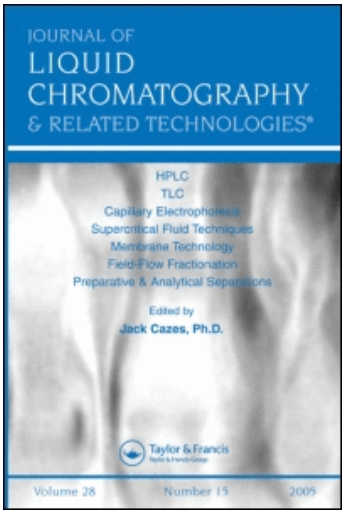

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Nanonization of Insulin from Dimethylsulfoxide Solution Using Supercritical Carbon Dioxide as an Antisolvent

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Abstract: Insulin was precipitated from solution in dimethylsulfoxide using supercritical carbon dioxide as an antisolvent. Solution enhanced dispersion by supercritical fluids (SEDS) process was applied to produce nano-size insulin particles. We used dimethylsulfoxide to help the supercritical carbon dioxide to extract water from the aqueous protein solution. Various sizes of insulin nano-particles were successfully prepared with a narrow particle size distribution from aqueous dimethylsulfoxide solution without using any additive. The theoretical particle sizes were deduced from the calculated droplet sizes based on a modified Jasuja's equation. The calculated mean particle sizes and the experimentally obtained ones were compared and the results showed an excellent correlation coefficient (R^2) of 0.9738.

Keywords: Nano-particle, Supercritical fluid, Insulin

INTRODUCTION

The administration of drugs to the lung via inhalation is a promising alternative to injection or oral administration. Small nano-particles with a narrow particle size distribution are desirable for applications in pulmonary delivery and controlled release systems.[1,2] In the case of protein drug administration through the lung by way of inhalation, the small particle size is especially important, because not only particles larger than 5 μm tend to

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fall out of the gas stream and deposit in the mucosa of the upper airways before reaching the alveoli of the lungs but incompletely dissolved protein particles can cause undesired side effects such as inflammation or immune response against the drug protein itself. Pulmonary delivery of a protein drug can avoid the hepatic first pass effect of and the digestive process typical for an oral administration, as well as the discomfort of the needle injection associated with a parenteral one. A good example where such pulmonary delivery can significantly improve the patient's comfort is the insulin inhalation therapy for diabetics, an issue which has already been investigated in clinical studies.[3] Formulation technology developed for manufacturing nanoparticles of a protein drug for a pulmonary delivery system, in addition, has a far reaching potential as a general tool for developing protein drug formulations that enable hitherto unrealized applications, such as an oral or topical delivery of protein drugs with or without controlled release features.[1] In many cases, sustained release formulations of drugs are desirable in order to alleviate immediate concentration bursts of the therapeutic agents.

Insulin is a peptide hormone composed of 51 amino acid residues and has a molecular weight of 5808Da. It is produced in the Islets of Langerhans in the pancreas.

It consists of two chains, A (21 residues) and B (30 residues) and is the post translational product of a single chain precursor, namely proinsulin, which possesses an extra linking peptide of varying lengths depending on the species. The three disulphide bonds are essential in maintaining the folding and stability of the protein.

Proteins, such as insulin, are often prone to denaturation or degradation if exposed to a high temperature. Therefore, their nanonization through mechanical comminution or milling is almost impossible, since these processes generally produce heat raising temperature significantly above the ambient temperature. Furthermore, milling or grinding often results in electrostatically charged particles with rather a broad particle size distribution. Thus, conventional and commonly used nondestructive processes for producing protein powder has relied on lyophilization, spray drying, crystallization, or precipitation from a target protein solution. Protein crystallization may be induced by applying a temperature gradient, by the addition of a liquid antisolvent, or by adjusting the pH value of the solution to the isoelectric point of the protein. All of these processes, however, suffer from various drawbacks. Spray drying may often induce denaturation of the proteins and, thus, entail low yields. Lyophilization often produces protein particles that are not small enough for either pulmonary delivery or encapsulation into controlled release microspheres.[4] Other processes necessitate employment of large amounts of organic solvents and subsequent downstream separation and recovery steps of the solvents. Moreover, these processes may also lead to high residual solvent contents in the resultant protein powders.

