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# Preparation of Large Porous Deslorelin–PLGA Microparticles with Reduced Residual Solvent and Cellular Uptake Using a Supercritical Carbon Dioxide Process

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***Purpose.*** The purpose of this study was to prepare large-porous peptide-encapsulating polymeric particles with low residual solvent that retain deslorelin integrity, sustain drug release, and exhibit reduced epithelial and macrophage uptake. We hypothesized that supercritical carbon dioxide (SC $CO_2$) pressure-quench treatment of microparticles prepared using conventional approach expands these particles and extracts the residual organic solvent.
***Methods.*** Initial studies with crystalline L-lactide (L-PLA) and amorphous copolymers of lactide-co-glycolide (PLGA) 50:50, 65:35, and 75:25 indicated that PLGA 50:50 was the most amenable to morphological changes upon SC $CO_2$ treatment. Therefore, we prepared deslorelin–PLGA (50:50) microparticles using the conventional emulsion-solvent evaporation method, and in a second step equilibrated with SC $CO_2$ at various temperatures (33–37°C) and pressures (1200–2000 psi) for discrete intervals followed by rapid isothermal depressurization. The particles were then characterized for morphology, polymer thermal properties, particle size, porosity, bulk density, and residual solvent content. Also, deslorelin integrity, conformation, release, and cellular uptake before and after SC $CO_2$ treatment was determined.
***Results.*** Upon SC $CO_2$ treatment (1200 psi, 33°C for 30 min), the mean particle size of the deslorelin PLGA microparticles increased from 2.2 to 13.8 μm, the mean porosity increased from 39 to 92.38%, the mean pore diameter increased from 90 to 190 nm, the mean bulk density reduced from 0. 7 to 0.082 g/cc, mass spectrometry indicated structural integrity of released deslorelin, the circular dichroism spectrum indicated stabilization of β-turn conformation, and the scanning electron microscopy confirmed increased particle size and pore formation. The deslorelin release was sustained during the 7-day study period. Also, the peak Tg of PLGA decreased from 51 to 45°C, and the residual solvent content was reduced from 4500 ppm to below detection limit (< 25 ppm). The accumulation of drug from SC $CO_2$ treated particles in cell layers of Calu-3, A549, and rat alveolar macrophages was reduced by 87, 91 and 50%, respectively, compared to untreated particles.
***Conclusion.*** An SCF-derived process could be successfully applied to prepare large porous deslorelin–PLGA particles with reduced residual solvent content, which retained deslorelin integrity, sustained deslorelin release, and reduced cellular uptake.

**KEY WORDS:** deslorelin, supercritical, porous particles, macrophage uptake, A549 and Calu-3.

## INTRODUCTION

The pulmonary route is gaining interest as a viable non-invasive route for the systemic delivery of peptide and protein drugs that exhibit little or no oral bioavailability and require parenteral administration (1). Indeed, inhaled insulin is currently undergoing Phase III clinical trials for systemic delivery via the respiratory route. The absorption of macromolecules via the pulmonary route is rapid and facilitated by the highly vascularized alveolar epithelium constituted by a single layer of cells, which offers a large absorptive surface (80–100 $m^2$; Refs. 2,3). The pulmonary route allows significant systemic delivery of macromolecules even in the absence of penetration enhancers and the bioavailability of peptides administered by the pulmonary route is greater compared to the nasal or the oral route (2,3). However, the advantages of rapid and efficient absorption into the systemic circulation are offset by poor dose deposition and clearance by alveolar macrophages, which limit the use of the pulmonary route to deliver drugs (4). The therapeutic aerosols deliver a maximum of 10–20% of administered dose to the deep lungs, limiting the systemic drug absorption.

