Recent Advances Using Supercritical Fluid Techniques for Pulmonary Administration of Macromolecules via Dry Powder Formulations

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Abstract: Growing demands on a suitable formulation method that ensures the stability of the active compound coupled with the limitations of current methods (milling, lyophilization, spray drying, and freeze spray drying) has brought wide attention to supercritical fluid (SCF) technology. Advantages of using the SCF technology comprise its high abilities, adaptability in providing alternative processing methods, high compressibility and diffusivity of the supercritical fluid, capability as an alternative for conventional organic solvents, and the option to attain different processing parameters which would be otherwise difficult to conduct with traditional methods. This review proposes to present an up-to-date outlook on dry powder pulmonary formulations of macromolecules using SCF technology.

Keywords: Dry powder, inhalation, macromolecules, microparticles, peptide, protein, super critical fluid.

1. INTRODUCTION

Over the past two decades a rapid growth of innovative technologies for producing novel therapeutic agents has arisen. This growth has mostly been initiated by the discovery of new therapeutic agents, such as macromolecules, together with an increased understanding and knowledge of pathophysiology [1]. Consequently, novel macromolecules (also known as biotechnology-derived pharmaceuticals, biotherapeutics, or biological drugs and include recombinant therapeutic proteins, monoclonal antibody based products and nucleic acid-based medicinal products) can now be produced with preferential selectivity for definite targets [1, 2]. To date many macromolecule based drugs are in clinical trials or at approval phase (Table **1**) [3]. Over the past decade pharmaceutical research and development has been focused on developing macromolecules for treatment of many diseases, and are preferred due to their greater selectivity; lower disruption of normal biological processes; effective substitutional therapy in mutated or deleted normal protein and less clinical development time in addition to a shorter FDA approval period [4,5].

However, most of these macromolecules must be administered repeatedly using an invasive manner to reach a therapeutic concentration, even though these procedures would be painful to the patients [6-8]. As an alternative to repeated injection, pulmonary inhalation could enhance macromolecule administration because it is a non-invasive technique for local and systemic drug delivery. The lungs own many favorable features, including large surface area for absorption, highly vasculatures, thin epithelium in the alveolar tissue and short path of gas–blood exchange movement [8]. However, formulating macromolecules into suitable pulmonary delivery systems remains a challenge.

A variety of micro/nanoparticulate systems such as polymer based micro/nanoparticles, liposomes and solid lipid micro/nanoparticles have been used for the encapsulation of macromolecules serving specific therapeutic purposes such as controlled release or targeted drug delivery. Major concerns on the formulation method of macromolecules must be addressed when dealing with these molecules [7]. The stability and biological activity of macromolecules are extensively dependent on their entire structures, and can be easily altered by physical means (e.g. denaturation, adsorption, or noncovalent aggregation) or chemical means (e.g. oxidation, deamidation, or peptide cleavage). The impact of modifications can be very complicated (losses in therapeutic activity, changes in absorption, biodistribution, elimination, toxicity and immunogenecity). In addition, these intact structures are important characteristics which have impact on the physicochemical properties of the final therapeutic products, especially in the case of pulmonary drug delivery that require careful manipulation of the particle size, shape, density, and surface properties (Table **2**). Many different routes are employed to achieve solid-state formulations, including milling, freeze drying (lyophilization), spray drying (SD), and spray–freeze drying (SFD) (Table **3**).

Growing demands on a suitable formulation method that ensures the stability of the active compound coupled with the limitations of current methods (milling, lyophilization, spray drying, and freeze spray drying) brought wide attention to supercritical fluid (SCF) technology [2]. SCF are gases and liquids above their critical pressure (P_c) and critical temperature (T_c), and under these conditions, the molecules exhibit the flow, polarity, and solvency properties common of liquids but have the diffusion and reactivity characteristics of gases. SCF technique can be performed using carbon dioxide (CO_2), water, propane, acetone, nitrous oxide (N_2O),

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Table 1. Examples of Therapeutic Macromolecules being Investigated for Systemic Delivery via the Pulmonary Route

