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Electrohydrodynamic atomization of protein (bovine serum albumin)

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Bovine serum albumin (BSA) was chosen as a model protein. Three solutions of different concentrations of 5, 20 and 50 mg/ml were prepared, characterised and subjected to electrohydrodynamic atomization (EHDA). The 5 and 20 mg/ml solutions were atomized successfully and mode selection (M-S) maps were drawn for both concentrations to find out regions of stable cone-jet mode atomizaton. Droplet relics of these two solutions were investigated by electron microscopy. Samples were investigated by UV spectroscopy and circular dichroism (CD) spectroscopy before and after electrohydrodynamic atomization. We conclude that, particularly at the higher concentration of protein, EHDA does not result in significant structural change of BSA, and therefore is a processing route that can be considered for encapsulating drugs in proteins.

1. Introduction

Serum albumins are the most abundant proteins found in blood plasma. They have an amazing ability to bind a wide variety of biological molecules, e.g. hydrophobic, hydrophilic, cationic and anionic substances. The concentration of albumin in human serum is about 45 mg/ml. Its principal functions are considered to be regulating osmotic pressure in blood and transporting fatty acids and other lipophilic compounds [1, 2]. The physiological and pharmacological properties of serum albumins have been studied extensively over several decades [1–4]. In addition to its ordinary clinical applications, such as hypovolemic shock treatment, many investigators have attempted to utilize it as a drug carrier [5, 6]. Many drugs, including anti-coagulants, tranquilizers and general anesthetics, are transported in blood while bound to albumin [7].

Bovine serum albumin (BSA) is a globular protein with a molecular weight of 66.3 kDa. The structure of bovine serum albumin is very similar to human albumin, with about 80% homology [8–10]. It is composed of 583 amino acids and its sequence contains 17 disulphide bonds [11–13].

Electrohydrodynamic atomization (EHDA), also referred to as electrostatic atomization or electrospraying, is a process in which a liquid is forced through a nozzle and a potential difference of the order of kV is applied. The electric field induces a surface charge in a drop, which forms at the tip of the nozzle and as a result of the electric field, it is transformed into a cone. A jet with a high charge density emanates from the cone apex and subsequently breaks down into tiny droplets [14–16]. This phenomenon was first reported by Zeleny [17, 18] with subsequent analysis by Taylor [19, 20]. Compared to other atomization techniques of liquids, EHDA has some significant advantages, e.g. relatively easy generation of droplets and avoiding coalescence of droplets due to electric charge of same polarity in the droplets, achievement of a narrow size distribution of droplets via the cone-jet mode [21]. The technique has been applied to paint and crop spraying [22], emulsion production [23] and to mass spectroscopy [24] and more recently for materials forming [25–27], including the processing of nano-biomaterials [28].

The cone-jet mode of EHDA allows the preparation of near-monodispersed droplets of a few micrometers in size [25, 26] and is attractive as a method of encapsulating drug in protein-based drug delivery systems. Reyderman and Stavchansky [29] formed cholesterol micro-spheres by EHDA of molten cholesterol solution for the application of drug delivery. Kaufman *et al*. [30] electrosprayed various protein solutions to form singly charged protein ions. It is claimed that protein nanoparticles were produced by electrospray drying [31]. A hand-held, multidose, pulmonary drug delivery prototype device was developed based on the principle of EHDA [32]. Recently, EHDA of drug solutions with the model drug methylparahydroxybenzoate was carried out to explore the possibilities of pulmonary drug delivery [33].

In this work, we investigate EHDA of BSA to identify the cone-jet mode region of spraying .We also study by

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using spectroscopy how the structure of BSA is affected by EHDA, as this is a crucial factor if this route is to be used in drug encapsulation applications. Relics of droplets were investigated by electron microscopy.

2. Experimental details

2.1. Materials

BSA (lyophilized powder, essentially fatty acid free and essentially globulin free and supplied by Sigma-Aldrich, Poole, UK) was chosen for this investigation as a model protein. A solution of 10 vol% ethanol (HPLC grade) in de-ionised water was prepared, BSA was dissolved in these solutions to obtain samples of concentration 5, 20 and 50 mg/ml. The solutions prepared were filtered using 0.2 μm cellulose acetate membrane syringe filters (low hold up volume, grade F 5023 and supplied by Sigma-Aldrich, Poole, UK) to get rid of any undissolved BSA.

