CHAPTER III

Retained integrity of encapsulated protein in spray-dried chitosan microparticles

Introduction

It is estimated that in excess of 160 biopharmaceuticals, most of which are protein-based, have now gained medical approval and several hundred are in the pipeline (Walsh, 2005). Accordingly, there have been increasing needs for development of efficient formulations of such labile therapeutics, which seem to have greater stability in the solid forms than in the liquid forms. One approach is to incorporate them into polymeric biodegradable micro-/nanoparticles. This could help simplify product storage and subsequently improve product stability. In addition, presence in solid dosage forms might be essential for application of some particular products. Several methods have been employed for micro-/nanoparticle productions, including spray drying (Blanco-Prieto *et al.*, 2004; Mumenthaler *et al.*, 1994; Tzannis and Prestrelski, 1999; Youan, 2004), spray freeze-drying (Costantino *et al.*, 2004), emulsion-solvent evaporation method (Blanco and Alonso, 1997; Cegnar *et al.*, 2004; Kim and Bae, 2004; Tobio *et al.*, 1998), and precipitation in supercritical antisolvents (Winters *et al.*, 1996). Of these, spray drying appears to be one of the most promising methods, because of its capacity to produce particles with controlled characteristics and narrow particle size distribution. Furthermore, it is considered as a fast, simple and energy-efficient process.

Numerous studies have reported so far on the development of biodegradable micro-/nanoparticles for peptide and protein delivery, in particular, those made of hydrophobic or amphiphilic polymers, such as poly(lactic acid), poly(lactic-co-glycolic acid) (Blanco and Alonso, 1997; Youan, 2004), poly(ε-caprolactone) (Youan, 2004) and their derivatives (Kim and Bae, 2004; Sinha *et al.*, 2004; Tobio *et al.*, 1998). However, these particles have a limitation in their preparation procedure, which requires the use of organic solvents and surfactants as well as sonication or homogenization. Such components and preparation process have been reported to

impose deleterious effects on the activity of encapsulated proteins (Cegnar *et al.*, 2004; van de Weert *et al.*, 2000).

Chitosan has been recognized as a promising material for delivery of drugs and labile macromolecular compounds, attributed to its excellent physicochemical and biological properties. It is regarded as a biocompatible and biodegradable polymer of low toxicity (Illum, 1998; Mi *et al.*, 2002). Furthermore, it is extremely important to note that chitosan is soluble in aqueous mild acidic solution, which is an obvious advantage over other biodegradable polymers. Therefore, preparation of chitosan microparticles intended for protein delivery generally requires none of those components or preparation procedures, which impose detrimental effects on the activity of proteins and are required when most biodegradable polymers are used.

As aforementioned, spray drying is a very efficient one-step process for production of microparticles. In order to prepare chitosan microparticles by this technique, it is inevitable that high temperature, at least above 100 °C must be applied, since the system is totally in aqueous environment (He *et al.*, 1999). How heat-labile proteins can resist such thermal stress and be encapsulated in chitosan microparticles by spray drying process is then in question. Therefore, feasibility of preparing chitosan microparticles for delivery of proteins by spray drying technique was investigated in this study. In order to control the physicochemical characteristics of the resultant microparticles, the effect of some formulation and process parameters on those was explored. Importantly, integrity of encapsulated bovine serum albumin, as a model protein, was also examined.

Materials and methods

Materials

Chitosan at molecular weight of 37 kDa with 94% degree of deacetylation (LCS) and 100 kDa with 95% degree of deacetylation (HCS) were obtained from Seafresh Chitosan (Bangkok, Thailand). Bovine serum albumin, Cohn Fraction V (BSA) was purchased from Sigma-Aldrich (Saint Louis MO, USA). All other chemicals were of analytical grade and used as received.

Methods

Preparation of chitosan microparticles

Chitosan was dissolved in 0.5% acetic acid solution and spray dried in a bench-top spray dryer (Büchi model 190, Büchi Labortechnik, Flawil, Switzerland). The liquid feed was pumped peristaltically and fed through a two-fluid nozzle (0.5 mm internal diameter) where it was atomized into fine droplets. Cooling water was circulated through the jacket around the nozzle throughout the process. The standard processing parameters comprised an atomizing air volumetric flow rate of 750 l/hour and an aspirator vacuum of 25 mbar. The inlet drying air temperature was varied at 80, 100 and 120 °C. Since it was not possible to control the outlet air temperature, it was thus only observed. The liquid feed rate was studied at 3, 5 and 7 ml/min. To incorporate protein into microparticles, BSA was first dissolved in a few milliliters of distilled water, subsequently mixed with chitosan solution, in order to obtain different protein loading levels, and spray dried as described above.

Particle size measurement

Particle size and size distribution of chitosan microparticles were measured by laser light-scattering method (Mastersizer 2000, Malvern Instrument, Malvern, UK). Small amount of chitosan microparticles was dispersed in a few milliliters of absolute ethanol and sonicated with a 3-mm-tip diameter standard probe at output control of 60 (Vibra Cell model VC 130 PB, Sonics and Materials, Newtown CT, USA) for about 30 seconds, in order to deaggregate the microparticles. The dispersion was loaded into a stirred sample cell, containing water as a measuring medium. Calculation of particle size was made from the intensity of light scattered at different angles, based on Mie's theory (Zimmerman, 1997). The particle size was presented in the volume-weighted mode and the 50% undersize diameter d (v, 0.5) was referred to as the particle diameter.

Zeta potential measurement

Chitosan microparticles were dispersed in 1 mM NaCl solution with sonication, as described above. The measurement was carried out on a Zeta-Meter System 3.0+ model 331 (Zeta-Meter, Staunton VA, USA). The result was averaged from 50 observations.

