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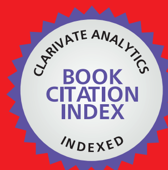

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Chapter

Effect of Spray-Drying and Electrospraying as Drying Techniques on Lysozyme Characterisation

Ijeoma Abraham, Eman Ali Elkordy, Rita Haj Ahmad, Zeeshan Ahmad and Amal Ali Elkordy

Abstract

The production of biopharmaceutical formulation incorporates several difficulties embracing their physical and chemical instabilities. In this study, two drying techniques, namely, spray-drying and electrospraying, were used to assess their application on lysozyme (as a model protein) without and with the use of betacyclodextrin. Samples were prepared in the ratio of 1:1 w/w (protein/betacyclodextrin), and several characterisation methods were applied to study the percentage (%) yield, morphology of the produced partials, thermal stability and biological activity of the protein. The results show the two drying methods led to different particle morphology as spherical-like shape was produced by spray-drying, while rodlike shape was generated by electrospraying with larger particle size. Lysozyme formulations produced by electrospraying were stable just directly after preparation, but after few weeks, those formulations showed visible aggregates. The biological activity of lysozyme was preserved by both drying techniques. In conclusion, both drying methods have different effects on the protein integrity and biological activity in which spray-drying shows more promising results.

Keywords: lysozyme, spray-drying, electrospraying, thermal stability, biological activity

1. Introduction

The majority of the FDA-approved protein-based medicines are delivered via conventional injection route (e.g. subcutaneous, intramuscular or intravenous). The pharmaceutical industries are faced with one of the most significant problems in protein manufacturing. One of the setbacks is the stability in the processing, manufacturing and storage of these therapeutic drugs. Solid dosage forms of therapeutic proteins could improve protein's bioavailability and stability during processing and storage. Various formulation techniques were applied utilising drying process aiming to develop a stable protein formulation (e.g. spray-drying, freeze drying, electrospraying, electrospinning, etc.). This chapter will be looking at the stability of lysozyme as a model of protein using spray-drying technique and

electrohydrodynamic atomisation (EHDA) also known as electrospray. Very few publications have looked at the stability of lysozyme using EHDA technique. This challenge has necessitated the contribution towards this area.

1.1 Proteins

Proteins are macromolecules which require their native structure to be biologically active, and their conformation is very important in the development of protein drugs. They may denature with structural changes under stress, and there will be a loss of activities in the molecules. Examples of stresses are heat, elevated temperature, pressure, surface adsorption and pH [1]. Proteins undergo physical and chemical degradation; examples of physical degradation include aggregation, precipitation and unfolding as updated in Hui et al. [2] which involves the transition of protein from its native state to an unfolded state and will follow a significant loss in the function of a protein which generally will cause an unstable solution during the processing, manufacturing and storage.

1.2 Structure of proteins

Proteins consist of chains or small units of amino acid also known as amino acid polymers or building blocks [3] which contain the backbone or main chain of repeated units with attachments of variable side chains and are linked by peptide bonds. Each protein has a unique sequence of the side chains which determines the characteristics of the individual chain. There is a free carboxyl terminal at the end and a free amino terminal at the other end of every protein except for few cyclic polypeptides. The amino acid sequence is given in order of N-terminal to the C-terminal [4].

They are macromolecules heterogeneous in their native environment and are in most cases unstable. The ordering sequences in amino acids are referred to as the primary structure of protein and the secondary structure (α helix and β sheet); these are three-dimensional elements which all have an orientation of the protein backbone; tertiary structure is formed from secondary structural elements [5]; and quaternary structure comprises of several subunits with tertiary structures [6]. The configuration is determined by the native form followed by the assignment of α helix and β sheet which produces secondary conformation as these molecules are all linked by hydrogen bond [7].

This stability of the protein structure should include the three-dimensional state, the folded and the tertiary state which are all required for the biological activity. Although conformational stability is not enough rather, the protein must be able to find the folding pathway or its state within a short time from a denatured, unfolded conformation [8]. Folding maximises exposure of polar groups to the solvent and minimises exposure of non-polar groups. Protein unfolding is the transition from a native form to a denatured state [9]. When molecules are in aqueous solution, there is an equilibrium between folded which is the native state conformation and unfolded known as denatured. Native conformation stability is based on the relationship thermodynamically between ΔS and ΔH and the extent of ΔG-Gibbs free energy of the system. When the magnitude of ΔG is negative, it shows that there is a high stability of the native conformation than the denatured state which means that the greater the stability, the more negative is the ΔG. During protein unfolding, the main critical bonds required for protein stabilization are broken. Unfolding of proteins can take place at high temperatures, where the entropy is the main factor and the conformational entropy overpowers stabilizing forces. Hence, protein unfolding takes place. DSC takes measurement of ΔH of unfolding as a result of heat denaturation [10, 11].

1.3 Stability of proteins

Stability of protein is the achievement of a balance between stabilising and destabilising forces. The stabilising forces are caused by protein-solvent and intra-protein interaction, while the other is caused by substantial increase in entropy of unfolding [12].

