Protein Spray-Freeze Drying. Effect of Atomization Conditions on Particle Size and Stability[1]

Henry R. Costantino,[2,4] Laleh Firouzabadian,[2] Ken Hogeland,[2] Chichih Wu,[2] Chris Beganski,[2] Karen G. Carrasquillo,[3] Melissa Córdova,[3] Kai Griebenow,[3] Stephen E. Zale,[2] and Mark A. Tracy[2]

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Purpose. To investigate the effect of atomization conditions on particle size and stability of spray-freeze dried protein.

Methods. Atomization variables were explored for excipient-free (no zinc added) and zinc-complexed bovine serum albumin (BSA). Particle size was measured by laser diffraction light scattering following sonication in organic solvent containing poly(lactide-*co*-glycolide) (PLG). Powder surface area was determined from the N_2 vapor sorption isotherm. Size-exclusion chromatography (SEC) was used to assess decrease in percent protein monomer. Fourier-transform infrared (FTIR) spectroscopy was employed to estimate protein secondary structure. PLG microspheres were made using a non-aqueous, cryogenic process and release of spray-freeze dried BSA was assessed *in vitro.*

Results. The most significant atomization parameter affecting particle size was the mass flow ratio (mass of atomization N_2 relative to that for liquid feed). Particle size was inversely related to specific surface area and the amount of protein aggregates formed. Zinc-complexation reduced the specific surface area and stabilized the protein against aggregation. FTIR data indicated perturbations in secondary structure upon spray-freeze drying for both excipient-free and zinc-complexed protein.

Conclusions. Upon sonication, spray-freeze dried protein powders exhibited friability, or susceptibility towards disintegration. For excipient-free protein, conditions where the mass flow ratio was > ~0.3 yielded sub-micron powders with relatively large specific surface areas. Reduced particle size was also linked to a decrease in the percentage of protein monomer upon drying. This effect was ameliorated by zinc-complexation, via a mechanism involving reduction in specific surface area of the powder rather than stabilization of secondary structure. Reduction of protein particle size was beneficial in reducing the initial release (burst) of the protein encapsulated in PLG microspheres.

KEY WORDS: particle size; PLG microspheres; protein delivery; spray-freeze drying; stability.

INTRODUCTION

For proteins and peptides that require frequent injections, a sustained-release form, e.g., biodegradable microspheres (1), provides an attractive alternative and may improve patient convenience, comfort and compliance. An example is Nutropin Depot™, a sustained-release form of recombinant human growth hormone (rhGH) in poly(lactide-*co*-glycolide) (PLG) microspheres. To produce this formulation, the protein is spray-freeze dried and subsequently encapsulated in PLG using a non-aqueous, low-temperature methodology which maintains protein integrity (2).

Spray-freeze drying is the process of atomizing a liquid to form droplets, freezing the droplets, and ice subliming at low temperature and pressure. The atomization step and the extremely rapid freezing of droplets impose stresses on proteins distinct from those provided by lyophilization. Although the effects of lyophilization on proteins have been discussed (3–5), there are few studies regarding protein spray-freeze drying (6).

MATERIALS AND METHODS

Chemicals

BSA (M_w~68 kDa, fraction V powder, approx. 99% purity, protease-free, essentially γ-globulin free) was purchased from Sigma Chemical Co. (St. Louis, MO) and was approximately 71% monomeric. PLG (Resomer RG502H, 50:50 lactide:glycolide nominal, M_w ~ 10 kDa) was supplied by BI Chemicals (Wallingford, CT).

Zinc-Complexation

BSA (25 mg/mL in 25 mM sodium bicarbonate, pH 7.2) was mixed with 73 mM zinc acetate to yield 50:1 zinc:protein (mol:mol) at a final concentration of 20 mg/mL protein. This resulted in a zinc-protein precipitate similar to rhGH at a lower ratio (2).

Spray-Freeze Drying

BSA solutions (excipient-free) or suspensions (zinc-complexed) (~ 250 mL) were atomized via a two-fluid nozzle in a stainless steel chamber (all nozzles and accessories were purchased from Spraying Systems Co. (Wheaton, IL)). At the chamber top, atomizing N_2 gas and liquid feed streams were pressure-driven to the nozzle assembly (model JBC). Liquid feed was drawn through the fluid cap (defined by its inner diameter) and external atomization was achieved by N_2 gas which exited the air cap (annulus defined by the air cap diameter and the fluid cap outer diameter). Different atomization conditions (n = 21) were used for both sample types. Three different air caps (model 64 for conditions 1, 4, 7, 10, 13, and 16; model 70 for conditions 2, 5, 8, 11, 14, 17, and 19; model 120 for other conditions) and fluid caps (model 1650 for conditions 3, 4, 8, 10, 14, and 18; model 2050 for conditions 1, 5, 9, 11, 15, and 16; model 2850 for other conditions) were used. Liquid droplets were frozen via liquid N_2, which was delivered by pressure (22–45 psi) into four single-fluid nozzles. Atomizing N_2 gas flow rate was measured using a compensated differential pressure flowmeter (Model 32915-72, Cole Parmer, Vernon Hills, IL). Liquid flow rate was calculated from volume and time. Frozen slurries were collected in stainless steel beakers, poured into glass dishes, and placed into a lyophilizer (model Durastop μp, FTS Systems Inc., Stone Ridge, NY) with shelves pre-cooled to –40 °C.

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[2] Alkermes, Inc., 64 Sidney Street, Cambridge, MA 02139.

[3] Department of Chemistry, University of Puerto Rico, Río Piedras Campus, P.O. Box 23346 San Juan, PR 00931.

[4] To whom correspondence should be addressed. (e-mail: rick.costantino@alkermes.com)

Drying was a single condition of 300 mTorr chamber pressure and 10 °C shelf temperature. The product temperature was initially at about –30°C, and increased up to the shelf temperature over the course of lyophilization.