Particle morphology

Chitosan microparticles were mounted onto double-faced adhesive tape, which was attached on a sample stub. The samples were sputtered with gold and viewed under a scanning electron microscope (Jeol model JSM-5410 LV, Tokyo, Japan) at a voltage of 15.0 kV. The photomicrographs were then taken at a magnification of 5000.

Protein content determination

Chitosan microparticles were dissolved in 0.5% acetic acid solution. BSA content was determined by using bicinchoninic acid kit for protein determination (Sigma-Aldrich, Saint Louis MO, USA). The reaction was run at room temperature for two hours. Optical density of the sample solution was read at 562 nm on a spectrophotometer (Jasco model V-530, Jasco, Tokyo, Japan). Protein loading and entrapment efficiency were then calculated. The experiment was performed in triplicate.

In vitro release test

Ten milligrams of microparticles were suspended in 1 ml of pH 7.4 phosphate buffered saline (PBS) and then shaken horizontally at 120 rpm and 37 °C. At predetermined time intervals, supernatant and microparticles were separated by centrifugation (Eppendorf model 5810, Eppendorf AG, Hamburg, Germany) at 7500 rpm for 10 minutes. BSA remained in the microparticles was recovered by dissolving microparticles in 0.5% acetic acid solution and assayed as described above. The experiment was carried out in triplicate.

Protein integrity

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

BSA was recovered by dissolving microparticles in 0.5% acetic acid solution. One part of the samples was mixed with one part of reducing sample buffer (5% β-mercaptoethanol in Novex® Tris-Glycine SDS sample buffer, Invitrogen, Calsbad CA, USA) and heated at 95 °C for one minute. The mixture equivalent to 7.5 μg of BSA was loaded onto a pH 8.8 12% Bis-Tris polyacrylamide gel and subjected to

electrophoresis (Bio-Rad model Mini-PROTEAN II, Bio-Rad Laboratories, Hercules CA, USA) in pH 8.3 Novex® Tris-Glycine SDS running buffer (Invitrogen, Calsbad CA, USA) at 80 V for about two hours. The gel was stained with SimplyBlue™ SafeStain (Invitrogen, Calsbad CA, USA) for one hour and destained several times with distilled water until the protein bands were visualized.

Circular dichroism (CD) BSA was recovered as described above. Sample solutions were diluted with ultrapure water to obtain about 50 μg/ml of BSA in solution. Ellipticity (θ, mdeg) of the solutions was recorded between 190-350 nm on a spectropolarimeter (Jasco model J-715, Jasco, Tokyo, Japan). Molar ellipticity ([θ], deg cm^2/decimol) was then calculated by the following equation

$$[\theta] = \theta \, Mp / 10\,000 \, n \, C' \, l \qquad (1)$$

where Mp was the molecular weight of BSA (66 430 Da), n was the number of amino acid residues of BSA (583 residues), C' was the concentration of BSA in sample solution (g/ml) and l was the path length of cell (0.5 cm). CD spectra were obtained by plotting molar ellipticity against wavelength. The solution of corresponding blank chitosan microparticles of each sample was prepared as described above and run as background.

Results

Characterization of chitosan microparticles

Chitosan microparticles were prepared by spray drying technique. Effects of formulation and process variables on particle size, morphology and yield percentage of chitosan microparticles were investigated. The results are shown in Table 3.1, Figures 3.1 and 3.2.

Spray drying of 1% w/v LCS solution at inlet air temperature and spray rate of 100 °C and 5 ml/min, respectively, resulted in smaller microparticles (3.910 μm, Table 3.1 and Figure 3.1B) than those prepared from 1% w/v HCS solution under the same condition (5.502 μm, Table 3.1 and Figure 3.2B). The higher the concentration

of chitosan, the larger were the microparticles, except those prepared at low temperature of 80 °C.

Table 3.1 Effects of formulation and process parameters on particle size and yield percentage of chitosan microparticles

Chitosan	Concentration (% w/v)	Temperature* (°C)	Spray rate (ml/min)	Particle size† (μm)	Yield (%)
LCS	1	80/37	5	6.640 (1.617)‡	34.01
	2	80/44	5	5.340 (0.925)	23.50
	1	100/56	5	3.910 (0.513)	48.38
	2	100/55	5	4.490 (0.476)	26.90
	1	120/65	5	3.760 (0.596)	60.29
	2	120/73	5	4.280 (0.566)	50.86
HCS	0.5	100/61	3	4.011 (0.314)	19.96
	1	100/59	3	7.096 (0.441)	29.99
	0.5	100/55	5	4.108 (0.691)	26.19
	1	100/54	5	5.502 (0.408)	37.28
	1	100/48	7	5.094 (1.980)	23.35

