Feasibility of Electrospraying Fully Aqueous Bovine Serum Albumin Solutions

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Abstract

Electrospraying or electrohydrodynamic atomisation, i.e. the formation of tiny droplets from a jet of conductive liquid under the influence of an electric field, has been gaining in popularity as a particle engineering technique in recent years. In addition to general benefits for particle engineering, e.g. the ability to generate nanometre sized particles with a very narrow size distribution, electrospraying also possesses a number of characteristics, like its applicability at ambient conditions, which could make it especially interesting for formulating therapeutic proteins. However, as fully aqueous solutions of proteins tend to have relatively high electrical conductivities and surface tensions, obtaining a stable Taylor cone-jet mode for these solutions is inherently challenging. This is why in the majority of studies reporting the successful electrospraying of proteins either emulsions, aqueous suspensions or a mixture of water and one or more organic solvents were used instead of fully aqueous solutions. Therefore, an ab initio electrospraying formulation development study was conducted, using only fully aqueous feed solutions containing protein stabilising excipients commonly used in spray- and freeze-drying of therapeutic

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proteins. The study included bovine serum albumin (BSA) as a model protein and consisted out of two parts: (1) a one parameter at a time screening study, designed to improve the understanding of how various formulation components influence relevant physicochemical properties and the electrospraying process and (2) two subsequent mixture design of experiments (DoE) studies, designed to aid in the statistical description and prediction of the influence of different protein-excipient combinations on the electrospraying process. Additionally, the influence of physicochemical properties relevant to the electrospraying process, i.e. the volumetric mass density, electrical conductivity, kinematic viscosity and surface tension, was assessed for all feed solutions included in the study.

Keywords:

Electrospraying, Electrohydrodynamic atomisation, Taylor cone-jet mode, Fully aqueous protein solutions, Protein formulation, Bovine serum albumin, Physicochemical characterisation, Volumetric mass density, Electrical conductivity, Kinematic viscosity, Surface tension, Design of experiments

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1. Introduction

The first observations regarding the formation of tiny droplets from a jet of conductive liquid under the influence of an electric field, a phenomenon that would later be identified as electrospraying or electrohydrodynamic atomisation, were made over four hundred years ago [1]. Yet, research into the potential of electrospraying as a novel particle engineering technique only started gaining momentum recently, following the Nobel prize being awarded to John B. Fenn for his work on an ionisation method for large biomolecules [2, 3, 4]. Electrospraying owes its general popularity as a particle engineering technique to, i.a., its relatively simple experimental set-up (see Figure 1), its potential to generate nanometre sized particles with very narrow size distributions, its applicability at ambient conditions and the possibility to incorporate it as an atomiser into other drying set-ups like a spray-dryer. However, feed flow rate and thus the throughput of an electrospraying process will be inherently lower than other

15 available drying techniques due to the nature of the atomisation process. Re-
16 cent developments have been made to help remedy the low throughput, such as
17 using multiple nozzles in parallel within a continuous process [5, 6, 7, 8].

18   Aside from aforementioned general benefits, electrospraying also has a num-
19 ber of characteristics which make it interesting for formulating therapeutic pro-
20 teins. A main advantage is the applicability of electrospraying at ambient tem-
21 peratures, which could help minimise the risk of heat induced protein degra-
22 dation. Additionally, the low feed flow rates, which are generally seen as a
23 disadvantage of the technique, and the principle of atomisation, make that the
24 overall shear stress to which proteins are subjected is relatively low. Finally, the
25 technique is reported to have a very high encapsulation efficiency when using
26 a coaxial set-up. As coaxial electrospraying allows encapsulation without the
27 need for emulsification, it could help limit the interactions between the, often
28 protein denaturing, solvents needed to dissolve the coating material and the
29 protein. This is because an aqueous protein solution could be fed to the inner
30 needle, while the coating solution could be fed to the outer needle, thus limiting
31 contact between both phases [9, 8, 4].

32   A major hurdle in the use of electrospraying for protein formulations is that
33 aqueous solutions of proteins tend to have a relatively high electrical conductiv-
34 ity and surface tension, making it generally more challenging to obtain a stable
35 Taylor cone-jet mode (see Figure 3a) compared to the organic solvent-based sys-
36 tems commonly used for electrospraying. The short (cm range) drying distance
37 between the tip of the spray nozzle and the collector, necessary for maintaining
38 an adequate electric field, the high boiling point, and therefore slow evaporation
39 of water, is another challenging factor of using fully aqueous instead of organic
40 solvent based feed solutions [8, 4, 10]. It is because of these hurdles that the
41 majority of the available literature on the electrospraying of biomolecules re-
42 port processes that either use emulsions [11, 12], an aqueous suspension [13]
43 or a mixture of water and one or more organic solvents [14, 15]. Moerman
44 et al. [16] reported successfully creating protein spots from fully aqueous so-
45 lutions containing lactate dehydrogenase, glucose-6-phosphate dehydrogenase

46 and pyruvate kinase, while López-Rubio and Lagaron [17] succeeded in creating
47 whey protein capsules starting from a fully aqueous solution of this mixture of
48 globular proteins. While the latter publication could serve as a proof of concept,
49 little information is given about relevant physicochemical properties of the feed
50 solution aside from the surface tension and composition.