Particle Sizing

Samples were processed in a manner relevant to their encapsulation in PLG microspheres (2). Powders were suspended (1.5% w/v) in methylene chloride containing 10% PLG and sonicated 4 min using a model VibraCell (Sonics and Materials, Danbury, CT) unit. Particle sizing was conducted using static light scattering (model LS130 with small volume module, Coulter Corporation, Miami, FL) in acetone. Particle size distribution was deconvolved using the software provided. Particle data are presented as diameter at 10% ($D_{v,10}$), 50% ($D_{v,50}$) and 90% ($D_{v,90}$) of the volume distribution.

Moisture Content Determination

Moisture content was determined by Karl Fischer titration (AquaStar C2000, EM Science, Cherry Hill, NJ). Powders (typically 5–10 mg) were directly added to the titrator cell. BSA was sparingly soluble in the methanol-based reagents used. The range in data for the residual moisture content of spray-freeze dried BSA samples likely reflects the environmental conditions during sample handling.

Scanning Electron Microscopy (SEM)

Samples were affixed with double-sided carbon tape to an aluminum stub and sputter coated with a layer of gold. SEM images were obtained using a JEOL model 6400 scanning electron microscope.

Size-Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) was conducted as previously described (2) using a G3000SW XL TSK Gel Column (TOSO HAAS, Japan). Data are reported as the decrease in percent monomer compared to the protein prior to spray-freeze drying. In cases where the monomer content was higher in the dried protein than the starting material, the decrease in percent monomer is reported as zero. Decrease in percent monomer was observed concomitantly with increased dimers and soluble aggregates.

Density and Surface Area Measurements

Samples were stored in glass vials over desiccant in a vacuum desiccator at room temperature prior to measurements. Skeletal density (also referred to as true or absolute density), defined as the density of the material excluding pores and interparticle spaces, was determined at room temperature by He pycnometery using a Quantachrome Micro-Ultrapycnometer 1000 (Boynton Beach, FL). Samples (40–60 mg) were run in triplicate.

A Quantachrome NOVA 2000 surface area analyzer (Boynton Beach, FL) was used to measure N_2 sorption at 77 K. The surface area per unit powder mass (specific surface area) was calculated from the fit of adsorption data (0.05–>0.3 relative pressure) to the Brunauer, Emmett, and Teller (BET) equation (7).

Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR studies were conducted with a Nicolet Magna-IR System 560 optical bench (8–11). A total of 256 scans at 2 cm^{-1} resolution using Happ-Ganzel apodization were averaged to obtain each spectrum. Spray freeze-dried samples were measured at least five times as KBr pellets (~1 mg protein per 200 mg KBr pressed at 5 kpsi pressure); these conditions were shown not to induce any artifactual alterations in secondary structure for rhGH (12). Correction of FTIR spectra, data analysis and assignments of secondary structural elements were described previously (9–11).

Preparation of PLG Microspheres and Determination of Initial *In Vitro* Release

For encapsulation (16% (w/w) target BSA load), particles were dispersed by 4 min sonication (model Vibra-Cell VC600 (Sonics and Materials, Danbury, CT)) in methylene chloride containing 10% (w/v) PLG. This suspension was processed into PLG microspheres using a non-aqueous, low-temperature technique as described previously (2,14). This suspension was atomized through an ultrasonication nozzle (Sonics and Materials probe model CV26 with atomizer model 630-0434) into a vessel containing frozen ethanol overlaid with liquid N_2. The vessel was transferred to a –80 °C freezer and incubated for 3 days to allow for microsphere hardening. The microspheres were then collected by vacuum filtration (filter type SV, 5-μm pore size, Millipore, Bedford, MA) and dried under vacuum in a lyophilizer. *In vitro* initial release was assessed by incubation of 10 mg microspheres in 1 mL of buffer (50 mM HEPES, 85 mM KCl, 0.01% sodium azide, pH 7.2) at 37 °C for 18 h. Sample aliquots were analyzed for amount of protein using the BioRad assay (BioRad Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Protein Spray-Freeze Drying

Various atomization conditions (Table I) were used to spray-freeze dry bovine serum albumin (BSA) as a model protein. Atomizing N_2 gas and liquid feed flow rates were important variables likely to influence droplet size and freezing rate (discussed below). At each condition, both excipient-free and zinc-complexed BSA were atomized, frozen in liquid N_2, and lyophilized. We decided to examine zinc complexation since this formulation strategy was successfully employed for rhGH microencapsulation (2,13). The resulting spray-freeze dried powders were very fluffy, exhibiting a tap density ranging from about 0.01 to 0.02 g/cc and a skeletal density of about 1 g/cc. Such a low tap density indicates that the BSA particles were highly porous, similar to other spray-freeze dried therapeutic proteins (6). The residual moisture content for spray-freeze dried BSA ranged from about 2–7% (w/w).

Effect of Atomization Variables on Spray-Freeze Dried Protein Particle Size

Particle size impacts the amount of encapsulated drug with accessibility to the microsphere surface, and hence avail-

Table I. Atomization Conditions Used to Produce Spray-Freeze Dried BSA

Atomization condition	Atomization N_2 pressure (psi)	Atomization N_2 flow[a](L/min)	Liquid feed pressure (psi)	Liquid feed flow (L/min)	Mass flow ratio[b]
1	20	20	3	33	0.76
2	40	38	30	568	0.084
3	2	20	120	198	0.13
4	120	70	30	129	0.68
5	82	100	120	498	0.25
6	14–18	100	3	131	0.95
7	102	74	102–120	857	0.11
8	120–130	130	3	58	2.8
9	26–28	140	30	217	0.81
10	12–18	20	110	244	0.10
11	6	20	5	[c]	[c]
12	2	20	30	250	0.10
13	118–120	93	3	145	0.80
14	32	100	30	[c]	[c]
15	12	100	118–120	426	0.29
16	120	80	30	138	0.72
17	112	127	120	834	0.19
18	28	140	3–6	100	1.7
19	92	120	3	222	0.68
20	84–88	331	3	116	3.6
21	84	[d]	3	143	[d]

[a] Atomization N_2 flow was measured for atomization of zinc-complexed BSA only. The output from the flowmeter was given for standard conditions.
[b] Calculated from equation (1).
[c] Zinc-complexed protein did not spray due to clogging of fluid cap.
[d] Not determined.

ability for release immediately upon hydration (14,15). Therefore, it is desirable to minimize particle size during encapsulation in order to minimize the fraction of drug released initially. Herein, protein particles were dispersed in an organic solvent containing 10% (w/v) PLG and sonicated immediately prior to encapsulation. The size of the drug particles and stability of the dispersion at this point in the process are directly relevant for microsphere performance. It was our aim to use various atomization conditions to spray-freeze dry BSA and to evaluate how the particles would break up upon sonication and remain dispersed in the polymer solution, conditions suitable for producing microspheres for sustained release.