*Inlet air temperature/outlet air temperature

†Volume-weighted median diameter, $d(v, 0.5)$

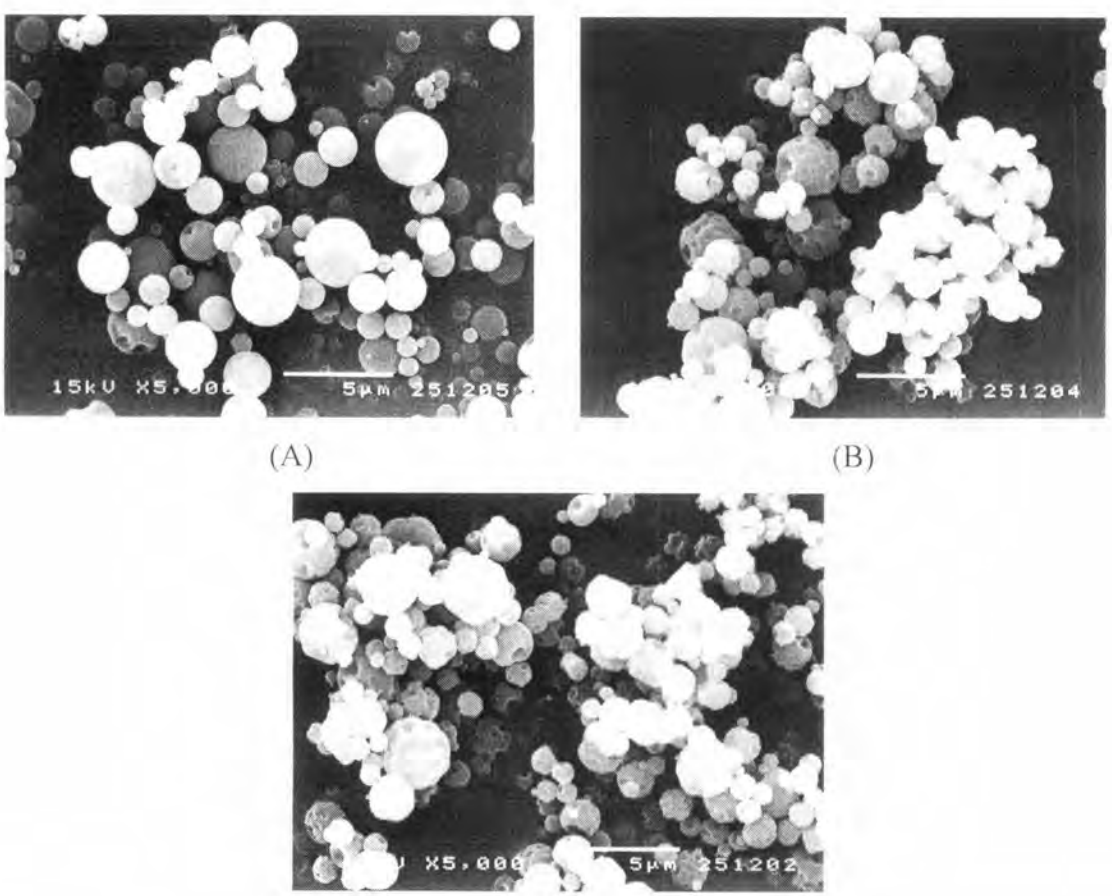
‡Absolute deviation from the median (Uniformity)

Taking into account the process variables, increasing the drying air temperature from 80 °C to 100 °C resulted in smaller size and size distribution of microparticles and/or apparent higher proportion of small particles (Figure 3.1A and 3.1B). However, further increasing the drying air temperature to 120 °C did not show much effect on the size and size distribution of microparticles (Table 3.1 and Figure 3.1C). It was clearly seen from Figure 3.1 that microparticles with more dented surface were obtained, as the inlet air temperature was increased. Although varying

spray rate did not obviously affect the average size of microparticles, it caused a broadened size distribution of microparticles, prepared at the spray rate of 7 ml/min. It was noted in Figure 3.2 that increasing the spray rate resulted in higher proportion of microparticles with less dented surface. The yield percentage was affected only by the inlet drying air temperature. As the drying air temperature was increased, the higher yield percentage was obtained.

Characterization of BSA-loaded chitosan microparticles

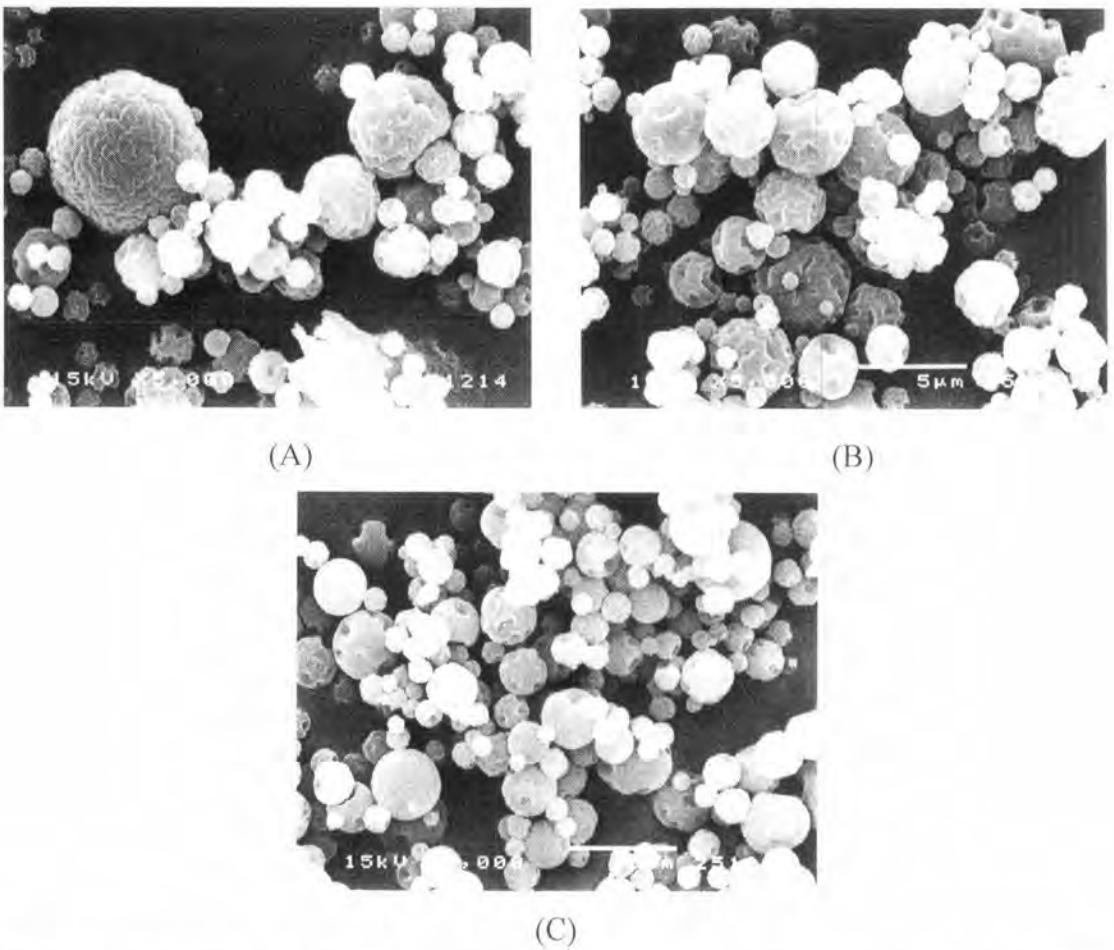
BSA was incorporated into chitosan microparticles at various loading levels. Physicochemical characteristics of the resultant microparticles are presented in Table 3.2.