One of the major challenges with the biopharmaceuticals is achieving protein stability.

There are three (3) types of stability:

1. Conformational stability
2. Chemical stability
3. Colloidal stability

Conformational stability describes the ability of the protein to maintain its native structure and be properly folded. Chemical stability is the stability among amino acid, covalent bond and different protein domains, while colloidal stability is the ability of the native structure of protein in a solution to avoid precipitation, aggregation and phase separation. However, it is necessary to maintain all types of stability during all the stages of development, processing and manufacturing.

The instability of protein necessitates the production in solid forms as solid proteins can be crystallised and dried [13] and its administration as a drug injection rather than through oral means like other drugs. Therefore, excipients also known as stabilisers are introduced to preserve the state and folding reversibility of proteins and reduce aggregation [14]. In effect to tackle protein degradation, necessary precautions and measures are taken to select the appropriate formulations for any excipients as well as the right technique for optimisation.

Proteins produced in different forms, like liquid formulations for medicines, are injectable due to the ease of preparation and are the most preferred, in manufacturing as well as the end users [15]. Unfortunately, it is not convenient due to the susceptibility of these proteins to the risk factors. This has instigated the consideration of the use of excipients as earlier mentioned in a dry solid state as important. Glass-forming agents such as saccharides, polyols and organic acids have been studied extensively over the years to stabilise spray-dried proteins in the solid state [16, 17]; these excipients stabilise the macromolecules by two primary mechanisms. Usually, the glass-forming ability of these excipients preserves the structure of proteins by trapping it in a rigid amorphous glass matrix [18, 19].

1.4 Lysozyme as a protein model

Lysozyme (1,4-β-N-acetylmuramidase) is the model of protein used in this study because lysozyme is one of the most potent proteins containing about 129 amino acids and has a high ionic strength. pH and the enzymes depend highly on their tertiary structure for maintaining their biological activity [20]. Lysozyme is folded into globular compact structure with a long cleft in the surface of the protein. The binding of the bacterial carbohydrate chain and the cleaving take place in the active site known as the cleft [21]; it has a molecular weight of 14.3 kDa and an isoelectric point of 10.7.

Lysozyme can be protected against bacteria through its activity as an enzyme as the role of an enzyme is to speed up the rate of chemical reaction in the body. Usually, polysaccharides are found in the cell walls of the affected bacteria which

contain amine groups (NH_2) with sugar and side chains of sugar. Addition of water molecules to the sugar linkage causes lysozyme to degrade the polysaccharide, thereby causing it to break open. Its activity is a function of the ionic strength and pH. It is active between a pH of 6.0 and pH 9.0. Maximum activity is observed at a pH of 6.2 and ionic strength 0.02–0.100 M compared to 0.01–0.06 M at pH 9.2.

1.5 Drying techniques

1.5.1 Electrohydrodynamic atomisation

EHDA also known as electrospray is a drying technique used in the production of dry powder with the help of charges. It was first observed and recorded by Williams Gilbert in 1600 [22]. It is a process where a liquid imposes through a nozzle (which is connected to a voltage supply) at a certain flow rate, subjecting to high potential electric field. As a result, the meniscus is elongated to form a jet that breaks up into droplet under electrical force influence. Particles can then be collected at a collecting platform that is located ~20 cm under the spraying nozzle (**Figure 1**). Various spraying modes can be obtained depending on the strength of the electric stress and on the kinetic energy of the liquid leaving the nozzle for nanoparticle production. It is a well-practised technique for generating very fine droplets with monodispersed size from the liquid under the influence of electric forces [23]. It can achieve controlled monodispersity and morphology of particles without denaturation of bioactive molecules throughout the process, and this is possible with the use of emulsion. However, it has the potential to reduce or stop degradation of protein drugs and offer a strict control over particle morphology and size distribution. The principle of electrospray is based on the theory of charged droplets, 'stating that an electric field applied to a liquid droplet exiting a capillary is able to deform the interface of the droplet' [24]. Some literatures published state that electrospray is better than the other drying techniques because of its advantages as it does not require a high temperature, might use little or no emulsion also and may not need further drying. In a study [25], lysozyme as a model protein was encapsulated within biodegradable microparticles using coaxial electrospray and the authors concluded that electrospray could be a promising approach to encapsulate biomolecules [25]. Bock et al. have also done a review on electrospraying of polymer with therapeutic