51 To the best of our knowledge, no literature exists on electrospraying fully
52 aqueous protein solutions containing commonly used stabilising excipients. There-
53 fore, an ab initio electrospraying formulation development study was conducted,
54 using only fully aqueous feed solutions containing protein stabilising excipients
55 commonly used in spray- and freeze- drying of therapeutic proteins. The study
56 uses bovine serum albumin (BSA) as a model protein. BSA, being a relatively
57 small and stable protein, is a poor reference model for therapeutic proteins
58 when protein stability is in scope. However, assessment of protein stability was
59 beyond the scope of this work, since the focus was on improving the under-
60 standing of how various formulation components influence the electrospraying
61 process and relevant physicochemical properties. Therefore, BSA was used as a
62 model protein for this work, as it was deemed to be adequately comparable to
63 therapeutic proteins regarding its impact on the physicochemical properties rel-
64 evant to the electrospraying process, i.e. the volumetric mass density, electrical
65 conductivity, kinematic viscosity and surface tension. The study is made up of
66 two parts, the first being a one parameter at a time screening study, designed
67 to improve the understanding of how various formulation components influence
68 relevant physicochemical properties and the electrospraying process. Next, a
69 selection of excipients was used in two subsequent mixture design of experi-
70 ments (DoE) studies, which were designed to aid in the statistical description
71 and prediction of the influence of different protein-excipient combinations on
72 the electrospraying process.

73 2. Materials

74 Bovine serum albumin (BSA), sodium phosphate monobasic monohydrate,
75 sodium phosphate dibasic anhydrate, D(+)-trehalose dihydrate, L-histidine, L-
76 arginine monohydrochloride, (2-Hydroxypropyl)- β -cyclodextrin (HP β CD) and
77 polysorbate 20 were all obtained from Sigma-Aldrich (St. Louis, MO, USA)
78 Hydrochloric acid (1 N) was purchased from VWR Chemicals (Radnor, PA,
79 USA). Phosphoric acid 85 % (analytical reagent grade) was obtained from
80 Chem-Lab (Zedelgem, Belgium). D(+)-sucrose was obtained from Acros Or-
81 ganic (Geel, Belgium). Hydroxypropylmethylcellulose (HPMC) (type E5) and
82 polyvinylpyrrolidone (PVP) K30 were obtained from Janssen Pharmaceutica
83 (Beerse, Belgium). Sulfobutylether- β -cyclodextrin sodium salt (Na SBE β CD)
84 was purchased from CyDex Inc. (Lenexa, KS, USA). Polyethylene glycol 300
85 (PEG 300) was obtained from TCI Chemicals (Portland, OR, USA). PVP K17
86 and PVP K25 were provided by BASF ChemTrade GmbH (Ludwigshafen, Ger-
87 many). All electrospraying feed solutions were prepared with ultrapure water
88 (Type 1, $\rho \geq 18.2 \text{ M}\Omega \text{ cm}$ at 298.15 K) as the only solvent, i.e. no other solvents
89 or co-solvents were used to prepare the solutions described in this work.

90 3. Methods

91 3.1. Electrospraying

92 3.1.1. Preparation of Feed Solutions

93 All feed solutions were prepared at ambient temperature ($293 \pm 5 \text{ K}$), us-
94 ing ultrapure water and were magnetically stirred at low speed until a clear
95 solution, free from visible particles, was obtained. Feed solutions were subse-
96 quently filtered using either $0.45 \mu\text{m}$ cellulose acetate syringe filters (Macherey-
97 Nagel, Düren, Germany) or $0.45 \mu\text{m}$ cellulose acetate membrane filters (Sar-
98 torius, Göttingen, Germany) under vacuum for the formulations described in
99 Section 4.1 and were not filtered in case of the DoE formulations described in
100 Section 3.7.

101 3.1.2. *Electrospraying Process*

102 Electrospaying was done with a climate-controlled electrospinning machine
103 (EC-CLI) (IME Technologies, Geldrop, the Netherlands) in combination with a
104 PHD 4400 syringe pump (Harvard Apparatus, Holliston, MA, USA) to deliver
105 constant flow rates (see Figure 1). All formulations were electrosprayed using
106 25 gauge passivated stainless steel dispensing tips (Nordson, Westlake, OH,
107 USA) connected to either 10 mL or 5 mL BD PlastiPak syringes with Luer
108 Lock (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in case of
109 the formulations described in Section 4.1 or Section 3.7, respectively.

110 Because optimal electrospaying process parameters were expected to differ
111 for each of the screened formulations, process parameters were varied within
112 the ranges shown in Table 1 until the (visibly) most stable cone-jet and driest
113 powder bed were obtained. For the experiments described in Section 4.1, cone-
114 jet stability or powder bed quality related responses are defined as the values
115 obtained at the optimal process parameter combination for a given formulation.

116 To visualise the spraying mode at the nozzle during the electrospaying pro-
117 cess of the DoE formulations described in Section 3.7, a Fastcam SA2 high speed
118 camera (Photron, Tokyo, Japan) was used, equipped with a K2 DistaMax long
119 distance microscope system and CF-4 objective (Infinity Photo-Optical Com-
120 pany, CO, USA). Recordings were made at a resolution of 2048 x 2048 pixels,
121 a shutter speed of $4 \cdot 10^{-5}$ seconds and a frame rate of 1080 frame per second
122 (fps).

123 3.2. *Density*

124 The volumetric mass density is defined as the mass per unit volume. A glass
125 pycnometer with a volume of 10.239 cm³ was used to measure all feed solu-
126 tions, with each measurement conducted at ambient temperature in a climate
127 controlled room with the temperature set to 293 K.