(A) (B)

(C)

Figure 3.1 Scanning electron photomicrographs of LCS microparticles, obtained by spray drying at a spray rate of 5 ml/min and the inlet air temperature of (A) 80 °C, (B) 100 °C, and (C) 120 °C



(A) (B)

(C)

Figure 3.2 Scanning electron photomicrographs of HCS microparticles, obtained by spray drying at an inlet air temperature of 100 °C and the spray rate of (A) 3, (B) 5, and (C) 7 ml/min

BSA loading and entrapment efficiency

BSA was successfully encapsulated into chitosan microparticles with very high encapsulation efficiency of 86-103%, implying that most of BSA was incorporated into microparticles.

Particle size, zeta potential and morphology of microparticles

When BSA was incorporated into chitosan microparticles, larger average particle size was obtained, compared with those of corresponding blank chitosan microparticles in Table 3.1. This effect was independent of all formulation and process parameters investigated. Furthermore, BSA-loaded chitosan microparticles seemed to lose their sphericity in some degree and had rough along with deep dented

and/or distorted surface (Figure 3.3). The effect was more pronounced when BSA was loaded at increasing percentage from 1% to 5% and 10%, as illustrated in Figure 3.3A, 3.3B and 3.3C, respectively. In contrast to the blank chitosan microparticles, BSA-loaded chitosan microparticles prepared from both LCS and HCS were not much different, in terms of average particle size, size distribution, particle morphology, and particle topography (Figures 3.3A and 3.3D). Increasing the spray rate from 3 to 5 ml/min and/or decreasing the inlet drying air temperature from 120 °C to 100 °C led to more spherical and less dented-surface particles in some extent, as depicted in Figure 3.3C in comparison with those in Figures 3.3E and 3.3F, respectively.

Surface property of microparticles was also investigated by determining their zeta potential. All particles exposed positive surface charge (Table 3.2), due to amino functional groups on the structural backbone of chitosan. Incorporation of BSA into microparticles led to slightly decreasing in zeta potential of particles, independent of protein loading level. It was likely that BSA accumulated at particle surface with maximum effect, even at as low as 1% loading level.

In vitro release test

Chitosan microparticles of LCS and HCS at different protein loading levels were subjected to *in vitro* release test at 37 °C in pH 7.4 phosphate buffered saline. The results are illustrated as a plot of percentage of BSA remained in microparticles against time in Figure 3.4.

LCS microparticles of 10% BSA loading released protein at the fastest rate and reached the maximum release at about 60% within 24 hours. The release was slower when the protein loading was decreased to 5 and 1%, with maximum release within three and six days, respectively. In case of HCS microparticles, the maximum amount of BSA released (about 30%) was reached within the first day of the test and no further increase in release was observed throughout the time course of release study. It was noted that the LCS microparticles could release more amount of the encapsulated protein than the HCS microparticles.

Table 3.2 Effects of protein loading and process parameters on physicochemical properties of BSA-loaded chitosan microparticles, prepared by spray drying of 1% w/v chitosan solution

	BSA load (% w/w)	T* (°C)	Spray rate (ml/min)	Actual load (% w/w)	Entrapment efficiency (%)	Particle size‡ (μm)	Zeta potential (mV)	Yield (%)
LCS	0	100/56	5	-	-	3.910 (0.513)¶	19.15 (2.49)§	48.38
	10	100/63	3	9.61 (0.24)§	93.17	6.134 (0.563)	-	37.15
		100/54	5	9.03 (0.14)	88.22	7.124 (0.506)	15.75 (1.62)	25.68
		120/76	3	9.05 (0.38)	86.20	6.428 (0.668)	16.62 (1.62)	38.81
		120/69	5	9.08 (0.46)	88.91	7.021 (0.561)	16.46 (1.61)	40.22
	5	100/62	3	4.59 (0.34)	89.57	6.813 (0.587)	-	33.03
		120/76	3	4.98 (1.35)	97.91	8.681 (0.565)	17.08 (2.12)	23.26
	1	120/76	3	0.96 (0.20)	88.34	6.407 (0.382)	15.64 (1.60)	36.54
HCS	0	100/54	5	-	-	5.502 (0.408)	19.04 (2.14)	37.28
	1	120/75	3	1.08 (0.03)	103.44	6.790 (0.530)	15.12 (1.76)	55.80