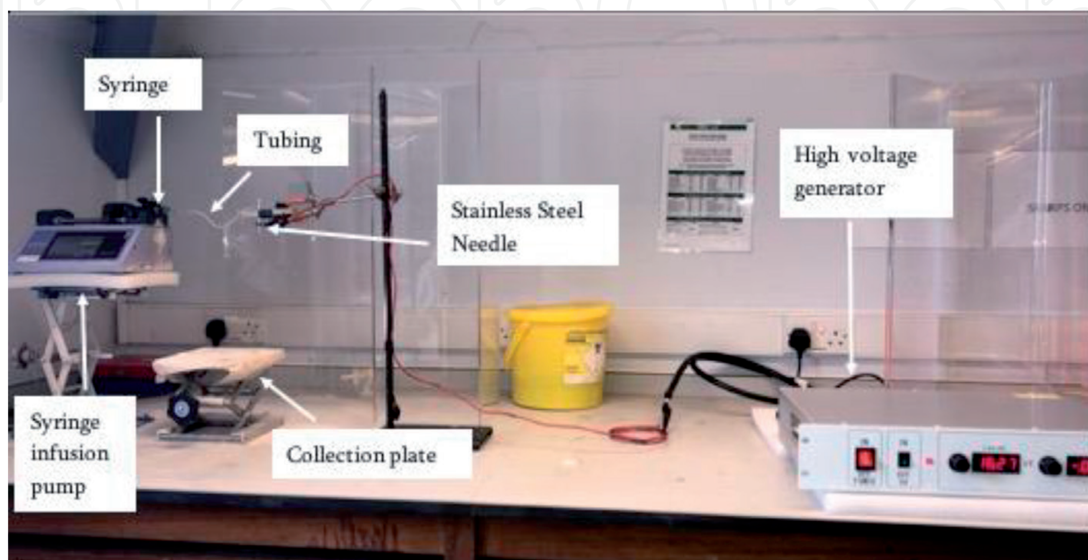

Figure 1.
Picture shows electrospray technique.

molecules as a state of the art and have concluded also that electrospraying technique may emerge as promising in the production of particles with entrapped therapeutic molecules that may be released as particle degradation occurs. So far, most of the research reports for electrospraying technique using proteins were based on using polymeric systems in order to encapsulate the protein either in protein carriers or nano- or microcapsules. For example, Suksamran et al. [26] successfully synthesised alginate microparticles (0.9–4 μm in diameter) with ~50% entrapment efficiency of bovine serum albumin that raised promises for oral drug delivery of proteins. Moreover, tristearin nanoparticles (~100–300 nm in diameter) were developed to aid the delivery of angiotensin II alongside clear core-shell particles with ~92 ± 1.8% encapsulation efficiency [27]. Electrosprayed core-shell microspheres (2–8 μm) with encapsulated bovine serum albumin as the core and an amphiphilic biodegradable polymer (poly(ε-caprolactone)-poly(aminoethyl ethylene phosphate) block copolymer) as the shell for protein delivery were generated by a single-step electrospraying. The protein release profiles of the microspheres exhibited steady release kinetics for a period of 3 weeks without a significant initial burst [28].

1.5.2 Spray-drying

Spray-drying is an established technology for the production of dried products from the liquid state. This method has gained increased interest in dry power formulation over the past decade, due to its potential simplicity, adaptability, scalability and cost-effectiveness [29, 30]. It is a method that has been studied in dry powder protein production involving the use of high temperature during the drying process. The principle of spray-drying method relies on atomisation of a drug solution that is pumped into a dry hot chamber in the form of droplets. By the influence of the heat, these droplets evaporate leading to dry solids in either powder, granules or agglomerated particles based on the chemical and physical properties of the feed in addition to the design of the used spray-dryer (**Figure 2**). One of the challenges of producing protein formulation utilising spray-drying method is the ability to destabilise the protein due to hot temperature and pressure during the production process compared to electrospray which uses charges for drying.

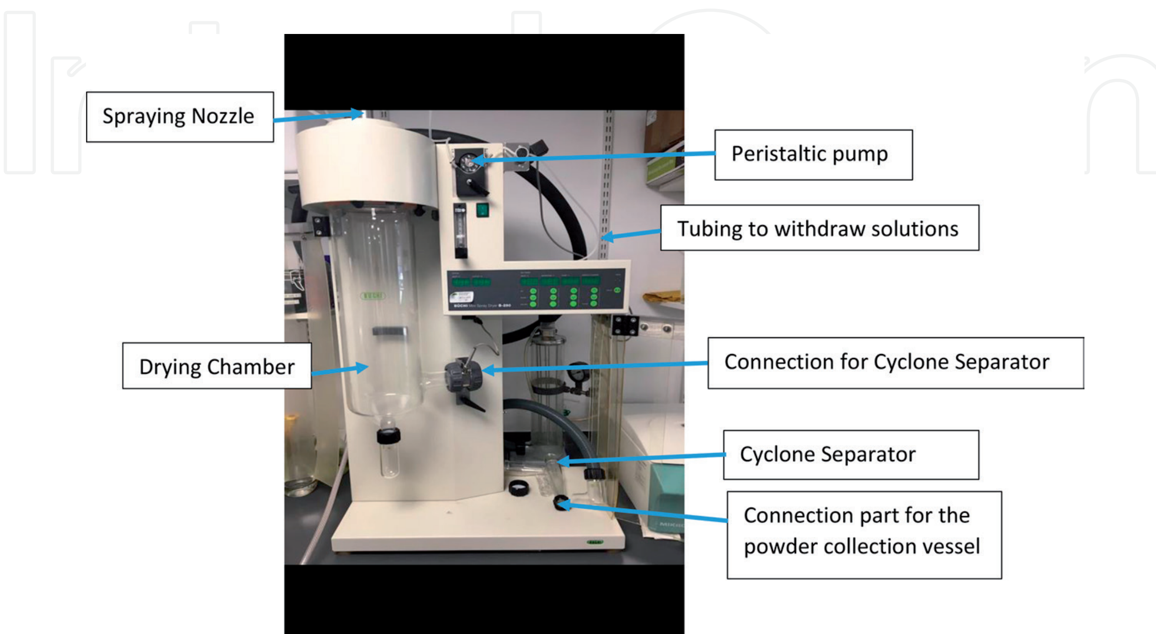

Figure 2.
Picture shows the spray-drying technique.