Type	Active Agent	Disease	Ref.
Hormone	Erythropoeitin	Anaemia	[15]
	Insulin	Diabetes	[15]
	Parathyroid hormone	Osteoporosis	[14, 15]
	Cetrorelix	LH-RH antagonist	[15]
	Follicle stimulating hormone	Fertility treatment	[15]
	LH-RH analogues	Endometriosis, prostate cancer	[15]
Growth factor	Sargramostim	Sarcoma	[15]
	Growth hormone releasing factor	Pituitary dwarfism	[14]
Blood derivative	Immunoglobulin	Trigger/modulate immune response	[15]
Interferon-beta	Interferon	Multiple sclerosis	[14, 15]
Natural extract	Calcitonin-salmon	Osteoporosis	[14, 15]
	Heparin	Inhibit thrombosis	[14, 15]
	Cyclosporine	immunosuppression	[15]
Recombinant protein	Human recombinant F.IX	Hemophilia B	[15]
Vaccine	HBsAg	Hepatitis B	[16]
	P30B2, (NANP)6P2P30	Malaria	[16]
	SPf66	Malaria	[16]
	Hemagglutinin	Influenza	[16]
	Mtb8.4 peptide	Tuberculosis	[16]
	HLA-A*0201 plasmid	Tuberculosis	[16]
	Diphtheria toxoid	Diphtheria	[16]
	Tetanus toxoid	Tetanus	[16]
	Formalin- inactived rotovirus	Rotavirus	[16]

Table 2. Product Parameters and Pharmaceutical Considerations in Drug Product Design

Parameter	Importance/Effect	Ref.
Particle size and distribution	Precise targeting Content uniformity Rates of dissolution, Rates of release, Dose delivery	[18-20]
Particle shape (morphology)	Flow property Dispersibility	[21]
Particle density	Flow property Dispersibility	[22]
Particle surface charge	Optimal surface properties of particles must be obtained to prevent particles agglomeration Flow property Dispersibility Precise targeting	[16, 21, 23]
Crystal form	Crystal form influences all steps of the development from discovery to marketing	[10]

Table 3. Common Methods for Formulations of Macromolecules

Method	Description	Disadvantages	Excipients	Ref.
Jet Milling	Bulk particles are introduced into the milling chamber. Air or nitrogen, fed through nozzles at high pressure, accelerates the solid particles to sonic velocities. The particles collide and fracture. While flying around the mill, larger particles are subjected to higher centrifugal forces and are forced to the outer perimeter of the chamber. Small particles exit the mill through the central discharge stream	Broad particle size Physical/chemical degradation	e.g. trehalose	[21]
Freeze drying (Lyophilization)	The formulation is frozen and the bulk water is removed by sublimation. The resulting cake is composed of the protein, any nonvolatile excipients, and a small amount of residual water tightly associated with the protein	Time consuming Broad particle size distribution	e.g. trehalose, inulin, dextran	[6, 11]
Spray drying	Uses atomization to form microdispersed droplets. The water in these droplets quickly evaporates when passed through a stream of hot gas, resulting in the formation of a fine powder of microparticles containing protein and excipients	Degradation Low process efficacy Time consuming	e.g. leucine, lactose, trehalose, dipamitoylphosphatidylcholine (DPPC), albumin	[8, 11, 22, 24, 25]
Spray-freeze drying	The microdispersed liquid particles are generated through the jet nozzle in the absence of heat, then collected and frozen in liquid nitrogen before sublimation occurs. The frozen particles are then lyophilized	Degradation Low process efficacy Time consuming	e.g. inulin, trehalose, manitol	[6, 8, 21, 24]
Supercritical Fluid	SCF are gases and liquids above their critical pressure and critical temperature. SCF technique can be performed using carbon dioxide (CO_2), water, propane, acetone, nitrous oxide (N_2O), trifluoromethane, chlorodifluoromethane, diethyl ether, water, or CO_2 with ethanol	Solubility of molecules in solvent Degradation Particle size control Residue of organic solvent Scale up of this process is restricted by particle aggregation and nozzle blockage due to rapid expansion cooling.	e.g. sucrose, trehalose, manitol	[2, 8, 11, 13]

trifluoromethane, chlorodifluoromethane, diethyl ether, water, or CO_2 with ethanol [13]. In addition SCF possess several fundamental advantages as solvents and/or anti-solvents for processing heat-labile solutes at low temperature. For example, supercritical CO_2 is a good solvent for water-insoluble as well as water-soluble compounds under suitable low critical conditions (T_c = 31.2 °C, P_c = 7.4 MPa) [8]. In addition, compressed CO_2 is accessible in ample proportions with a higher degree of purity [9]. Moreover, CO_2 is non-toxic, nonflammable, and inexpensive [8]. Therefore, supercritical CO_2 has potential as an alternative for conventional organic solvents used in solvent-based processes for forming solid dosage forms. Furthermore, the general consideration of SCF as a "green" substitute has become very significant as the harmful effects of residual organic solvents, from both a processing and environmental point of view, have been known, and in addition, the regulatory specifications for the utilization and residual amounts of organic solvents in the final pharmaceutical product become more stringent [10].