128 3.3. Surface Tension

129 Surface tension was measured using a CAM 200 goniometer and the ac-
130 companying CAM 200 software (KSV Instruments, Helsinki, Finland) using the
131 pendant drop method. All solutions were measured at ambient temperature in
132 a climate controlled room with the temperature set to 293 K, using a 25 gauge,
133 passivated stainless steel dispensing tip (Nordson) and surface tension was mea-
134 sured in triplicate for each formulation, i.e. by dispensing three different drops
135 from which 10 pictures were taken at a frame interval of 975 ms, unless explicitly
136 stated otherwise.

137 For the DoE discussed in Section 3.7.2, an alternative method was used,
138 consisting of collecting 60 frames at 1 fps per droplet and this for five droplets
139 per formulation using an 18 gauge polypropylene dispensing tip (Nordson) in
140 order to avoid wetting the tip’s external surface. Subsequently a t_0 was defined
141 as the point where a droplet is formed and the Young-Laplace equation is valid
142 and a point was determined where a plateau was reached in the surface tension
143 versus time plot, i.e. the point where polysorbate 20 had reached the air-water
144 interface. These points were then compared for all formulations in the DoE and
145 the longest time, i.e. the point where the “slowest” solution reached a plateau
146 and the surface tension values for this time point, relative to t_0 , were entered
147 in the DoE.

148 3.4. Viscosity

149 The kinematic viscosity of all feed solutions was measured using an iVisc
150 LMV 830 capillary viscometer (Lauda, Königsshofen, Germany) using Ubbelo-
151 hde capillaries with known volume, diameter and length. Temperature was set
152 at 293 K, using a glass panelled liquid temperature-controlled bath equipped
153 with a Lauda ECO silver immersion thermostat (Lauda).

154 3.5. Electrical Conductivity

155 An Orion A150+ conductivity meter, equipped with a 013016D conductiv-
156 ity probe with an actual K-value of 0.0923 cm^{-1} (Thermo Fisher Scientific,

Walthman, Massachusetts, USA) was used for all electrical conductivity measurements. All solutions were measured at ambient temperature (293 ± 5 K) following a two point calibration with $100 \mu\text{S cm}^{-1}$ and $1413 \mu\text{S cm}^{-1}$ conductivity standards, which were provided by the manufacturer.

3.6. Scanning Electron Microscopy

A JSM-IT300 InTouchScope® scanning electron microscope (SEM) (Jeol, Tokio, Japan), combined with version 1.110 of the IT300 operation software (Jeol), was used to further assess powder bed characteristics. Particle samples were fixed onto a cylindrical mount with double-sided carbon tape and a gold layer was subsequently applied using a Q150R ES Rotary-Pumped Sputter Coater/ Carbon Coater (Quorum Technologies, Lewes, U.K.).

For powder resulting from the DoE formulations described in Section 3.7, an XL30 FEG SEM combined with Genesis 4.61 software (Philips, Amsterdam, The Netherlands) was used, sample preparation was similar to that of the JSM-IT300, except for a platinum layer applied instead of the gold layer.

3.7. Mixture Design of Experiments (DoE)

In mixture experiments, a factor's value is its proportion in the mixture, which falls between zero and one. Mixture experiments differ from other experimental types in that one cannot vary factors independently of one another. When the proportion of one factor is changed, the proportion of one or more other factors must also change. In our case, we had mixture experiments with either five (Table 3) or three (Table 4) factors, with the sum of these factor proportions being equal to one (100 %). The final response levels included assessment of both the cone-jet stability and the powder bed quality. An unstable cone-jet should be interpreted as any spraying mode deviating from a Taylor cone-jet mode, i.e. dripping, micro dripping, spindle or multi-jet, but also any droplet elongation or "jerking" of the Taylor cone-jet mode within the time frame observed with the high speed camera or the slow motion recordings

(> 100 frames at 5 fps) made herewith. The latter were included in the supporting information of the publication [4]. The powder bed quality was assessed based on whether or not a powder could be obtained and on the number and size of droplet residue visibly present in the powder bed.

3.7.1. Mixture DoE with Ordinal Responses

One ordinal response, which combined the observation of both cone-jet and powder bed quality, was of interest and set to be evaluated at five distinct levels, as summarised in Table 5. The suggestions given in the doctoral thesis by Mancenido [18] were used as a starting point to generate the custom design with mixture factors displayed in Table 3 using the JMP 11.0 software package (SAS Institute, Cary, North Carolina, USA). BSA, glycerol, trehalose, HP β CD and PVP K25 concentrations were selected as the five factors for the subsequent mixture DoE, which was based on a custom design generated in JMP 11.0. The resulting DoE is summarized in Table 3. Only main effects can be estimated with the generated design. DoE encoding used in Table 3 represents the fraction of a 40 % m/V (40 g of solute per 100 mL of aqueous solution) excipient content, not including polysorbate 20 (1 % m/V), that each formulation factor assumes. As will be shown in Section 4.2, two binary responses were used instead of an ordinal response in the final data analysis. A detailed description of the statistical methodology is included in the supporting information.