Recent interest to sustain systemic drug delivery in the lungs presents additional challenges because the lung efficiently clears drug-containing particles via mucociliary clearance in the airway and via phagocytosis by the alveolar macrophages in the deep lungs (5). Particles deposited in the lungs are primarily cleared by mucociliary clearance in the trachea and bronchi and by macrophage clearance in the alveolar space. Although particles deposited in the ciliary region are removed within hours, those deposited in alveolar space can be retained for several days. The coordinated movement of the cilia moves the mucus blanket containing the foreign particles at a rate of 2–5 $cm \cdot min^{-1}$ outwards toward the pharynx, where they are swallowed (6). In the alveolar space, alveolar macrophages phagocytose particles, sequester them into phagolysosomes, and eventually leave the organ primarily via the airways and mucociliary clearance and partially via the lymphatic system. Engulfed particles may be digested by the proteases in the phagolysosomes, where the pH is 5.1–5.5 (7). Also, alveolar macrophages have a number of endopeptidases capable of digesting peptide drugs. Particles can also be transported from the alveoli to the ciliated airways where they are removed by the mucociliary clearance. The epithelial cells in the airways as well as alveolar space can engulf particles. Particles taken up by epithelial cells may be translocated to the connective tissue, where they are phagocytosed by interstitial macrophages. Thus, uptake of particles into any of the cells is undesirable, as this would clear the particles, thereby shortening the release and the effect. We believe that systemic delivery can be better sustained by retaining drug-polymer composite particles in the alveolar fluid without macrophage entry or epithelial entry. The released peptide from such particles can enter systemic circulation either via the paracellular pathway or receptor-mediated processes.

Therapeutic dry powder inhalers to date comprise drug particles with mass densities (ρ) of ≈1.0 ± 0.5 g/$cm^3$ and mean geometric diameters (d) of <5 μm adsorbed onto carrier particles to facilitate deep lung deposition (8). These therapeutic aerosols suffer from a number of disadvantages including poor flow properties, high dose variation, and rapid clearance from the deep lungs (9). To overcome these limitations, some investigators have proposed the use of large porous particles

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for use in lieu of the conventional small high-density particles adsorbed onto carriers (10,11). Large porous particles are particles with mean diameters >5 μm and mass densities <0.1g/cm$^3$ (10). Although these particles have large geometric diameters by virtue of their low density, they exhibit aerodynamic diameters comparable to smaller particles having higher densities. Due to their large sizes, these particles will likely avoid clearance by alveolar macrophages, enabling sustained drug delivery through the lungs (11). Furthermore, increasing the geometric diameters would increase the dispersibility and entrainment of the drug particles from the inhaler, making it possible to efficiently deliver the dose using a dry powder inhaler (9).

In this study, we used a novel supercritical fluid based approach for the preparation of large porous peptide-polymer microparticles. For the purpose of this study, poly (lactide-co-glycolide) (PLGA) series of biodegradable polymers, primarily PLGA (50:50) was used. The peptide drug used in our studies is deslorelin, a nonapeptide, with the amino acid sequence, pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-ethylamide. Deslorelin is a potent leuteinizing hormone-releasing hormone (LHRH) agonist that acts by binding to LHRH receptors, which are now known to be present in various tissues including the ovary, prostate, endometrium, and breast tissues besides the pituitary gland (12). Initial binding of an LHRH agonist to the pituitary gland results in a brief period of increased gonadotrophin synthesis and release, followed by a more prolonged decrease of pituitary hormone secretion due to receptor down regulation. This receptor desensitization is clinically useful in the treatment of various sex hormone dependent disorders such as uterine fibroids, menorrhagia, endometriosis, polycystic ovarian disease, and central precocious puberty. Also, hormone-dependent malignant tumors including breast ovarian endometrial and prostate carcinoma can be treated using LHRH agonists (12). For several of these disorders, a local action of LHRH agonists in the affected tissues is also being hypothesized. Thus, after lung administration, deslorelin has to first enter the systemic circulation to begin exerting its effects at various target tissues. Systemic entry of small peptides such as deslorelin can occur via the paracellular pathway.

Supercritical fluids (SCFs) are substances that at temperatures and pressures simultaneously higher than their critical points (Tc and Pc) exist as a single phase with properties intermediate between gases and liquids. SCFs have liquid like densities and solvating characteristics and gas-like diffusivities and mass transfer properties (13,14). Carbon dioxide ($CO_2$) is the most widely used SCF for pharmaceutical applications, and offers the advantages of easy availability, low critical temperature (31°C) and pressure (1080 psi) suitable for macromolecule processing, non-flammability, and low toxicity and reactivity. Different SCF-derived processes, including rapid expansion of supercritical solution, gas antisolvent, and aerosol supercritical extraction of solvents have been used for the preparation of particulate drug delivery systems (14). Although, most of these processes are in laboratory and/or early development phases some of these processes have been scaled-up. For instance, Thies and Muller developed a scaled-up process of aerosol solvent extraction system aerosol supercritical extraction of solvents capable of producing 200 g of biodegradable poly(lactide) (PLA) microparticles (15). Furthermore, industrial units such as Bradford Particle Design have resources for the production of up to one ton per year of cGMP particulate material, using solution enhanced dispersion by supercritical fluids.