There are two major principles based on SCF. The first process utilizes supercritical fluids as a solvent. The Rapid Expansion of a Supercritical Solution (RESS) process is the method that represents this first principle. Initially the solute must dissolve in a SCF followed by sudden decompression, after which the solution is rapidly expanded at low pressure by passing through an orifice. The restrictions facing application of RESS for macromolecules formulations include: it is restricted to molecules that are soluble in SCF CO_2, relatively high temperature required for the rapid expansion (typical temperature of 40 ºC) which can destroy proteins, lacking control of particle size, and scale up of this process is restricted by particle aggregation and nozzle blockage due to rapid expansion cooling [8].

The second process involves the solute being insoluble in SCF and hence utilizes SCF as an antisolvent. Many macromolecules which are suitable as therapeutic agents are slightly soluble in SCF and have high solubility in water. These processes use SCF as an antisolvent where a solute is dissolved in an organic solvent then precipitated by SCF. Precipitation develops when the SCF is absorbed by the organic solvent followed by expansion of the liquid phase and decreases in the solvent power leading to particle formation. The Gas Anti-Solvent (GAS), Aerosol Solvent Extraction System (ASES), Supercritical Fluid Antisolvent (SAS), Precipitation with Compressed Antisolvent (PCA), Solution Enhanced Dispersion by Supercritical Fluids (SEDS), and

supercritical fluid extraction of emulsion (SFEE) are the processes that exemplify this second group Fig. (**1**). The main disadvantage of the second process that applies SCF as antisolvent is the difficulty to remove the residual organic solvent completely [8].

Thorough discussions of differences in the above techniques have been recently published elsewhere [8-13]. This review proposes to present an up-to-date outlook on the progression of dry powder formulations for inhalation of macromolecules using SCF techniques, covering its challenges, possibilities and recent developments.

2. SUPERCRITICAL FLUID APPLICATIONS IN PREPARATION OF MACROMOLECULES FOR DRY POWDER INHALATION

Unlike conventional methods of particle formation such as milling, lyophilization, spray-drying, and freeze-spray drying, where large particles are reduced to the intended size, SCF technology includes developing the particles in a controlled approach to achieve the intended size. Therefore the negative effect of the energy transmitted to the system such as denaturation of protein to produce the desired size can be avoided. Ideally, the particles once formed must not be subjected to further treatment and this property makes SCF technology suitable to produce macromolecules in their native pure state and/or encapsulating these agents [13].

Advantages of using the SCF technology comprise its high abilities, adaptability in providing alternative processing methods, the high compressibility and diffusivity of SCF, capability as an alternative for conventional organic solvents, and the ability to reach different processing parameters (such as pressure and the rate of solvent evaporation) which would otherwise be difficult to conduct with conventional methods [10].

2.1. Encapsulation of Insulin

SCF have unique properties as mentioned above that makes them applicable for various processing methods such as particle formation and extractions. Recently, a significant interest in SCF technology has been revealed in an attempt to find other preferable approaches of insulin processing.

Todo *et al.* [26] examined the absorption of insulin dry powders processed with mannitol (carrier) and citric acid (an absorption enhancer) by the SCF process known as solution enhanced dispersion by supercritical fluids (SEDS) with that prepared by spray drying (SD) technique. In this investigation insulin powder was precipitated by dispersing the insulin aqueous solutions through V-type nozzles into supercritical CO_2/ethanol/water ternary system. Water feed rate, liquid CO_2 feed rate, ethanol feed rate, pressure, and temperature were 0.035 mL/min, 5.7 g/min, 0.665 mL/min, 15 MPa, and 35 ºC, respectively. These operating conditions enabled the water to be miscible with the non-polar CO_2. A manual injector was used to introduce aqueous 0.25 % insulin solution with 0.2% citric acid and 5.0 % mannitol into the water stream. For dry powders (0.25 % insulin solutions containing 5 % mannitol and 0.20 % citric acid) prepared by SD technique the following standard operating parameters were used: an inlet temperature of 90 ºC, a drying air flow rate of 0.75 m^3/min, a solution feed rate of 5 mL/min, and an atomizing air pressure of 100 kPa. The authors found that insulin