Particle formation techniques based upon the use of near- or supercritical fluids are an interesting alternative to the processes mentioned above as they

overcome most of the drawbacks of conventional particle size reduction techniques and allow, at the same time, production of submicron particles with controlled particle size distributions under mild and inert conditions. This is particularly the case when nontoxic, low cost carbon dioxide is used as near critical fluid solvent or antisolvent ($T_c = 31.1^{\circ}C$, $P_c = 73.8$ bar). In the rapid expansion of supercritical solutions (RESS) process, supercritical CO_2 is used as a solvent to achieve particle formation by expanding binary mixtures of solvent and solute across capillary or ultrasonic nozzles. The application of RESS to protein precipitation is hindered by the drastically low solubility of proteins of practical interest in high pressure carbon dioxide. Antisolvent techniques, on the other hand, make use of the low solubility of most proteins and many pharmaceutical compounds in CO_2. In fact, CO_2 is used as an antisolvent for the solute, which is initially solubilized in a conventional organic solvent that is completely miscible with carbon dioxide. Upon carbon dioxide addition to the initial solution, the solute precipitates due to reduced solubility in CO_2. Carbon dioxide and the solution can be brought into contact in different ways, namely by either gradually adding compressed carbon dioxide to the solution, or by spraying the solution into a vessel initially pressurized with carbon dioxide. The latter alternative, referred to as the solution enhanced dispersion by supercritical fluids (SEDS), was used in this study to precipitate insulin proteins from modified aqueous solutions.[5,6] Proposed by York and coworkers, this method could produce spherical precipitates of lysozyme or trypsin with mean particle sizes below 1 μm from their respective aqueous solutions, where dimethylsulfoxide was used as a secondary modifier solvent.[5] In another study, fairly large agglomerates consisting of primary particles of mean diameters between 1 and 5 μm were obtained through precipitation of lysozyme from DMSO and those retained biological activities between 50 and 100%, depending on the choice of operating conditions.[6] A SEDS process has also been applied to the precipitation of an aqueous formulation of recombinant human immunoglobin G, using carbon dioxide with dimethylsulfoxide as a modified supercritical fluid antisolvent.[7]

Sievers and coworkers consistently obtained particles of various pharmaceutical agents, peptides, and proteins, with mean particle sizes in the range of 0.1–3 μm, from their respective aqueous solutions through rapid decompression of emulsions formed by mixing the solutions with carbon dioxide.[8,9] Product particle sizes and their distributions could be controlled to some extent by varying concentration of the protein solution. Again, no significant loss of protein activity after the CO_2 assisted nebulization process has been observed.[8]

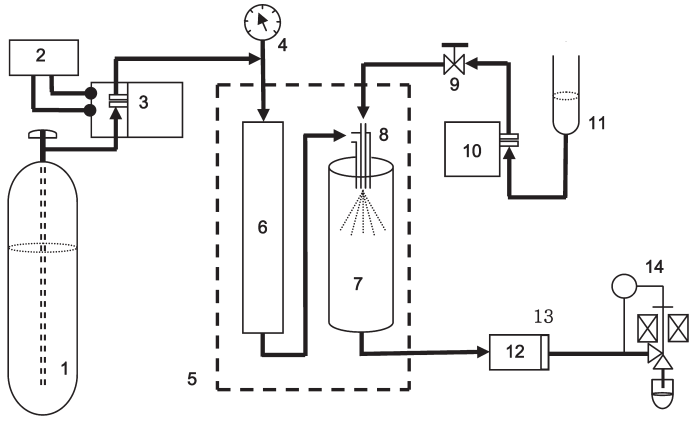
In this paper, we show that insulin nanoparticles of desired sizes and narrow size distributions can be generated from aqueous dimethylsulfoxide solutions of insulin using a CO_2 based SEDS process. More significantly, in conjunction with accommodating the maximum amount of insulin in a given volume, we achieved this without the aid of any additive. We also show that the theoretical particle sizes calculated from the droplet sizes

based on modified Jasuja's equation concurred excellently with the experimentally obtained particle sizes.