3.7.2. Mixture DoE with Three Factors for Binary Responses

As for the method described in Section 3.7.1, two binary responses are registered for this design: cone-jet stability and powder bed rating. The levels of the responses are described in Table 6. BSA, HP β CD and PVP K25 concentrations were selected as the three factors for the subsequent mixture DoE, which was based on a simplex lattice algorithm generated in JMP 11.0. The resulting DoE is summarised in Table 4 where DoE encoding represents the fraction of a 20 % m/V excipient content, not including trehalose (20 % m/V) and polysorbate 20 (1 % m/V), that each formulation factor assumes. Similarly to the

method described in Section 3.7.1, logistic regression was applied and model estimation was done using the maximum likelihood. A detailed description of the statistical methodology is included in the supporting information.

4. Results and Discussion

4.1. Electrospraying Feasibility Screening

A large set of excipients commonly used for stabilising spray- or freeze-dried protein formulations, either in solid state or in liquid state during drying, was selected in an ab initio formulation development for fully aqueous feed solutions for electrospraying. The relevant physicochemical characteristics of the feed solutions, i.e. volumetric mass density, electrical conductivity, kinematic viscosity and surface tension, were determined where possible to increase understanding of the influence of different formulation components. In this regard, it is worth pointing out that the pH of the different solutions was not determined during the experiments, even though the pH of a solution determines the total amount of charged groups a protein carries and thus influences the protein’s molecular organization and stability in solution. But, as mentioned in the introduction (see Section 1), assessment of the protein stability was beyond the scope of the present work. The main responses used for the electrospraying feasibility screening experiments were the quality of the powder bed, e.g. whether or not solid particles could be obtained, if there were any droplets deposited on the collector during the process, and visual assessment of the cone-jet stability. Overall, the main focus of this part of the study was on whether or not solid particles could be collected, since directly assessing the cone-jet stability was not always feasible with the set-up used for these experiments.

Results of the broad electrospraying feasibility screening study (see supporting information) corroborated findings of Kriegel et al. [19] and Pérez-Masiá et al. [20], which stated that decreasing the surface tension results in a lower electrical field strength being required for jet initiation, thus improving cone-jet feasibility. The findings also confirm the theoretical surface tension “cut-off”

243 value of circa 50 mN m^{-1} [21]. Nevertheless, one of the biggest challenges of
 244 electrospraying aqueous protein solutions lies in their (relatively) high electrical
 245 conductivity, which could be countered by reducing the overall surface tension of
 246 the liquid, as surface tension and conductivity have counteracting effects regard-
 247 ing the electrospraying process. Furthermore, the electrospraying experiments
 248 summarised in the supporting information revealed that an increase in PVP
 249 K25 concentration positively affected the formulation’s electrospraying feasibil-
 250 ity, i.e. a visibly more stable cone-jet was observed with less droplets deposited
 251 in the powder bed. Yet, little difference could be observed between different types
 252 of PVP concerning the electrospraying process characteristics. Improvement of
 253 the powder bed quality with concentration of the non-ionisable PVP K25 was
 254 reminiscent of the results observed for increasing BSA concentrations. However,
 255 contrary to PVP, electrical conductivity increased with increasing BSA concen-
 256 trations (see supporting information). These findings emphasized the impor-
 257 tance of the total solids content present in the feed solution as an additional
 258 requirement for ensuring adequate drying efficiency during electrospraying.

259 With regard to process parameters, it was found that the best results were
 260 generally obtained when a feed flow rate of 0.2 mL per hour and a tip to collec-
 261 tor (TTC) distance between 6 and 12 cm were applied. Larger TTC distances
 262 resulted in an increased amount of droplets on the collector, as the electric field
 263 strength required for jet initiation became too weak at these distances. Gener-
 264 ally, a voltage between 19 kV and 25 kV was needed to obtain solid particles on
 265 the collector. However, as illustrated by the SEM images obtained for different
 266 fractions of formulation K25_5 (see supporting information), the powders result-
 267 ing from the one parameter at a time screening study still displayed substantial
 268 heterogeneity.

269 4.2. Mixture DoE with Ordinal Responses

270 A mixture DoE was chosen to allow for a more in depth assessment of how
 271 the different formulation components influence the process and the resulting
 272 powder quality. Following the execution of the DoE, it was found to be hard to

273 divide the defined response, which combined cone-jet stability and powder bed
274 quality observations, into five categories as originally intended (see Table 5).
275 Therefore, as differences in cone-jet stability and powder bed quality were less
276 pronounced than expected, two separate responses were recorded and, for each
277 response, two factor levels were defined (see Table 6). Given this encoding
278 revision of the responses, a logistic regression was applied during data analysis.

279 To further improve the resolution of our process quality assessment, a high
280 speed camera was used to assess cone-jet stability instead of the previously
281 used visual observation method. Table 7 summarises the physicochemical char-
282 acteristics and response values obtained for the DoE given in Table 3. Values
283 obtained for the cone-jet stability and powder bed responses were identical for
284 all runs, implying that the estimations for both models are identical as well. The
285 response values were subsequently processed according to the method described
286 in Section 3.7.1, but among the fifteen runs performed for this DoE, only five
287 gave a level 2 response. This resulted in the obtained estimators (Table 8) being
288 “unstable”, i.e. they could not be considered as reliable due to the very large
289 standard error of the obtained parameter estimates.