Another important attribute of supercritical carbon dioxide (SC $CO_2$) that can be used in the preparation of drug delivery systems is its ability to modify polymers. Rapid expansion of $CO_2$ using a pressure drop has been shown to expand polymers and induce pore formation (16). In addition, SC $CO_2$ has a high affinity for organic solvents and enables their removal from drug delivery systems (17). Therefore, the purpose of this study was to prepare pilot-scale large-porous peptide-encapsulating polymeric particles with low residual solvent that retain deslorelin integrity, sustain drug release, and exhibit reduced epithelial and macrophage uptake.

In this study, we prepared deslorelin–PLGA 50:50 microparticles using a conventional solvent evaporation technique and rendered these particles porous by SC $CO_2$ treatment and characterized them for their morphology, thermal properties, particle size, porosity, bulk density, residual solvent content, drug release, deslorelin integrity and conformation, and cellular uptake. Previous studies indicated that porous particles sustain drug delivery and one of the reasons for this is that these particles avoid extensive clearance by macrophages by virtue of their large size. However, there is no previous *in vitro* study characterizing porous particle uptake by macrophages. For the first time, we have investigated the alveolar macrophage and respiratory epithelial cell uptake of porous particles and compared it with non-porous particles.

## MATERIALS AND METHODS

### Drugs and Chemicals

Deslorelin was a gift from Balance Pharmaceuticals, Inc. (Santa Monica, CA, USA). L-PLA and copolymers of poly-(lactide-co-glycolide) (D-PLGA) with different lactide:glycolide ratios-PLGA 50:50, PLGA 65:35, and PLGA 75:25 with molecular weight (intrinsic viscosities in dL/g are shown in parentheses) of 160 (1.07), 96 (0.66), 10.05 (0.68), and 97.1(0.68) kDa, respectively, and PLGA 50:50 with acid end groups and molecular weight of 23.2 (0.26) kDa were obtained from Birmingham polymers, Inc. (Birmingham, AL, USA). Although the preliminary studies used all the polymers for preparing blank particles, studies with the peptide were performed using PLGA 50:50 with acid end groups. Poly(vinyl alcohol) (average molecular weight 30,000–70,000) and trifluoroethanol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methylene chloride and acetonitrile of high-performance liquid chromatography (HPLC) grade were obtained from Fisher Scientific (Pittsburgh, PA, USA). Carbon dioxide (99.95%) obtained from Linweld Company (Lincoln, NE, USA) was used as the supercritical fluid.

### Microparticle Preparation

Polymeric microparticles with and without deslorelin were prepared using an emulsion-solvent evaporation method. Weighed quantities (90–110 mg) of the polymer were placed in methylene chloride (0.5–1 mL) and allowed to dissolve under intermittent vortexing at room temperature. Deslorelin (10–20 mg) was dissolved in methanol (0.5–1 mL) and this solution was then added to the polymer solution to form

the dispersed phase. This dispersed phase was then added over 1 min to 10 ml of aqueous continuous phase containing 2% w/v poly(vinyl alcohol) on ice under sonication (Misonix Inc. Farmingdale, NY, USA) at 50 W to form an O/W emulsion. For the preparation of polymeric drug particles devoid of deslorelin, an equal volume of methanol devoid of the drug was used in the dispersed phase. This emulsion was then added dropwise to 100 mL of 2% w/v aqueous poly(vinyl alcohol) solution under rapid stirring and allowed for solvent evaporation at 25°C for 12 h. Subsequently, the microparticles were separated by ultracentrifugation at 100,000 g for 30 min at 4°C. The microparticle pellet was suspended in distilled water and lyophilized for 24–48 h to obtain dry particles.