2.2. Characterisation

The physical properties of the solvents and BSA solutions were characterised. Kinematic viscosity was measured using a AVS 360 viscometer (Camlab, Schutt-Gerate, Mainz). DC electrical conductivity and pH measurements were assessed using a HACH SensION^{Tm} 156 probe. Surface tension was measured using a Kruss Tensiometer K9 (Du Nouy's ring method). The densities of the samples were estimated using the standard density bottle. All measurements were performed at ambient temperature ($\sim$26 °C).

2.3. Electrohydrodynamic atomization (EHDA)

Fig. 1 depicts the apparatus used for EHDA. Briefly, it consists of an infusion pump (Harvard Apparatus PHD 4400), a 500 μl gastight syringe (Hamilton-microliter series), a stainless steel capillary (outer and inner diameter of 710 and 410 μm, respectively), a high voltage d.c. power supply (Glassman Europe). The distance between the tip of the capillary and ring ground electrode was 10 mm. Silicone tubing was used to connect the needle inlet and syringe pump outlet. A high-speed camera (Weinberger AG, Dietikon, Switzerland) was used to observe the jet modes and capture images. Mode Selection (M-S) maps, i.e. modes for applied voltage versus flow rate, were established for two concentrations of BSA, 5 and 20 mg/ml.

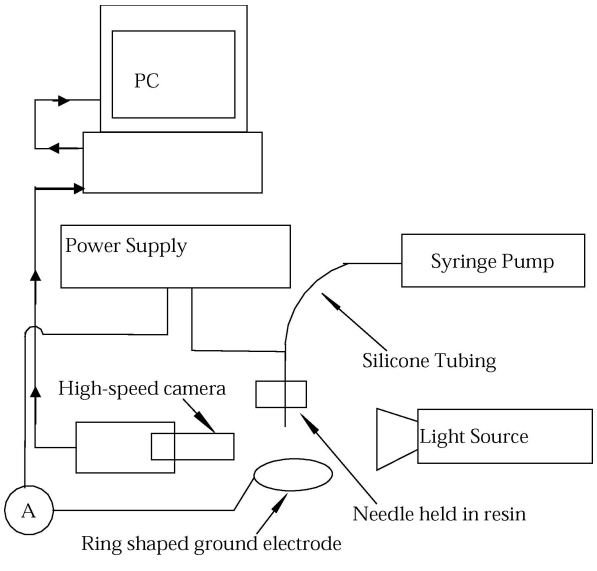


Figure 1 EHDA set up.

Samples of the two BSA solutions were subjected to EHDA at 6.5 kV and 8×10^{-11} m^3s^{-1} for 5 mg/ml and 6.5 kV, and 4×10^{-11} m^3s^{-1} for 20 mg/ml, respectively. These samples were collected just above the ring ground electrode in an eppendorf for UV and CD spectroscopy. Sprayed droplets of these samples were also collected just below the ring ground electrode on a carbon coated aluminium stub for investigation by scanning electron microscopy.

2.4. UV spectroscopy

This assay is based on the binding of the protein-specific dye, coomassie brilliant blue to proteins [34]. The experiments were performed using a U-2010 spectrophotometer. The concentration of BSA in solution before and after electrospraying was determined colorimetrically using Bradford's reagent, which was purchased from Sigma-Aldrich (for 1–1400 μg/ml protein, grade B 6916). The Bradford's dye shifts the maximum absorbance from 465 to 595 nm. This shift is thought to be due to formation of a protein-dye complex. A reference curve as a function of protein concentration was established and then by detecting the absorbance of the protein solution used in our experiments, concentration was determined, taking care of the dilution factor. The BSA solutions were kept in disposable cuvettes. Blank samples for calibration were prepared by mixing 10% ethanol solution in de-ionised water and Bradford's reagent in 1:30 ratio.

2.5. Circular Dichroism (CD) spectroscopy

Optical activity of α-helix in far-UV permits the use of CD studies for investigations of conformational changes in protein solutions [35]. The CD spectra were recorded on a Model 202, Aviv Instruments Inc. CD spectrometer at room temperature under constant nitrogen purge. A quartz cell of 1 mm path length was used. The spectra were scanned between 185 and 300 nm with 0.5 nm resolution and averaging for 3 s. Four scans were recorded for each sample and two separate dilutions of each BSA solution were prepared and tested. Each sample was diluted to 0.1 mg/ml ($\pm$0.01).

