Preparation of Active Proteins, Vaccines and Pharmaceuticals as Fine Powders using Supercritical or Near-Critical Fluids

Stephen P. Cape,1,5 Joseph A. Villa,1 Edward T. S. Huang,1 Tzung-Horng Yang,4 John F. Carpenter,2 and Robert E. Sievers1,3

INTRODUCTION

Preparing protein therapeutics as dry powders is usually required in order to overcome stability problems that commonly plague liquid formulations. The most common process for making dry solid formulations of therapeutic proteins is freeze-drying, also known as lyophilization (1). Another fairly common process is spray-drying, especially when the goal is to produce dry powders of therapeutic proteins and peptides (such as insulin) for pulmonary delivery (2). In the past decade or so, several supercritical fluid (SCF) or dense gas processes have received considerable attention as methods for producing particles containing a therapeutic agent or agents of interest that are suitable for pulmonary delivery or controlled release applications. (“Dense gas” is here defined as a gas in a supercritical, near-critical or liquid state.) In order for a powder to be suitable for pulmonary delivery, the aerodynamic size requirements are that particles must be in the 1 to 5 µm range (3), but preferably in the 1 to 3 µm range, with optimal size being ≤2 µm (4). Production of particles in this size range is generally possible by applying one of the various SCF processes, provided that the pharmaceutical is soluble in a compatible solvent. An elegant and extensive review (albeit with a disclaimer by the authors that it is not exhaustive) has earlier been published that surveys the literature and patents covering the field of particle preparation using SCF (5). Jovanovic et al. (6) have summarized the narrower literature regarding the stabilization of proteins and drying by SCF technologies. In their review they discuss effervescent atomization, which includes in their terminology CAN-BD and supercritical assisted atomization (SAA). Shoyele and Cawthorne (7) have recently reviewed inhaled biopharmaceuticals manufactured by SCF technologies.

In the present review, we survey the application of various supercritical or near-critical fluid techniques to the preparation of protein powders and particles, and the progress to date and the limitations. For proteins and vaccines, the CO2-assisted nebulization with a Bubble Dryer® (CAN-BD) process (8–12) appears to be a very promising new technology for the preparation of dry fine powders. This is due to the fact that CAN-BD can nebulize an aqueous solution without the need to use an organic solvent. Successful application of CAN-BD to both small-molecule and protein macromolecule particle preparations is reviewed. Case studies on the CAN-BD processing of three proteins, two of which are of clinical therapeutic interest, are
Table I: Summary of Protein-Containing Particles Produced by Supercritical/Dense-Gas Anti-Solvent Processes

| Protein          | Solvent                                  | Process                   | Anti-solvent | Particle Sizes (µm, unless otherwise noted) | Biological activity | Miscellaneous results or notes                                                                 | References |
|------------------|------------------------------------------|---------------------------|--------------|---------------------------------------------|--------------------|-----------------------------------------------------------------------------------------------|------------|
| Albumin          | Methanol (mixed with methylene chloride and TFE containing polymer) | ASES                      | CO₂          | 5–140                                        | Not determined     | Protein encapsulated in PLGA or L-Lactide/D, L-lactide/glycolide three-blockpolymer. Agglomeration tendency of primary particles | (25)       |
| Albumin          | Water                                    | ASES with coaxial nozzle (similar to SEDS) | CO₂ modified with ethanol | 0.05–0.5                                       | Not determined     |                                                |            |
| Antibody Fragment (Fab) | Water (with ethanol in intermediate nozzle channel) | SEDS                      | CO₂          | Not reported                                | 21% of original activity retained | 50% to 75% monomer content compared to 86% in original | (26,27)   |
| Antibody Fragment (Fab) | Water (with isopropanol in intermediate nozzle channel) | SEDS                      | CO₂          | Not reported                                | 12% to 46% of original activity retained | Retained activity depends on processing temperature | (28,29)   |
| Antibody fragment (Fv) | Water (with methanol in intermediate nozzle channel) | SEDS                      | CO₂          | Not reported                                | 20% of original activity retained |                                                | (28,29)   |
| β-lactamase      | Water (with ethanol in intermediate nozzle channel) | SEDS                      | CO₂          | Not reported                                | 3% of original activity retained |                                                | (28,29)   |
| Calcitonin       | DMSO                                     | SAS                       | CO₂          | 0.5–1.0                                      | “The particulate product formed retained substantial enzymatic activity” | Protein loaded in HYAFF microspheres | (30,31)   |
| Catalase         | Ethanol (with 10% water)                 | ASES (reported as continuous GAS) | CO₂          | ~1                                           | Not determined     | Possible partial crystallinity                  | (32)       |
| α-Chymotrypsin   | Water                                    | PCA                       | CO₂ modified with ethanol | 0.2 µm primary particle size by SEM          | 59% of original activity retained | Significant agglomeration of primary particles to form 250–400 µm particles | (33,34)   |
| Protein Type | Solvent | Method (reported as) | CO$_2$ Pressure (bar) | Activity | Loading Method |
|--------------|---------|----------------------|----------------------|----------|----------------|
| Chymotrypsin (HIP to AOT) | DCM | ASES (reported as SAS) | CO$_2$ | 1–2 | Not reported |
| Chymotrypsin (HIP to AOT) | DCM | ASES (reported as GAS) | CO$_2$ | 2–3 | Not reported |
| Chymotrypsin (HIP to AOT) | iso-octane | ASES (reported as GAS) | CO$_2$ | 1–10 | Not reported |
| Cytochrome C (HIP to SDS) | Ethanol | ASES (reported as GAS) | CO$_2$ | ~5 | Not reported |
| GMCSF | DMSO | SAS | CO$_2$ | 0.5–1.0 | Protein loaded in HYAFF microspheres |
| Immunoglobulin G (IgG) | Aqueous | SEDS | CO$_2$ modified with ethanol | Not determined | 38% to 48% of original activity retained |
| Insulin | DMF | ASES (reported as continuous GAS) | CO$_2$ | 1.0–4.0 | Full retention of original activity |
| Insulin | DMSO | GAS | CO$_2$ | 0.15–8 | Not determined |
| Insulin | DMSO | ASES | CO$_2$ | 0.05 (primary particle size) | Not determined |
| Insulin | DMSO | GAS | CO$_2$ | 1.4–1.8 | Not determined |
| Insulin | DMSO | ASES (reported as continuous GAS) | CO$_2$ | 1.0–4.0 | Not determined |
| Insulin | DMSO | ASES (reported as SAS) | CO$_2$ | 1–5 | Not determined |
| Insulin | DMSO | ASES (reported as SAS) | CO$_2$ | Not reported | Not determined |
| Insulin | DMSO | ASES (reported as semicontinuous GAS) | CO$_2$ | 0.4–0.6 | Greater than 80% activity maintained |
| Insulin | DMSO | SAS | CO$_2$ | 0.8 | Protein loaded in PEG/PLA nanoparticles |
| Insulin | DMSO (with 50% DCM) | ASES (reported as semicontinuous SAS) | CO$_2$ | 0.5–5 | Full retention of original activity |
| Insulin | Ethanol | GAS | CO$_2$ | 0.05–0.3 | Not determined |
| Protein                  | Solvent                          | Process                  | Anti-solvent | Particle Sizes (µm, unless otherwise noted) | Biological activity | Miscellaneous results or notes                                                                 | References |
|-------------------------|----------------------------------|--------------------------|--------------|---------------------------------------------|---------------------|-----------------------------------------------------------------------------------------------|------------|
| Insulin                 | Ethanol (with 10% water)         | ASES (reported as continuous GAS) | CO₂          | ≤1 (spheres); ~5×1 (length×width; needles)   | Not determined      | Possible partial crystallinity                                                              | (33,34)   |
| Insulin                 | Ethyl acetate                    | GAS                      | CO₂          | 0.3–0.7                                     | Not determined      | Potency fully retained (as determined by a chromatographic method)                           | (42,44)   |
| Insulin                 | HFIP                              | GAS                      | CO₂          | 1.0–7.9                                     | Not determined      | Slight chemical degradation and reversible structural changes of insulin                      | (49)      |
| Insulin                 | Methanol                          | GAS                      | CO₂          | 0.05–1                                      | Not determined      | Influence of various operating conditions examined                                           | (42,43)   |
| Insulin                 | Methanol                          | ASES                     | CO₂          | 0.2 (primary particle size)                 | Not determined      | Agglomerated with irreproducible morphology from primary particle                            | (42,43)   |
| Insulin                 | Methanol                          | GAS                      | CO₂ modified with ethanol                    | 0.2–0.7            | Not determined      | 93% monomer content retained                                                                | (42,44)   |
| Insulin (HIP to SDS)    | Water                             | GAS                      | NH₃          | 0.2–0.3                                     | Not determined      | Protein denaturation                                                                       | (42,44)   |
| Insulin (HIP to SDS)    | DCM                               | ASES (reported as SAS)   | CO₂          | 1–5                                         | Not reported        | Protein loaded in PLA particles                                                              | (36)      |
| Insulin (HIP to SDS)    | Methanol                          | ASES (reported as SAS)   | CO₂          | Not reported                                | Not reported        | Amorphous nanoparticles                                                                    | (37,38)   |
| Insulin (HIP to SDS)    | pyridine                          | ASES (reported as SAS)   | CO₂          | Not reported                                | Not reported        | Spheroidal particles                                                                       | (37,38)   |
| Insulin (HIP to SDS)    | THF                               | ASES (reported as SAS)   | CO₂          | 1–5                                         | Not reported        | Influence of various operating conditions examined. Water content unchanged by processing: 10% H₂O | (42,43)   |
| Lysozyme                | DMSO                              | GAS                      | CO₂          | 0.02–1                                      | 60% to 100% at 45°C to 25°C processing, respectively. Activity reduced by 25% to 50% upon storage over 3 months at 20°C | (42,43)   |
| Lysozyme                | DMSO                              | GAS                      | CO₂          | 0.05–0.2                                    | Not determined      | Influence of various operating conditions examined. Water content unchanged by processing: 10% H₂O | (42,44)   |
| Lysozyme                | DMSO                              | GAS                      | CO₂          | 0.2–0.3                                     | Up to 75% of original activity                                   | Amorphous nanoparticles | (50) |
| Lysozyme                | DMSO                              | ASES (reported as SAS)   | CO₂          | 1–5                                         | 88 to 100% of original activity                                   | Minimal secondary structure perturbation in dry solid; reversible upon reconstitution | (45)      |