*Inlet air temperature/outlet air temperature

‡Volume-weighted median diameter, $d(v, 0.5)$

¶Absolute deviation from the median (Uniformity), §Standard deviation

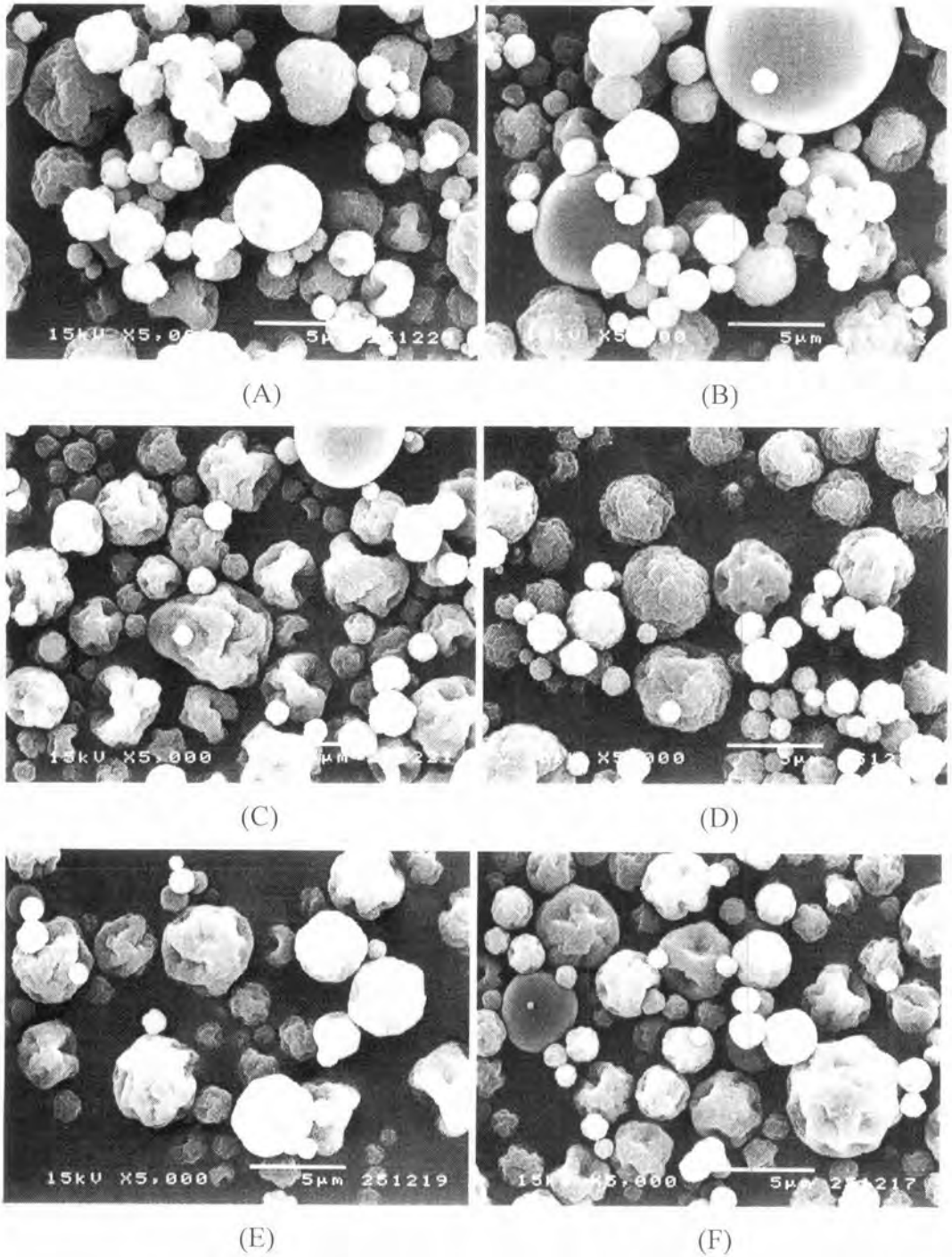


Figure 3.3 Scanning electron photomicrographs of BSA-loaded chitosan microparticles, obtained by spray drying of (A) 1%, (B) 5%, (C) 10% w/w BSA in 1% w/v LCS solution, (D) 1% w/w BSA in 1% w/v HCS solution at the spray rate of 3 ml/min and the inlet air temperature of 120 °C, and 10% w/w BSA in 1% w/v LCS solution at a spray rate of 5 ml/min and the inlet air temperature of (E) 120 °C, and (F) 100 °C, respectively

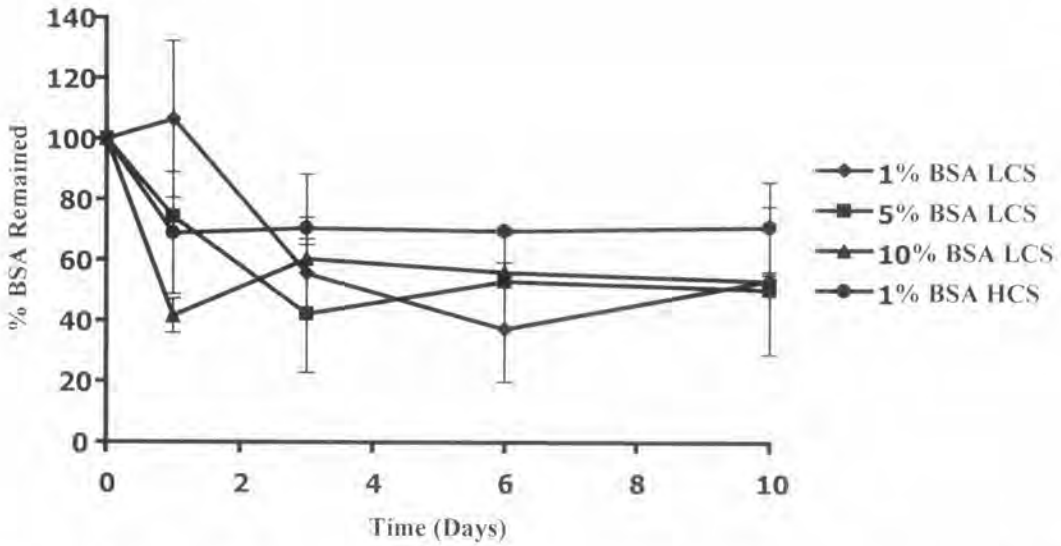
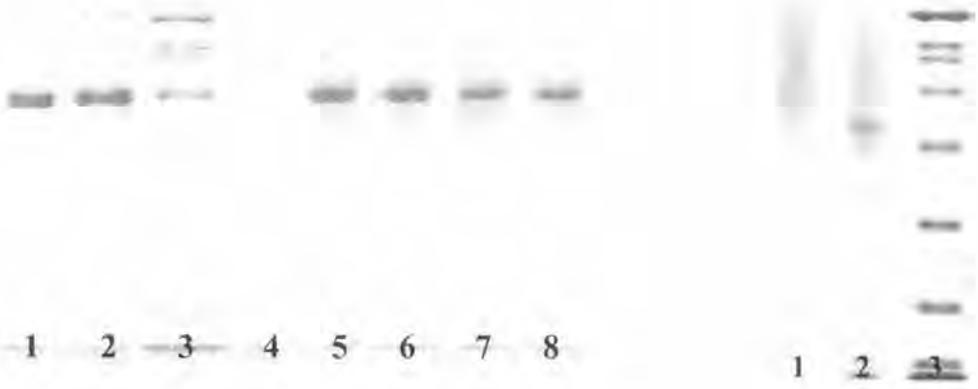