CD spectra were baseline corrected and data are presented as θ, where θ is the observed ellipticity in degrees. The experimental spectra were fitted with 1 nm resolution in the 185–300 nm range and no constraints were used in the procedure.

2.6. Scanning electron microscopy

Stubs containing droplet relics were sputter coated with gold for 120 s. Subsequently, these were examined in the secondary electron mode using a Jeol JSM 6300F field emission scanning electron microscope operating at 10 kV with the working distance set at 10 mm.

TABLE I Physical properties of BSA solutions

Sample	Viscosity (mPa s)	Conductivity ($Sm^{-1} \times 10^{-4}$)	Surface tension (mNm^{-1})	pH	Density (kgm^{-3})
De-ionised water	1	2	68	6.1	1000
Ethanol	1.3	0.3	20	6.0	800
10 vol% Ethanol in de-ionised water	1.1	1	49	6.1	920
5 mg/ml BSA	1.3	70	52	7.0	1010
20 mg/ml BSA	1.4	230	50	7.0	1020
50 mg/ml BSA	1.7	540	47	7.0	1030

3. Results and discussion

3.1. BSA solution characteristics

Table I shows the properties measured. Ethanol was added as it lowers the electrical conductivity and the surface tension of the protein solution and helps in obtaining the cone-jet mode. In EHDA, the droplet size is dependent on the liquid flow rate, applied voltage and the liquid properties (i.e. viscosity, electrical conductivity, and surface tension) [25, 26]. Viscosity of a protein solution depends on its intrinsic characteristics, such as molecular mass, size, volume, shape, surface charge and ease of deformation [36, 37]. In addition, viscosity is influenced by environmental factors such as pH, temperature, ionic strength and ion type [38–40]. Surface tension of protein solutions decreases as the protein concentration increases and then becomes platonic [41].

As expected the viscosity of BSA samples increased with increasing concentration of BSA in the solution. Electrical conductivity of the BSA samples also increased with the increase of BSA concentration. The surface tension of the BSA samples was found to be virtually independent of protein concentration, as reported by McClellan and Franses [41]. The pH of the BSA solutions was ∼7.

The solution containing 5 mg/ml of BSA flowed smoothly throughout the atomization process but in the 20 mg/ml BSA solution, a small amount of protein deposited in the silicone tubing leading to the needle, increasing with time, but atomization was possible. At 50 mg/ml BSA solution, there was significant deposition of the protein in the silicone tubing, which prevented the flow, making it impossible to electrospray. Figs. 2 and 3 show the mode selection maps for 5 and

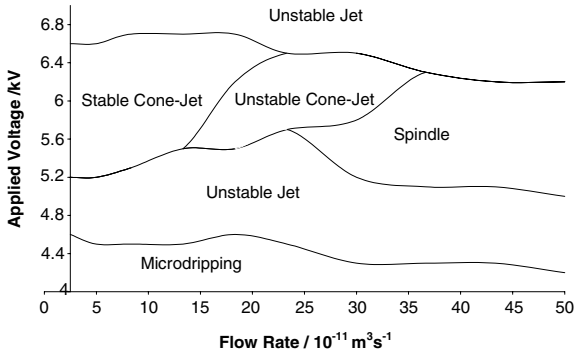


Figure 2 M-S map for the 5 mg/ml as-prepared BSA solution. In the case of unstable jet, a clear mode classification was not possible.

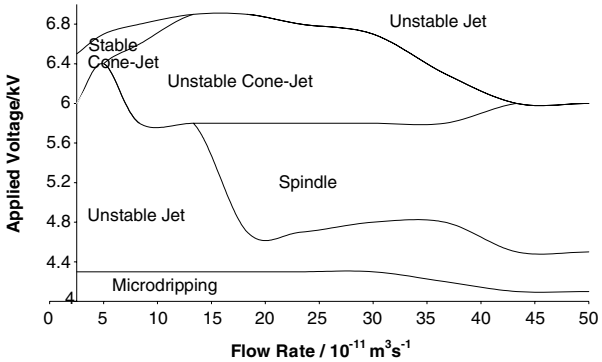


Figure 3 M-S map for the 20 mg/ml as-prepared BSA solution. In the case of unstable jet, a clear mode classification was not possible.