Table I. (continued)
| Lysozyme | DMSO   | ASES (reported as SAS) | CO₂ | Not reported | 90%±5% of original activity | Secondary structure in dry solid and activity maintained during storage (±12 months at −25°C to 20°C or 3 days at 60°C). Moisture=2.5–7.4% H₂O | Enhanced mass transfer (using ultrasound to enhance droplet atomization). Particle size depends on ultrasound intensity |
|----------|--------|-----------------------|-----|--------------|-----------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Lysozyme | DMSO   | ASES (reported as SAS) | CO₂ | 0.19–1.2     | 87% of original activity retained | Retained activity depends on processing conditions | Attempt to encapsulate protein in PLA that was dissolved in methylene chloride. Low protein loading observed |
| Lysozyme | DMSO   | SEDS                  | CO₂ | 1–5          | 44% to 100% of original activity | | |
| Lysozyme | DMSO (with methylene chloride in intermediate nozzle channel) | ASES with coaxial nozzle (similar to SEDS) | CO₂ | Nanospheres: about 0.1–0.2 (primary particle size) | Not determined | |
| Lysozyme | DMSO (with 30% DMF) | GAS                  | CO₂ | 0.1          | Not determined | | | | Protein loaded in PLA particles Partially crystalline |
| Lysozyme | DMSO (with 50% DCM) | GAS                  | CO₂ | 0.02-0.04    | Not determined | Complete drying difficult; agglomeration |
| Lysozyme | DMSO (with 8% acetic acid) | GAS                  | CO₂ | 1–2          | ~30% of original activity | Complete drying difficult; agglomeration |
| Lysozyme | Ethanol (with 2% water) | GAS                  | CO₂ | 0.05 width x 0.25 length | Not determined | Complete drying difficult; agglomeration |
| Lysozyme | Ethanol (with 5% water) | GAS                  | CO₂ | 0.05–1       | Not determined | |
| Lysozyme | Ethanol (with 10% water) | GAS                  | CO₂ | 0.05–0.1    | Not determined | |
| Lysozyme | Ethanol (with 15% water) | GAS                  | CO₂ | 0.05–0.07   | Not determined | |
| Lysozyme | Methanol | GAS                  | CO₂ | 0.01–0.05   | Not determined | |
| Lysozyme | Water   | GAS                   | NH₃ | 0.05–0.2    | Not determined | |
| Lysozyme | Water   | ASES with coaxial nozzle (similar to SEDS) | CO₂ modified with ethanol | 0.05–0.5 | 96% to 98% of original | 96% to 98% of original | <6% undissolved upon reconstitution; undissolved material |
| Lysozyme | Water   | ASES with coaxial nozzle (similar to SEDS) | CO₂ modified with ethanol | <1 (primary size), up to ~20 (agglomerated size) | 96% to 98% of original | | |
Table I. (continued)

| Protein       | Solvent | Process                  | Anti-solvent | Particle Sizes (µm, unless otherwise noted) | Biological activity                                      | Miscellaneous results or notes                                      | References |
|---------------|---------|--------------------------|--------------|---------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------|------------|
| Lysozyme     | Water   | ASES with coaxial nozzle (similar to SEDS) or ultrasonic nozzle | CO₂ modified with ethanol | 0.1–0.5 (primary size), 3–20 (agglomerated size) | Not determined                                              | removed before further analysis; no structural changes observed by CD and fluorescence spectroscopy | (55)       |
| Lysozyme     | Water (with ethanol in intermediate nozzle channel) | SEDS          | CO₂          | 0.47–1.6 (mean = 0.78)                       | 95% of original activity retained                         | Moisture content = 10.18%                                        | (28,56–58) |
| Lysozyme     | Water   | SEDS                      | CO₂ modified with ethanol | Aggregates with primary particle sizes of 1–5 μm (polydisperse) | “minimal loss of biological activity”                     | Some secondary structure changes observed by FT-Raman spectroscopy | (59)       |
| Myoglobin    | DMSO    | GAS                      | CO₂          | 0.05, 0.3                                  | Not determined                                            | Polydisperse                                                   | (42,43)    |
| Myoglobin    | DMSO    | ASES                     | CO₂          | 0.05                                       | Not determined                                            | Polydisperse                                                   | (42,43)    |
| Myoglobin    | Methanol| GAS                      | CO₂          | 0.03, 0.4                                 | Not determined                                            | Polydisperse                                                   | (42,44)    |
| Myoglobin    | Water   | ASES with coaxial nozzle (similar to SEDS) | CO₂ modified with ethanol | 1–20                                      | Not reported                                               | >35% (unformulated) and 8–12% (formulated with sucrose or trehalose) remained undissolved upon reconstitution; undissolved material removed before further analysis | (54)       |
| RhDNase      | Water   | ASES with coaxial nozzle (similar to SEDS) | CO₂ modified with ethanol | 0.05–0.5                                  | Not determined                                            | 0% to 35% monomer content retained                              | (26,27)    |
| Ribonuclease (HIP to SDS) | Methanol | ASES (reported as GAS)    | CO₂          | ~ 50                                       | Not reported                                               |                                                                  | (37,38)    |
| Ribonuclease (HIP to SDS) | Methanol | ASES (reported as GAS)    | CO₂          | 0.5–1 (spheroidal particles), 10 µm x 1 mm (fiber-like particles) | Not reported                                               | Protein loaded in PEG particles                                 | (37,38)    |
Anti-CD4 antibody is a Primatized® monoclonal antibody that has potential clinical application in autoimmune and inflammatory diseases (13). Alpha-1-antitrypsin (AAT or α1-AT), also known as α1-proteinase inhibitor (API or α1-PI), is a serine proteinase inhibitor in plasma, the primary physiological function of which is to protect the connective tissue of the lungs from excessive protease activity by neutrophil elastase (14,15). AAT has been under clinical investigation (for both intravenous and aerosol pulmonary administration) as a therapeutic for α1-antitrypsin deficiency related emphysema and cystic fibrosis, diseases in which an imbalance of AAT relative to elastase is recognized (15,16). Finally, trypsinogen was selected as a protein model for examining the effects of formulation conditions and CAN-BD processing on the biological activity of enzymes.