Various reports documented the use of spray-drying that produced protein formulations with high protein activity. For instance, the fabrication of uniform trehalose microparticles immobilised with trypsin showed ~97.7 ± 2.6% biological activity using an optimal trypsin/trehalose mass ratio of 1:1 [31]. Insulin microparticles were also designed by spray-drying method to bypass deposition in the extrathoracic region (mouth-throat). The total lung dose of >95% was achieved indicating a high degree of lung targeting [32]. Newly developed microencapsulated solid lipid nanoparticles containing papain (as a model protein) were prepared by spray-drying method. Papain was adsorbed onto glyceryl dibehenate and glyceryl tristearate solid lipid nanoparticle. The protein was found to be released from the particles to a large extent. Moreover, protein stability was reserved throughout spray-dried microsphere production [33].

This chapter aims to describe the effect of spray-dried and electrospray-dried formulations on lysozyme with and without excipients using various characterisation techniques like differential scanning calorimetry (DSC) to determine the thermal stability with lysozyme solid samples, UV-Vis spectroscopy for the enzymatic activity, dynamic light scattering for particle size, scanning electron microscope (SEM) for morphology of the particles and high-sensitivity DSC for thermal stability using lysozyme solutions and two drying (electrospray and spray-drying) techniques, to dry powders for characterisation.

2. Materials and methods

2.1 Materials

Chicken egg white lysozyme in lyophilised powder (90%), ethanol, *Micrococcus lysodeikticus* and betacyclodextrin were purchased from Sigma-Aldrich Chemicals Company. Sodium phosphate monobasic anhydrous, disodium hydrogen orthophosphate anhydrous and sodium chloride were obtained from Fisher Scientific Company.

2.2 Methods

2.2.1 Preparation of spray-dried protein

Aqueous lysozyme solutions (1% w/v) were spray-dried without and with an excipient (betacyclodextrin) via a BÜCHI Mini Spray Dryer B-290. Excipients were used at the concentrations of 1% w/v. The protein solution run through a silicone tubing of inner diameter of 4 mm, peristaltic feed pump (35%) to an 0.5 mm diameter atomising nozzle at rate of 20 ml/min and compressed air at rate of 600 l/h. In a glass chamber, protein solutions were sprayed at an inlet temperature of 130 ± 3°C, and outlet temperature was 73 ± 4°C. To minimise the effect of heat stress on the protein, a cooling water was distributed through a jacket around the nozzle. Spray-dried protein powder were collected by a high-performance cyclone separator and stored in glass vials at 3–4°C.

2.2.2 Preparation of electrosprayed lysozyme

Lysozyme solutions (1% w/v) were prepared using (80/20 v/v) ethanol/water. Electrosprayed formulations were prepared without and with excipients (1% w/w of betacyclodextrin). A syringe containing 5 ml of the protein solution was attached to a syringe infusion pump (Harvard Apparatus, Pump 11 Elite, USA) attached

to a high-power voltage supply (Glassman High Voltage Supply, UK). The protein solution was run through a silicone tubing attached to a stainless steel needle (inner diameter 0.3 cm) at a flow rates of 15 μL/min. Atomised particles were collected on microscope slides which fitted 20 cm below the tip of the nozzle. A high voltage was used to maintain the voltage range of 9–18 kV. The electrospraying process was conducted at ambient temperature (23°C).

2.2.3 Characterisation of spray-dried and electrosprayed lysozyme

2.2.3.1 Scanning electron microscopy

Microscopic examination of spray-dried and electrosprayed formulations was investigated utilising scanning electron microscope (SEM) (Hitachi S3000-N variable pressure scanning electron microscope, Japan). Around 2–3 mg of dried lysozyme formulations were applied to a double-sided carbon tape (Agar Scientific, Stansted, UK), fixed on an aluminium stub. The dried powder was sputter coated with a mixture of gold/palladium using a Quorum Technology (Polaron Range) SC760 by subjecting powder to an argon atmosphere at about 10^{-1} mbar.

2.2.3.2 Differential scanning calorimetry (DSC)

The thermal stability of lysozyme, before and after processing, was evaluated in the solid form using DSC Q1000M TA instrument, England. Pure indium standard was utilised to calibrate the DSC instrument. Unprocessed, spray-dried and electrosprayed formulation (2–3 mg) was sealed in a DSC aluminium pan with lids and loaded under nitrogen at a flow rate of 50 ml/min. An empty sealed pan was used as a reference. Pans were scanned in the range of 0–300°C at a rate of 10.0°C/min.