An example of a time course for sonicating spray-freeze dried BSA powder is presented in Fig. 1A. The data are shown for excipient-free protein atomized using condition 8. Without sonication (t = 0), this powder exhibits a relatively monodisperse particle size distribution with a $D_{v,50}$ of about 14 μm (Fig. 1B). After sonication, the particle size distribution is markedly lower and indicates a multi-modal distribution, with a $D_{v,50}$ of less than 0.2 μm (Fig. 1C). Therefore, the particle size of interest herein is not the geometric size of the dried droplets, but rather reflects the powder friability, or susceptibility to disintegration upon sonication.

The presence of PLG in the methylene chloride resulted in a slightly more efficient particle size reduction upon sonication compared to methylene chloride alone. A possible explanation for this trend is that the presence of polymer served to increase the viscosity and allowed the fluid to generate greater viscous force to the suspended particles. To further test this, we also sonicated the particles in another PLG-dissolving solvent, namely dimethylformamide. Dimethylformamide has a viscosity which is markedly higher (0.802 cP at 20 °C) than that of methylene chloride (0.449 cP at 20 °C) (16). Indeed, the time course data (Fig. 1A) show that the more viscous medium demonstrated a more efficient particle size reduction.

Data for the particle size distribution for all spray-freeze dried BSA samples are presented in Table II. The $D_{v,10}$, $D_{v,50}$ and $D_{v,90}$ indicate the measured particle size at 10%, 50% (or the median) and 90% of the volume distribution, respectively, for the powder sonicated in methylene chloride/10% PLG. Generally, the same atomization conditions yielded a similar sonicated particle size for the excipient-free and zinc-complexed protein. Values for the median volume diameter ($D_{v,50}$) upon sonication spanned a wide range; several atomization conditions yielded sub-micron particles and the largest size achieved was about 10 μm. When atomization conditions were replicated, good reproducibility in terms of particle characteristics was observed. For instance, the average and standard error of four excipient-free samples produced using atomization condition 19 yielded a volume median particle size of 0.25±0.05 μm and a protein monomer loss of 9.4±0.7%.

To explore the effect of atomization conditions in producing such a broad span of particle sizes, we considered the effect of the atomization mass flow ratio. The mass flow ratio is defined as the ratio of atomization gas to liquid feed:

$$\frac{Q_{atom.N_2}}{Q_{liquid}} = \frac{M_{atom.N_2} \cdot \rho_{atom.N_2}}{M_{liquid} \cdot \rho_{liquid}} \quad (1)$$

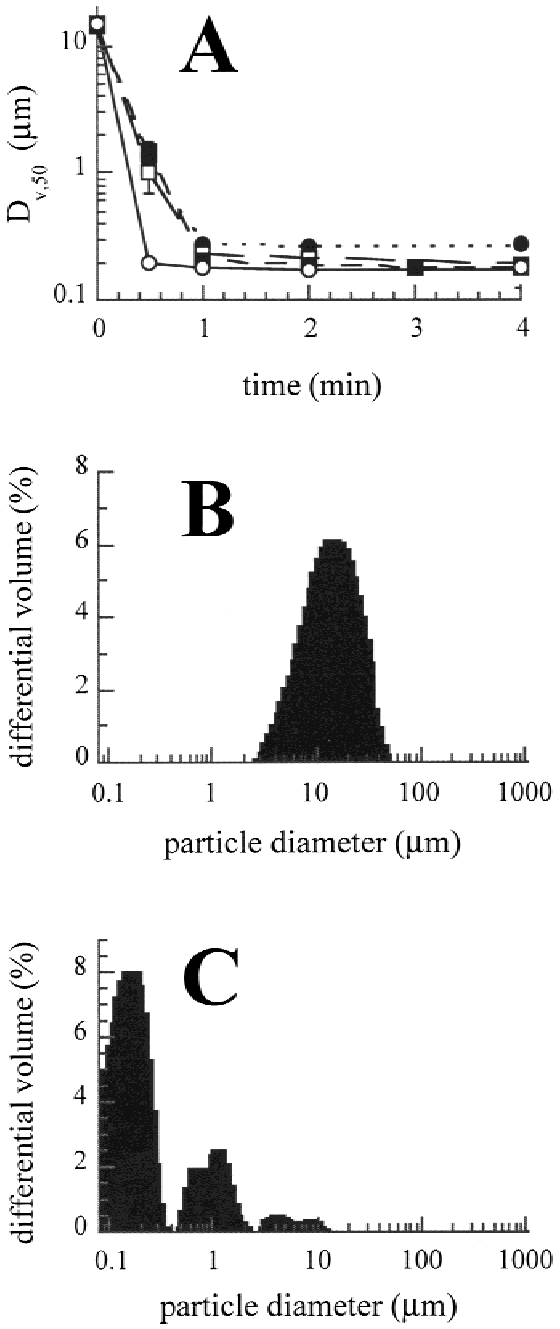

Fig. 1. Particle size ($D_{v,50}$) of sonicated spray-freeze dried BSA (excipient-free protein atomized using condition 8). (A). Time course for powder suspended in (○) dimethylformamide or methylene chloride containing 0% (●) , 5% (□), and 10% (■) PLG (w/v). Representative volume distributions are shown for the sample suspended in methylene chloride/10% PLG both before (B) and after (C) 4 min of sonication.

where Q is the mass flowrate, M is the volumetric flowrate and ρ is density and the subscripts *atom.* N_2 and *liquid* refer to the atomization N_2 gas and liquid feed streams, respectively. It was observed that the mass flow ratio was a reasonable predictor of the particle size of the sonicated spray-freeze dried powder (Fig. 2A). The most friable powders, e.g., $D_{v,50}$ <1 μm, were produced using atomization conditions that resulted in mass flow ratios of above about 0.3. It has been described for aqueous protein formulations that the droplet size produced by a two-fluid atomizer decreases as the ratio of atomization gas to liquid feed increases (17).