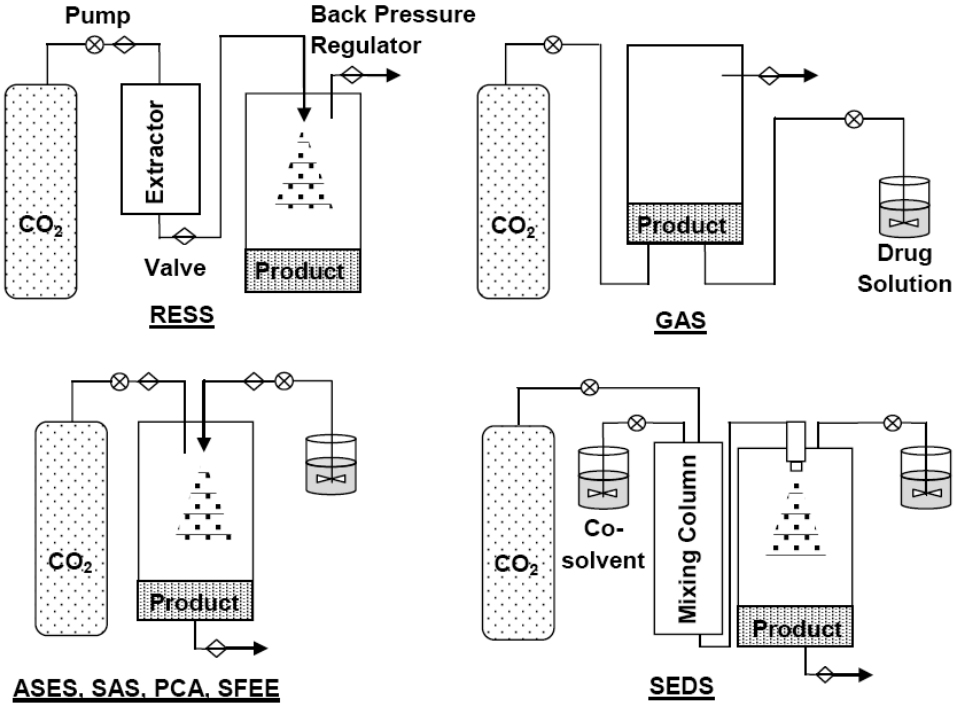

Fig. (1). Schematic diagrams of various particle-formation processes using supercritical fluid technology. RESS: Rapid Expansion of a Supercritical Solution, GAS: Gas Anti-Solvent, ASES: Aerosol Solvent Extraction System, SAS: Supercritical Fluid Antisolvent, PCA: Precipitation with Compressed Antisolvent, SEDS: Solution Enhanced Dispersion by Supercritical Fluids, and SFEE: supercritical fluid extraction of emulsion.

powder prepared by the SCF method showed enhanced aerosolisation efficiency at 28 L/min using an Andersen Cascade Impactor in comparison with insulin powder prepared by the SD method. For example, SCF insulin powders had the greatest quantity of powder retrieved from stages 2–7 and a mass median aerodynamic diameter of 3.2 μm with respirable fraction of 47.6 %. The hypoglycemic effect of insulin powder processed by SCF after intratracheal application was higher than that produced by SD. In addition, they found that SCF technique provided the largest yield of insulin powder (>80 %) although it was on a laboratory scale production. These results demonstrated that the SCF process would be beneficial to prepare inhalable insulin powder.

