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# A solid-state NMR study of molecular mobility and phase separation in co-spray-dried protein–sugar particles

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**Abstract**

Molecular mobility and physical form of co-spray-dried sugar–lysozyme formulations were evaluated. Co-spray-dried trehalose:lysozyme and sucrose:lysozyme formulations in 1:9, 1:1 and 9:1 ratios (w:w) were stored at 0% RH and 75% RH for 5–6 days. Molecular mobility and physical form of the co-spray-dried formulations after storage were determined by using $^{13}C$ and $^{1}H$ solid-state NMR as well as X-ray powder diffractometry. The results showed that increasing sugar content in co-spray-dried formulations stored at 0% RH decreased molecular mobility of the amorphous formulations indicating a close association of the protein and sugar. Exposure of sugar–lysozyme 1:1 and 9:1 formulations to 75% RH led to separation of sugar and protein phases, where the sugar phase was crystalline. The intimate sugar–lysozyme interaction of the formulations stored at 0% RH and the phase separation of the sugar-rich formulations stored at 75% RH were also confirmed by using $^{13}C$ solid-state NMR spin-lattice relaxation time-filter ($T_1$-filter) measurements. The propensity of sucrose and trehalose to crystallise was similar; however, the results suggest that part of the sugar in the phase-separated formulations remained amorphous and in close association with lysozyme.


*Keywords:* Protein formulation; Spray-drying; Solid-state NMR; Molecular mobility; Phase separation

## 1. Introduction

Proteins are very labile macromolecules, susceptible to chemical and physical degradation. The preferred way of storing, and nowadays more and more, administration of therapeutic proteins is in the form of dried powders. However, stresses that proteins may undergo during drying and subsequent storage in the dry state may change the protein conformation exposing it to physical and chemical degradation leading to loss of biological activity. Use of stabilising excipients, such as sugars, can prevent physical and chemical degradation of protein formulations by establishment of hydrogen bonds between protein and excipient and by forming an amorphous glassy matrix around proteins (Carpenter and Crowe, 1989; Arakawa et al., 1993; Angel, 1995; Allison et al., 1999). These two mechanisms maintain the protein's native conformation with decreased molecular mobility. However, under stress, such as high humidity or temperature, some protein–excipient formulations may undergo phase separation of the protein and excipient due to crystallisation of the excipient (Forbes et al., 1998; Sun and Davidson, 1998). The phase separation is a potential precursor for inactivation of the protein.

The lungs are potential administration site for proteins since proteins have poor absorption properties by other non-invasive routes (Niven et al., 1994; Gonda, 2000; Codrons et al., 2003). Spray-drying is a potential method for preparing stable protein particles for inhalation therapy (Broadhead et al., 1992; Mumenthaler et al., 1994; Adler and Lee, 1999; Tzannis and Prestrelski, 1999). Spray-dried materials typically contain relatively high levels of residual moisture (Adler

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and Lee, 1999). Spray-dried powders with a moisture level equivalent to freeze-dried materials could only be prepared using high-temperature spray-drying conditions or subsequent vacuum-drying (Maa et al., 1998). Either of these procedures would not be feasible for large-scale manufacturing. In addition, dry powders would eventually equilibrate with the subsequent processing and storage environments. Particle size, shape and structure of spray-dried particles are advantageous for inhalation therapy (Broadhead et al., 1994; Liao et al., 2003). However, several workers have identified that the surface of co-spray-dried protein–excipient particles is protein-rich (Fäldt and Bergenståhl, 1994; Adler et al., 2000), rendering the excipient as the main constituent of the core of the particles. The surface excess of proteins in the spray-dried particles has been reduced by adding surfactants to the formulation to further enhance the stability of the protein (Adler et al., 2000). Although there are many reports of protein conformation and stability in spray-dried protein–excipient particles, to our knowledge, there are no reports of molecular mobility.

In the present study, solid-state NMR has been used to investigate the effect of sugars and humidity on protein and sugar mobility in spray-dried powders. Previously, solid-state NMR has been used successfully to study hydration and stability of proteins (Gregory et al., 1993; Separovic et al., 1998) as well as molecular mobility of freeze-dried excipient–protein formulations (Yoshioka et al., 1993, 1999, 2002; Lam et al., 2002).