290 Figure 3 illustrates how a difference in cone-jet stability resulted in a dif-
291 ferent powder bed morphology, as formulation M2 yielded nanometre-sized and
292 highly monodisperse particles, resemblant of the carbamazepine nanoparticles
293 obtained by Wang et al. [22], while the unstable cone-jet resulting from formu-
294 lation M14 gave particles more similar to those obtained with a spray-drying
295 process. Additionally, slow motion recordings (> 100 frames at 5 fps) of the
296 cone-jet modes made with the high speed camera are included in the supporting
297 information of the publication. It should be noted that the overall integrity of
298 powder/particle morphology was relatively low. This resulted in a large part
299 of the formulations being converted to agglomerates or homogeneous layers on
300 the aluminium foil (used for collection) awaiting SEM analysis, despite being
301 stored in a desiccator at 278 K. The low storage integrity of these powders is
302 believed to be caused by a combination of the very small particle sizes obtained
303 with the process and high residual water content in the powder bed, both in-

side and around the particles. In order to confirm this hypothesis, additional characterisation of the powders, e.g. Karl Fischer titration, thermogravimetric analysis, ... will have to be performed, which was beyond the scope of the present study due to sample quantity restrictions. To resolve this issue, a different set-up could be used to increase the drying distance without having to sacrifice electric field strength. An example could be the electrospraying set-up used by Ciach [5], who used a nozzle-ring set-up inside a glass chamber under air flow, a process very much resembling spray-drying, where electrospraying functions as the atomiser. Although promising, feed flow rates for this process will still be much lower than those obtainable with a standard spray-drying set-up, and the added effect of the air flow on the electrospraying process should not be underestimated. Alternatively, the electrospraying process could be performed at elevated temperatures and decreased humidity. Even though this would increase the rate of water evaporation, temperature changes could heavily impact the physicochemical properties of the feed solution, e.g. viscosity, electrical conductivity, ..., making process optimisation and stringent temperature control necessary for such processes. The use of higher temperatures would also nullify the advantage of working at ambient conditions and could introduce additional solid state stability concerns. Finally, a more aggressive post-drying step than storage in a desiccator could be added to the method, like freeze-drying the final product, but this would once again nullify some of the advantages that electrospraying has compared to more traditional drying processes like spray- and freeze-drying. Regardless of which of these approaches is selected, a thorough understanding of the impact of formulation and process parameters on the electrospraying process will be indispensable.

In addition to assessing the influence of formulation factors on the cone-jet stability and powder bed responses, the influence of the physicochemical (uncontrolled) factors shown in Table 7 was evaluated by both plotting the responses versus physicochemical factors and by including them as factors in regression models. However, neither method was able to show a correlation between, or a significant influence on, the different responses for any of the

335 included physicochemical parameters.

336 Despite not yielding any statistically significant model, the collected data
337 (see Table 7) did show that glycerol seemed to impact the responses, more
338 specifically, that the absence of glycerol in the mixture correlated to a response
339 equal to 2. Additionally, it can be observed that all responses which equal 2
340 contain 20 % m/V trehalose. Therefore, glycerol was eliminated from formula-
341 tions for the mixture DoE described in Section 3.7.2, while 20 % m/V trehalose
342 was added to all formulations, thus leaving only BSA, HP β CD and PVP K25
343 as factors of the resulting mixture DoE with three factors for binary responses
344 (see Section 3.7.2) of which the results are given in Section 4.3.

345 4.3. Mixture DoE with Three Factors for Binary Responses

346 Following the analysis of the mixture design defined in Section 3.7.1, three
347 formulation factors were selected to be further analysed: BSA, HP β CD and
348 PVP K25, according to the mixture DoE, based on simplex lattice algorithm
349 generated in JMP 11.0. The resulting DoE is summarised in Table 4 where DoE
350 encoding represents the fraction of a 20 % m/V excipient content, not including
351 trehalose (20 % m/V) and polysorbate 20 (1 % m/V), that each formulation
352 factor assumes.

353 Table 9 summarises the results obtained for the DoE given in Table 4. Con-
354 trary to Section 4.2, there are discrepancies between the “Cone-jet stability
355 rating” and “Powder bed rating” response values, which is likely due to the
356 resolution of powder bed observation being too low to distinguish between sub-
357 tle differences in powder bed quality, resulting from slightly different cone-jet
358 stability modes observed with the high speed camera. This was within expec-
359 tations as the formulations included in the DoE in scope could be considered
360 as being more optimised for electrospraying than those discussed in Section 4.2,
361 thus making the difference between different formulations less pronounced. Fol-
362 lowing data acquisition, response values were processed according to the method
363 described in Section 3.7.2. Additionally, slow motion recordings (> 100 frames
364 at 5 fps) of the cone-jet modes made with the high speed camera are included

365 in the supporting information of the publication. Representative SEM images
366 could not be taken for any of the formulations due to powder/particle integrity
367 issues, as was discussed in Section 4.2 and illustrated by the SEM images shown
368 in Figure 6.

369 Estimator values for the cone-jet stability response are given in Table 10,
370 initial analysis of the DoE showed BSA to be borderline significant with a p -
371 value (Likelihood Ratio (LR) test) of 0.0519 (see Table 10). Figure 4 represents
372 the JMP prediction profiler and associated desirability functions output which
373 were used to determine the factor value combination, which, according to the
374 model, would have the highest probability of getting a response equal to 2. The
375 optimum mixture combination that should theoretically maximise the odds was
376 determined to be 30.7 % (DoE encoding) or 6.2 % m/V BSA, 69.3 % (DoE
377 encoding) or 13.8 % m/V PVP K25 and 0 % HP β CD (see Figure 4).