EXPERIMENTAL

Apparatus

Figure 1 is the schematic diagram of the continuous SEDS apparatus used in the present study. It consists of three main parts: feeding, precipitation, and particle collection units. An HPLC pump (model: SYSTEMGOLD Programmable Solvent Module 126, Beckman, USA) was used to deliver the insulin solution and another high pressure liquid pump (model: NS-500, Nihon Seimitsu, Japan), for carbon dioxide. Through a coaxial injection tube, carbon dioxide and insulin solution were diverted, respectively, into the precipitator. The precipitator is a stainless steel chamber (JASCO, Japan) with an inside diameter of 20 mm, a length of 250 mm, and an internal volume of 78.5 cm^3. The coaxial injector was composed of a 3.175 mm (OD) stainless steel tube with an insert of PEEK capillary tube. The inside diameters (i.d.) of the capillary tube are commercially available at 1.587 mm. While insulin solution was being sprayed into the chamber via the capillary tube, carbon



1. CO_2 cylinder	2. Cooling circulator	3. Liquid pump	4. Pressure gauge
5. Oven	6. Heat exchanger	7. Precipitator	8. Nozzle
9. Valve	10. HPLC pump	11. Protein solution	12. Particle collector
13. Metal frit	14. Back pressure regulator		

Figure 1. Schematic diagram of particle formation apparatus with a continuous solution enhanced dispersion of supercritical fluids (SEDS) process.

dioxide was charged simultaneously through the stainless steel tube (outside the capillary tube). A 0.2 μm metal frit was mounted at the particle collector to retain protein particles. The precipitator was immersed in a thermostatic oven, which was regulated to within ± 0.1 K. A pressure transducer (model: 312 SS, ranging 0–10000 psi, WIKA, USA) measured the operation pressure in the precipitator.

The experimental procedure is described as follows. At a given bath temperature, the mass flow rate of carbon dioxide was adjusted to about 30 mL/min during each precipitator run and the precipitator was maintained at a constant pressure by manipulating the liquid pump and the needle valve at the back pressure regulator (Model: 880–81, JASCO, Japan). While keeping a steady state with the influx of CO_2, the protein solution was introduced into the precipitation chamber via the capillary Teflon tube at a prespecified flow rate. Upon the droplet of protein solution contacting supercritical carbon dioxide in the precipitator, the solvent vaporizes instantaneously into the carbon dioxide rich phase and the protein particles form due to the supersaturation of the solute. Even after the period of particle formation, the flow of carbon dioxide was maintained for about 1–1.5 h to remove the residual solvent inside the precipitator. The precipitation chamber was then depressurized gradually to atmospheric pressure. The collected samples at different positions, including the sample on the metal frit, were collected from the chamber and observed with a field emission scanning electron microscope (FESEM) (model: S-4300, HITACHI, Japan).

Materials

Dimethylsulfoxide (purity 99.9%) and water (purity 99.9%) were HPLC grade and were bought from Aldrich (Milwaukee, WI). The insulin was supplied by Regeron, Inc. (Chunchon, Korea). CO_2 (purity 99.9%) was supplied by Goldstar Oxygen Ltd. (ChunChon, Korea).

RESULTS AND DISCUSSION

To make insulin nano-particles with supercritical fluid, we employed the solution enhanced dispersion (SEDS) technique in this study. The nanonization of heat labile proteins, such as insulin, is difficult to achieve by conventional high temperature drying techniques. SEDS process uses an additional polar solvent to help the supercritical carbon dioxide to extract water from the aqueous protein solution. For this reason, dimethylsulfoxide was used as an additional polar solvent. Dimethylsulfoxide also helped insulin protein nanoparticles to improve on their dissolution rate in water. The typical SEM photograph of insulin particles obtained in this study is presented in Figure 2. Figure 2 shows insulin nanoparticles in spherical shapes with a

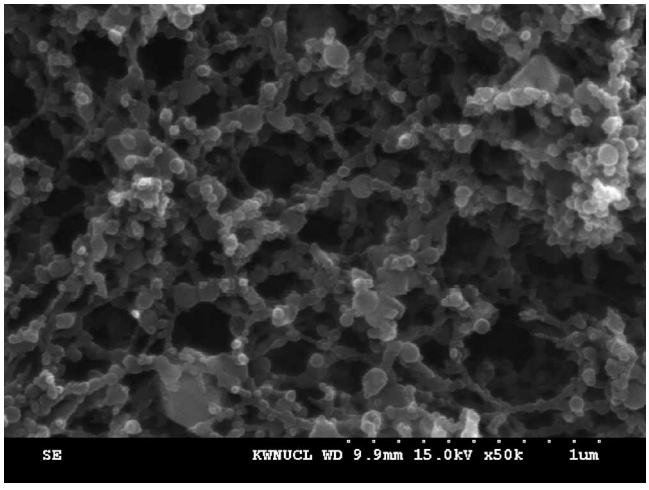


Figure 2. FE-SEM image of insulin nano-particle.