An empirical statistical model (JMP, SAS Institute, Cary, NC) was used to analyze the effect of the various operational inputs on the particle size. The model indicated that particle size decreased with decreased liquid feed flow rate (or liquid feed pressure) and fluid cap inner diameter, and decreased with increasing atomization N_2 flow rate (or atomization N_2 pressure). These conditions are apt to decrease the size of atomized droplets. The model also suggested that the other operational variables tested, namely air cap diameter, zinc complexation, and liquid N_2 pressure, did not have a significant impact on spray-freeze dried particle size. Overall, the information provided by the empirical statistical model indicates that atomization conditions which produce smaller droplets also result in a smaller diameter for the dried particle upon sonication.

Next, we endeavored to rationalize the influence of mass flow ratio on the sonicated particle diameter. We hypothesize that as droplet size decreases the freezing rate should increase. Increasing freezing rate, in turn, tends to decrease the size of ice crystals, as discussed elsewhere in the protein lyophilization literature (18–21). For instance, it has been reported that rapid freezing can promote supercooling which produces small ice crystals and a smaller pore size in the layer of dried protein (18). In this case, atomization conditions that produce smaller droplets would result in more rapid freezing leading to formation of smaller ice crystals, a finer microstructure following sublimation of ice, and thus a more friable spray-freeze-dried powder.

To test this hypothesis, we started by examining the median particle size of the suspended powder both before and after the sonication step (Fig. 2B). The particle size for the unsonicated powder should reflect the size of the frozen droplet. The particle size upon sonication, or rather the particle size reduction upon sonication, reflects the friability and microstructure of the powder. In every case, the data show a substantial reduction in the median particle size after sonication. The data for $D_{v,10}$ and $D_{v,90}$ reveal a similar reduction (data not shown). Moreover, the data indicate that the extent of particle size reduction increased with increasing mass flow ratio (Fig. 2C).

Therefore, increasing the mass flow ratio resulted in decreased droplet size and an increased friability of the dried powder. The former result is consistent with current atomization theory (22). The latter finding, to our knowledge, has not been previously reported for a spray-freeze dried powder.

To further probe our hypothesis regarding the effect of mass flow ratio on ice crystal size and morphology of the dried powder, we examined powder SEMs (Fig. 3). For atomization conditions yielding sub-micron ($D_{v,50}$~0.2 μm) particle diameters (condition 8), the SEM for both excipient-free (Fig. 3A) and zinc-complexed (Fig. 3D) protein powder revealed a very fine, porous morphology. Somewhat less fine structures were observed for powders made using atomization condition 10 (SEMs shown in Fig. 3B and 3E) which yielded larger particles ($D_{v,50}$ ~ 4 μm). In addition, a noticeably different, thicker microstructure was seen for condition 12 (Fig. 3C and 3F) where the largest particle diameter of about 10 μm was achieved. Thus, the most friable spray-

Table II. Particle Size Distribution of Spray-Freeze Dried BSA[a]

Atomizing condition	Excipient-free			Zinc-complexed		
	Sonicated particle size (μm)			Sonicated particle size (μm)		
	$D_{v,10}$	$D_{v,50}$	$D_{v,90}$	$D_{v,10}$	$D_{v,50}$	$D_{v,90}$
1	0.14	1.39	6.83	0.16 ± 0.02	0.70 ± 0.29	2.2 ± 0.4
2	0.24 ± 0.02	4.29 ± 0.14	12.7 ± 0.4	1.5 ± 0.1	6.47 ± 0.14	16.9 ± 0.5
3	0.41 ± 0.02	5.47 ± 0.58	15.1 ± 1.6	1.3 ± 0.2	4.60 ± 0.39	12.2 ± 1.0
4	0.11 ± 0.01	0.19 ± 0.01	1.4 ± 0.1	0.13 ± 0.01	0.24 ± 0.05	1.3 ± 0.2
5	0.16 ± 0.01	2.27 ± 0.06	8.4 ± 0.1	0.25 ± 0.02	3.64 ± 0.75	12.6 ± 0.6
6	0.13 ± 0.02	0.27 ± 0.06	4.1 ± 1.0	0.16 ± 0.01	0.80 ± 0.47	2.6 ± 0.2
7	0.32 ± 0.03	4.81 ± 0.65	13.1 ± 2.5	1.7 ± 0.3	7.00 ± 0.59	17.7 ± 0.4
8	0.11 ± 0.01	0.18 ± 0.01	1.1 ± 0.1	0.13 ± 0.02	0.23 ± 0.04	1.03 ± 0.06
9	0.13 ± 0.01	0.25 ± 0.01	3.6 ± 0.1	0.16 ± 0.01	0.62 ± 0.08	3.7 ± 0.4
10	0.26 ± 0.02	4.14 ± 0.02	11.5 ± 0.3	0.62 ± 0.39	3.60 ± 0.20	10.3 ± 1.1
11	0.14 ± 0.01	0.68 ± 0.38	4.9 ± 0.7	[b]	[b]	[b]
12	2.8 ± 0.5	9.21 ± 1.33	23.4 ± 3.3	3.7 ± 0.3	12.6 ± 0.2	31.6 ± 2.1
13	0.13 ± 0.01	0.28 ± 0.03	4.5 ± 0.7	0.14 ± 0.01	0.28 ± 0.02	1.9 ± 0.6
14	0.13 ± 0.08	0.32 ± 0.08	4.8 ± 1.2	[b]	[b]	[b]
15	0.23 ± 0.02	3.90 ± 0.47	11.9 ± 1.3	0.27 ± 0.02	2.8 ± 0.1	8.5 ± 0.4
16	0.14 ± 0.01	1.37 ± 0.04	6.4 ± 0.1	0.14 ± 0.01	0.25 ± 0.03	1.22 ± 0.08
17	0.18	2.87	9.13	0.26	3.68	9.77
18	0.12 ± 0.01	0.20 ± 0.01	2.3 ± 0.7	0.13 ± 0.01	0.25 ± 0.05	1.4 ± 0.2
19	0.13 ± 0.01	0.31 ± 0.01	5.9 ± 1.9	0.13 ± 0.01	0.26 ± 0.05	1.2 ± 0.2
20	0.12 ± 0.01	0.21 ± 0.02	2.0 ± 0.5	0.15 ± 0.01	0.34 ± 0.14	1.4 ± 0.1
21	0.12 ± 0.01	0.21 ± 0.03	1.9 ± 0.9	0.14 ± 0.02	0.22 ± 0.14	1.0 ± 0.3

