 2003) than corresponding values for PVA and accordingly T_g^2 (glass transition of the dextran phase) would decrease. However, T_g^1 was unaffected while T_m decreased 7° upon addition of trehalose. This indicates that trehalose interacts with PVA, possibly to change the structure of the polymer in the solid state. The change in melting temperature and in recrystallisation temperature may indicate that the crystalline structure is altered. Trehalose clearly interacted with dextran as expected, which is reflected as a reduction of T_g^2 to approximately 210 °C.

The thermal effects obtained by trehalose, in the ATPS, were not accompanied by any changes in the

morphology of the particles (data not shown). However, the appearance of the powders was clearly affected by the presence of PVA, in that the particles become smoother and are without dimples (Fig. 6). Presumably, this is due to the state of the surface film at the droplet surface during drying. The properties of the surface film seems thus to be affected by the level of PVA. Moreover, the particles with higher levels of PVA appeared to be slightly larger than particles without PVA. This may be because the surface film solidifies at a higher level of residual water in the case of high content of PVA, or that the effects of PVA on the viscosity and atomisation are such that larger droplets are formed initially. It has previously been shown that an increase in the droplet size result in larger particles (Elversson et al., 2003).

Variation in the properties of the surface film could possibly explain the differences in apparent particle density observed from gas pycnometry. The apparent particle density of the spray-dried powder was lower than the estimated true density (approximately 1.5 g/cm^3) indicating that the particles were hollow or porous (Fig. 7). The apparent particle density decreased as the PVA content increased. In PVA-continuous particles the apparent particle density was approximately half that of the true density or less. Assuming a hollow interior gas pycnometry will describe the "permeability" of the particle shell. Film forming polymers such as PVA are likely to form a dense ("gas-resistant") shell during spray drying and with an increasing PVA content less N_2 reaches the interior void resulting in decreasing apparent particle density. In dextran-continuous particles, the apparent particle density decreased from 1.4 to 0.7 g/cm^3 as the particle shape changed from fully collapsed (with small or no void volume) to slightly dent (Fig. 6). Dextran is likely to be permeable to N_2 and since the interior void is more or less reduced, the apparent particle density will be close to the true density. As more and more of the particle surface is covered by PVA (and particles achieve a spherical shape) the apparent particle density decreased. This supports our assumption that spray-dried ATPS particles are hollow (at least when spherical) and that data derived from gas pycnometry describe a range of densities from true density (the material as such) to apparent particle density, depending on the gas permeability of the particle surface.

4.3. Encapsulation effects on protein (FTIR, SEC-HPLC, DSC)

The protein spectra of a rehydrated formulation is normally derived from subtraction of background (buffer) and if excipients show absorbance in the amide I region, by subtraction of a placebo formulation. In solid FTIR, only the excipients are subtracted. In the case of PVA–dextran, both PVA and dextran showed some absorbance between 1620 and 1680 cm^{-1}. This was subtracted from the sample spectra by making physical mixtures of PVA and dextran in amounts corresponding to the studied formulations. However, the sample spectra were distorted by a broad medium-intensity band at 1670–1680 cm^{-1}. When a spray-dried placebo of PVA and dextran was used instead of the physical mixture, the resolution of the spectra was improved and a proper protein related band between 1680 and 1690 cm^{-1} (β-sheet) was revealed.

When comparing the absorbance spectra of the placebo formulations (50–50) (physical mixture and spray-dried mixture, respectively), two unresolved bands at 1700–1760 cm^{-1}, only present in the spray-dried mixtures, were discovered. This indicates that some interaction between the excipients was present or that a rearrangement occurred either in PVA or in dextran. In another comparison, between spray-dried BSA in pure PVA (100–0) and spray-dried BSA in pure dextran (0–100) with corresponding placebos (physical mixtures), the two unresolved bands were only present in the spray-dried BSA–PVA sample. To sum up, the bands were present in spray-dried BSA–PVA and in spray-dried BSA-free PVA–dextran but neither in spray-dried BSA–dextran nor in a physical mixture of BSA-free PVA–dextran. Three conclusions can be drawn from these facts: (i) the band does not belong to BSA solely, (ii) PVA must be present for the band to occur and (iii) the bands represent an interaction between BSA and PVA, and dextran and PVA, respectively. Considering the structure of the species involved, hydrogen bonding between these different species is the most likely cause.

FTIR analysis demonstrated that considerable loss of native structure of BSA occurred during spray drying although the protein was embedded in a matrix preventing it from partitioning to the surface of the drying droplet (Fig. 3). The spectral features appearing in the dried samples, corresponding to primary β-

turns (1660–1685 cm^{-1}) and to some extent β-sheets (1620–1640 cm^{-1}), increased in intensity as the α-helix content decreased. This indicates that some of the native helix structure of BSA has converted to loops, turns and extended strand conformation. This is in accordance with (Imamura et al., 2003) who reported changes in secondary structure of freeze-dried BSA. A 42% loss in helix structure was detected with FTIR when BSA was freeze-dried without sugar, but addition of trehalose minimized this denaturation to 18%. Other major rearrangements found occurred in the 1660–1690 cm^{-1} region (unordered structure and β-sheet).