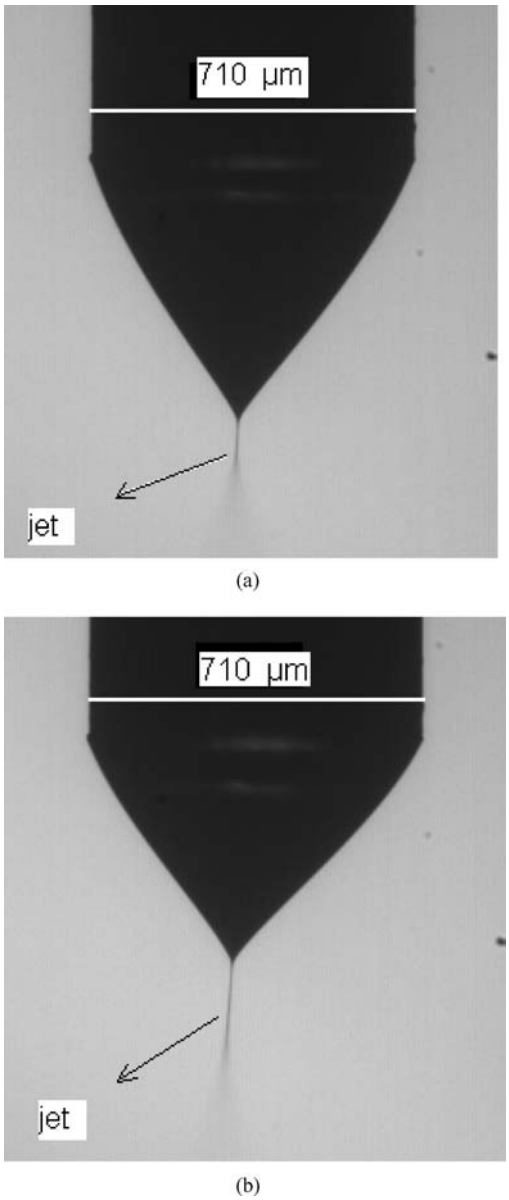
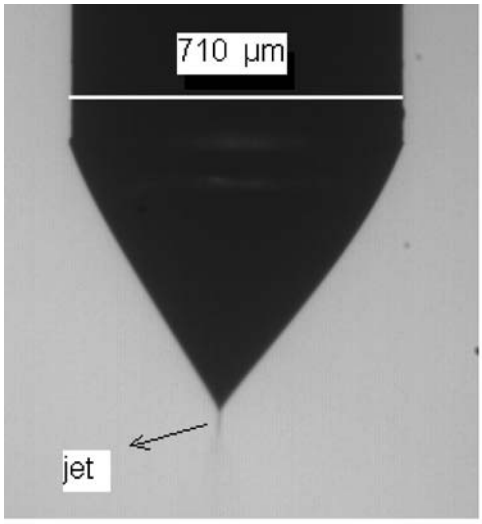
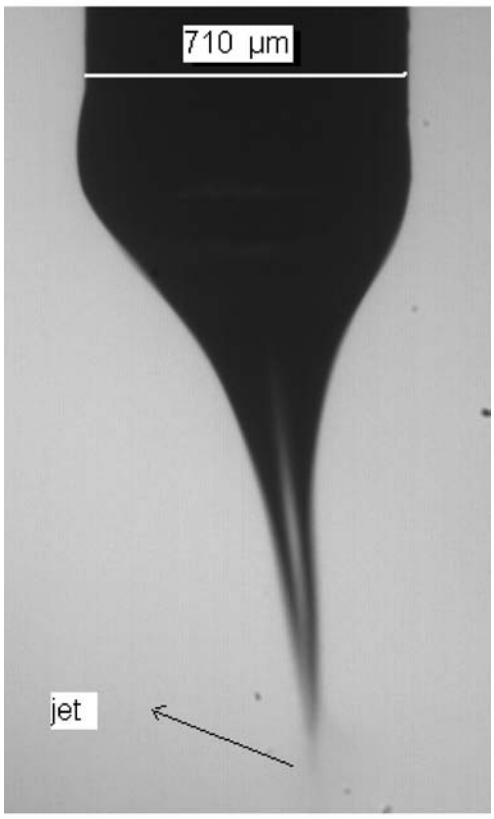


Figure 4 (a) Typical cone-jet mode EHDA of the 5 mg/ml BSA solution electrosprayed at 6.5 kV and 8×10^{-11} m^3s^{-1}, (b) mode observed during collecting the sample at 6.5 kV and 8×10^{-11} m^3s^{-1} for uv and cd spectroscopy.