[a] Particle size distribution was determined by light scattering of the powder sonicated in methylene chloride containing PLG. Particle size data are given as average and standard error for at least two determinations.
[b] Sample was not available due to clogging of nozzle during atomization.

freeze dried powders were those that had the finest microstructures.

In order to quantitatively describe the relationship between powder morphology and sonicated particle size, we measured the specific surface area of selected samples spanning a wide range of particle diameters. The data reveal an inverse relationship between sonicated particle size and specific surface area (Fig. 4A). Therefore, the most friable spray-freeze-dried samples were those that had with the highest specific surface areas. Such extremely high specific surface areas for spray-freeze dried protein powders has been previously reported by Maa *et al.* (6) and are consistent with an extremely fast freezing rate compared to the freezing rate achieved during typical lyophilization. It was interesting in the present investigation that zinc complexation markedly lowered the specific surface area in the spray-freeze-dried powder concomitant with improved protein stability as discussed below.

Stability of Spray-Freeze-Dried Protein

The observations regarding the surface area have implications for the stability of spray-freeze-dried protein. It has been reported that proteins are susceptible to denaturation at the ice-water interface and that the rate of denaturation increases with freezing rate and interfacial surface area (19,23–25). For example, Hsu *et al.* (23) have shown that for recombinant human tissue-type plasminogen activator, a faster freezing rate results in increased turbidity in the freeze-dried protein upon storage. Furthermore, it has been shown that upon lyophilization BSA is susceptible to drying-induced structural alteration (26) and solid-state aggregation which may impact its release when encapsulated in sustained-release dosage forms (27).

For these reasons, it was of interest to examine the aggregation of spray-freeze dried BSA and the correlation, if any, with particle size of the sonicated powder and its specific surface area. For the excipient-free protein, there were significant decreases in percent protein monomer, up to about 17% for condition 8 (the data for decrease in percent protein monomer are presented in Table III).). It was found that the decreases in percent monomer increased with decreasing particle size (filled symbols in Fig. 4B). Moreover, a marked correlation was observed between specific surface area and decrease in percent protein monomer for all data (filled symbols in Fig. 4C). The data strongly suggest that the surface area plays a role in aggregate formation in spray-freeze dried BSA. It has been hypothesized that the ice interfacial area can denature proteins which may lead to deterioration (3,23–25).

Complexation with zinc stabilized the protein upon spray-freeze drying (Table III). Similar to the case for excipient-free BSA, there was a slightly larger decrease in percent monomer for zinc-complexed protein spray-freeze dried using conditions resulting in lower sonicated particle diameters (open symbols in Fig. 4B). However, the extent of damage was far lower compared to the excipient-free protein (and in numerous cases essentially complete stabilization was achieved). For instance, at condition 21 there was 16.5% decrease in percent protein monomer for the excipient-free pro-

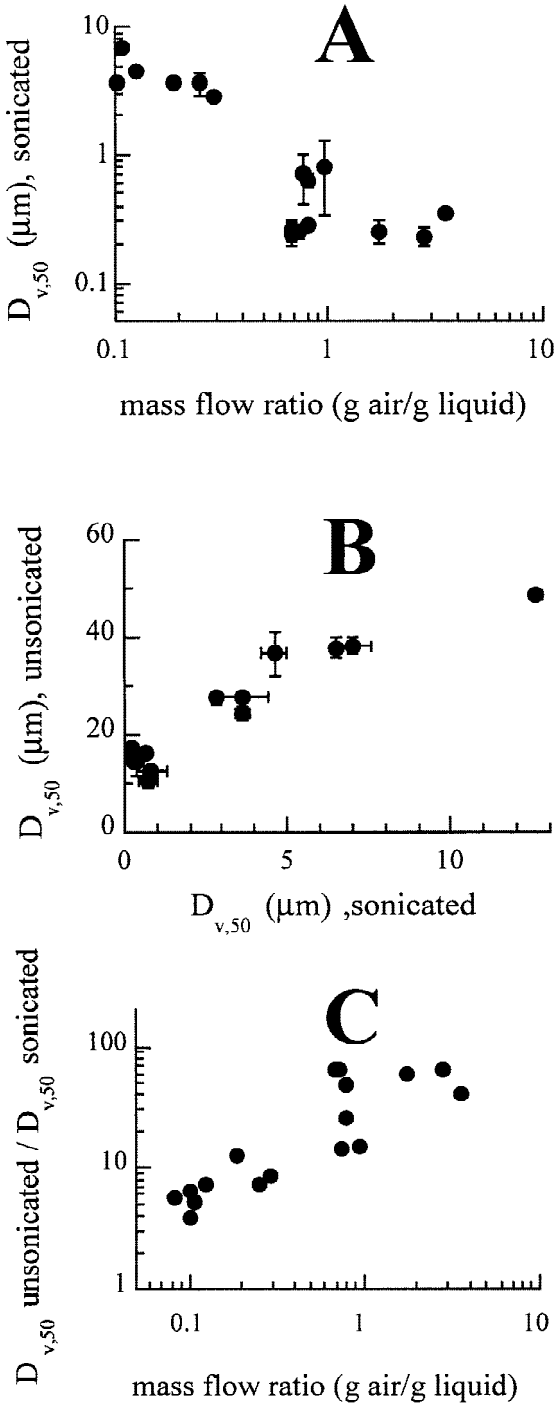

Fig. 2. Effect of mass flow ratio on friability of spray-freeze dried protein. (A) Effect of mass flow ratio on sonicated particle size. (B) Correlation between sonicated and unsonicated particle size. (C) Effect of mass flow ratio on reduction in particle size (expressed as the ratio of $D_{v,50}$ before to that after sonication). Data shown for zinc-complexed protein (atomization conditions in Table I, $D_{v,50}$ data from Table II).

tein compared to 2.7% for its zinc-complexed counterpart, even though both powders had a $D_{v,50}$ of approximately 0.2 μm.