Furthermore, broadening of the helix band illustrates transformation to a non-native structure. The extent of preserved native conformation can be quantified by using the method of area overlap (Kendrick et al., 1996). An overlap in area between the normalized spectra of a sample relative to the solution spectra of native BSA of 1.0 indicates an identical spectrum whereas 0.0 means that there is no similarity at all. Here, the simpler method of measuring the depth of the α-helix band at 1657 cm^{-1} to quantify the degree of non-native structure (Fig. 4) was used (Heller et al., 1997). The data showed that the presence of PVA incurs a loss of helix structure, which is directly related to the level of PVA. Thus, the encapsulation of BSA in the ATPS cannot prevent the interactions between protein and PVA that distort the protein structure. This may allow the extensive aggregation observed (Fig. 5), although it can be assumed that protein denaturation at the air–liquid interface is prevented through the efficient encapsulation. Dextran alone failed to protect BSA during drying, a fact that has been reported by others (Allison et al., 1999). Only 57% of the helix structure of BSA was preserved which is comparable with Allison et al.'s (1999) data on lysozyme (60% preservation). Steric hindrance was presented as one reason that hydrogen bonding between dextran and protein in the dried state is not effective (Allison et al., 1999). The native conformation was slightly improved by adding trehalose to the ATPS, as was expected. The reduced T_g^2 indicated that trehalose and dextran interact and form a true mixture and thus stabilisation with trehalose may benefit from higher dosage. However, all samples supplemented with trehalose had helix bands more narrow than the corresponding PVA–dextran samples, indicating less structural variability within the trehalose samples (data not shown). The present data further indicate that the presence of a glassy matrix in which the protein is embedded is not sufficient for preservation of the protein structure, but slightly improved in the presence of trehalose.

The BSA polypeptide chain is constrained by 17 sulphide bridges, which makes the protein prone to refolding upon rehydration. However, not all chains refold properly but arrange in aggregates of dimers and trimers. As a result, appreciable unfolding in the solid state, as detected in all spray-dried samples often correlates with increased tendency to aggregation upon rehydration (Dong et al., 1995), which was confirmed by SEC-HPLC (Fig. 5).

4.4. ATPS as formulation concept for proteins

The ATPS concept was successful in terms of protein encapsulation during spray drying, thus minimizing the exposure of protein to the large air–liquid interface of the drying droplets. However, the system dried from the ATPS presented here suffered from extensive aggregation of BSA, and thus PVA cannot be considered appropriate for the purpose. However, other systems using materials approved for the desired delivery route can be formulated, although considering the requirements stated above it may be a time-consuming task to find a suitable ATPS. Nevertheless, a possible application of the ATPS concept could be for, e.g. controlled release formulations. By proper usage of polymers, control of droplet size (of the dispersed phase) and behaviour upon reconstitution various wetting and dissolution profiles can be obtained. For example, particles covered by dextran dissolved easily (approximately in 10 min) in water while particles covered by PVA needed nearly an hour, depending on the polymer ratio, to dissolve (data not shown).

5. Conclusions

Successful encapsulation of BSA in aqueous two-phase systems of PVA and dextran was demonstrated. Phase separation and exclusion of BSA from the air–liquid interface were confirmed after spray drying. By variation of the components in the ATPS, specific surface properties were established. However, the

secondary structure of BSA was not stabilised as efficiently as expected. In fact, PVA increased the loss of native structure and dextran was not sufficient as stabilizer. The destabilisation may be due to specific interactions between PVA and BSA. The well-documented effect of trehalose as a protein stabilizer during freeze-drying was confirmed after spray drying, but the total effect was lower than expected, presumably due to the specific interactions between PVA and BSA. The particles were well separated with a low apparent density, and could thus be considered for inhalation.

Acknowledgements

The authors thank Dr. J.F. Carpenter (University of Colorado Health Sciences Centre, Denver, CO) for introduction and use of FTIR instrumentation. Former and present students in the "Carpenter lab" are acknowledged for helpful discussions and laboratory assistance: Dr. K. Sampatkumar (FTIR), Dr. Y.-S. Kim (FTIR) and T. Estey (SEC-HPLC). Thanks also to Paul Smith (YKI, Institute for Surface Chemistry) for linguistic advice. Financial support was received from AstraZeneca R&D Lund, Sweden, and the IF Foundation, Swedish Academy of Pharmaceutical Sciences for student grant and travel, respectively.

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