The aim of this study was to evaluate moisture-induced changes in molecular mobility, physical form and excipient–protein interactions of co-spray-dried trehalose–lysozyme and sucrose–lysozyme formulations. Trehalose–lysozyme and sucrose–lysozyme powders in different sugar–protein ratios were co-spray-dried and thereafter stored at 0 or 75% RH. Molecular mobility and changes in physical structure of the co-spray-dried formulations were determined by using solid-state NMR and X-ray powder diffractometry.

## 2. Materials and methods

### 2.1. Materials

Lysozyme was purchased from Biozyme Laboratories (U.K.), trehalose was a donation from Hayashibara Biochemical Labs. Inc. (Okayama, Japan) and sucrose was purchased from Sigma Aldrich Co. Ltd. (Poole, U.K.). All materials were used as received.

### 2.2. Spray-drying

Sucrose:lysozyme and trehalose:lysozyme both in 1:9, 1:1 and 9:1 weight ratios as well as neat sucrose, trehalose and lysozyme aqueous solutions were spray-dried (Büchi 190 Mini Spray Dryer, Büchi Labortechnik AG, Switzerland) under the following conditions; inlet temperature $120 \pm 5\,^{\circ}C$, outlet temperature $70 \pm 5\,^{\circ}C$, solution feed rate 8 ml/min, air flow 600 L/min, aspirator control reading 20 (100%). The spray solutions contained 2% (w/w) total dissolved solids. Physical sugar:lysozyme mixtures (1:1, w/w) were blended in a mortar by using the spray-dried neat materials, which were amorphous (halo XRPD patterns, results not shown).

### 2.3. Sample storage

After spray-drying all samples were stored in vacuum over $P_2O_5$ (relative humidity, 0% RH) at least 6 days before further analysis or change in storage conditions. Samples that were stored at 75% RH were placed in a desiccator containing a saturated NaCl solution for 5–6 days prior further analysis. Appearance of the sample powders after storage at 0% was dry powder whereas after storage at 75% RH appearance of the formulations were moist cohesive powder, and in case of the co-spray-dried sucrose:lysozyme 1:1 formulation the powder was sticky.

### 2.4. Solid-state NMR

Solid-state NMR (SSNMR) spectroscopy was performed using a Varian Unity Inova spectrometer (Palo Alto, CA) operating at 75.43 MHz for carbon ($^{13}C$) and 299.947 MHz for protons ($^{1}H$).

Cross-polarisation/magic angle spinning (CP/MAS) $^{13}C$ solid-state NMR spectra were obtained using a Doty CP/MAS probe (Columbia, SC). Measurements were performed at ambient temperature ($22 \pm 2\,^{\circ}C$) Approximately 250 mg samples were spun at 3.9–4.6 kHz in a 7 mm outside diameter rotor. Cross-polarisation with flip back was achieved with a contact time of 1 ms. This process was followed by data acquisition time which varied from 20 to 60 ms with continuous wave (CW) decoupling. Relaxation delays varied from 5 to 60 s (the choice of value was determined from the measured proton $T_1$, see Table 1). In general, the sugar-rich materials required the longer values. The number of transients accumulated varied from 76 to 672, depending on sample properties. Spectra were referenced (indirectly) to tetramethylsilane (by setting the high-frequency line of adamantane to 38.4 ppm).

Proton relaxation times were determined using a Varian static probe (5 mm coil diameter) using approximately 150 mg sample size. Static $^{1}H$ spectrum was also collected (without decoupling) for each sample using 1 ms data acquisition time, recycling times varying from 10 to125 s, and the number of repetitions ranging from 4 to 32.

Spin-lattice relaxation time, $T_1$, measurements were used to determine dynamics on the higher frequency (MHz) timescale, associated with motions of the protein side chains and surface groups (Cornell et al., 1983). The spin-lattice time in the rotating frame ($T_{1\rho}$) of protons, which is sensitive to motions on kHz timescale were also determined for some samples. $T_1$ relaxation times were measured with a