Protein Nanoparticles

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ABSTRACT

Protein nanoparticles can be utilized for the pulmonary delivery of protein therapeutics or can be incorporated into biodegradable polymer microspheres/nanospheres for controlled release depot or oral delivery. With proteinaceous therapeutics, the generation of nanoparticles is particularly problematic. Existing practices have difficulty achieving the desired particle size distribution, expose the protein to denaturing conditions such as heat, organic solvents or air and often require stabilizing excipients or leave residual product contamination that necessitates further processing. Our process utilizes supercritical, critical or near-critical fluids with or without polar entrainers (*SuperFluids™*) to penetrate the protein aggregates and then utilizes the expansive energy of *SuperFluids™* to disaggregate protein crystals into monodisperse nanoparticles.

BACKGROUND

With proteins, the generation of nanoparticles by conventional processing methods is particularly problematic (Table 1).

Method	Drawbacks
Mechanical milling	Protein denaturation by mechanical shear and/or heat
Spray drying	Protein denaturation at gas-liquid interface and/or heat for solvent vaporization
Fluid energy grinding	High velocity gas can yield electrostatically charged powders, size reduction inefficient for soft proteinaceous powders
Lyophilization	Broad size distribution, protein specific applicability and protocols
Antisolvent precipitation	Protein denaturation by organic solvents, solvent removal, solvent residuals, particle size control difficult
Salt precipitation	Protein specific applicability, salt removal or contamination, particle size control difficult

Table 1. Conventional Protein Size Reduction Methods

Over the past decade, it has been demonstrated that compressed gases, liquefied gases and materials intermediate to gases and liquids known as supercritical fluids can be used to create fine uniform powders.

As shown by the pressure-temperature diagram in Figure 1, a pure compound enters its supercritical fluid region at conditions that equal or exceed both its critical temperature and critical pressure. These critical parameters are intrinsic thermodynamic properties of all sufficiently stable pure component compounds. Carbon dioxide, for example, becomes supercritical at conditions that equal or exceed its critical temperature of 31.1°C and its critical pressure of 7.38 Megapascals (MPa). In the supercritical or near-critical fluid region, normally gaseous substances become dense phase fluids that have been observed to exhibit greatly enhanced thermodynamic properties of solvation, penetration, selection and expansion. At a pressure of 21 MPa and a temperature of 40°C, carbon dioxide has a density around 0.8 g/ml and behaves very much like a nonpolar organic solvent. Selectivity can be modified by utilizing small quantities of polar entrainers.

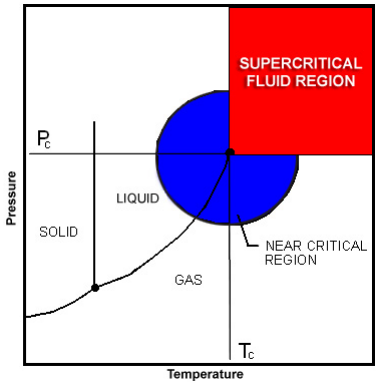

Figure 1: Supercritical Fluid Phase Diagram

A supercritical fluid solvent can simultaneously exhibit a liquid-like density and gas-like properties of viscosity and diffusivity. The latter increases mass transfer rates, significantly reducing processing times. Additionally, the ultra-low surface tension of a supercritical fluid allows facile penetration into microporous materials, increasing contact efficiency and overall process yields. Supercritical fluids, critical or near-critical solvents with/without polar cosolvents such as an alcohol are jointly referred to as *SuperFluids™* [SFS].

SUPERCRITICAL FLUIDS NANOPARTICLES TECHNOLOGIES

The readily adjusted solubility of compounds in supercritical fluids is the basis of a number of precipitation processes that have appeared in the literature [1]. The materials of interest are first dissolved in the supercritical fluid and then rapidly expanded into a lower pressure chamber (usually at atmospheric pressure but could be under vacuum). This rapid expansion of supercritical fluid solutions (REES) technique [2] is limited, however, to compounds that have significant solubility in the supercritical fluid for at least some temperature and pressure conditions. Many materials of interest, such as proteins and peptides, are too polar to dissolve to any extent in supercritical fluids and hence do not fulfill this criterion. It is this limitation that has led to the development of the gas antisolvent (GAS) technique, which name refers to the fact that compressed gases (or liquefied or supercritical gases) are used as antisolvents [3-5].