OVERVIEW OF RAPID EXPANSION OF SUPERCRITICAL SOLUTIONS (RESS)

The SCF method first used for particle preparation is RESS, rapid expansion of supercritical solutions. As reported by Jung and Perrut (5), the basic concept of RESS is actually more than a century old, starting with the work on metal salts by Hannay and Hogarth (17) in 1879, while the modern practice and applications to pharmaceuticals have been developed and patented over the past two decades. Particle formation by RESS is accomplished by dissolving the substance of interest in a supercritical fluid and then rapidly expanding the solution through a nozzle, thereby causing solute nucleation and particle growth. Successful application of this process is obviously limited to that category of substances soluble in a SCF; proteins are not appreciably soluble in pure carbon dioxide, liquid or supercritical. In fact, the anti-solvent processes discussed below use supercritical carbon dioxide (scCO2) to precipitate proteins.

While a variety of supercritical fluids such as pentane, propane and nitrous oxide have been examined in particle formation processes, carbon dioxide is overwhelmingly the fluid of choice, particularly in the anti-solvent methods (5). It is relatively cheap, has readily accessible critical temperature (31.1°C) and critical pressure (7.38 MPa or 1,070 psi), has relatively low toxicity, and is environmentally benign.

![Fig. 1. Schematic diagram of a CAN-BD system.](image-url)
| Substance                        | Solvent               | Particle Sizes (µm) | Miscellaneous Results or Notes                                                                 |
|---------------------------------|-----------------------|--------------------|------------------------------------------------------------------------------------------------|
| Albuterol sulfate               | Water                 | 0.7 (mean size)    | “Static” CAN-BD method; narrow size distribution typically in 0.1–3 mm range; amorphous spherical particles |
| Aluminum sulfate                | Water                 | 1–2                | Amorphous spherical particles                                                                  |
| Amikacin                        | Water                 | Resp. fraction=84%<5 µm | Full retention of antibiotic activity                                                           |
| Ammonium chloride               | Water                 | Number distribution: 95%<3.9 | Spherical particles with microcrystallinity.                                                   |
| Amphotericin B                  | Ethanol               | 0.3–2 (mean=0.65)  | Amorphous spherical particles; nanometric particles also observed                              |
| Ampicillin                      | Water                 | 1–2                | Particles produced by CAN-BD with a cross from a 2% ethanolic solution of betamethasone and a 2% aqueous solution of lactose |
| Betamethasone-17,21-dipropionate| Ethanol               | 0.5–6.6            | Various processing parameters (nebulizing pressure, solute concentration, restrictor nozzle ID, etc.) were examined |
| Betamethasone-17,21-dipropionate| Ethanol               | Mean=0.8; 95%<1.2; MMAD=1.1 µm, GSD=2.0 | Particles produced by CAN-BD with a cross from a 2% ethanolic solution of betamethasone and a 2% aqueous solution of lactose |
| Betamethasone-17,21-dipropionate, heterogeneous particles with lactose| Ethanol/Water | Mean=1.2; 95%<2.0; MMAD=1.6 µm, GSD=1.7 | Particles produced by CAN-BD with a cross from a 2% ethanolic solution of betamethasone and 0.2% stearic acid, and a 2% aqueous solution of lactose |
| Betamethasone-17,21-dipropionate, heterogen. particles with stearic acid and lactose| Ethanol/Water | Mean=1.0; 95%<1.5; MMAD=1.2 µm, GSD=1.4 | Particles produced by CAN-BD with a cross from a 2% ethanolic solution of betamethasone and 0.2% stearic acid, and a 2% aqueous solution of lactose |
| Budesonide                      | Ethanol               | 0.4–3 (mean=1)     | Amorphous spherical particles                                                                  |
| Capreomycin                     | Water                 | Resp. fraction=77%<5 µm | Full retention of antibiotic activity                                                           |
| Carbamazepine                   | Methanol              | Not reported       | Needle-like micronic crystals                                                                  |
| Ciprofloxacin                   | Water                 | Resp. fraction=89%<5 µm | Full retention of antibiotic activity                                                           |
| Cromolyn sodium                 | Water                 | 0.58 (mean size)   | Narrow size distribution typically in 0.1–3 mm range                                           |
| Dexamethasone                   | Acetone               | Submicron particles | Amorphous spherical particles                                                                  |
| Dipalmitoyl phosphatidylcholine (DPPC) | Ethanol               | Mean=0.9; 95%<1.6 (by number distribution) Mean=3.7; 95%<15 (by volume distribution) | |
| Doxycycline                     | Water                 | Mean=1.0; 95%<1.6  | Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs |
| Erythromycin                    | Methanol or ethanol   | Methanol: mean=1.0; ethanol: 0.1–2.0 | |
| Erythromycin                    | Methanol, ethanol, or acetone | Volume distributions: methanol: 0.1–3.0 ethanol; 0.1–2.5 acetone: coalescing particles | |

References:

(86) Cape et al.
(90,92,99,100)
(93) Cape et al.
(94)
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(100)
(101)
(102)
(103)
(104)
(105)
(106)
(107)
| Compound                  | Solvent       | Volume distribution: | Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs |
|---------------------------|---------------|-----------------------|--------------------------------------------------------------------------------------------------|
| Griseofulvin              | Acetone       | mean = 0.5–2.5, 100% < 5.5 |                                                                                                   |
| HMR1031 (new chemical entity by Aventis Pharma; C_{35}H_{41}N_{5}O_{6}, 628 Da) | Methanol | 0.5–6; MMAD = 1.6 to 4.0 | MMADs were calculated from particle size distributions measured by laser diffraction                |
| myo-Inositol              | Water         | 0.6–4.5 (mean = 1.0 to 1.7) | Various processing parameters (drying temperature, nebulizing pressure, solute concentration, restrictor nozzle ID, etc.) were examined |
| Lactose                   | Water         | 0.5–5                 |                                                                                                   |
| Lactose                   | Water         | Mean = 1.2; 95% < 2.3; MMAD = 1.9 μm, GSD = 1.5 | Amorphous spherical particles                                                                   |
| Mannitol                  | Water         | Mean = 1.2; 95% < 3.2 |                                                                                                   |
| Mannitol                  | Water         | 0.6–5.6 (Mean = 1.0 to 2.1) | Various processing parameters (drying temperature, nebulizing pressure, solute concentration, restrictor nozzle ID, etc.) were examined |
| Mixed iron oxides (Fe_{3}O_{4} and FeO) | Water         | 0.1–0.7               | Particles formed by pyrolysis of aqueous Fe(II) acetate in CO_{2} aerosol Full retention of antibiotic activity |
| Moxifloxacin              | Water         | Resp. fraction = 56% < 5 μm |                                                                                                   |
| Naproxen                  | Water         | 0.4–2 (mean = 1)      |                                                                                                   |
| Palmitic acid             | Ethanol       | 0.4–3 (mean = 3)      |                                                                                                   |
| Palmitic acid             | Ethanol       | Mean = 1.1; 95% < 2.1; MMAD = 8.7 μm, GSD = 2.2 |                                                                                                   |
| Palmitic acid, heterogeneous particles with sodium chloride | Ethanol/Water | Mean = 1.8; 95% < 5.0; MMAD = 17.8 μm, GSD = 2.2 |                                                                                                   |
| Palmitic acid, heterogeneous particles with sodium chloride | Ethanol/Water | 0.6–6                |                                                                                                   |
| Palmitic acid, heterogeneous particles with lactose | Ethanol/Water | Mean = 1.8; 95% < 5.0; MMAD = 17.8 μm, GSD = 2.2 |                                                                                                   |
| Pigment Red 60 (DR60)     | Acetone       | Number distribution: 0.25–3.0 |                                                                                                   |
| Potassium Iodide          | Water         | Number distribution: Mode = 0.65–1.35, 95% < 1.65–4.24 | Cubic crystals; particle size dependent on starting solution concentration. Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs |
| Potassium Iodide          | Methanol      | Micron-sized particles | Cubic crystals, somewhat coalesced                                                                 |
| Rifampin                  | Methanol      | Number distribution: Mode = 0.3–1; Volume distribution: Mean = 0.7–1.8; 100% < 3.2 | Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs |
| Rifampin                  | Ethyl Acetate | Resp. fraction = 86% < 5 μm; MMAD = 1.2 μm, GSD = 2.1 | Supercritical nitrogen used for nebulization instead of near-critical CO_{2}. Full retention of antibiotic activity |
Table II. (continued)