Amidi *et al.* [27] demonstrated that PCA-type SCF technology is an appropriate method to manufacture inhalable insulin-loaded microparticles with specific particle properties and retained structure of insulin. The authors used N-trimethyl chitosan (TMC), as a mucoadhesive absorption enhancer, and dextran as carriers for insulin, and prepared two formulations of 10 % (w/w) of insulin/TMC or insulin/dextran. The particles were formed by spraying an acidic water/dimethyl sulfoxide (DMSO) solution of insulin and polymer within supercritical CO_2. The flow rates of polymer-insulin solution and CO_2 were 4.5 mL/min and 333 g/min, respectively. The pressure and temperature were maintained at 110 bar and 40 °C. The mass median aerodynamic diameter was 4 μm and the percentages of the emitted dose were 62 and 67 % for insulin/TMC and insulin/dextran respectively. The water content of the particles was 4 % (w/w), and neither crumpled nor agglomerated after formation and storage. The intact structure of insulin in the freshly formulated dried insulin powders were monitored by circular dichroism spectroscopy, which indicated the secondary and tertiary structures of insulin was retained in all preparations. Furthermore, at the end of one-year storage at 4 °C, the particle properties were preserved and the insulin structure almost retained in the TMC powders.

Kim *et al.* [28] compared micronized insulin using mannitol as a stabilizer alone or with the addition of trehalose as a second stabilizer using a process known as aerosol solvent extraction system (ASES) SCF technology. They injected co-currently the solution of insulin/mannitol (15/85 wt.%) with or without 10 and 15 wt.% trehalose with the CO_2 into the precipitation vessel using the solvent, N,N-dimethylformamide (DMF) at 35 °C, 180 bar, 8 mg/mL solution concentration, and 5 ml/min solution flow rate. Results indicated that when trehalose was used as a second stabilizer, the particles were almost uniform, more spherical, less adhesive, and less aggregated in air flow, in comparison to insulin mannitol particles alone. The mass median aerodynamic diameter of the insulin/mannitol particles was ~5 μm and of the insulin/mannitol/trehalose particles was ~2.32 μm, which are appropriate for inhalable dosage from. *In vitro* aerosolisation deposition test conducted with a micro-orifice uniform deposit impactor (MOUDI-II™Impactor) at 30 L/min revealed 69 % by weight of the insulin/mannitol and 41 % by weight of the insulin/manitol/trehalose particles was deposited on stages 3, 4, 5 and 6. This study showed the ASES process was able to retain the secondary structures of insulin in both insulin/mannitol and insulin/manitol/trehalose particles.

2.2. Gene Powders

Polymer based non-viral gene delivery systems have been shown to offer protection from nuclease degradation, increased plasmid DNA (pDNA) uptake and sustained duration of pDNA action [29]. These gene delivery systems can be prepared from biologically compatible and degradable polymers e.g. poly (d,l-lactic-co-glycolic) acid (PLGA). The effectiveness of gene therapeutic systems depends on the ability of such system to deliver nucleic acid into the target cells. Gene dry powders are anticipated to have further advantage of prolonged shelf life of the preparation [29].

Okamoto *et al.* [29] compared stability of a chitosan–pDNA complex powder processed by a SEDS-type SCF technique and gene solution alone for inhalation. They precipitated the gene powder by dispersing an aqueous chitosan–pDNA complex solution with mannitol into the stream of a SCF CO_2/ethanol admixture. In the mixing column, the CO_2 was admixed with ethanol at a flow rate of 5.7 g/min and 0.665 mL/min respectively. Using these operating conditions resulted in complete miscibility of ethanol, water, and CO_2. The admixture was dispersed into the particle formation vessel at 35 ºC and 15 MPa using one end of the V-shaped nozzle, while the other end of the V-shaped nozzle was used to disperse water at a flow rate of 0.035 mL/min. The aqueous chitosan–pDNA complex solution (0.4 mL) was manually injected into the water stream and the dry powder was collected from the depressurized vessel. This procedure resulted in powder yields of ~80 % and aerodynamic particle sizes of ~3 μm, which was appropriate for pulmonary delivery. Scanning electron microscopy examination revealed the powders to have rectangular shape. Gene integrity and transfection potency were determined by electrophoresis and *in vivo* pulmonary transfection test in mice. The SCF technique minimized the supercoiled DNA during the formulation processing; although, the reduction in the remaining supercoiled and open circular DNA in the powders during storage was more prolonged than in solutions. As a result, the powders generated using SEDS had increased transfection potency in comparison to the gene solutions for the same quantity of DNA.