In the GAS method, a compressed fluid is added to a conventional organic liquid solvent containing the solute to be crystallized. If compressed gas is used as the antisolvent, its dissolution into the solvent is typically accompanied by a reduction in density and change in polarity of the solvent mixture, and consequently, a reduction of the liquid's solvation power relative to a particular substance. As a result, the mixture becomes supersaturated, which causes crystals to form. If a supercritical fluid or liquefied gas is used as the antisolvent, however, the mixture density may actually be greater than that of the neat solvent. In this case, precipitation is presumably caused primarily by the polarity change of the mixture. In the GAS crystallization process, it is essential that solids have low solubility in the selected compressed fluid and bioactivity is retained [3,5].

Yeo *et al.* [3] carried out *in vivo* testing of the supercritical fluid processed insulin in rats, finding full retention of activity. Winters *et al*. [5] carried out secondary structure analysis of their supercritical fluid processed proteins using Raman and Fourier transform infrared spectroscopy and found major conformational changes. However, upon re-dissolution in aqueous solution, the proteins appeared to largely regain their native configuration. The conclusion to be drawn from these studies is that, at least for some proteins, exposure to high pressure, supercritical solvents and shearing during decompression does not significantly damage proteins. Limitations to the GAS technique include use of organic solvents that could be denaturing to proteins, limited solubility of proteins in organic solvents and consequential use of large volumes of organic solvents that can create environmental disposal problems.

SUPERFLUIDS™ GENERATED PROTEIN NANOPARTICLES

Supercritical fluids are not generally expected to solvate proteins. Disaggregation, however, does not necessarily require dissolution. The secondary and tertiary structure of proteins and protein aggregates are partially dependent on hydrophobic interactions. If these interactions can be weakened by a surrounding and penetrating supercritical fluid, protein particles may become susceptible to breaking apart by the flow shear, mechanical impact and/or expansion of the interstitial supercritical fluid that occurs during rapid depressurization. Furthermore, breakage of the protein particles may be aided by the low temperatures resulting from the expansion process. The low temperatures can make the protein particles relatively brittle and susceptible to fracture. Also, rapid expansion of carbon dioxide (or other supercritical fluid) leads to the formation of small crystals of dry ice. If these crystals form within the interstices of the protein agglomerates, they can aid in the breakage process. Thus, supercritical fluids may effect the disaggregation of protein particles by several possible mechanisms. This process is not limited by the solubility of proteins in supercritical fluids and does not require the use of organic solvents [6].

Protein nanoparticles can be generated in the *SuperFluids*™ protein nanoparticles apparatus shown in Figure 2. This apparatus consists of three major components: a supercritical fluid pumping or pressurizing system (3); a contacting chamber (8); and a de-pressurization and product recovery receptacle (15).

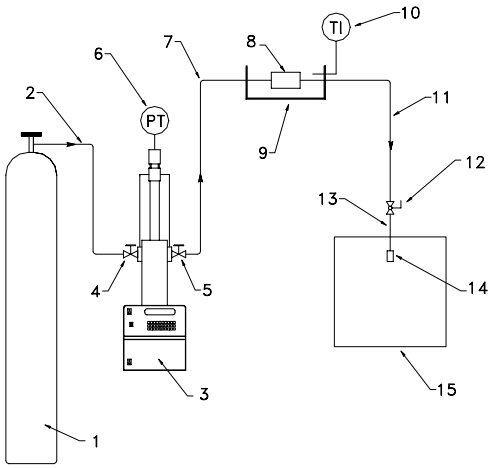

Figure 2: Schematic Diagram of Experimental *SuperFluids*™ Protein Nanoparticles Apparatus

After depressurization, protein nanoparticles are collected from the depressurization receptacle (15) for analysis and further processing.