2.2.3.3 High-sensitivity differential scanning calorimetry (HSDSC)

High-sensitivity DSC also known as VP-DSC was used in this study to determine the thermal behaviour and folding reversibility of lysozyme. The fresh sample 5 mg of each formulation (1 ml 0.1 M phosphate buffer at a pH of 6.24) and reference (0.1 M sodium phosphate buffer at a PH of 6.24) was degassed and injected into the cells using a syringe. The sample and reference were complete for maintenance of equal volumes, and the same amount of lysozyme was used in each run. The sample and reference were heated between 20 and 90°C at 1°C/min under pressure. The folding reversibility each, of the denatured protein, was evaluated by cycling temperature by carrying out three scans (up scan, 20–90°C; down scan, 90–20°C; and another up scan, 20–90°C). These engaged two or more scans at different temperatures. Furthermore, before the measurement of each sample, a baseline was run by loading both cells (sample and reference cells) with the reference; the baseline was later subtracted from the protein thermal data using the MicroCal VP-DSC, USA. The sample was analysed with Origin DSC analysis software by normalising the concentration and excesses in heat capacity. The calorimetric enthalpy changes which is the area under the peak (ΔH) and midpoint of the transition peak (T_m) were calculated.

2.2.3.4 Biological activity analysis of lysozyme

Utilising M501 Single Beam Scanning UV/Visible Spectrophotometer Camspec (Biochrom, UK), the enzymatic activity of lysozyme was investigated by determining the hydrolysis rate of β-1,4-glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid in bacterial cell walls. Following a procedure described

by Haj Ahmad et al. [34], the activity of lysozyme was assessed. A 100 ml of *Micrococcus lysodeikticus* suspension was prepared by adding 20 mg of the bacteria added to 10 ml of 1% sodium chloride solution and 90 ml of potassium phosphate buffer 0.067 M, pH 6.6. The biological reaction of lysozyme was initiated by adding 0.5 ml of protein solution (1.5 μg/ml), prepared using the same buffer, to 5 ml of the bacterial suspension. The unit activity of the protein was monitored by measuring the reduction rate in the absorbance at 450 nm. The biological activity of lysozyme was assessed using the equation suggested by Shugar [35]:

$$\text{Activity (units/mg)} = \Delta 450_{nm/min}/0.001 \times \text{mg enzyme present in the mixture.} \quad (1)$$

2.2.3.5 Particle size distribution analysis

The particle size distribution of the dried particles was determined by dynamic light scattering technique using the Malvern Zetasizer (Nano ZSP, Malvern Instruments, UK). The particles were dispersed in ethanol containing 0.1% Tween® 80, which was selected to achieve suitable deagglomeration. The average particle size was measured at a scattering angle of 90° in three replicates for each sample.

3. Results and discussion

3.1 Determination of percentage yields for spray-dried and electrosprayed lysozyme

Table 1 shows the percentage yield of the spray-dried and electrosprayed lysozyme formulation collected. For spray-dried lysozyme, 29% of the preparation was achieved. The percentage yield was higher (~53%) for spray-dried lysozyme with betacyclodextrin compared to the spray-dried lysozyme in the absence of excipient. The addition of betacyclodextrin reduced the deposition of spray-dried lysozyme on the wall of the spray-drying chamber and cyclone separator. With no significant differences, lysozyme formulation produced with electrospraying produced ~30%. However, a major reduction was noticed for electrospraying lysozyme with betacyclodextrin (~25%). Around 30–40% of product yield is typically expected for spray-drying formulations utilising benchtop spraying system [36]. Increasing the percentage yield can be achieved by introducing polymeric excipients with high glass transition temperature in addition to optimise the drying condition used during the spray-drying [37]. A large-scale production is feasible in pharmaceutical industries for spray-drying formulations by using a large scalable spray-drier that would generate the highest possible yield [38]. This is also feasible for electrospraying by using, for example, a multi-tip emitter to improve the potential upscale electrospraying [39]. For electrospraying technique, the process parameters, such as feeding flow rate, voltage supply and the distance between the tip of the nozzle and the collecting platform, would affect the physical properties of the produced particles. It's worth mentioning that the time consumed by each process to generate the quantity outlined in **Table 1** was significantly different. Spray-drying process required 15–20 min for the whole sample, while more than 6 h was consumed to finish with all of the electrosprayed sample.