20 mg/ml BSA solutions, respectively. Fine jets were obtained in the cone-jet mode spraying of both these solutions (Figs. 4(a) and 5(a)). As the protein concentration was increased, the stable cone-jet mode region shrinks and shifts to a lower flow rate.



(a)



(b)

Figure 5 (a) Typical cone-jet mode EHDA of the 20 mg/ml BSA solution electrosprayed at 6.5 kV and 4×10^{-11} m^3s^{-1}, (b) mode observed during collecting the sample at 6.5 kV and 4×10^{-11} m^3s^{-1} for uv and cd spectroscopy.

The samples for spectroscopy were collected at the highest possible applied voltage and lowest flow rate in the stable cone-jet mode region (Figs. 2 and 3), in order to subject the protein solutions to the maximum effect of the electric field. In the case of the 5 mg/ml BSA solution, which showed a larger stable cone-jet mode region (Fig. 2), there was no change of mode during collection (Fig. 4(b)). In contrast, under these conditions the spray mode changed to an unstable jet in the case of the 20 mg/ml BSA solution due to the influence of introducing a plastic eppendorf into the electric field (Fig. 5(b)).

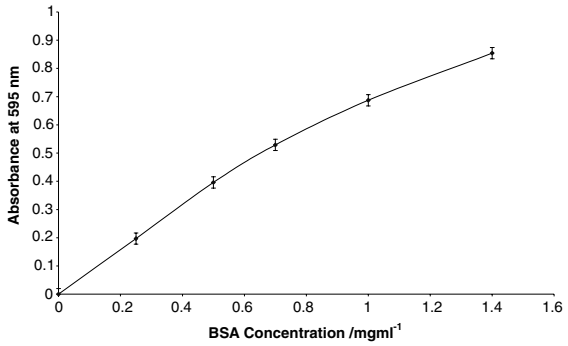


Figure 6 Calibration curve of absorbance versus BSA concentration used for UV spectroscopy.

3.2. BSA concentration

Fig. 6 shows the calibration graph for BSA used in uv spectroscopy measurements. The concentration of BSA in the samples before and after EHDA is given in Table II. As expected the protein concentration increased slightly after electrospraying and this is due to evaporation of ethanol and water from the fine droplets produced.

3.3. Structural features

Figs. 7 and 8 compare the CD spectra of BSA before and after electrospraying at concentrations of 5 mg/ml

TABLE II Concentration measured by UV Spectroscopy (standard deviation in parenthesis)

BSA solution	Conc. before electrospraying (mg/ml)	Conc. after electrospraying (mg/ml)
5 mg/ml	5.5 (±0.2)	6.1 (±0.2)
20 mg/ml	20.8 (±0.4)	25.9 (±0.4)

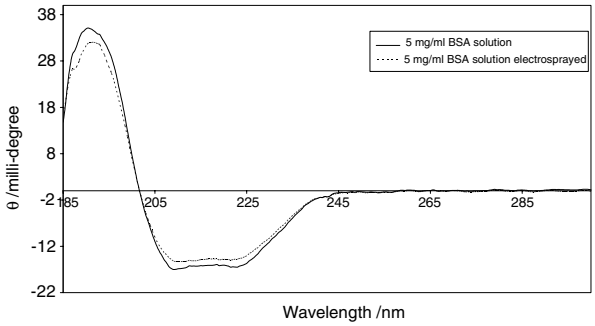


Figure 7 CD spectra of the 5 mg/ml BSA sample, before and after electrospraying.