Impact of Pressure: As shown in Figure 3 for bovine serum albumin (BSA), protein size reduction increases non-linearly with pressure, approaching an asymptote between 21 and 28 MPa for carbon dioxide. This trend is supported by the mechanism that higher pressures will result in higher expansive forces. The data indicates that extremely high pressures are not required for the efficient generation of protein nanoparticles utilizing this technology since the density of most fluids approaches an asymptote at a value of 3 times the critical pressure.

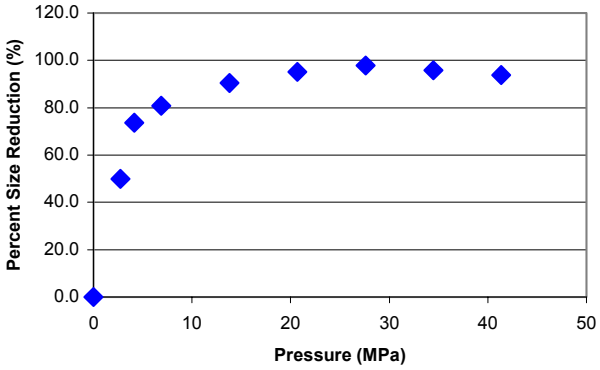

Figure 3: Protein (BSA) Nanoparticles Formation as a Function of Pressure

Impact of Protein Type: The *SuperFluids™* protein nanoparticles technology was optimized in terms of fluid type, pressure, temperature and residence time for the generation of insulin nanoparticles with a size range 1.5 to 4.0 μm (for pulmonary delivery) from 10 to 20 μm insulin crystal aggregates. The optimal conditions established for the generation of insulin nanoparticles were utilized to evaluate the impact of the *SuperFluids™* protein nanoparticles (PNP) technology on different protein types and molecular weights. This evaluation, summarized in Table 2, indicates that the technology is relatively robust for a wide range of proteins, molecular weights and particle sizes.

Protein	MW	Particle Size Before (μm)	Particle Size After (μm)
Insulin	5,500	10-20	1.5-4.0
α-amylase	50,000	150-850	1.5-7.0
Lysozyme	14,000	50-600	1.5-6.5
α-chymotrypsin	25,000	50-700	1.0-5.0
Alkaline Phosphatase	140,000	500-1000	1.0-4.0

Table 2: Impact of *SuperFluids™* PNP Technology on Different Proteins

Impact on Bioactivity: The impact of the *SuperFluids™* PNP technology on the biological activity of different proteins was evaluated by a number of different techniques. The impact of *SuperFluids™* PNP on alkaline phosphatase (which is very sensitive to denaturation and provides a stringent test of the technique) is shown in Table 3.

Sample No.	Particle Size (μm)	Change in Absorption (ΔA) @ 410 nm			ΔA_{avg} @ 410 nm
Ref.	500-1000	3.077	3.126	3.075	3.093±0.033
PNP-109	1.0-4.0	3.014	3.075	3.261	3.117±0.144

Table 3: Impact of *SuperFluids™* PNP Technology on Alkaline Phosphatase

The data indicates that *SuperFluids™* PNP has negligible impact on alkaline phosphatase. On the other hand, *SuperFluids™* PNP appears to have a significant positive impact (~ 3 fold) on the enzymatic activity of α-amylase as shown in Table 4.

Sample No.	Particle Size (μm)	Activity (U/L) at 37°C			$Activity_{avg}$ (U/L) at 37°C
Ref.	150-850	1,209	1,792	1,119	1,373±419
PNP-106	1.5-7.0	4,189	4,427	3,268	3,961±693

Table 4: Impact of *SuperFluids™* PNP Technology on Alpha Amylase

The biological activity of recombinant insulin, measured by quantitative HPLC analysis, is shown in Table 5. The chromatograms (not shown) indicate that the retention time and UV spectral characteristics are almost identical. However, as shown in Table 5, there is an increase of 15 to 20% in biological activities of the nanoinsulin particles. This increase could be the result of enhanced exposure of the bioactive sites of insulin nanoparticles.