Interestingly, there were markedly lower specific surface areas for the zinc-complexed protein samples (compare the open and closed symbols in Fig. 4A). Apparently, complexation with zinc resulted in significant reduction in specific surface area concomitant with protection against aggregation while not significantly impacting powder friability. It should be noted that complexation of BSA with zinc resulted in a suspension. Thus, instead of the protein in solution becoming progressively more concentrated upon ice crystal formation (with an intimate contact between the ice crystal surface and aqueous protein molecules), the zinc-complexed protein was already removed from the bulk water phase and thus potentially protected against any damaging influence of the ice interface.

Additional evidence for the deleterious role of interfacial area was provided by examination of the protein's stability in the presence of a surfactant. BSA was spray-freeze dried (using atomization conditions 19) in the presence of 0.005%, 0.01% and 0.05% Tween-20. At the lowest concentration tested (0.005%), below the critical micelle concentration (CMC) (28), the monomer loss upon spray-freeze drying was 6.6±0.5% compared to 10% loss for the same atomization conditions in the absence of the surfactant. In the presence of 0.01% Tween-20 (somewhat above CMC) the monomer loss was further reduced to 5.1±0.5%, similar to the level of protection afforded at 0.05% Tween-20. The improvement in stability afforded by addition of Tween-20 and the observation that the maximal effect was observed at or above the CMC are consistent with the deleterious role of interfacial area in BSA degradation.

Examination of Spray-Freeze Dried Protein Secondary Structure

The data implicate surface area as a significant factor in aggregation of spray-freeze dried BSA (Fig. 4C). Therefore, we also examined the solid-state protein structure to determine if there was a link between surface area and procedure-induced conformational changes in BSA. Fourier-transform infrared (FTIR) spectroscopy permits the non-invasive determination of protein secondary structure for proteins both in solution and in the amorphous dehydrated state (9–12). Previous FTIR analysis of BSA has shown that upon freeze-drying the α-helix content dropped from 54±6 % for the aqueous, native protein to 31±1 % in the lyophilized powder along with an increase in β-sheet structure (26). Similar results were observed herein for spray-freeze drying of BSA (Table III). (The spectra obtained were strikingly similar to those of freeze-dried BSA shown previously (10).) The overall α-helix and β-sheet contents were 29±5 % and 19±4 %, respectively, for the excipient-free spray-freeze dried powder (n = 18). For the powder that was spray-freeze dried following complexation with zinc (n = 18), the overall α-helix content was 32±5 % and the β-sheet content 16±3%, and thus the structural data were not statistically different from that for the excipient-free protein. No correlation was observed between secondary structure (percentages of α-helix and β-sheet) in the powders and either specific surface area or protein monomer loss. This observation is similar to that reported for freeze-dried recombinant human albumin exposed to moisture, namely, that there was no correlation between secondary structure determined by FTIR spectroscopy and