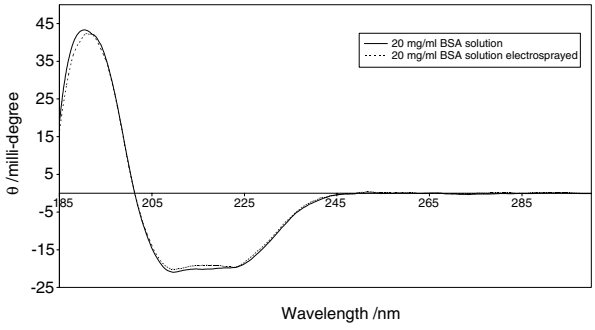


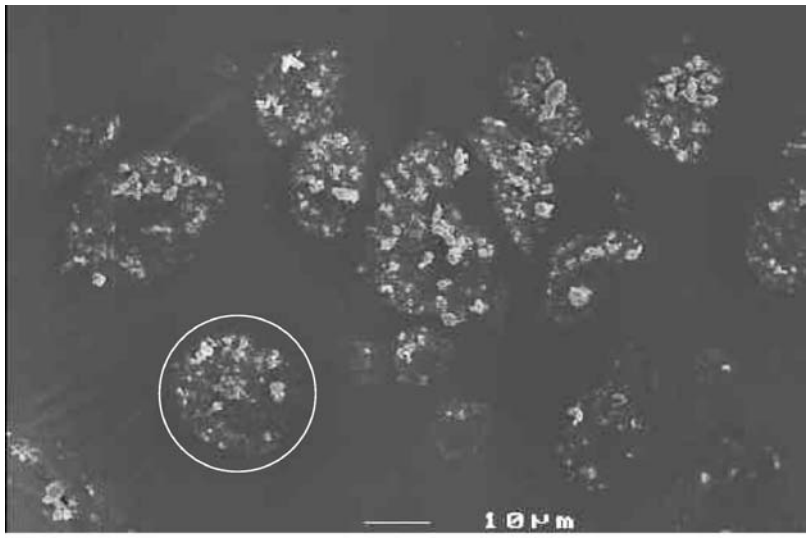
Figure 8 CD spectra of the 20 mg/ml BSA sample, before and after electrospraying.

TABLE III Analysis of secondary structure elements obtained from CD spectra of BSA

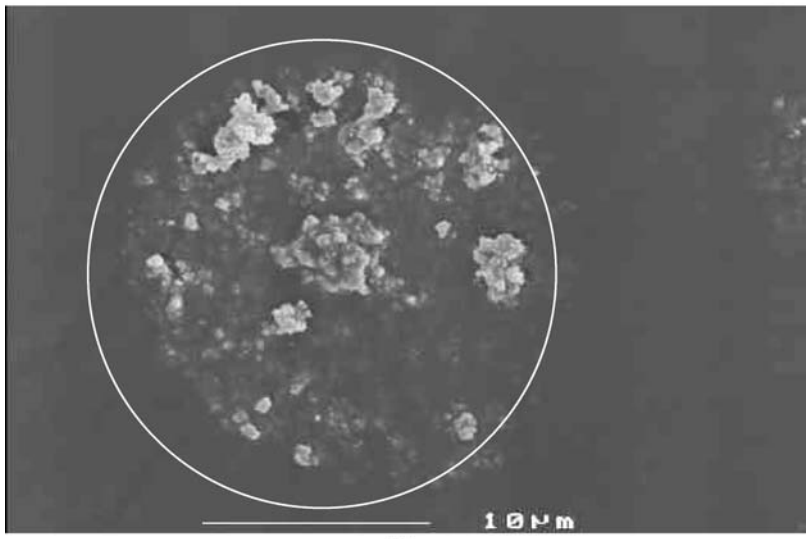
BSA solution	α-Helix % ($\pm$1)	β-Sheet % ($\pm$0.5)	β-turn % ($\pm$0.5)	Unordered % ($\pm$0.5)	NRMSD
5 mg/ml before electrospraying	65.5	7.5	8.5	19	0.010
5 mg/ml after electrospraying	58	9	10	23	0.013
20 mg/ml before electrospraying	67.5	7	7.5	18	0.005
20 mg/ml after electrospraying	63.5	8	9	20	0.008

and 20 mg/ml, respectively. The curves in Figs. 7 and 8 were fitted using Dichroweb, CDSSTR (Reference set 4) [42–46]. Analysis of the CD results (Table III) show that the deviations (NRMSD) of the solutions before and after electrospraying are comparable and <0.005. Hence the BSA solutions subjected to EHDA are not denatured. The differences in CD spectra are due to the change in concentration of the solutions as detected by UV spectroscopy (Section 3.2). However, the results in Table III indicate that the 20 mg/ml BSA solution was more stable than the 5 mg/ml BSA solution in terms of withstanding the electric field. Thus, more concentrated protein samples may be more suitable for EHDA although an upper limit prevails due to actual practical processing considerations (i.e. observations with the 50 mg/ml BSA solution).