378 The estimated optimal feed solution was then prepared in triplicate (L22,
379 L23 and L24), characterised and electrosprayed. All three iterations gave a
380 value of 2 for both responses. Response values and physicochemical characteris-
381 tics can be found in Table 9. The results of these three confirmatory runs were
382 subsequently added to the original DoE and results were reanalysed with these
383 values included. Estimator and p -values of this expanded DoE are summarised
384 in Table 11 for the cone-jet stability response, confirming the statistical signifi-
385 cance of the BSA factor effect, which now has a p -value (LR test) of 0.0319 (see
386 Table 11). The JMP prediction profiler and the associated desirability function
387 are presented in Figure 5 and reconfirm the previously found optimum values
388 for the cone-jet stability response at 31.4 % (DoE encoding) or 6.3 % m/V BSA,
389 68.5 % (DoE encoding) or 13.7 % m/V PVP K25 and 0 % HP β CD.

390 Data processing of the powder bed response was conducted in the same
391 manner as for the cone-jet stability response, but estimators for the powder bed
392 response (see supporting information) were “unstable” due to the very large
393 standard error of the obtained parameter estimates, making their values un-
394 reliable. p -Values resulting from the Wald and LR test are thus included for
395 information purposes only. The three confirmatory runs all gave a level 2 powder

396 bed response, confirming that a good powder bed was obtained at the theoret-
397 ical mixture optimum, but the addition of the confirmatory runs to the dataset
398 did not result in a reliable model as the standard error remained large (see
399 supporting information).

400   Assessment of the physicochemical data (see Table 9) was performed in the
401 same way as for the first DoE (see Section 4.2) for both responses, i.e. by
402 both plotting responses versus physicochemical factors and by including them
403 as factors in regression models. However, neither method was able to show a
404 correlation between or a significant influence on the different responses for any
405 of the physicochemical parameters included in the study.

406   Overall, the presented results provide evidence that electrospraying of fully
407 aqueous BSA solutions is feasible and that, using the correct statistical ap-
408 proach, the atomisation process can be predicted and optimised for formula-
409 tions containing BSA. Even so, the used statistical approach did not allow for
410 prediction of powder bed quality or estimation of the impact of, or interaction
411 between, different physicochemical characteristics of the feed solution. The lat-
412 ter was likely due to the fact that physicochemical factors had to be analysed
413 as “uncontrolled factors” in the DoE, rather than formulating feed solutions
414 possessing a range of different physiochemical properties. Visual observation
415 of the powder bed quality was probably insufficiently discriminatory between
416 good and bad processes, but was deemed the most feasible for this study, due to
417 the high number of experiments and low process throughput. Hence, it is sug-
418 gested that future work should include a more complete set of characterisation
419 methods like particle size, morphology, water content, . . . to increase the overall
420 response resolution. Protein stability of the formulations was not taken into
421 account for the present work, inclusion of these parameters in future work, with
422 a more therapeutically relevant model protein, will likely introduce a number of
423 additional constraints and requirements to ensure production of a high quality
424 protein powder [23].

425 5. Conclusions

426 The present body of work has confirmed the feasibility of electrospraying
427 fully aqueous solutions containing BSA in order to obtain a powder, albeit
428 one with limited storage integrity. Extrapolation of the general conclusions of
429 this study to other, more therapeutically relevant, proteins seems permissible,
430 provided that there is sufficient similarity with BSA for relevant physicochemical
431 characteristics. A caveat here is that protein instability and aggregation could
432 be disruptive factors. A design of experiments approach was successfully used
433 to screen and optimise the electrospraying performance of a formulation based
434 on the Taylor cone-jet stability. No valid statistical model could be obtained
435 for the powder bed quality, but experiments did suggest the powder bed quality
436 being dependent on the amount of dissolved solids present in the feed solution.
437 The overall (storage) integrity of the obtained powders was low, presumably as
438 a consequence of a high residual water content still being present after drying, a
439 hypothesis that still needs to be confirmed, which could be resolved by further
440 optimising the process parameters or making alterations to the used set-up.
441 Finally, an extensive dataset containing physicochemical properties relevant to
442 electrospraying was gathered for a large number of excipients commonly used
443 to stabilise proteins in spray- or freeze-drying, which can be used as a reference
444 work for future studies.

445 6. Acknowledgements

446 The authors would like to thank UCB Pharma for providing the equipment,
447 materials and funding to make this study possible.

448 References

449 References

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Table 1: Range of electrospraying process parameters scanned in search for the experiments. Parameter values denoted with an * were not varied, but fixed at the value shown in the table.

Parameter	Screening study	Mixture DoE studies
Voltage	0-25 kV	19-25 kV
Flow rate	0.1-3 mL/hour	0.1 mL/hour *
Tip to collector distance	6-15 cm	9 cm *
Temperature	298 ± 2 K*	303 ± 2 K*
Relative humidity	35 ± 5 % (fixed)	20 ± 5 % *

Table 2: Aqueous formulations containing different concentrations and types of PVP in the presence of BSA.

Formulation	PVP		BSA	Polysorbate 20
	Type	% m/V	% m/V	% m/V
K25_2	K25	2.0	10	1.105
K25_3	K25	3.0	10	1.105
K25_5	K25	5.0	10	1.105
K17_5	K17	5.0	10	1.105
K30_5	K30	5.0	10	1.105

Table 3: Mixture DoE with replicate runs (factors in DoE encoding).