| Substance                                | Solvent          | Particle Sizes (µm) | Miscellaneous Results or Notes                                                                 | References |
|-----------------------------------------|------------------|--------------------|------------------------------------------------------------------------------------------------|------------|
| Sodium chloride                         | Water            | 0.5–2              | Cubic crystals; size dependent on starting solution concentration                                 | (86)       |
| Sodium chloride                         | Water            | 0.6–4              | Spherical cluster of cubic crystals                                                              | (92,94,97) |
| Sodium chloride                         | Water            | Volume distribution: 0.2–2.5 | Cubic crystals; size dependent on starting solution concentration; Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs | (102)      |
| Sodium chloride coated with PLGA        | Acetone          | Mean=1.3; 95% < 2.6 | Spherical cluster of cubic crystals                                                              | (105)      |
| Sodium chloride coated with PLGA        | Acetone          | Mean=2.0; 95%<5.2   | Particles produced by CAN-BD from a suspension containing 0.5% NaCl particles and 2% dissolved 50/50 PLGA | (105)      |
| Sodium chloride with PLGA               | Water/Acetone    | Mean=1.3; 95% < 2.5; MMAD=2.2 µm, GSD=1.6 | Particles produced by CAN-BD from a 10% sodium chloride solution                                 | (95)       |
| Sodium chloride heterogeneous with PLGA | Water/Acetone    | Mean=1.3; 95% < 2.4; MMAD=2.0 µm, GSD=1.6 | Particles produced by CAN-BD with a cross from a 10% sodium chloride solution (water) and a 0.5% PLGA solution (acetone) | (95)       |
| Sodium chloride heterogeneous with palmitic acid | Water/Acetone | Mean=1.5; 95% < 2.9; MMAD=2.6 µm, GSD=1.8 | Particles produced by CAN-BD with a cross from a 10% sodium chloride solution (water) and a 2% palmitic acid solution (acetone) | (95)       |
| Sodium chloride heterogeneous with palmitic acid | Water/Acetone | Mean=1.5; 95%<3.4; MMAD=3.6 µm, GSD=1.8 | Particles produced by CAN-BD with a cross from a 2% sodium chloride solution (water) and a 2% palmitic acid solution (acetone) | (95)       |
| Terbutaline                              | Water            | Number distribution: Mode=0.3–0.7; Volume distribution: mean=0.7–2.6; 100%<4.0 | Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs | (115)      |
| Tetracycline                             | Water            | Number distribution: mode=0.3–0.5; Volume distribution: mean=0.5–1.2; 100%<2.4 | Particle size distribution determined from a statistical analysis of ~1,000 particles in scanning electron micrographs | (103,114) |
| Tobramycin sulfate                       | Water            | 0.5–3.2            |                                                                                                  | (100,116) |
| Triclabenzadol                           | Methanol         | 1–2                | Irregular crystals                                                                               | (86)       |
| Yttrium acetate                          | Water            | <0.25–3.5          | Amorphous spherical particles                                                                     | (86,103)   |
| Yttrium acetate                          | Methanol         | <0.1–1.0           | Amorphous spherical particles                                                                     | (86,103)   |
| Zanamivir (Relenza®)                      | Water            | Resp. fraction=73%<5 µm; (MMAD=2.4 µm) |                                                                                    | (69,70,101) |
| Zinc acetate                             | Methanol         | <0.5               | Amorphous spherical particles                                                                     | (86)       |
| Zirconyl nitrate hydrate                  | Water            | 1.5–3              | Amorphous spherical particles                                                                     | (86)       |
| Protein | Solvent | Particle Sizes (μm) | Biological Activity | Miscellaneous Results or Notes | Reference |
|---------|---------|---------------------|---------------------|--------------------------------|-----------|
| Alpha-1-antitrypsin | Water | Mean=1.9–2.2 (95% <5.3–5.4) | Full retention of enzymatic activity. | Formulated in buffered solution with trehalose and Tween 20 as stabilizing excipients | This work |
| Anti-CD4 | Water | Mean=1.4–1.8 (95% < 3.5–5.5) | Full retention of antigen binding activity. | Formulated in buffered solution with saccharide and surfactant as stabilizing excipients | This work |
| Hepatitis B surface antigen protein (HBsAg), aluminum hydroxide adjuvanted | Water | Not reported | Full retention of in vitro (ELISA) activity even after storage at −20°C or 66°C for 43 days. Full retention of immunogenicity in mice after processing | Formulated with trehalose or trehalose plus polyvinylpyrrolidone (PVP) | (69,70) |
| IgG (human) | Water | Resp. fraction=94% <5 μm | Full preservation of total human IgG content and full retention of activity against influenza A as measured by ELISA assays | Formulated as the commercial Polygam® S/D Immune Globulin Intravenous, a lyophilized formulation containing sodium chloride, human albumin, glycine, glucose, polyethylene glycol, and other trace components | (101,105) |
| Lactate dehydrogenase | Water | 1–3 | 15% of original activity (no stabilizing excipients added); 40% to >95% of original activity depending on stabilizing excipients added | Various stabilizing excipients (type and amount) tested | (91,92) |
| Lactate dehydrogenase, Lysozyme | Water | 0.5–5 (mean= ~3) | Full retention and even enhancement of original activity possible | Various stabilizing excipients (type and amount) tested | (11,117) |
| Measles vaccine virus, live-attenuated | Water | Resp. fraction=94% < 5 μm; (MMAD= 1.9 μm) | Full retention of virus activity as measured by a standard plaque assay | Trehalose was added to a commercial lyophilized measles vaccine formulation for processing. Particle sizing was conducted on a corresponding virus-free placebo formulation | (69,70) |
| Measles vaccine virus, live-attenuated | Water | Resp. mass fraction= 42% to 50% < 5.8 μm, 17% to 30%<3.3 μm | 50% to 80% of original activity as measured by a standard plaque assay. Less than 1 log loss in viral activity of the powder after 7 days at 37°C | The vaccine virus was formulated with myo-inositol as the primary stabilizing excipient. Other stabilizers included hydrolyzed gelatin, amino acids, and a buffer | (71–75) |
| Ovalbumin | Water | 0.3–5 | Not determined | Original aqueous solution contained 5% ovalbumin and 5% trehalose | (8,92,94) |
| Ovalbumin, with DPPC and lactose | Ethanol/ water (80:20) | Mean=0.56 (95%<0.96) | Not determined | Particles produced from an ethanol/water solution (80:20 volume ratio) containing 0.06% DPPC, 0.02% lactose, and 0.02% chicken egg albumin | (105) |
| Small peptide new drug entity | Ethanol | Mean=0.93 (95%<1.8) | Not reported | Low density particles with much larger geometric diameters (~4 μm) observed by SEM | (106) |
| Trypsinogen | Water | 0.5–7 (mean= ~3) | Full retention and even enhancement of original activity possible | Various stabilizing excipients (type and amount) tested | (117) |
OVERVIEW OF DENSE GAS ANTI-SOLVENT PROCESSES

While RESS is not applicable to the formation of protein particles, processes that take advantage of the ability of supercritical fluids or compressed gases to precipitate proteins have been investigated for this purpose. These processes all work on the same principle: the dense gas acts as an anti-solvent when it dissolves in and expands a solvent containing the target solute(s), causing supersaturation and precipitation of the solute(s) in a high pressure chamber, usually operating at pressures >1,200 psi. The solvent and supercritical or near-critical fluid must be miscible. Unfortunately, the preferred solvent for most proteins, water, is very poorly miscible with dense CO$_2$: at 21°C and 1,200 psi, only $\sim$0.3 mole% of water dissolves in CO$_2$ and only $\sim$2.5 mole% of CO$_2$ dissolves in water (18). Therefore, the application of dense gas anti-solvent processes is largely restricted to pharmaceuticals (such as lipophilic compounds) dissolvable in organic solvents that are miscible with the dense gas. Although the basic principle is the same in several processes, the various specific implementations of this principle have acquired different names and acronyms in the literature.

Gas anti-solvent (GAS) precipitation and Supercritical fluid anti-solvent (SAS) precipitation are essentially synonymous and refer specifically to the batch expansion of a solute-containing solvent by a dense gas. Aerosol solvent extraction system (ASES) is a modification of GAS/SAS in which the solute-containing solvent is sprayed through an atomization nozzle into the compressed anti-solvent. Precipitation from compressed anti-solvent (PCA) is another designation found in the literature for what is essentially the ASES process. Solution enhanced dispersion by supercritical fluids (SEDS) is a refinement and modification of ASES in which a special nozzle with two (or three) coaxial channels is used to combine the SCF with the other solvents and spray the mixture into the same SCF. The three-channel nozzle allows aqueous solutions to be processed by SEDS by using an organic solvent, such as ethanol, that is miscible in both the aqueous solution and scCO$_2$, to promote miscibility between scCO$_2$ and water.