Figure 3.4 *In vitro* BSA released from different chitosan microparticles, as percentage of BSA remained in microparticles plotted against time

Integrity of encapsulated BSA

After preparation process, the integrity of encapsulated protein in chitosan microparticles, prepared at different protein loading levels and under different conditions, was confirmed by SDS-PAGE. Unprocessed BSA was also run as control. It was apparent that the spray drying process, especially at high inlet air temperature of 120 °C, did not deteriorate the integrity of BSA. As illustrated in Figure 3.5A, all bands of BSA, recovered from chitosan microparticles with 5% and 10% protein loading (Lanes 5-8), moved downwards the electrophoretic gel in a comparable distance to control (Lane 1). No band was observed in either higher or lower molecular weight region, suggesting that neither aggregate formation nor peptide backbone clipping occurred. It was, therefore, conceivable that the spray drying process conditions used in this study, even at the supposedly high thermal stress, did not deteriorate the integrity of encapsulated BSA.

In contrast, BSA-loaded at 1% into LCS microparticles obviously showed some degradation, since some bands of lower molecular weight protein than BSA were apparent (lane 2, Figure 3.5B), although BSA content and entrapment efficiency were determined to be as high as 0.96% and 88.34%, respectively (Table 3.2).



(A) (B)

Figure 3.5 SDS-PAGE of (A) Lanes 1 and 2: unprocessed BSA, Lane 3: molecular weight standards, broad range (Bio-Rad Laboratories, Hercules CA, USA), Lane 4: sample buffer, Lanes 5 and 6: BSA recovered from 10% w/w BSA-loaded LCS microparticles, prepared at a spray rate of 3 ml/min and the inlet air temperature of 120 °C and 100 °C, respectively, Lanes 7 and 8: BSA recovered from 5% w/w BSA-loaded LCS microparticles, prepared at a spray rate of 3 ml/min and the inlet air temperature of 120 °C and 100 °C, respectively, and (B) Lane 1: blank chitosan microparticles, Lane 2: BSA recovered from 1% w/w BSA-loaded LCS microparticles, prepared at the spray rate of 3 ml/min and the inlet air temperature of 120 °C, and Lane 3: molecular weight standards, broad range

Smear appeared on the lanes of BSA-loaded chitosan microparticles was believed to be the staining of chitosan (Lanes 5-8, Figure 3.5A and Lane 2, Figure 3.5B), since it was also found on the lane of blank chitosan microparticles (Lane 1, Figure 3.5B).

Because all biological phenomena involve process of molecular recognition, it is essential that the delivery systems are able to release protein content in its native structure. Therefore, the structural conformation of BSA encapsulated in chitosan microparticles was studied with CD. The CD spectra region of a single protein molecule can be observed within two regions, *i.e.* far ultraviolet (UV) (below 250 nm) and near-UV (250-300 nm) which correspond to secondary and tertiary structure of proteins, respectively (Kelly and Price, 1997; Sreerama and Woody, 2000). Unfortunately, concentration of BSA recovered from chitosan microparticles was too

low to reveal the CD spectra in the region of tertiary structure. Only investigation of secondary structure was then reported. BSA, which was recovered from chitosan microparticles at 5% and 10% protein loading level, yielded similar CD spectral shape to that of the unprocessed BSA (Figure 3.6A). All three spectra exhibit minima at *ca.* 208 and 222 nm, which were indicative of predominantly α-helical secondary structure (Pelton and McLean, 2000; Sreerama and Woody, 2000). It was noticed that the CD spectra of BSA recovered from 5% and 10% protein-loaded microparticles showed lower molar ellipticity in some degree than that of the unprocessed BSA. This might lead to the conclusion that encapsulation of BSA into chitosan microparticles by spray drying would have some detrimental effect on secondary structure of protein. In order to recover BSA incorporated in chitosan microparticles, it is inevitable that acidic solution had to be applied. Unprocessed BSA was then dissolved in 0.5% acetic acid solution and subsequently determined their secondary structure. It was obvious that dissolving protein in acidic solution resulted in decreased molar ellipticity (Figure 3.6B). Nevertheless, CD spectral shape was still maintained. This could help postulate that the lower molar ellipticity found in the CD spectra of BSA recovered from 5% and 10% protein-loaded microparticles resulted from dissolving the microparticles in acidic solution rather than the spray drying process.

In case of 1% BSA loading in both LCS and HCS microparticles, CD spectra of the recovered protein changed dramatically, in terms of both shape and molar ellipticity, from that of the unprocessed BSA. This indicated that α-helical secondary structure was lost in some extent, corresponding well with the degradation of protein found in SDS-PAGE.