mean diameter of about 50 nm that was prepared from aqueous dimethylsulfoxide solution.

Although the potential of SEDS in manufacturing protein nanoparticles has been recognized and successfully realized, very little is known about the nature of the process that enables the particle size prediction for each system, partly due to complexity of the process parameters. Hydrodynamics, phase behavior, and crystallization thermodynamics of the system change throughout the process and all need to be considered simultaneously. The parameters that may influence the particle characteristics precipitated by the SEDS process include the nature of the solvent, solution concentration, operating temperature and pressure, flow rates of the solution and CO_2, and the type and size of the nozzle used for spraying.

The efficiency of the SEDS nanonization system is determined by droplets size produced in the nozzle and by the manner in which the supercritical medium mixes with the droplets. A nozzle is a device that renders liquid to disintegrate into droplets of a specified size range and controls their spatial distribution. Droplet sizes are correlated with individual system dimension as well as the ratio of the flow of the supercritical fluid to that of the solution.

Several equations have been proposed to predict the size of droplets in a two fluid nozzle spray. Among those, we chose Jasuja's droplet size equation[10] and modified it. The modified Jasuja's equation gave the best fits with the particle sizes obtained from our SEDS experiments. The modified Jasuja's equation calculates initial droplet size as the following:

$$\begin{aligned} d_{\text{droplet}} = {} & 0.0087(\sigma/\rho_S)^{0.45}(1/U_{SL})^{0.9}(1+L/S)^{0.5}(d_{\text{nozzle}})^{0.55} \\ & + 0.0045[(\mu_L)^2/(\sigma\rho_L)]^{0.5}(d_{\text{nozzle}})^{0.5}(1+L/S) \end{aligned} \quad (1)$$

Table 1. The operating conditions and results of eight insulin particle formation experiments using SEDS technique

	Experiment							
	1	2	3	4	5	6	7	8
Pressure (bar)	100	100	100	100	100	100	100	100
Temperature (°C)	40	40	40	40	40	40	40	40
Flow rate of CO_2 (g/min)	15	20	25	15	15	15	20	20
Flow rate of solvent (mL/min)	0.5	0.5	0.5	0.5	0.5	0.3	0.2	0.3
Consitution of solvent (DMSO:H_2O)	50:1	50:1	50:1	75:1	100:1	60:1	60:1	60:1
Concentration of insulin in solvent (mg/mL)	0.071	0.071	0.071	0.047	0.036	0.060	0.060	0.060
Mean particle diameter (nm)	**115**	**65**	**47**	**102**	**87**	**79**	**48**	**55**

where L/S is the mass ratio of liquid flow to supercritical fluid flow, ρ_L is liquid density(kg/m^3), $d_{droplet}$ is the diameter of the supercritical fluid discharge tube, and $d_{droplet}$ has the same surface to volume ratio as the total droplet population. The last term of Equation (1) is called viscosity term. Equation (1) is dimensionally consistent; any set of consistent units on the right hand side yields the droplet size in units of length on the left hand side.

When droplets are produced in a precipitation vessel, the droplet size and size distribution depend on the interfacial tension of the droplet. Interfacial properties have a fundamental influence on dense gas/liquid separation processes, with the interfacial tension being an important parameter associated with mass transfer.