Anti-solvent processes have been applied to the preparation of protein particles with only limited success. Success is defined here as the formation of fine protein particles with mean aerodynamic diameter of less than 5 μm and essentially full retention of biological activity. Table I summarizes the various proteins that have been processed into particles using anti-solvent methods. Particles of hydrophobic proteins such as insulin or generally robust proteins such as lysozyme that tolerate dissolution in or contact with organic solvents have been successfully prepared using GAS/SAS or related methods. Essentially full retention of activity has repeatedly been possible with lysozyme and insulin, which are relatively robust when compared with other proteins and peptides. For example, both of these proteins can be dried without stabilizing excipients and fully recover native structure and function after rehydration (19–21). In contrast, more labile proteins such as lactate dehydrogenase or Factor XIII are irreversibly denatured if they are subjected to drying without stabilizers (22–24). Success in stabilization and micronization is generally much more limited for other proteins and peptides. The literature review presented in Table I demon-
strates that in the overwhelming majority of attempts to produce protein particles by anti-solvent techniques, post-processing biological activity was either low or not determined or not reported.

Lysozyme is often chosen as a model protein for proof of concept testing of new techniques or processes because it is robust and is arguably the most investigated protein of all time for processing, formulation and drug delivery systems. This holds true in the developmental history of anti-solvent processes. Lysozyme is a good model to start with, but success with it far from guarantees success with any other protein, especially more labile ones. Before general claims of applicability to protein processing are made, a number of proteins should be tested, particularly ones that might be sensitive to stresses (such as organic solvent exposure and dehydation) encountered in the process.

It is well established in the published literature [see reviews by Carpenter et al. (60) and Wang (1)] that successful preparation of proteins in a dry solid form by lyophilization generally requires the addition of stabilizing excipients such as disaccharides (e.g., sucrose or trehalose) and/or surfactants (e.g., Tween 20 or 80). Careful pre-formulation studies are often undertaken in order to identify the optimal formulation: buffer type, amount, and pH; disaccharide type and amount; surfactant type and amount; etc. The dense gas anti-solvent processes discussed here do not lend themselves to such careful formulation endeavors. Differential precipitation of the various excipients during expansion by the dense gas would complicate the formulation efforts. Carpenter et al. (60) make a strong case that long-term storage stability of proteins in a dry solid form generally hinges on whether or not native protein secondary structure is retained. In most of the cases in Table 1, protein secondary structure was not considered nor investigated. Winters et al. (45) did examine secondary structure and found that there was minimal secondary structure perturbation in the dry solid due to ASES processing in the case of lysozyme, but moderate and appreciable perturbation in the cases of trypsin and insulin, respectively. Protein contact with or dissolution in the organic solvent(s) required for anti-solvent processing will usually significantly complicate the attempts to maintain native secondary structure, since such solvents are often protein denaturants. With a few exceptions, those investigators that did determine biological activity after processing did not, however, determine the long-term stability of the protein subjected to anti-solvent processing. Winters et al. (46) observed that the activity and secondary structure of lysozyme, trypsin and insulin in particles produced by ASES were maintained upon long-term storage at various temperatures relative to the activity and secondary structure determined immediately after processing (45). Thiering et al. (42,44), however, observed a 25% to 50% reduction in lysozyme activity upon storage over 3 months at 20°C of lysozyme particles produced by GAS. Despite the success by Winters et al. (45,46), given the relatively poor track record or lack of data for proteins to date, particularly for proteins other than lysozyme, insulin and trypsin, one should not expect that dense gas anti-solvent processes will be broadly applicable to preparations of particles of stable protein formulations. The instability of many proteins and enzymes in contact with organic solvents (61) should lead one to conclude that processes requiring organic solvents will only be useful for a limited number of these biomolecules.

A few approaches to protein particle formation have involved dense gas processing of solid protein cakes or suspensions. Castor and Hong (62) obtained a patent for a process in which solid protein (e.g., from a lyophilized cake) is contacted with a supercritical fluid and then rapidly depressurized to achieve size reduction. Resulting size distributions were usually very broad with sizes typically ranging from tens of microns to several hundred microns. Although it was stated in the patent that retention of full activity was expected, no supporting activity data was presented. Young et al. (63) used a variation of ASES to encapsulate lysozyme in polymer microspheres. Lysozyme, spray-dried from aqueous solution to form 1–10 μm particles, was suspended in a solution of poly(lactic acid) (PLA) or poly(lactic acid-co-glycolic acid) (PLGA) in dichloromethane and then sprayed through a nozzle into a CO₂ vapor phase over a CO₂ liquid phase, leading to precipitation of the polymer and encapsulation of the lysozyme. Particle sizes were typically 5 to 60 μm. The biological activity of the processed lysozyme was not reported. Mishima et al. (64) used a variation of RESS to encapsulate lysozyme and lipase into various polymers. Solid protein was suspended in scCO₂ containing a cosolvent (e.g., ethanol) and dissolved polymer (e.g., polyethylene glycol (PEG)) and then rapidly expanded through a capillary nozzle to atmospheric conditions. Particles containing encapsulated protein with primary diameters ranging from 8 to 62 μm were reported. No biological activity data were reported. In more recent reports (65–68), dry particles (produced by milling and sieving of lyophilized material) or dry microcrystals of several proteins and enzymes have been coated or encapsulated using SCF processes; full or very good retention of activity was reported for these proteins.

CO₂-ASSISTED NEBULIZATION WITH A BUBBLE DRYER® (CAN-BD)

CAN-BD is a process patented by Sievers et al. (8–12). This invention covers two versions of the process, static and dynamic. The static version involves the pre-mixing of scCO₂ and a solution containing a solute of interest at a pressure higher than the critical pressure of CO₂. After equilibrium is established or approached, the mixture in a high pressure chamber is allowed to expand to atmospheric pressure through a flow restrictor (or a capillary tube) by expansion into a drying chamber.

The dynamic version involves continuous intimate mixing of a solution containing a solute of interest and scCO₂ or near-critical CO₂. In one version of this process, the two fluid streams become intimately mixed in a low dead volume tee and are then expanded through a flow restrictor to atmospheric pressure, where the plume of microbubbles and microdroplets are rapidly dried. This dynamic version of CAN-BD has been consistently, repeatedly and broadly successful in preparing protein particles that are usually stable, active and in the size range suitable for pulmonary delivery. This success has been achieved because the aqueous solution or suspension containing a protein or vaccine virus can be formulated to contain the appropriate stabilizers.
Recently, the CAN-BD process has been used to produce dry powders of live-attenuated measles vaccine virus (Edmonston-Zagreb) with good mechanical yield and with retention of viral activity as measured by a plaque forming unit assay that is comparable to commercial lyophilization (69–75). CAN-BD has also been used to dry siRNA nucleotides (71). Depending on formulation and laboratory processing conditions, typical lab scale yields range between 50% and 90%. In traditional spray drying, yield usually increases with scale, and the same may be realized for CAN-BD, in which droplet drying and particle collection is similar to traditional spray drying.

In papers by Abdul-Fattah et al. (76, 77) and patent applications by Truong-Le et al. (78–81), drying process methods are described that are similar to the CAN-BD process patents (8–12). Truong-Le et al. called this process, which uses compressed fluids of carbon dioxide, nitrogen, helium, or argon, “high pressure effervescent atomization” or “high pressure spray drying”. Both carbon dioxide and nitrogen were used at similar temperatures and pressures in the 1997 patent (8). In some of the examples given by Truong-Le et al. (78–81), particles of stable live attenuated B/Harbin influenza virus can be stored with only about one log loss of activity over one year at 25°C. High pressure spray drying was also used to prepare dry powders of an IgG monoclonal antibody and a live attenuated virus vaccine of a para influenza strain (76,77). The spray dried vaccine preparation was less stable compared to that from foam drying but more stable than that obtained by freeze drying. Additionally, in a recently issued patent by Shekunov et al. (82) and in patent applications by Truong-Le et al. (83,84), spray freeze drying methods are described that employ the nebulization method patented earlier (8,9,11), combined with a freeze drying or lyophilization step. Shekunov et al. (82,85) showed that >80% of the biological activity of trypsinogen (formulated with trehalose) could be retained using their modification of the CAN-BD process. B/Harbin influenza virus processed with the Truong-Le et al. (83,84) spray freeze drying method was reported to have only lost about one log
of activity over 13 months at 25°C or 67 days at 37°C for certain formulations. One variant of the CAN-BD process has also been referred to by Reverchon et al. (86–88) as supercritical-assisted atomization (SAA) in the published literature. In SAA, carbon dioxide is intimately mixed with the solution to be dried in a large high pressure chamber containing packing that provides a large surface area, to achieve effervescent atomization.