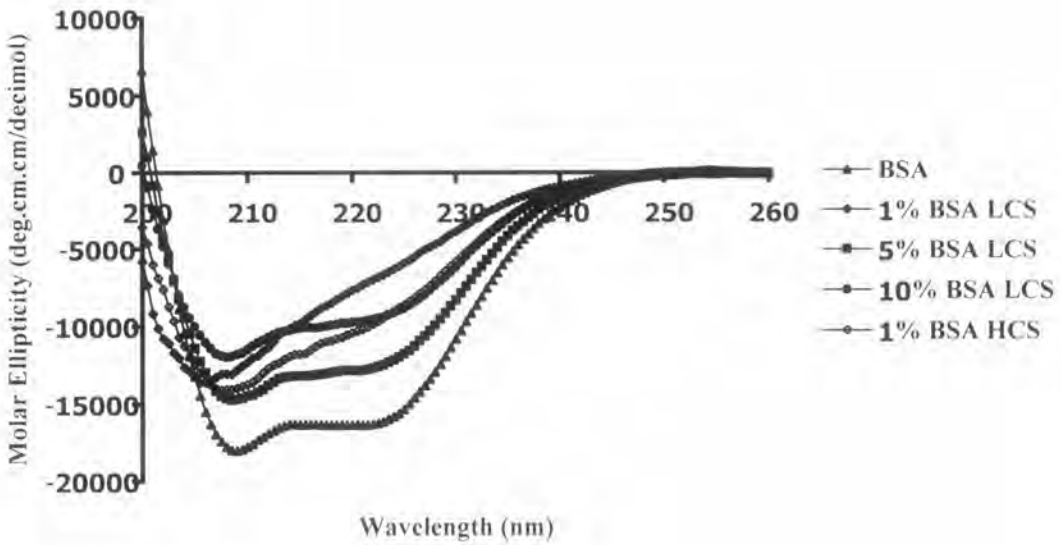
Discussion

It was evident in this study that the size of chitosan microparticles was mainly affected by composition and concentration of liquid feed. As all process parameters were kept constant, increasing the solid content and/or the viscosity of the formulations either by increasing the chitosan concentration or by the incorporation of higher molecular weight chitosan or other components, such as BSA, would result in

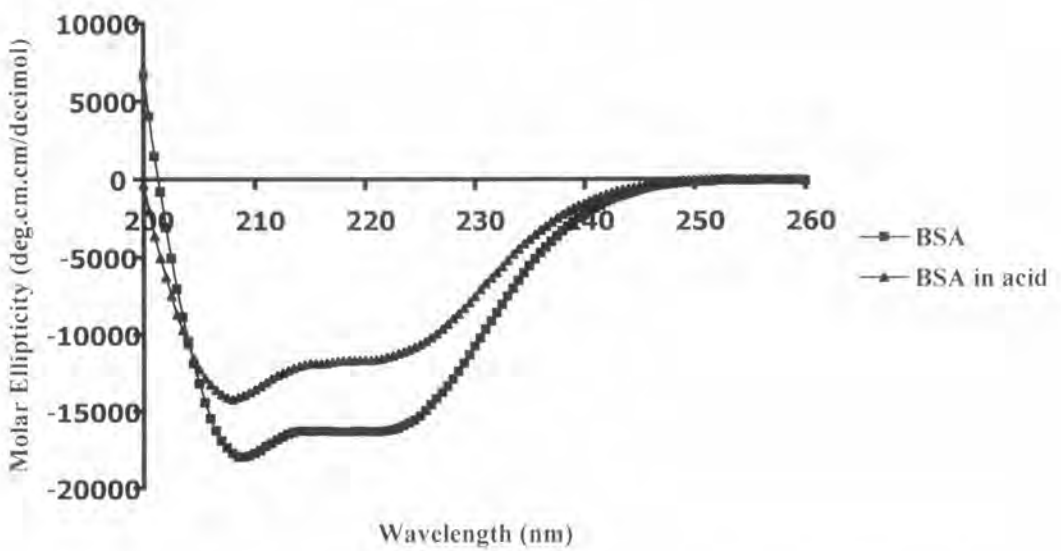
larger droplet size upon atomization. Thus, larger particles were obtained when dried (He *et al.*, 1999). Meanwhile, the particle size distribution was clearly influenced by the rate of droplet evaporation. At either the low inlet air temperature of 80 °C or the high spray rate of 7 ml/min, insufficient supply of heat energy and/or excessive volume of liquid to be evaporated would likely lead to an un-uniformed drying of droplets, resulting in the broadened size distribution of microparticles (Ståhl *et al.*, 2002).

Particle morphology and topography upon spray drying are determined by two factors, including rate of droplet drying and composition of liquid feed (Ameri and Maa, 2006). Since the temperature within drying chamber was reported to be close to the outlet air temperature, rather than the inlet air temperature (Lee, 2002), the rate of droplet drying, therefore, depended mainly on the outlet air temperature. Either increasing the inlet air temperature or decreasing the spray rate would result in the increase of outlet air temperature and hence the fast drying. As the atomized droplet dries, a hypothetical film is formed at the external surface. Such film could prevent the outward evaporation of liquid medium and subsequently cause the increase of vapor pressure inside the droplets. The film is prone to disrupt before complete drying could occur, resulting in deformed or defective particles as shown in Figures 3.1 and 3.2, especially in the fast drying process (Ameri and Maa, 2006).

The composition of the formulations to be spray dried had very strong influences on the observed particle morphology and topography, especially when a polymer and/or protein were included in the formulations. It was conceivable that chitosan also formed a film or crust upon droplet drying, hindering water inside the droplets to evaporate. Additionally, the intrinsic viscosity of chitosan solution inside the droplets possibly aggravated the outward diffusion of water. Thus, the film disruption was more pronounced, resulting in the microparticles with rougher surface.