Table 2. The parameters used for the calculation of the modified jasuja's equation

	L/S	U_{SL} (m/s)	σ (N/m)	ρ_s (Kg/m^3)	ρ_L (Kg/m^3)	μ_L (Pa · s)	d_{nozzle} (m)	C (mg/mL)
1	0.03645	0.10755	0.0041	629	1098	0.00112	0.0002	0.071
2	0.02762	0.10605	0.0041	629	1098	0.00112	0.0002	0.071
3	0.02170	0.29562	0.0041	629	1098	0.00112	0.0002	0.071
4	0.03649	0.10755	0.0041	629	1098	0.00113	0.0002	0.047
5	0.03652	0.10755	0.0041	629	1098	0.00110	0.0002	0.036
6	0.02196	0.17476	0.0041	629	1098	0.00112	0.0002	0.060
7	0.01098	0.40531	0.0059	486	1098	0.00112	0.0002	0.060
8	0.01664	0.26326	0.0041	629	1098	0.00112	0.0002	0.060

Modified Jasuja's equation: $d_{droplet} = 0.0087\ (\sigma/\rho_S)^{0.45}\ (1/U_{SL})^{0.9}\ (1 + L/S)^{0.5}\ (d_{nozzle})^{0.55} + 0.0045\ [(\mu_L)^2/(\sigma\ \rho_L)]^{0.5}\ (d_{nozzle})^{0.5}\ (1 + L/S)$.

Table 3. The comparison of calculated and experimentally obtained insulin particle size

		Modified Jasuja's	Equation
	$d_{experiment}$ (m)	$d_{droplet}$ (m)	$d_{calculated}$
1	1.15×10^{-7}	2.86×10^{-6}	1.11×10^{-7}
2	6.45×10^{-8}	1.67×10^{-6}	6.52×10^{-8}
3	4.73×10^{-8}	1.16×10^{-6}	4.53×10^{-8}
4	1.02×10^{-7}	2.86×10^{-6}	9.71×10^{-8}
5	8.71×10^{-8}	2.86×10^{-6}	8.88×10^{-8}
6	7.92×10^{-8}	1.85×10^{-6}	6.80×10^{-8}
7	4.85×10^{-8}	1.15×10^{-6}	4.22×10^{-8}
8	5.51×10^{-8}	1.28×10^{-6}	4.73×10^{-8}

We assumed that the initial droplet was in equilibrium with the surrounding supercritical fluid CO_2; therefore, the pressurized CO_2 can be exchanged readily with the droplet solution to produce insulin particles. The diameter of the insulin particle can be calculated from the following equation:[11]

$$d_{calculated} = d_{droplet\times}(C/1200)^{1/3} \tag{2}$$

where C is the concentration of insulin solution. The insulin molecules were assumed stable through the SEDS process and the molecular weight of insulin, likewise, unchanged during particle formation.

Eight experiments were conducted to produce insulin nanoparticles. The operating conditions and results of each of the experiments are summarized in Table 1. The mean particle diameter ranges from 47 to 115 nm at 40°C and 90 − 100 bar. The parameters used for the calculation of Jasuja's equation are shown in Table 2. To compare the calculated and experimentally

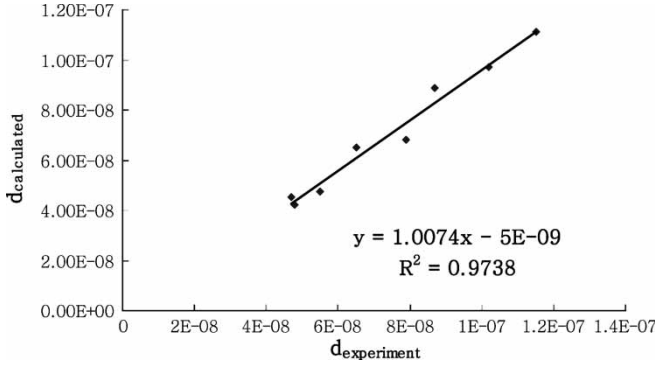


Figure 3. Correlation between $d_{experiment}$ and $d_{calculated}$.

obtained insulin particle sizes, both results are tabulated in Table 3. The calculated and experimental results correlated superbly with the correlation coefficient (R^2) of 0.9738 (Figure 3).

In conclusion, insulin nanoparticles were produced successfully from dimethylsulfoxide solution using supercritical carbon dioxide as an antisolvent, and our results show that the mean particle sizes calculated from the modified Jasuja's equation excellently correlates those obtained empirically from the SEDS experiments. The same principle could be applicable to the particle size prediction of the proteins other than insulin, and to what extent our findings can be generalized to protein particle nanonization employing the SEDS process.

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