Formulation	BSA	Glycerol	Trehalose	HP β CD	PVP K25
M1	0.025	0.05	0.5	0.175	0.25
M2	0.025	0	0.5	0.475	0
M3	0.25	0	0	0.5	0.25
M4	0.25	0	0.5	0	0.25
M5	0.25	0.05	0.2	0.5	0
M6	0.25	0.05	0.45	0	0.25
M7	0.025	0.05	0.175	0.5	0.25
M8	0.25	0	0	0.5	0.25
M9	0.25	0	0.5	0	0.25
M10	0.025	0	0.5	0.475	0
M11	0.25	0.05	0.5	0.2	0
M12	0.25	0.05	0.2	0.5	0
M13	0.025	0	0.5	0.475	0
M14	0.25	0	0	0.5	0.25
M15	0.025	0.05	0.175	0.5	0.25

^a DoE encoding represents the fraction of a 40 % m/V excipient content, not including polysorbate 20 (1 % m/V), that each formulation factor assumes.

Table 4: Mixture DoE following a simplex lattice (factors in DoE encoding).

Formulation	BSA	HP β CD	PVP K25
L1	0.2	0.4	0.4
L2	0.6	0	0.4
L3	0	0.2	0.8
L4	0	0	1
L5	0.2	0.6	0.2
L6	0.6	0.2	0.2
L7	0.2	0.8	0
L8	0	0.4	0.6
L9	0.4	0.6	0
L10	0	0.6	0.4
L11	0.2	0.2	0.6
L12	0.4	0.2	0.4
L13	0.8	0.2	0
L14	0.6	0.4	0
L15	0.2	0	0.8
L16	0	0.8	0.2
L17	0.8	0	0.2
L18	0.4	0.4	0.2
L19	0	1	0
L20	0.4	0	0.6
L21	1	0	0

^a DoE encoding represents the fraction of a 20 % m/V excipient content, not including trehalose (20 % m/V) and polysorbate 20 (1 % m/V), that each formulation factor assumes.

Table 5: Original response level encoding for the mixture DoE summarised in Table 3.

Level	Observation of cone-jet and powder bed quality
1	Thick water jet, no mist
2	Sprayed mist, no solid
3	Sprayed mist, solid with thick droplets
4	Sprayed mist, solid with fine droplets in powder bed
5	Sprayed mist, dry powder without visible droplets

Table 6: Final response level encoding for the mixture DoE summarised in Table 3 and Table 4.

Level	Cone-jet stability rating	Powder bed rating
1	Unstable within the observed time frame	Many and/or large droplets present
2	Stable within the observed time frame	Visually dry

Table 7: Results for the mixture DoE (see Table 3) with replicate runs and the measured physicochemical properties associated with each formulation.

	Density		Surface tension		Kin. viscosity		El. conductivity		Cone-jet	Powder
	g cm^{-3}		mN m^{-1}		$\text{mm}^2 \text{s}^{-1}$		$\mu\text{S cm}^{-1}$		stability	bed
	$n = 3$	$\pm \text{SD}$	$n = 3$	$\pm \text{SD}$	$n = 3$	$\pm \text{SD}$	$n = 3$	$\pm \text{SD}$	rating	rating
M1	1.12637	0.00076	31.412	0.161	18.06	0.34	154.1	1.1	1	1
M2	1.13513	0.00064	34.385	0.339	5.55	0.01	93.8	0.4	2	2
M3	1.10676	0.00085	37.601	0.204	33.96	0.24	471.7	1.2	1	1
M4	1.12339	0.00091	34.806	0.371	22.97	0.03	431.3	2.5	2	2
M5	1.11992	0.00140	35.843	0.224	8.35	0.02	427.0	1.0	1	1
M6	1.12049	0.00051	34.128	0.698	23.52	0.15	436.3	1.5	1	1
M7	1.11427	0.00050	35.444	0.357	20.25	0.01	185.4	0.2	1	1
M8	1.10466	0.00056	37.192	0.603	31.78	0.20	477.0	3.5	1	1
M9	1.12321	0.00050	34.442	0.899	23.26	0.32	432.7	1.2	2	2
M10	1.13660	0.00013	34.826	0.211	5.73	0.02	93.6	0.5	2	2
M11	1.13144	0.00020	31.709	0.432	7.14	0.00	399.3	0.6	1	1
M12	1.11931	0.00036	36.005	0.390	8.11	0.00	433.3	0.6	1	1
M13	1.13584	0.00099	34.813	0.450	5.78	0.04	94.2	1.1	2	2
M14	1.10356	0.00012	37.434	0.211	30.03	0.14	474.7	1.5	1	1
M15	1.11408	0.00070	35.913	0.044	20.82	0.26	185.1	0.5	1	1

Table 8: Parameter estimates, Wald and Likelihood Ratio (LR) test for the mixture DoE with replicate runs.

Term	Estimate	Standard error	p -value		Remark
			Wald test	LR test	
BSA	17.424016	8966.3688	0.9984	0.9978	Unstable
Glycerol	660.165053	50255.443	0.9895	0.0012*	Unstable
Trehalose	-49.607099	4271.6647	0.9907	0.9738	Unstable
HP β CD	15.4189488	4391.8467	0.9972	0.9969	Unstable
PVP K25	17.1407206	8264.7876	0.9983	0.9976	Unstable

Table 9: Results for the mixture DoE following a simplex lattice (see Table 4) with replicate runs and the measured physicochemical properties associated with each formulation including 3 additional confirmation points (L22, L23 and L24).