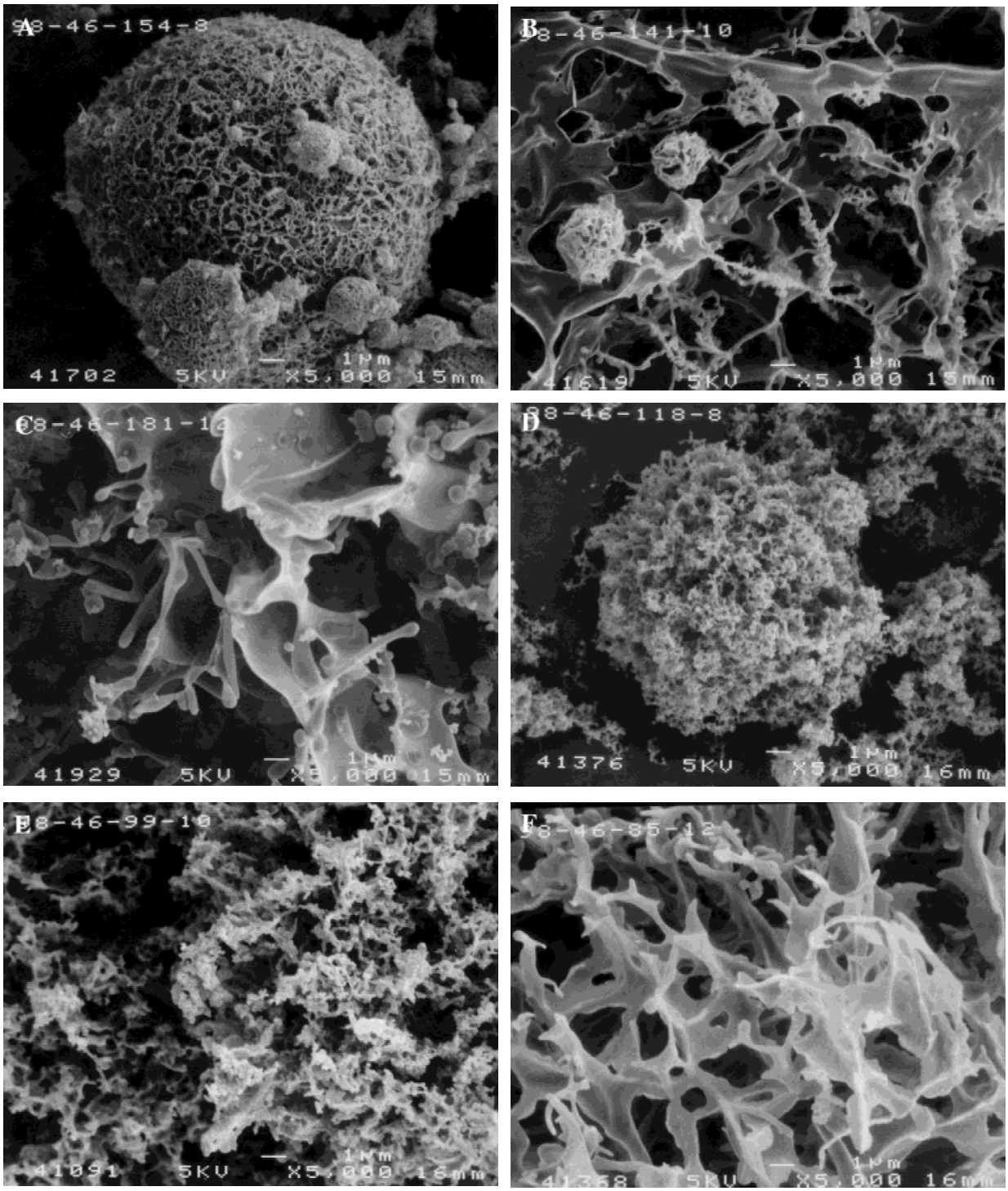

Fig. 3. SEM of spray-freeze dried BSA. Excipient-free protein spray-freeze dried using atomization conditions 8 (A), 10 (B), and 12 (C). Zinc-complexed protein spray-freeze dried using atomization conditions 8 (D), 10 (E), and 12 (F).

aggregation via thiol-disulfide interchange (29). In this case, either the mechanism of aggregation is not dependent on overall secondary structure, or, if there is a structural change leading towards instability, it is either transitory or not detectable by FTIR spectroscopy. An example of the latter would be formation of the molten globule state, which retains native secondary structure, but has non-native tertiary structure (30).

Effect of Spray-Freeze-Thaw and Annealing

Next, the relative impact of the different stress events, namely atomization, extremely rapid freezing, and drying, were explored. Such events can cause deterioration of proteins. For instance, recombinant human growth hormone may form aggregates upon atomization, and the extent of aggregation was shown to directly correlate with the surface area of

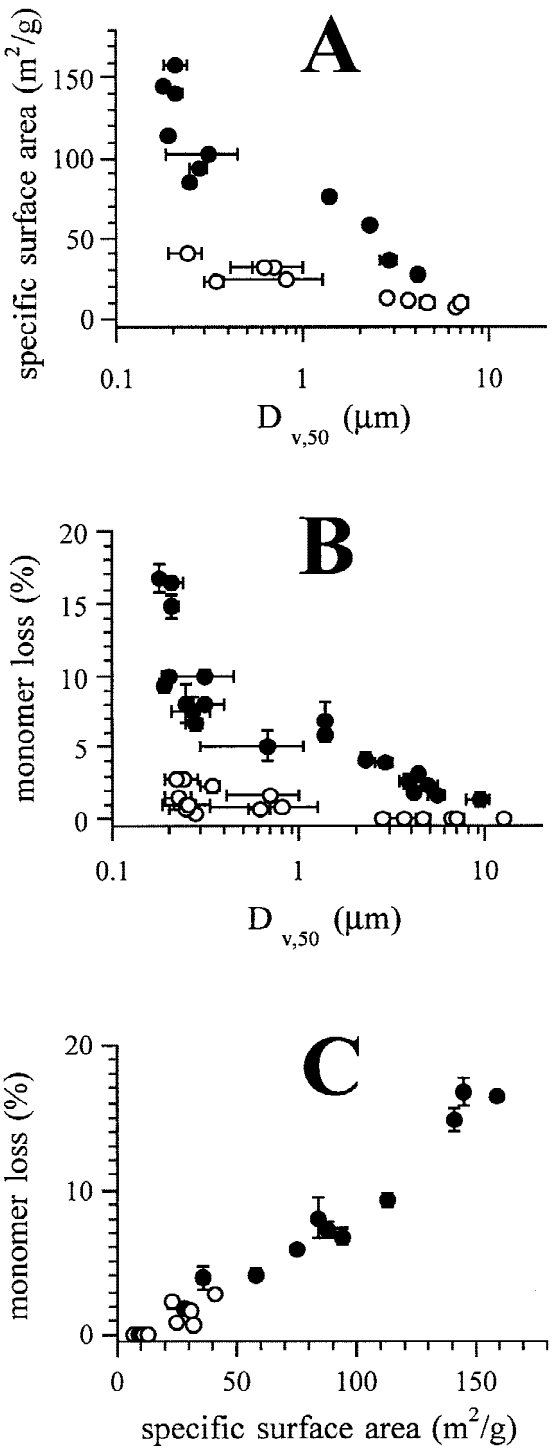

Fig. 4. Effect of surface area on spray-freeze dried protein stability and particle size. Correlation between $D_{v,50}$ and specific surface area (A), $D_{v,50}$ and decrease in percent protein monomer (B), and specific surface area and decrease in percent protein monomer (C) for spray-freeze dried BSA. The closed and open symbols are data for excipient-free and zinc-complexed protein, respectively.

the droplet (air-liquid interface) (17). In addition, it has been shown separately that freezing and drying can cause damage to enzymes such as phosphofructokinase and lactate dehydrogenase (5).

In the current investigation, excipient-free BSA was atomized using condition 19, and freeze-thaw and annealing studies were performed (Fig. 5). In the freeze-thaw experiment, the protein was spray-frozen and then thawed at two different conditions: (*i*) slower thawing by placing the container of frozen slurry at room temperature, and (*ii*) a more rapid thawing by immersing the container in a 37 °C water bath. After thawing using either regimen, we observed no change in the percent BSA monomer. In contrast, the spray-freeze-dried protein exhibited a substantial decrease in percent monomer (10% for the excipient-free protein using atomization condition 19, Table III). Therefore, the aggregation occurred neither during atomization nor during extremely rapid freezing, but rather upon drying (sublimation of water). This suggests that the ice formed during the rapid freezing step was not an amenable matrix from which to dry a protein.