Scanning electron micrographs in Figs. 9 and 10 show that droplet relics were in the size range 10–20 μm. These relics contained clusters of protein, each a few micrometers in size. Clustering is more pronounced in the sample containing a higher concentration of protein. BSA molecules are $\sim$7 nm in size [47–50], therefore each cluster contains several BSA molecules and the optimization of this number is an important aspect of any future work.

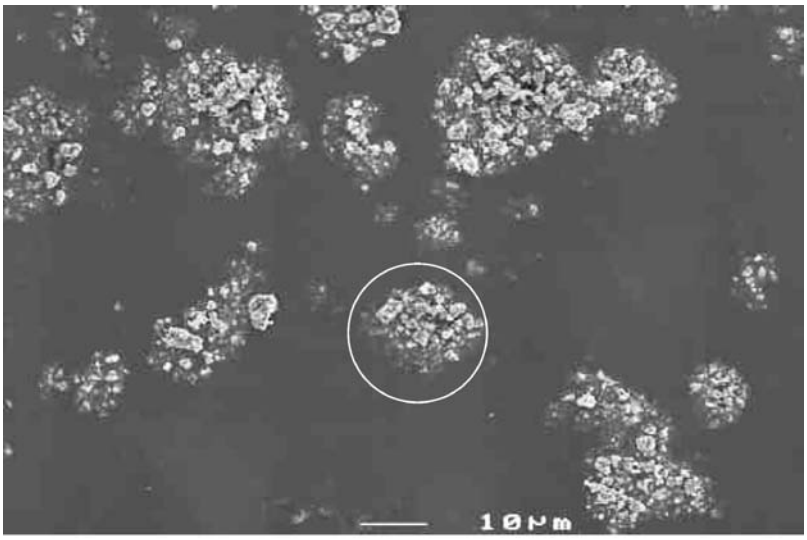


(a)

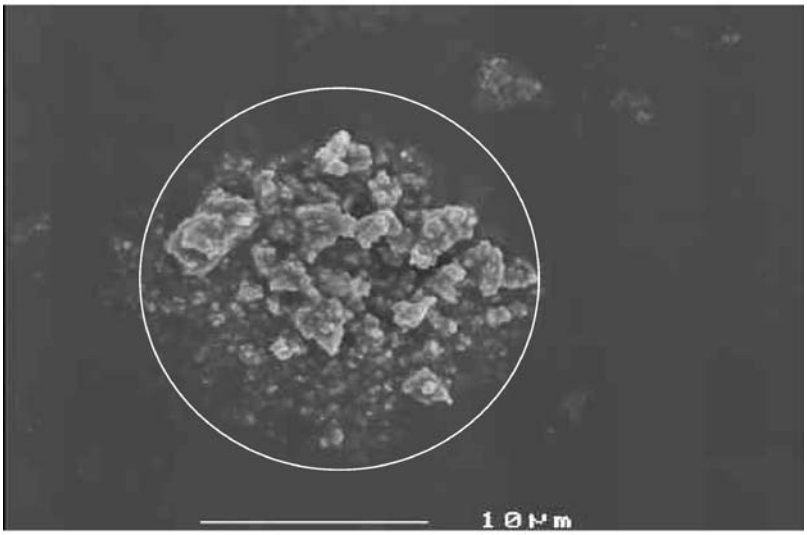


(b)

Figure 9 Scanning electron micrograph of 5 mg/ml BSA sample electrosprayed at 6.5 kV and 8×10^{-11} $m^3 s^{-1}$, (a) magnification of $\times$ 1000, and (b) magnification of $\times$3500.



(a)



(b)

Figure 10 Scanning electron micrograph of 20 mg/ml BSA sample electrosprayed at 6.5 kV and 4×10^{-11} m^3s^{-1}, (a) magnification of $\times$ 1000, and (b) magnification of $\times$ 3500.

4. Conclusions

The stable cone-jet mode of EHDA of BSA protein occurred at $<$6.5 kV and $<2 \times 10^{-10}$ m^3s^{-1} for 5 mg/ml BSA in solution, but as protein concentration is increased it becomes more difficult to electrospray the protein in this mode. BSA particle clusters of a few micrometers were produced by this method of processing. As expected the concentration of the protein in solution increases due to EHDA but there was no significant change in the secondary structure of the BSA due to processing by EHDA and this makes it a viable method for encapsulating bio-medicines in proteins, particularly at higher concentrations.

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