	Density g cm^{-3}		Surface tension mN m^{-1}		Kin. viscosity $\text{mm}^2 \text{s}^{-1}$		El. conductivity $\mu\text{S cm}^{-1}$		Cone-jet stability rating	Powder bed rating
	$n = 3$	$\pm \text{SD}$	$n = 3$	$\pm \text{SD}$	$n = 3$	$\pm \text{SD}$	$n = 3$	$\pm \text{SD}$		
L1	1.12793	0.00046	32.826	0.069	16.26	0.16	225.0	1.7	2	2
L2	1.12601	0.00079	35.044	0.122	20.45	0.04	472.3	0.6	2	2
L3	1.12222	0.00044	31.778	0.048	30.96	0.49	136.3	0.0	1	2
L4	1.11814	0.00036	32.191	0.349	38.97	0.59	145.8	0.9	2	2
L5	1.13124	0.00008	33.999	0.061	9.98	0.12	202.0	2.0	2	1
L6	1.12845	0.00046	32.763	0.047	13.11	0.06	464.0	2.6	2	1
L7	1.13487	0.00030	33.439	0.049	6.04	0.00	183.2	1.1	2	2
L8	1.12612	0.00068	32.585	0.151	20.20	0.21	120.0	1.1	1	2
L9	1.13402	0.00086	33.891	0.103	6.72	0.01	313.7	2.3	1	1
L10	1.12950	0.00032	33.650	0.059	14.59	0.02	104.4	1.1	2	2
L11	1.12614	0.00018	31.760	0.302	24.25	0.06	238.3	1.2	1	2
L12	1.12772	0.00028	32.402	0.051	18.29	0.04	338.3	1.2	2	2
L13	1.13150	0.00060	33.113	0.095	9.38	0.03	603.3	3.5	1	1
L14	1.13434	0.00029	32.014	0.095	7.99	0.00	452.3	1.2	1	1
L15	1.12085	0.00064	34.232	0.076	31.79	0.27	263.0	1.0	2	2
L16	1.13358	0.00031	33.554	0.061	9.41	0.17	85.4	0.2	1	1
L17	1.13339	0.00056	33.915	0.207	17.68	0.07	591.0	6.2	1	2
L18	1.12991	0.00032	32.697	0.052	11.41	0.31	330.3	1.5	1	1
L19	1.13768	0.00025	34.339	0.073	5.49	0.02	59.8	0.1	1	1
L20	1.12429	0.00048	34.089	0.064	26.44	0.11	356.3	0.6	1	2
L21	1.13112	0.00055	36.179	0.069	11.67	0.03	763.3	3.1	1	1
L22	1.12005	0.00098	33.889	0.220	29.00	0.17	327.0	1.7	2	2
L23	1.11789	0.00038	34.183	0.171	27.33	0.43	325.0	1.0	2	2
L24	1.11896	0.00019	34.855	0.087	27.29	0.43	326.7	2.1	2	2

Table 10: Parameter estimates, Wald and Likelihood Ratio (LR) test for the cone-jet response model.

Term	Estimate	Standard error	<i>p</i> -value	
			Wald test	LR test
BSA	5.1810408	3.8134782	0.1743	0.0519
HP β CD	0.89532763	1.7500357	0.6089	0.5963
PVP K25	-0.0589736	1.6572741	0.9716	0.9716
BSA * HP β CD	-9.6248058	9.7810755	0.3251	0.2879
BSA * PVP K25	-13.561715	10.341393	0.1897	0.1365
HP β CD * PVP K25	0.68869608	7.0188939	0.9218	0.9219

Table 11: Parameter estimates, Wald and Likelihood Ratio (LR) test for the cone-jet response model including 3 additional confirmation points (L22, L23 and L24).

Term	Estimate	Standard error	<i>p</i> -value	
			Wald test	LR test
BSA	5.99074798	4.0235338	0.1365	0.0319*
HP β CD	0.81006464	1.7376995	0.6411	0.6311
PVP K25	-0.284217	1.6713835	0.8650	0.8645
BSA * HP β CD	-10.539421	10.088892	0.2962	0.2591
BSA * PVP K25	-16.913351	10.381707	0.1033	0.0640
HP β CD * PVP K25	1.93661982	6.9737515	0.7812	0.7813

Figure 1: Schematic representation of the used electrospraying set-up.

Figure 2: SEM images of the powder resulting from the 5 % m/V PVP K25 formulation (K25_5) given in Table 2. a depicts a representative SEM image of the particles collected from the visibly dry area of the powder bed, while b depicts a representative SEM image of the particles collected from the rest fraction of the powder bed where residual droplet could still be observed.

Figure 3: Images captured using a Fastcam SA2 high speed camera of the Taylor cone-jet mode observed during the electrospraying and SEM images of the resulting powder beds captured for formulations M2 (Figure 3a and Figure 3c, respectively), being a stable cone-jet, and formulation M14 (Figure 3b and Figure 3d, respectively), being an unstable cone-jet, of the mixture DoE shown in Table 3.

Figure 4: JMP prediction profiler and desirability function for the cone-jet response model.

Figure 5: JMP prediction profiler and desirability function for the cone-jet response model including 3 additional confirmation points (L22, L23 and L24).

(a)

(b)

Figure 6: SEM images of the powders resulting from electrospraying formulations a L17 and b M14 from the mixture DoE following a simplex lattice (see Table 4).