3.2 Microscopic examination of spray-dried and electrosprayed protein particles

Figure 3 and **Table 1** show the SEM images and particle size of spray-dried and electrosprayed formulations, respectively. The process of protein preparation

Formulation	Percentage (%) yield	Biological activity (%)	Tm (°C)[a]	Apparent Tm (°C)[b]	Mean diameter (μm) (mean ± SD)
Unprocessed lysozyme		100	74.32 ± 0.16	199.06	1.4 ± 0.04
SD lysozyme	29	90.1	72.46 ± 0.38	201.30	2.3+ 0.06
ESR lysozyme	30	113	74.44 ± 0.22	222.18	1.6 ± 0.19
SD (1:1 w/w) lysozyme with betacyclodextrin	53	87.06	74.36 ± 0.26	No transition detected	2.7 ± 0.5
ESR (1:1 w/w) lysozyme with betacyclodextrin	25	100	74.51 ± 0.52	223.26	3.0 ± 0.91

[a]*For aqueous protein formulations.*
[b]*For solid protein formulations.*
SD, spray-dried; ESR, electrosprayed

Table 1.
Shows percentage (%) yield, biological activity, denaturation temperature, Tm and particle size of dried lysozyme particles.

had a significant impact on the morphology and size of the spray-dried and electrosprayed lysozyme particles. As a result, it will impact the choice of the protein delivery system and route of drug administration. Protein particles produced by spray-drying technique were smooth and spherical in shape (**Figure 3A**) with a size of 2.3 + 0.06 μm, while mainly rodlike shape particles with few spherical particles were produced via electrospraying process (1.6 ± 0.19 μm). The inclusion of betacyclodextrin had no significant effect on the shape of electrosprayed particles (3.0 ± 0.91 μm), while solid dimple spheres were observed for spray-dried lysozyme with betacyclodextrin formulation (2.7 ± 0.5 μm) (**Figure 3**). For spray-drying process, Prinn et al. [40] suggested four categories to classify the morphology of the spray-dried particles: (I) smooth spheres, (II) dimpled or collapsed particles, (III) raisin like particles and (IV) highly crumpled folded particles. Accordingly, spray-dried lysozyme without betacyclodextrin would fall into class I, and spray-dried lysozyme with betacyclodextrin would fall into class II. The rate of drying has a crucial impact on the morphology of the spray-dried particles; faster drying would result in more dimpled particles [41]. However, the spherical shape of the particles will not guaranty a 100% biological activity as will be discussed below. Moreover, it has been recognised that the optimal aerodynamic particle size distribution of particles intended for pulmonary delivery should be within the range of 1–5 μm [42]. Accordingly, all particles produced in this research would fit within this range and thus are suitable for pulmonary delivery.

The electrospraying configuration used in this study consists of one conducting needle, and one voltage was applied. A stable Taylor cone usually forms at the tip of the needle generating nearly uniform particles with distinct morphology. The incorporated protein disperses equally with the solution (without and with the excipient) improving the amorphous nature and bioavailability (Mehta et al. [22]). Flow rate is highly related to the particle size of the electrosprayed particles. It documented that larger flow rates tend to generate smaller particle size [43]. The flow rate used in this study considered as low flow rate that might explain the large particle size of electrosprayed lysozyme especially with betacyclodextrin (**Table 1**, **Figure 3**). Also, a study conducted by Gomez et al. [44], electrospraying of insulin, suggested reducing protein concentration in the