### SCF Process for the Preparation of Large Porous Polymeric Microparticles

Large, porous-polymeric particles with or without deslorelin were formed using the SCF set-up shown in Fig. 1. The SCF set-up consisted of a high-pressure vessel, a manual pump, a $CO_2$ cylinder, and valves, all obtained from High Pressure Equipment Company (Erie, PA, USA). Valves were located at appropriate locations for regulating the $CO_2$ flow. The pressure inside the high-pressure vessel was monitored using a pressure gauge (Psi-tronix, P&R Intra Supply, Inc., Atlanta, GA, USA) and the vessel was maintained in a temperature-controlled bath. For the preparation of large porous particles, the polymeric microparticles prepared in the previous step were placed in the high-pressure vessel, tightly sealed and equilibrated for 15 min to the desired temperature. During this stage, the valves $V_3$ and $V_4$ remained closed. The $CO_2$ from the cylinder was allowed to fill into pre-cooled manual pump by opening the valves $V_1$ and $V_2$. Once the pump was filled, the valve $V_1$ was closed. To allow $CO_2$ entry into the high-pressure vessel, valve $V_3$ was opened. The $CO_2$ was then pumped into the high-pressure vessel and pressure in the vessel was monitored using the pressure gauge (G). Once the desired pressure within the vessel was attained, valve $V_3$ was closed and the high-pressure vessel was allowed to remain at this level for discrete time intervals. Following the SCF treatment, the high-pressure vessel was depressurized over 1 min by opening $V_4$. For initial feasibility studies, SC $CO_2$ pressures ranging from 1100-2000 psi and temperatures ranging from 37–50°C were used. The exposure times ranged from 15-60 min. For the preparation of the final deslorelin–PLGA porous particles, SC $CO_2$ at 1200 psi and 33°C for 30 min was used. The mechanism of formation of porous polymeric particles by the pressure-quench method using SC $CO_2$ is shown in Fig. 1b.

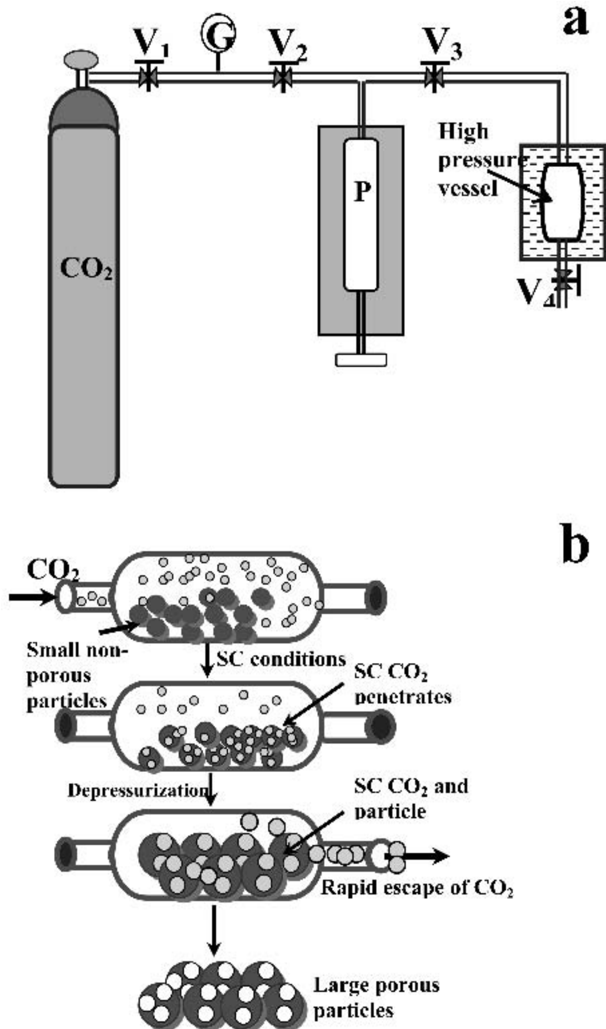


**Fig. 1.** a, Supercritical fluid setup for preparing large porous polymeric particles. $CO_2$, carbon dioxide source; P, pump; G, pressure gauge; high-pressure vessel; and $V_1$, $V_2$, $V_3$, $V_4$, valves 1–4, respectively. b, Mechanism of large porous polymeric particle formation by the pressure quench method using supercritical carbon dioxide.