(A)



(B)

Figure 3.6 CD spectra of (A) unprocessed BSA and BSA recovered from BSA-loaded chitosan microparticles, percent of which designates the BSA loading, and (B) unprocessed BSA dissolved in distilled water and 0.5% acetic acid solution

It is well known that proteins are surface-active molecules. Previous studies have shown that the surface of spray dried proteins/carbohydrates powders to a large extent was covered by proteins, even when small protein concentration as low as 0.1% was used (Adler and Lee, 1999; Fäldt and Bergenståhl, 1994; Landström *et al.*,

1999; Millqvist-Fureby *et al.*, 1999). Upon atomization, proteins are prone to accumulate at the droplet surface. Therefore, the nature of proteins appears to have a strong influence on the observed particle morphology. BSA tended to form particles with an undulate morphology (Ameri and Maa, 2006). The effect was more pronounced when loading of BSA in microparticles was increased. Surface accumulation of protein was also confirmed by the zeta potential measurement. At neutral pH, BSA (pI 4.7-4.9, Malamud and Drysdale, 1978; Righetti and Caravaggio, 1976) tended to expose negative charges on the surface of microparticles, resulting in a slight decrease in zeta potential, when compared with that of the corresponding blank chitosan microparticles.

Broadhead *et al.* (1994) reported that the product yield increased with increasing inlet air temperature. However, the collection efficiency was relatively low, *ca.* 20-60%, which was the common drawback of the bench-top spray dryer, due to unoptimized design of the system (Broadhead *et al.*, 1994; Labrude *et al.*, 1989; Maa *et al.*, 1998; Ståhl *et al.*, 2002). It was estimated that, depending on the process conditions, on average 15% and 24-77% of the spray dried material were deposited on the walls of the instrument and carried through the cyclone with the outgoing air, respectively (Ståhl *et al.*, 2002).

Spray drying is generally concerned as a harsh drying method, due to the application of high temperature, which could be detrimental to sensitive biological materials such as peptides and proteins. However, the drying droplets and the dried particles are usually maintained at a temperature of 15-20 °C lower than the outlet air temperature throughout the drying process, especially in the co-current flow drying process used in this investigation (Broadhead *et al.*, 1992). In addition, a cooling effect is achieved, due to the moisture uptake as water evaporates from the droplets (Lee, 2002). Furthermore, the droplet drying time and the total droplet residence time in the spray-dryer are generally in the order of milliseconds and seconds, respectively (Adler and Lee, 1999; Lee, 2002). Therefore, the thermal stress and hence the damage to proteins theoretically seem to be less likely. Nevertheless, it was reported that the thermal stress played a part in lactate dehydrogenase inactivation during spray drying (Adler and Lee, 1999).

There are two other possible sources of stress during spray drying, *i.e.* shear stress, due to atomization, and air-liquid interface (Ameri and Maa, 2006). Despite non-significant effect of high shear and shear rate on the aggregation of proteins, a slight conformational change and backbone clipping were found (Maa and Hsu, 1996). Large air-liquid interface, generated through atomization, could cause the adsorbed protein to unfold, expose hydrophobic regions and undergo aggregation by the interaction of the exposed hydrophobic regions with other unfolded molecules until precipitation occurs (Ameri and Maa, 2006). A direct correlation between the degree of aggregate formation of a protein and the total air-liquid interfacial area of spray droplets provided a strong support that surface denaturation at the air-liquid interface of spray droplets plays a major role in the degradation of protein during spray drying. (Maa and Hsu, 1997; Maa *et al.*, 1998; Mumenthaler *et al.*, 1994).

Additionally, protein denaturation also occurs during dehydration step (Griebenow and Klibanov, 1995; Prestrelski *et al.*, 1993; Tzannis and Prestrelski, 1999). Removal of hydration water molecules that are required to form hydrogen bonds with protein molecules can alter the native structural conformation of proteins and thus compromise the biological activity of proteins.

In spite of those potential stresses imposing on protein stability, it was conceivable in this investigation that spray-drying process was very efficient in entrapping stable protein within chitosan microparticles, according to the protein content. Nominal protein loading and actual protein loading were quite comparable (Table 3.2). Consequently, protein encapsulation efficiency was as high as 90-100%, implying stable incorporation of protein into microparticles. The release rate and the total release of protein were controlled with protein loading and molecular weight of chitosan, respectively, corresponding well with a previous study (Xu and Du, 2003).

It was apparent that percentage of protein loading in microparticles also determined the capability of protein in retaining its integrity after spray drying. In the case of chitosan microparticles with 5 and 10% of protein loading, no evidence of aggregate formation or backbone cleavage was observed on electrophoretic gel. In addition, the CD spectral shape was still maintained. Although, their molar ellipticity

was slightly changed, supposedly attributed to limitation in recovery of protein from microparticles. It was plausible that the secondary structure of protein was also retained. In contrast, some backbone cleavage and obvious change of CD spectra were found for microparticles with low protein loading at 1%. Due to a clear correlation between the degree of protein surface accumulation and the retained integrity of protein (Millqvist-Fureby *et al.*, 1999), the loss of protein integrity likely resulted from denaturation at the air-liquid interface. Based on the assumption that the same total interfacial area would result in about the same degree of denaturation, with an increasing protein concentration in the liquid feed, a decreasing fraction of protein would be present at the interface and hence smaller fraction of denatured protein. As a result, the total loss of protein integrity was found to be relatively high in case of low protein-loaded microparticles, while it was negligible in the other (Maa *et al.*, 1998; Millqvist-Fureby *et al.*, 1999).

Conclusion

Chitosan microparticles for delivery of protein could be prepared successfully by conventional spray drying technique. Particle size was mainly controlled by composition and concentration of spraying formulations, whereas particle morphology and topography were controlled by rate of droplet drying and composition of liquid feed. Protein incorporated into microparticles seemed to accumulate at particle surface, as indicated by zeta potential measurement. Capability of encapsulated protein in retaining its integrity after spray drying depended principally on protein loading level. Protein integrity was well maintained when BSA was loaded into microparticles at high loading level as 5 and 10%, while it was lost when protein was incorporated at low loading level as 1%. This investigation provided useful information supporting the potential of spray-dried chitosan microparticles to be utilized as protein delivery systems. Modification of the microparticles by incorporating some pharmaceutical excipients in order to achieve the delivery systems with desired properties is in progress.