Mayo *et al.* [30] developed a SCF extraction of emulsions (SFEE) technique based on CO_2 for formulating nanoparticles having high plasmid (pFlt23K, an anti-angiogenic pDNA capable of inhibiting vascular endothelial growth factor (VEGF) secretion) loading and loading efficiency. First they prepared a lipophilic phase by dissolving PLGA into ethyl acetate and the inner aqueous phase composed of pFlt23K Tris-EDTA buffer. The aqueous phase was sonicated for 1 min (15W) with the lipophilic phase to obtain a 1° emulsion (w/o). The outer aqueous phase consisted of 0.5 % (w/v) polyvinyl alcohol (PVA) saturated with ethyl acetate. The 1° emulsion was further sonicated with the outer aqueous phase for 3 min to obtain the 2° emulsion (w/o/w). The emulsion extraction and particle production was performed using coaxial SFEE apparatus consisting of a cylindrical pressure vessel, placed in a temperature controlled water bath at 45 °C. Supercritical CO_2 was injected via a syringe pump to the base of this vessel. The emulsion was introduced counter currently by means of a 100 μm capillary

tube within a 1/16" stainless steel high pressure tubing at the top of the vessel, permiting CO_2 to flow continually from the vessel. During the process the pressure and flow rate of the emulsion were kept at 8 MPa and 0.4 ml/ min, respectively. Results revealed spherical particles with smooth surface and 280 nm in diameter. In addition, a high loading of pFlt23K (19.7 %, w/w) and high encapsulation efficiency (>98 %) was achieved, while a low residual solvents (<50 ppm), attributed to rapid particle formation due to successful solvent removal afforded by the SFEE technique. *In vitro* transfection of pFlt23K-PLGA nanoparticles were able to significantly reduce secreted VEGF from human lung alveolar epithelial cells (A549) with normoxic and hypoxic status, with no cytotoxicty detected.

2.3. Vaccination

Recently immunization by inhalation has been investigated as a substituent for parenteral vaccination. Vaccines delivered via the lung route can initiate both systemic and local immune responses due to the presence of extensive dendritic cells and macrophages lining the respiratory epithelium.

Amidi *et al.* [31] examined the potential of N-Trimethyl chitosan (TMC) and dextran microparticles for pulmonary delivery of diphtheria toxoid (DT). DT-loaded microparticles were formulated by spraying an aqueous solution of DT/dextran (w/w) or DT/TMC into supercritical CO_2 and ethanol using SEDS type SCF technology. When the operating temperature (38 ºC) and pressure (100 bar) was achieved, CO_2 and ethanol were completely miscible forming a single supercritical phase. The ethanol/CO_2 mixture was immediately introduced via a concentric coaxial two-fluid nozzle and sprayed into the vessel for 5 min. This was subsequently followed by pumping the aqueous polymer-DT solution into the T-mixer at a flow rate of 0.5 ml/min using two syringe pumps. This was mixed with SCF-CO_2 (367 g/min) and ethanol (25 ml/min) and fed into the precipitation vessel through the concentric coaxial two-fluid nozzle. Smooth spherical particles were produced with median volume diameter of ~3 μm and the fine particle mass fractions less than 5 μm, as revealed by cascade impactor analysis at 30 L/min, were 35 % for the dextran and 56 % for the TMC formulations.

This study demonstrated that the generated powders had a significant mass fraction of particles less than 5 μm, suitable for inhalation. In addition, this investigation revealed that TMC microparticles were a potent pulmonary delivery system for DT antigen. Pulmonary immunization with DT-TMC microparticles containing 2 or 10 Lf of DT produced a strong immune response as manifested by the production of IgM, IgG, IgG subclasses (IgG1 and IgG2) antibodies similar to or significantly greater than those gained after subcutaneous application of alum-adsorbed DT (2 Lf). In addition, the IgG2/IgG1 ratio after pulmonary immunization with DT-TMC microparticles was significantly greater in comparison with subcutaneous administered alum-adsorbed DT. While pulmonary administrations of DT-dextran particles produced a very poor immune response.

3. CONCLUSION

There has been great progress in supercritical CO_2 techniques to increase performance of macromolecules without losing the biological activity of these sensitive molecules. However, there are still possibilities for optimizing processing parameters, such as temperature, pressure, flow rates, and concentration of ingredients. In addition, processing of therapeutic macromolecules with supercritical CO_2 permit a wide range of macromolecules to be processed, and in the future it is expected that SCF techniques will provide a more favorable alternative to producing formulations encapsulating macromolecules.

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