Principles of CAN-BD

Unlike the anti-solvent processes, CAN-BD does not employ dense gases to achieve precipitation by solubility reduction of the solute(s) to be micronized. Rather they are used to enhance or facilitate the nebulization or aerosolization of a liquid solution, which is then rapidly dried to form particles by solvent removal. Organic or aqueous solutions are both readily processed by CAN-BD, although neither solvent type needs to be present for the processing of the other. CAN-BD is broadly applicable to the processing of aqueous protein solutions and therefore lends itself readily to studies undertaken to create dry solid formulations optimized for protein storage stability and retention of biological activity, and to develop such protein particles with morphology and size suitable for pulmonary administration.

Table IV. Percentage of Binding Activity of Anti-CD4 Antibody to its Antigen as Measured by a Standard ELISA Method

| Sample description | % binding activity±SD |
|--------------------|-----------------------|
| Bulk solution      | 106±6                 |
| CAN-BD             | 91±10                 |
| Lyophilized        | 95±7                  |

A goat anti-human IgG HRP conjugate and ABTS were used to detect the bound antibodies on the soluble CD4 antigen-coated plate

Fig. 6. Size-exclusion chromatograms of rehydrated dry powders of anti-CD4 antibody produced by CAN-BD at 50°C (Run A) compared to the starting material. Numbers above or next to peaks refer to their area percents. Size-exclusion HPLC was performed using an HPLC system (Agilent Technology 1100 series) equipped with a TSK-Gel G3000SWXL column (Tosoh Biosep LLC, Pennsylvania, USA), using a phosphate based buffer for elution.

Fig. 7. Second-derivative infrared spectroscopy of unprocessed bulk solution and powders of anti-CD4 antibody produced by CAN-BD at 30°C (Run B) and 50°C (Run A). IR spectra were collected and analyzed according to the methods described by Dong et al. (119–121).

The principles of the CAN-BD process and the experimental setup have been previously described elsewhere (89–95). They are here briefly described again. Figure 1 is a schematic diagram of a typical CAN-BD system. A liquid solution (organic or aqueous), typically containing 1% to 10% total dissolved solids, is brought into intimate contact with supercritical or near-critical CO₂ (usually at 1,200 to 1,500 psi and 20°C to 35°C, although a wide variety of conditions can be used) in a low dead volume tee. The resulting emulsion or solution mixture is rapidly expanded to near atmospheric pressure through a capillary flow restrictor, which is usually fused silica, stainless steel or PEEK with an inner diameter of 50 to 175 μm and a length of ~10 cm. Upon expansion, the emulsion or solution forms a dense aerosol consisting of microdroplets and microbubbles. The aerosol is formed primarily due to the sudden physical dispersion of the liquid solution caused by the rapid expansion of compressed CO₂. Further break up of the microdroplets occurs due to the sudden release of any CO₂ that became dissolved in the liquid solution during intimate contact in the tee. At 1,000 to 2,000 psi, the solubility of CO₂ in water is about 2 to 2.5 mole % (18). The dense aerosol is delivered into a drying chamber (maintained at or near atmospheric pressure), into which preheated air or nitrogen gas is also delivered so as to maintain the chamber at a desired average drying temperature (typically 25°C to 65°C when processing aqueous protein solutions). Drying of an aerosol droplet is very fast. Adler and Lee (96) calculated that the total drying time in a Buchi spray-dryer (T_{inlet}=150°C, T_{outlet}=95°C) was less than 2 ms for a 8.6 μm droplet containing 10% (w/w) trehalose. In CAN-BD, the average residence time of a droplet/dry particle in the drying chamber has been estimated from chamber volume and flow rate calculations to be a few seconds (94). It should be noted that the droplet drying time will be shorter than the residence time. Microbubbles should dry even faster than microdroplets with the same diameter.

In drying some substances by CAN-BD, hollow dry particles are formed. Dry particles are collected on a filter membrane, with pore sizes between 0.2 and 0.45 μm, located
at the outlet of the drying chamber. CAN-BD can be operated as either a batch, semi-continuous, or continuous process. Typical flow rates on a lab-scale are 0.3 to 0.6 ml/min of liquid solution and 1 to 3 ml/min of dense CO2. We have successfully scaled up CAN-BD to process up to 20 ml/min of liquid solution (97), and have more recently used flow rates as high as 30 ml/min, which is commercial production scale for high value pharmaceutical products (98).

Organic solvents that are compatible with liquid carbon dioxide can be substituted in part or totally for water. Examples that the authors have used include ethanol, methanol, acetone, ethyl acetate and various mixtures of solvents, surfactants, buffers, stabilizers and other excipients. The solvent choice depends on the solubility and stability of the pharmaceutical to be micronized, and on the desired morphology and mean size of the particles.

**Review of Successful Applications of CAN-BD**

Application of the CAN-BD process to produce fine particles of a variety of substances dissolved in a variety of solvents has been broadly successful. Table II summarizes the application of CAN-BD to small-molecule substances such as salts, sugars and low-molecular weight pharmaceutical products. Particles in the respirable size range of 1 to 5 µm were consistently produced. The CAN-BD process has also been applied to the preparation of protein particles with broad success. Table III summarizes the examples of CAN-BD in protein processing to form stable active powders. To achieve the desired protein stability and retention of biological activity, addition of pH buffers and appropriate stabilizing excipients such as disaccharide sugars (e.g., sucrose or trehalose) or surfactants (e.g., Tween 20 or Tween 80) are usually required. However, addition of these solution components does not pose any extra processing difficulties for CAN-BD. Full retention of biological activity is readily and repeatedly observed for proteins appropriately formulated. An interesting example of a protein vaccine that has shown full retention of activity after CAN-BD processing, as indicated in both in vitro (ELISA) and in vivo (mice) tests, is the hepatitis B surface antigen (HBsAg) (69,70). As mentioned above, CAN-BD has been recently used to prepare fine dry powders of live attenuated measles vaccine virus (69–75). For a myo-inositol based formulation under development as an inhalable dry powder vaccine, it was determined that 72%±18% SD (n=8) of the viral activity was preserved through CAN-BD processing (74,75). This compares favorably with freeze drying of the commercial sorbitol based measles vaccine formulation, in which retention of activity is on the order of 50% to 60%. In addition, the CAN-BD prepared powder formulation passed the World Health Organization stability criterion of less than one log loss (or less than 90% loss) after 7 days of storage at 37°C. Specifically, the myo-inositol based formulation retained 20%±9% SD (n=4) of the viral activity after 7 days at 37°C. Aerodynamic diameters by cascade impaction were determined to be 45% to 50%<5.8 µm and ~20%<3.3 µm. In vivo viral replication was demonstrated by the observation by polymerase chain reaction (PCR) assay of measles virus nucleoproteins in the lungs of Cotton rats 7 to 14 days after the rats were allowed to inhale an aerosolized powder of the myo-inositol based CAN-BD powder formulation (75).

Under certain formulation conditions, apparent enhancement of the enzymatic activity of lactate dehydrogenase (LDH) and trypsinogen has been observed (11,117). This indicates that treatment with supercritical or near-critical CO2 can possibly refold the protein molecules in the original stock solution that are denatured or in a subactive folded conformation to a native and active structure. Independent investigators have reported similar results. Giessauf and Gamse (118) reported increases in the enzymatic activity of porcine

| Run | Drying temp. (°C) | Particle size (µm) | Water content (%) |
|-----|------------------|--------------------|-------------------|
| A   | 50               | 1.4                | 3.5               | 2.0               |
| B   | 25 to 30         | 1.5                | 5.5               | 1.7               |
| C   | 50               | 1.5                | 3.9               | 1.3               |
| D   | 50               | 1.8                | 4.9               | 1.5               |
| E   | 50               | 1.5                | 4.0               | 1.4               |

Aerodynamic particle size was measured as in Fig. 5. The powders were stored for one to several weeks in a vacuum chamber over calcium sulfate desiccant before their water content was determined using a methanol extraction method and a Denver Instruments Model 260 Titration Controller with a Model 275KF Coulometric Karl Fischer Titrator.