Relaxation in the frozen state may alter the ice structure and make it more amenable to protein lyophilization. To examine this, spray-frozen samples were annealed in the frozen state (to allow for ice crystal growth) for 2 h at –20 °C, –10 °C and –5 °C prior to initiating the drying step. It is important to note that all annealing temperatures tested were well above the product temperature during primary drying, which was measured to be about –30 °C. As depicted in Fig. 5, there was no effect upon annealing at –20 °C or –10 °C. However, after annealing at –5 °C the decrease in percent protein monomer and specific surface area were significantly lower and the particle size was markedly higher, both consistent with promotion of ice crystal growth.

Concluding Remarks

Finally, we wanted to establish whether controlling the particle size (via selection of atomization conditions) was useful in modulating the release of the encapsulated protein. It was expected that the sample with the largest encapsulated particle size would also have the highest initial release, and that this initial release would decrease with decreasing encapsulated particle size (14,15). Thus, we microencapsulated the zinc-complexed spray-freeze dried BSA sample with the largest sonicated particle diameter achieved, ($D_{v,50}$ ~ 12 μm) and compared its *in vitro* initial release to microspheres comprised of intermediate- (3.6 μm) and sub-micron-sized (0.2 μm) particles. There was about a 30% reduction in the *in vitro* initial release for microspheres comprised of intermediate-sized particles and an even greater reduction of 60% for the case where submicron particles were microencapsulated. The profound influence of particle size on microsphere performance underscores the importance of understanding the impact of atomization variables on the characteristics of spray-freeze dried proteins.

In summary, the effect of atomization variables on the particle size and stability of spray-freeze dried BSA has been studied. The protein powders exhibited friability, or susceptibility towards disintegration, which increased with increasing mass flow ratio during atomization. Powders with the smallest particle size upon sonication also tended to have the highest specific surface areas, which correlated with a decrease in percent protein monomer upon drying of the spray-frozen protein. Complexation with zinc stabilized the protein and resulted in reduced specific surface area of the spray-freeze dried powder without significantly affecting particle

Table III. Stability and Secondary Structure of Spray-Freeze Dried BSA[a]

Atomizing condition	Excipient-free			Zinc-complexed		
	Decrease in % monomer	α-helix	β-sheet	Decrease in % monomer	α-helix	β-sheet
1	6.8 ± 1.4	29 ± 2	21 ± 1	1.6 ± 0.1	30 ± 1	19 ± 2
2	3.1 ± 0.1	[b]	[b]	0	28 ± 0	17 ± 1
3	1.6 ± 0.1	29 ± 2	22 ± 1	0	32 ± 1	18 ± 1
4	9.2 ± 0.5	24 ± 1	20 ± 2	2.7 ± 0.3	28 ± 1	12 ± 1
5	4.1 ± 0.4	39 ± 5	21 ± 3	0	37 ± 3	13 ± 2
6	7.5 ± 0.9	35 ± 3	17 ± 0	0.8 ± 0.1	45 ± 2	19 ± 1
7	2.2 ± 0.3	24 ± 1	15 ± 1	0	42 ± 1	16 ± 1
8	16.8 ± 1.0	29 ± 2	9 ± 2	1.4 ± 0.1	30 ± 6	18 ± 1
9	8.0 ± 1.4	[b]	[b]	0.6 ± 0.2	29 ± 0	15 ± 1
10	1.8 ± 0.2	[b]	[b]	0	30 ± 3	15 ± 1
11	5.1 ± 1.0	33 ± 4	17 ± 2	[c]	[c]	[c]
12	1.3 ± 0.5	30 ± 5	22 ± 2	0	27 ± 1	17 ± 1
13	6.7 ± 0.6	32 ± 3	15 ± 2	0.4 ± 0.1	32 ± 6	14 ± 1
14	8.0 ± 0.3	28 ± 2	17 ± 2	[c]	[c]	[c]
15	2.6 ± 0.5	22 ± 1	24 ± 1	0	31 ± 4	19 ± 2
16	5.9 ± 0.1	29 ± 2	25 ± 1	0.9 ± 0.1	30 ± 3	12 ± 2
17	3.9 ± 0.1	24 ± 0	22 ± 1	0.8 ± 0.1	[b]	[b]
18	9.9 ± 0.1	21 ± 0	22 ± 1	0.7 ± 0.1	27 ± 3	12 ± 1
19	10.0 ± 0.4	31 ± 3	24 ± 2	1.0 ± 0.1	31 ± 1	16 ± 1
20	14.8 ± 0.8	29 ± 1	16 ± 1	2.2 ± 0.4	32 ± 1	15 ± 1
21	16.5 ± 0.1	26 ± 3	13 ± 2	2.7 ± 0.1	32 ± 3	13 ± 1

[a] Decrease in percent protein monomer was determined by SEC of the reconstituted sample. Data shown for at least two determinations. α-Helix and β-sheet were determined by Gaussian curve-fitting the Fourier self-deconvolved spectra in the amide I region (13). Data for four to five independent determinations.
[b] Not determined.
[c] Sample was not available due to clogging of nozzle during atomization.

size or protein structure under the conditions studied. Reduction of the protein particle size was beneficial in reducing the initial release of the protein encapsulated in PLG microspheres.

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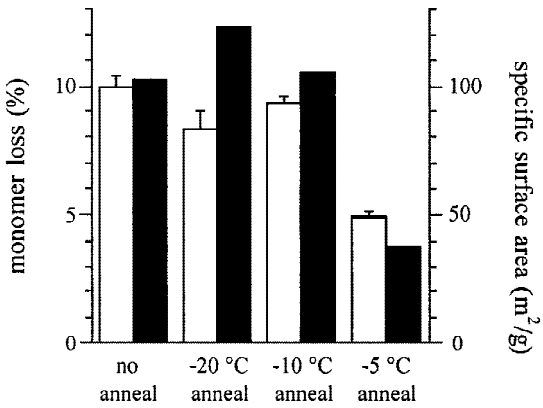

Fig. 5. Effect of annealing on spray-freeze dried BSA. The decrease in percent protein monomer (open bars) and specific surface area (filled bars) are depicted for excipient-free protein atomized using conditions 19. From left to right: not annealed, and annealed for 2 h at –20 °C, –10 °C, and –5°C.

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