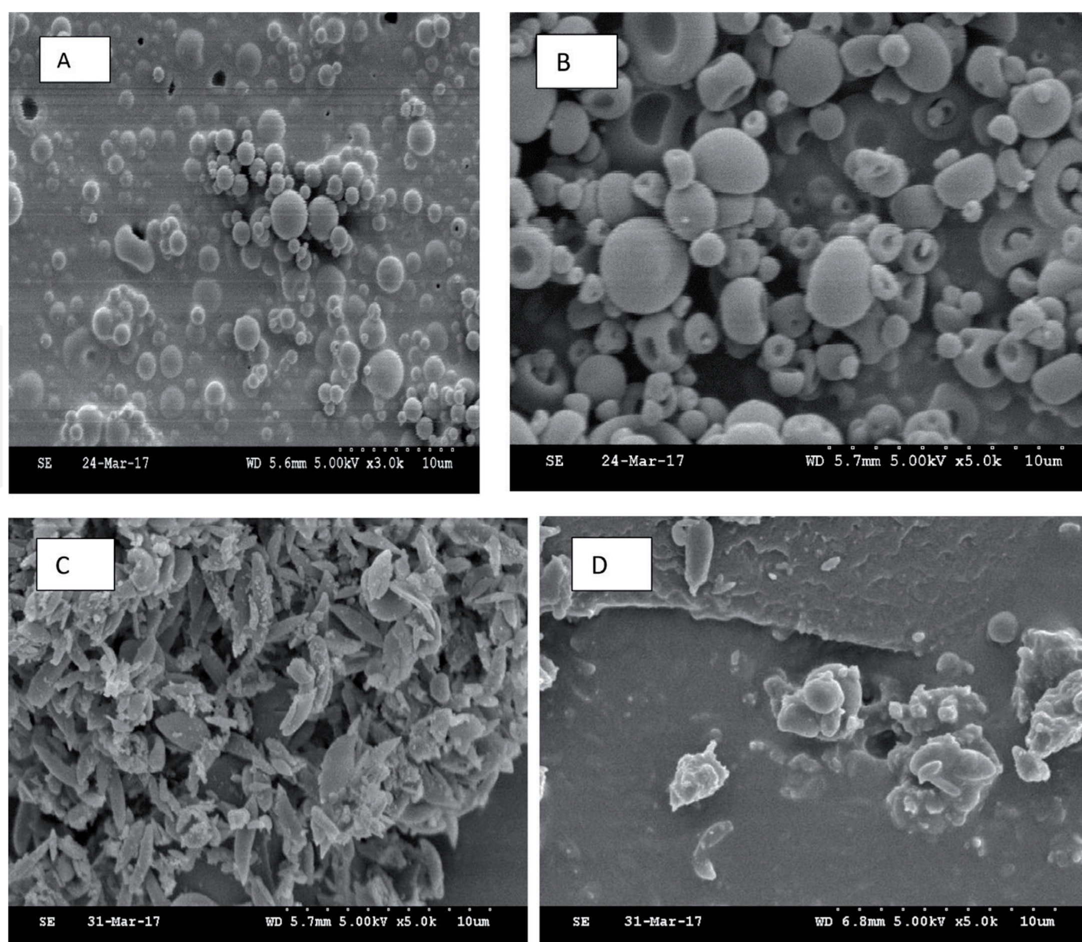

Figure 3.
SEM images for (A) spray-dried lysozyme, (B) spray-dried lysozyme with betacyclodextrin, (C) electrosprayed lysozyme and (D) electrosprayed lysozyme with betacyclodextrin.

solvent or the flow rate in order to achieve a smaller particle size. A future study with various lysozyme concentrations and higher and lower flow rates will be conducted. The particle morphology of protein formulations and size play a crucial role in the aerodynamic properties and performance of aerosol applications. Smooth spherical particles are more preferred than other particle shapes as they might result in much lower aerodynamic particle diameter in comparison with dense particles.

3.3 Differential scanning calorimetry (DSC)

DSC determines the variation in heat flow between the protein sample and an empty sealed pan that was used as a reference cell. Throughout a thermal event in the protein preparation, the operation of the system will conduct heat to, or from, the protein sample pan to maintain an equal temperature in both sample and reference pans. Thermal profiles of unprocessed, spray-dried and electrosprayed lysozyme formulations without excipients are presented in **Figure 4**. This represents the heat flow as a function of temperature and illustrates the apparent denaturation temperature T_m values of all protein preparations (**Table 1**). In the DSC thermogram scans, two endothermic peaks can be observed. The first endothermic broad peak (~120°C) indicates that the preparations contain some amount of water [45, 46]. The second endothermic peak around ~199°C represents the apparent denaturation transition of the protein in which the peak maximum was considered the apparent denaturation temperature [21].

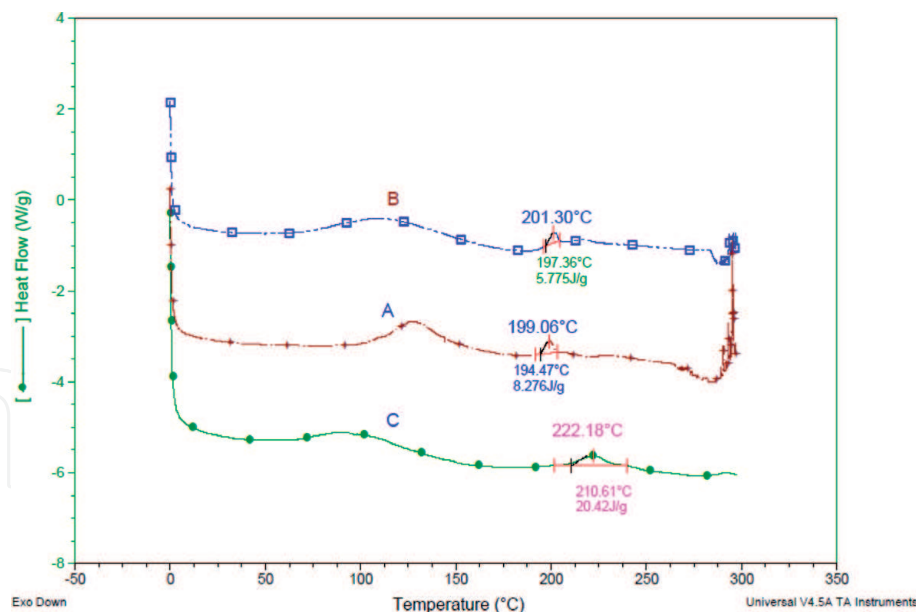

Figure 4.
DSC thermograms for unprocessed, spray-dried and electrosprayed lysozyme.

Spray-drying of lysozyme has improved the denaturation temperature of lysozyme by about 2°C as compared with unprocessed lysozyme (**Table 1**). However, this transition was not significant. The presence of betacyclodextrin within spray-dried sample indicated an amorphous protein content as no peak was detected around 199°C; this was in contrast with other publications which showed transition for spray-dried lysozyme with betacyclodextrin. Around 22°C increase of the denaturation temperature of lysozyme was observed for electrosprayed protein without any excipient (**Table 1**, **Figure 4**). Moreover, electrospraying of lysozyme with betacyclodextrin led to an increase (by ~23°C) of the apparent protein denaturation temperature compared to the unprocessed lysozyme. The results for electrospraying formulations suggest an increase of lysozyme thermal stability as they exhibited increase in denaturation temperatures indicating the effect of the process and the excipient on the protein's integrity, and this is in consistent with the biological activity results for freshly dried protein samples.