![Fig. 8. SEM image of AAT particles produced by CAN-BD at 40°C from an aqueous solution containing AAT and trehalose (3 to 5 mass ratio) in 0.1 M sodium phosphate, pH 7.0 buffer with 0.1% Tween 20.](image-url)
pancreatic lipase as high as 860% upon treatment with supercritical carbon dioxide, although they treated damp powders by hourly pressurization and depressurization cycling with supercritical carbon dioxide, while our studies were by CAN-BD of aqueous solutions, followed by dissolution of the dried LDH or trypsinogen for enzymatic activity assays.

### CAN-BD Case Studies

The CAN-BD process described above was used to obtain fine dry powders of anti-CD4 antibody, AAT and trypsinogen. An aqueous solution containing the given protein with excipient(s) was delivered to one inlet of a low dead volume mixing tee using an HPLC pump set at a constant flow rate, typically 0.3 to 0.5 ml/min. Supercritical or near-critical CO₂ (typically at room temperature and 1,200 psi) was delivered to the other inlet of the tee using an ISCO Model 260D syringe pump. The resulting fine emulsion of liquid near-critical CO₂ and aqueous protein solution was then rapidly expanded to near atmospheric pressure through a fused silica capillary tube (9 to 10 cm length, 74 μm I.D.) into a glass drying chamber (1 to 2.5 l) to form a very fine aerosol of aqueous droplets. Preheated nitrogen was passed through the drying chamber at 15 to 30 l/min to maintain the average temperature at a selected set point (usually between 25°C and 65°C), thereby drying the aerosol to form dry particles, which were collected on a filter (0.2 or 0.45 μm mixed cellulose ester, 142 mm diameter, Advantec MFS, Inc., Dublin, CA) located at the exit of the drying chamber.

#### Anti-CD4 Antibody

Using the CAN-BD process, we have successfully produced fine, dry powders of anti-CD4 antibody, a Primatized monoclonal antibody with potential clinical value in treating rheumatoid arthritis and other diseases. Anti-CD4 antibody was expressed in CHO cells and purified by several chromatographic steps to homogeneity. The material was formulated in a proprietary buffered solution containing saccharide and surfactant as stabilizing excipients and was used as provided by Biogen Idec Inc. (San Diego, CA).

Figure 2 (91) is a representative SEM image of particles produced at 50°C. The particles display dimpled “ping-pong ball” morphology. Figure 3 is an SEM image of anti-CD4 antibody particles dried at about 30°C and displays the same morphology, although the dimple effect is somewhat less pronounced. Such morphology was characteristic of all anti-CD4 antibody powders that we generated by CAN-BD and suggests that the particles are hollow, or at least began as

| Composition by weight percent Aqueous Solution (dry solid) | Trehalose Particle size (μm) | Sucrose Particle size (μm) |
|-----------------------------------------------------------|-----------------------------|---------------------------|
| Trypsinogen Sugar<sup>a</sup> | Mean | <95% | Mean | <95% |
| 1 (100) | 0.86<sup>b</sup> | 1.57<sup>b</sup> | 0.86 | 1.57 |
| 1 (80) | 0.86 | 1.47 | 0.87 | 1.47 |
| 1 (67) | 0.89 | 1.53 | 0.87 | 1.56 |
| 1 (50) | 0.90 | 1.56 | 0.87 | 1.56 |
| 1 (33) | 0.93 | 1.75 | 1.02 | 1.85 |
| 1 (20) | 1.02 | 2.06 | 1.06 | 1.93 |
| 1 (11) | 1.17 | 2.47 | 1.33 | 2.86 |
| 0 | 1.34 | 2.58 | 1.43 | 2.88 |

<sup>a</sup>The sugar used as an excipient was either sucrose or trehalose.

<sup>b</sup>These particles contained no sugar, only trypsinogen.

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Fig. 9. SEM image of commercial as-received lyophilized trypsinogen.

Fig. 10. SEM image of particles produced by CAN-BD from an aqueous solution containing 10 mg/ml trypsinogen (average aerodynamic diameter: 0.86 μm, 95%<1.57 μm).
hollow spheres that collapsed. TEM visualization, an image of which is presented in Fig. 4, lends additional credence to the suggestion of hollow character by demonstrating that the particles are less dense in the centers, as seen by the lighter region in the center of particles.

While the goal of producing dry powders of anti-CD4 antibody by CAN-BD was primarily as an alternative to the traditional freeze-drying or spray-drying processes, the size distribution shown in Fig. 5 indicates that these powders are also suitable for delivery by inhalation. Average aerodynamic size of the particles was 1.4 μm [squarely centered in the 1 to 3 μm range recommended by Corkery (4)] with 95% of the particles less than 3.5 μm in diameter. The distribution in Fig. 5 is representative of all the anti-CD4 antibody powders generated by CAN-BD. In addition, final moisture content of the powders as determined by Karl Fischer titration was 2% or less, a value targeted as desirable for stability of dry formulations of proteins produced by freeze-drying (1).

A concern during the production of dry protein formulations by any process is that of protein aggregation. Figure 6 shows that the anti-CD4 antibody formulation was successfully processed by CAN-BD without the irreversible formation of any aggregates. The starting material and the rehydrated CAN-BD powder display essentially identical size-exclusion chromatography (SEC) profiles. Protein aggregation due to CAN-BD processing was also not observed by SEC for any of the other anti-CD4 antibody powders produced by CAN-BD (data not shown).

Preparation of stable and active anti-CD4 antibody powders by CAN-BD is demonstrated by the full retention of biological activity. Table IV shows that the ELISA antigen binding activity of the reconstituted CAN-BD processed anti-CD4 antibody powder is statistically no different from that of the bulk starting solution. The data also demonstrate that in terms of binding activity retention, CAN-BD processing performs equally well as lyophilization. It should be noted that the binding assay was performed on CAN-BD particles rehydrated after 3 months of storage at room temperature, indicating that the CAN-BD anti-CD4 antibody powders are stable upon storage.

The reproducibility of generating anti-CD4 antibody powders by CAN-BD without significant structural perturbation is demonstrated by consistent and comparable secondary structure of the dried protein, not only for powders generated under very similar processing conditions, but also for powders
produced by CAN-BD at different drying temperatures. Second-derivative IR spectra in the conformationally sensitive amide I region (Fig. 7) (119–121) demonstrate that the secondary structure for anti-CD4 antibody powder dried at 50°C is essentially identical (within experimental error) to that dried at about 30°C. The spectra (not shown) for the other CAN-BD powders of anti-CD4 antibody also overlay within experimental error the spectra shown in Fig. 7. In addition, Fig. 7 shows that the secondary structure of anti-CD4 antibody in the dry CAN-BD powders is substantially the same as that of native anti-CD4 antibody in aqueous solution prior to CAN-BD processing.

CAN-BD powder generation reproducibility is also demonstrated by the summary of particles sizes and residual moisture content presented in Table V. Mean aerodynamic diameters of the anti-CD4 antibody CAN-BD particle size distributions were typically 1.5 μm with 95% of the particles almost invariably ≤ 5 μm. Equilibrium residual moisture content was in all cases 2% or less.

α1-antitrypsin (AAT)

We have successfully formed fine dry powders of the enzyme α1-antitrypsin (AAT) by CAN-BD. AAT (Cat. # A6150) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and used without further purification. Trehalose (Cat # T-104-1) was purchased from Pfanstiehl Laboratories, Inc. (Waukegan, IL, USA). AAT solutions were prepared by dissolving a known mass of the solid, commercially lyophilized powder in the desired buffer solution. Figure 8 is a representative SEM image of particles of AAT generated at 40°C drying temperature from an aqueous solution containing 2.8% (w/w) AAT and 4.6% (w/w) trehalose in 0.1 M sodium phosphate, at pH 7.0 with 0.1% (w/v) Tween-20. The resulting dry powder consisted of particles with an average aerodynamic diameter of 2.2 μm, while 95% of the particles had a diameter of <5.3 μm. Water content of the final dry powder was 1.8%. AAT processed by CAN-BD into dry powder retained 98%±2% of its original activity when reconstituted in water. Biological activity of AAT was determined using the elastase inhibitory activity assay described by Travis and Johnson (122).