Sample No.	Particle Size (μm)	Conc. (mg/ml)	Area	Computed Conc. (mg/ml)	Activity (% of Mass)
Ref.	10-20	1.025	2,517,795	1.025	100.0
PNP-96	1.5-6.0	0.250	715,422	0.291	116.4
PNP-98	1.5-4.0	0.225	662,761	0.270	120.0

Table 5: Impact of *SuperFluids™* PNP Technology on Recombinant Insulin

Impact on Structural Integrity: The impact of the *SuperFluids™* protein nanoparticles process on the structural integrity of insulin was evaluated by IR spectroscopy, zeta potential, SDS-PAGE and HPLC.

Fourier Transform Infrared (FT-IR) spectroscopic analyses of *SuperFluids™* insulin nanoparticles (PNP-105) and untreated insulin were performed on a Nicolet Magna 550 FT-IR by Shuster Laboratories, Inc., Smyrna, Georgia. The FT-IR spectra, data not shown, were spectrally similar suggesting that there were no significant changes in the structural integrity of insulin after SFS treatment at 21 MPa.

The zeta potential, electrophoretic mobility and particle sizes of *SuperFluids™* insulin nanoparticles and untreated insulin were analyzed by Beckman-Coulter Particle Characterization SAT Laboratory, Hialeah, Florida using a Beckman Coulter Delsa 440SX Zeta Potential-Electrophoretic Mobility Analyzer. 20 milli-grams of the samples were diluted in a 20 ml volume mixture of 95% methylene chloride and 5% acetone. The results of these analyses are summarized in Table 6.

Sample No.	Particle Size (μm)	Mean Mobility (μm-cm/V-s)	Mean Zeta Potential (mV)	Size (nm)
Ref.	10-20	0.435	21.7	---
PNP-105	1.5-4.0	-0.154	-8.3	558

Table 6: Zeta Potential and Electrophoretic Mobility of *SuperFluids™* Insulin Nanoparticles

The data indicates that the untreated insulin particles were trimodal with an average positive zeta potential of 21.7 mv whereas the *SuperFluids™* insulin nanoparticles were unimodal with an average negative zeta potential of 8.3 mv. The trimodal characterization agrees with microscopic observations that untreated insulin particles were in the 3 to 20 μm range (lots of 10 μm and 20 μm particles with a scatter of 3 to 5 μm particles). The unimodal characterization of the *SuperFluids™* insulin nanoparticles is also in agreement with microscopic observations that the insulin nanoparticles were monodisperse and in the 1.5 to 4.0 μm range. The latter, however, does not agree with the DELSA 440SX data that yielded a particle size of 558 nm with a standard deviation of 93 nm. This inconsistency could be the result of larger particles settling out of the suspending medium during zeta potential measurements.

In SDS-PAGE, both samples showed up just above the molecular weight band for the insulin, B-chain standard of 3 KDa (data not shown). The insulin nanoparticles bands were, however, much more intense than the untreated insulin band, even though similar quantities of proteins were used in all test wells.

The *in vivo* biological activity of insulin bears a strong correlation to its HPLC profile. HPLC analyses are often utilized as a QC method for the biological activity of insulin. HPLC analyses of untreated insulin and *SuperFluids™* insulin nanoparticles were measured on an insulin-specific, high molecular weight column (Waters Corporation, Milford, Mass.). The 2-D Photodiode Array spectra and 3-D HPLC chromatograms of the untreated insulin and the *SuperFluids™* insulin nanoparticles were identical. The data in Table 5 suggests that there was a 15 to 20% increase in the biological activity of the *SuperFluids™* insulin nanoparticles.

CONCLUSIONS

The *SuperFluids™* PNP technology can be used to form monodisperse protein nanoparticles without having to first dissolve the protein in a solvent. The process is thus not constrained by limited solubility of proteins in benign solvents such as ethanol that may be used with other supercritical fluid techniques, and the utilization of large volumes of liquids that limits other techniques such as spray drying. The process also produces a Joule-Thompson cooling effect and thus avoids heat generated by milling techniques that can result in thermal degradation as well as contamination by metal particles

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