HSDSC offers information about protein folding and stability by measuring the thermodynamic parameters in solution forms which have an impact on protein folding-unfolding transitions [47]. Subsequently, this method was used to assess the thermal stability of the prepared protein solutions after processing. **Table 1** displays the HSDSC results for denaturation temperature of unprocessed, spray-dried and electrosprayed lysozyme preparations. The results (**Table 1**) show that the transition temperature (T_m) of the spray-dried formulation in the absence of betacyclodextrin reveals lower thermal stability (T_m was ~72°C) than the unprocessed lysozyme and other formulations (T_m was ~74°C). Electrosprayed samples have maintained the thermal stability of the protein after processing (T_m was ~74°C). However, after 6 months of storage of electrosprayed samples in solid forms, it was noticed that those samples showed visible aggregates upon addition of aqueous solutions, while spray-dried lysozyme samples were soluble in the aqueous solutions.

3.4 Biological activity of lysozyme

The most crucial parameter in any protein formulation is to maintain the biological activity of the protein. This will give an indication about the integrity, foldability and stability of the protein. Biological activity test was run for lysozyme

before and after processing. Solid protein samples were reconstituted, and the biological activity was expressed as a percentage that is relative to the control protein (activity of unprocessed protein was 100%). **Table 1** displays the biological activity results for spray-dried and electrosprayed lysozyme formulations. Spray-drying of lysozyme without and with betacyclodextrin led to about 10% reduction of lysozyme activity as compared with the unprocessed lysozyme. On the other hand, electrospraying of lysozyme better maintained protein biological activity compared to spray-dried protein formulations (**Table 1**). However, aggregate formation in solutions was noticed after 6 months of storage of electrosprayed lysozyme solid samples, meaning that proper storage conditions require to be maintained for long-term stability of dried lysozyme samples prepared by electrospraying technique.

The outcomes of this study could be explained on the basis that spray-drying process has perturbed the tertiary structure of lysozyme, thus reducing the biological activity of the protein; these results are in agreement with Haj Ahmad et al. [21] and Hulse et al. [45]. Lu et al. [48] reported that the type and percentage of the excipient used would influence the stability of protein formulation. The addition of betacyclodextrin to the spray-drying formulation did not have any improvement as ~87% biological activity of lysozyme was achieved. Sustaining the tertiary structure of lysozyme is critical for its full activity. One of the vital factors for this is the level of hydration at the active site cleft of the protein. Nagendra et al. [49] suggested that the active site in the lysozyme is heavily hydrated and ~0.2 grammes of water/g protein is required for the protein to maintain its biological activity with at least 9.4% of moisture content. Otherwise, the active site cleft will shrink, and thus the protein will inactivate [49]. On the other hand, 100% of the protein biological activity was observed for freshly electrosprayed lysozyme without and with betacyclodextrin suggesting the full hydration of the active site cleft in the protein. A study conducted by Gomez et al. [44] using insulin as a model protein reported electrospraying technique was gentle not to hinder the biological activity of insulin. The overall results suggest that different ways of drying (e.g. heating like in spray-drying and electric charge like in electrospraying) have different influences on the protein biological activity. The structure of proteins embraces several weak interactions (hydrogen bonging and electrostatic). These interactions can easily be affected by subjecting to physical and chemical stimuli that would include various processing conditions of conventional processing techniques (e.g. spray-drying) where high temperature or stress is applied. High temperature tends to denature sensitive biomolecules, such as proteins. Atomisation of materials by electrospraying relies mainly on evaporation of the solvent to generate dry particles without affecting the characteristics of proteins such as biological activity. While denaturation by the effect of temperature can be avoided, some denaturation and degradation might be initiated by the shear stress in the nozzle tip through which the aqueous solvent is infused [22, 37, 50, 51]. In this study, the biological activity of freshly dried lysozyme forms was conserved by using electrospraying method compared with spray-drying technique. However, aggregate formation in solutions was noticed after 6 months of storage of electrosprayed lysozyme solid samples, meaning that proper storage conditions require to be maintained for long-term stability of dried lysozyme samples prepared by electrospraying technique. Accordingly, there is a need for further study of the stability of the protein particles at various temperature and humidity conditions.

4. Conclusion

It was observed that electrospray lysozyme formulations without and with the used excipient looked promising compared to unprocessed and spray-dried

formulations. However, it was observed that proper storage for electrosprayed samples is needed as the protein degraded after some time of storage. Spray-dry and electrospray seem to have some positive and negative effects on the lysozyme depending on the technique and its used parameters.

Acknowledgements

This study was partially supported by EPSRC EHDA network for part funding and the ability to use the EHDA equipment for this research.

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