Particles of AAT were also produced by CAN-BD at 50°C from an aqueous solution containing 4% (w/w) AAT and 4% (w/w) trehalose in 0.1 M sodium phosphate, pH 7.0 with 0.01% (w/v) Tween-20. The resulting dry powder displayed a qualitative morphology as observed by SEM (data not shown) that was very similar to that seen in Fig. 8 and consisted of particles with an average aerodynamic diameter of 1.9 μm (95% of the particles had a diameter of <5.4 μm). The water content of the final dry powder in this case was also determined to be 1.8%. As before, AAT in the CAN-BD processed dry powder retained full activity, displaying 106%±10% recovery of its original activity when reconstituted in water. For both dry powder samples, the size-exclusion chromatography profiles of CAN-BD processed AAT were nearly identical to the AAT in the original unprocessed sample.
indicating that CAN-BD processing caused no physical degradation or irreversible aggregation (data not shown).

**Trypsinogen**

The CAN-BD process was able to generate dry and stable particles of the model protein trypsinogen in the appropriate size range for pulmonary drug delivery. Trypsinogen (Cat # LS003649) was obtained from the Worthington Biochemical Corporation (Lakewood, NJ, USA) and was used without further purification.

The stabilizing effect of two disaccharide sugars, sucrose and trehalose, on trypsinogen processed by CAN-BD was investigated. Sucrose (Cat # S-124-1) and trehalose (Cat # T-104-1) were purchased from Pfanziehl Laboratories. Each sugar was combined with the protein in varying ratios of sugar to protein. Protein concentration in the pre-CAN-BD solution was kept constant at 10 mg/ml in formulations in which the sugar concentrations varied from 0%, 0.25%, 0.5%, 1%, 2%, 4%, and 8% (w/w; see Table VI). Another condition was also tested (protein concentrations and percentages of sugar refer to concentrations in the pre-CAN-BD solutions): 5 mg/ml trypsinogen with 25% sugar.

The average aerodynamic diameter of the particle was dependent on the concentration of total dissolved solids (TDS) in the initial aqueous solution. For concentrations ranging from 1% to 10% (w/w) TDS, the average aerodynamic diameters ranged from 0.8 μm to 1.4 μm, respectively. From Table VI it can be seen that as the TDS increased in the aqueous solution, the corresponding average particle aerodynamic diameter in the dry powders (either with trehalose or sucrose as an excipient) also increased. This dependence of the particle size upon the initial solution concentration has also been reported elsewhere (110). An SEM image of the as-received trypsinogen product is presented in Fig. 9 and shows it consists of very large flakes. Representative SEM images and particle size distributions of the CAN-BD processed trypsinogen formulations show the particles to be in the appropriate size range for the most effective pulmonary delivery (See Figs. 11, 12, 13, 14, 15 and 16 and Table VI). Fig. 10 shows an image of trypsinogen particles, generated from a 1% (w/w) aqueous solution. It shows that CAN-BD generated dimpled raisin-like particles with a mean aerodynamic diameter of 0.86 μm and 95% of particles less than 1.57 μm. Fig. 11 shows an image of trehalose particles, without any protein, generated from a 10% (w/w) aqueous solution. It shows spherical particles with a mean aerodynamic diameter of 1.34 μm and that 95% of the particles are less than 2.58 μm in diameter. All trypsinogen–trehalose mixtures processed by CAN-BD showed increasingly dimpled or raisin-like particle morphology with increasing trypsinogen concentration. Upon inclusion of 20% or more trypsinogen (mass percent in the dried solid), the morphologies of the particles were all very similar, that is, raisin-like (see Figs. 12 and 13). Similar morphologies were observed for particles generated from trypsinogen formulations containing sucrose instead of trehalose. Figure 14 shows an image of sucrose particles that were synthesized as spheres. Similar to the formulations containing trehalose, Figs. 15 and 16 show that trypsinogen–sucrose particles have raisin-like morphologies upon inclusion of 20% or more trypsinogen (mass percent in the dried solid).

The presence of the excipients (i.e., sucrose or trehalose) preserves the activity of the enzyme through the micronization and drying of the CAN-BD process (Fig. 17 shows the results for trehalose; similar results were obtained for sucrose) (123). In the absence of a stabilizing sugar, the activity of the enzyme was only 48% of its original activity when redissolved in water after CAN-BD. Previous experiments have shown that most of the damage experienced by the protein occurs during the drying process, after the aerosol is generated (91). The addition of either sucrose or trehalose in fractions constituting 80% to 89% (w/w) of the TDS was able to improve the retained activity of the trypsinogen processed by CAN-BD to near 100%. We have also measured the activity of the CAN-BD treated particles that have been stored in a vacuum chamber for 6 months over calcium sulfate desiccant. The activity of the particles remained virtually unchanged, indicating that the CAN-BD treated powders are stable for at least 6 months when kept dry at room temperature.

In formulations with very high sugar concentration (>95% of the TDS), we observed an apparent enzyme activity greater than 100% of the original. The trypsinogen was used in formulations as received, with no purification attempts prior to processing to remove any aggregated or denatured and inactive protein impurities. It is possible that some reversibly aggregated, denatured protein or improperly folded molecules were present in the as-received commercial product. During the CO₂-pressurization-depressurization cycling in CAN-BD, such inactive trypsinogen molecules may have become renatured or refolded to an active conformation, thus explaining the apparent enhancement of the originally measured enzyme activity. Similar enhancements relative to original enzyme activity have been previously observed in CAN-BD studies with a different enzyme, lactate dehydrogenase (11,117).

All trypsinogen–sugar formulations studied were analyzed by size exclusion chromatography (SEC) for the presence of soluble aggregates. No significant agglomeration of the protein after processing by CAN-BD was observed (data not shown).

To assist in the protection of the structural integrity of the protein in the dried state it is important to use excipients that will be in the amorphous rather than crystalline state (1). Both sucrose and trehalose are amorphous after processing by CAN-BD as confirmed by X-ray diffraction (data not shown).

**CONCLUSIONS**

After surveying the published literature, we conclude that SCF processes generally, and SCF anti-solvent processes particularly, have limited application to the preparation of powders and particles containing active proteins. This is due to the fact that all these SCF processes, excepting RESS, require the presence of organic solvents. One notable exception is the CAN-BD process, which can nebulize aqueous solutions without the use of an organic solvent, which almost invariably causes degradation of sensitive biomolecules and vaccines. CAN-BD (or supercritical-assisted atomization or effervescent atomization) uses a dense gas (near-critical or supercritical CO₂), not as a solvent or anti-solvent, but as a tool to facilitate aerosolizing a solution of interest to form.
microdroplets and microbubbles, which rapidly dry to produce microparticles and nanoparticles. CAN-BD has been successfully applied to a wide range of substances (e.g., small-molecule pharmaceuticals, protein therapeutics and various excipients) formulated as aqueous or organic (e.g., methanol, ethanol, acetone, etc.) solutions. The resulting powders routinely consist of particles with mean aerodynamic diameter between about 1 to 2 μm, with 95% of the particles usually less than 3 to 5 μm in diameter, making such powders suitable for use in dry powder inhalers or in metered dose inhalers. In addition to these desirable particle size characteristics, appropriately formulated protein-containing particles can be readily produced by CAN-BD without process-induced physical degradation (e.g., protein aggregation) or unacceptable loss of biological activity. This has been repeatedly demonstrated, in previous publications (11,69–75,91,92,101,105,116,117) with various biologicals (such as the enzymes lysozyme and lactate dehydrogenase, human IgG antibody, aluminum hydroxide adjuvanted hepatitis B surface antigen, and attenuated live virus measles vaccine) and in this publication with two therapeutically interesting proteins (anti-CD4 antibody and AAT) and a model enzyme (trypsinogen). Fine dry powders of anti-CD4 antibody or AAT, produced by CAN-BD from buffered solutions containing sugars and surfactants as stabilizing excipients, retained full biological activity upon redissolution with water and no detectable process-induced aggregation was observed by size-exclusion chromatography. Enzymatic activity of trypsinogen was essentially fully retained when sucrose or trehalose was included at 80% or more of the dry powder mass. Apparent activities greater than 100% of the original as-received enzyme were reproducibly achieved for trypsinogen formulations that contained very high sugar content (98–99% of the dry powder mass). Presumably, these enhanced activities occur when previously inactive protein molecules are returned to a native conformation through some as yet unknown mechanism during the CAN-BD processing (CO2 pressurization, depressurization, and aerosolization and drying) of trypsinogen in a favorable environment (i.e., high sugar concentrations). Similar activity enhancement results have been previously observed for another enzyme, lactate dehydrogenase (11,117).

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