### Scanning Electron Microscopy (SEM)

The effect of SC $CO_2$ on the shape and surface characteristics of drug-polymeric microparticles was assessed using a scanning electron microscope (JSM-5510, JEOL USA, Peabody, MA). Briefly, the samples were fixed to stubs using a double-sided copper tape, sputter coated with gold–palladium under an argon atmosphere using a gold sputter module in a high vacuum evaporator, and examined using SEM set at 15 kV.

### Differential Scanning Calorimetry (DSC)

The DSC analysis for deslorelin and all the polymeric microparticles with or without SCF processing was carried out using a differential scanning calorimeter (Shimadzu DSC-50 System, Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). For each scan, 1–2 mg of the sample was weighed and placed in a hermetically sealed aluminum pan. The samples were then heated at a heating rate of 5°C/min under a nitrogen atmosphere (flow rate 20 mL/min), using an empty aluminum pan as the reference. All the samples were heated in the temperature range, 25–300°C. Also the reversibility of the polymer transition was assessed, by heating the particles from 20 to 70°C, cooling it to 20°C and rescanning it. All the thermograms were analyzed using a TA thermal analysis software (version 1.14) for onset, endset and peak glass transition temperatures. The onset and endset temperatures were determined as the temperatures at the intersection of tangent to the baseline and the tangent of the steep descent and ascent portions of the endothermic glass transition curves, respectively. The peak temperature was determined as the intersection of the tangents to the steep ascent and descent portions of the endothermic transition.

**Powder X-ray Diffraction Analysis**

The powder X-ray diffraction data for L-PLA microparticles with or without SC $CO_2$ treatment were obtained using a Rigaku D/Max-B X-ray diffractometer (Rigaku, Slidell, LA, USA) with Bragg–Brentano parafocusing geometry, a diffracted beam monochromator, and a conventional copper target X-ray tube set to 40 KV and 30 mA. The 2Θ scan range was 5–35°, with a step size of 0.03° and a scan speed of 2.5°/min. The wavelength of X-ray radiation was 1.5418 and the spacing between diffracting planes was 2.56 Å.

**Drug Loading, Encapsulation Efficiency, and *in Vitro* Release from Microparticles**

The amount of deslorelin loaded within the microparticles was determined following extraction with an organic solvent. Briefly, a weighed quantity (2–5 mg) of microparticles was solubilized in 1 mL of methylene chloride under vigorous vortexing. Two milliliters of 0.1 M acetate buffer (pH 5) was added to the methylene chloride and the samples were agitated for 3 h. Samples were then allowed to stand for the separation of the layers. The drug in the aqueous layer was analyzed using a previously reported HPLC method (18). The % encapsulation efficiency was determined using the following expression:

Encapsulation efficiency =
(actual drug loading/theoretical drug loading) × 100

The *in vitro* release of deslorelin from the deslorelin–PLGA microparticles was carried out in a 15-mL centrifuge tube maintained at 37°C in a shaking water bath. A 0.5-mL suspension of the deslorelin–PLGA microparticles was placed into a floatable dialysis membrane unit obtained from Spectrum Laboratories (Molecular weight cut off: 10,000) and the unit was suspended in the centrifuge tube containing 10 mL of acetate buffer (pH 5) containing 0.025% sodium azide as a preservative. At various time points, 2-mL of the release medium was removed, and an equal volume of fresh release medium was immediately added to continue the release study until 7 days. The deslorelin released into the medium was analyzed using HPLC method (18).

**Determination of Deslorelin Conformation Using Circular Dichroism**

The CD spectra for deslorelin were recorded on an Aviv Model 202SF CD spectrophotometer at 25°C. Strain free quartz cuvette from Helma with a pathlength of 1 mm was used for all samples. Data collection was done from 320–175 nm, with a 1 nm step size, a bandwidth of 1–1.5 nm and an average time of 2 s per data point. In the case of deslorelin–PLGA microparticles, the conformation of deslorelin released from the particles into 1 mL of aqueous medium was monitored. Previously we have demonstrated that deslorelin does not adopt a definite conformation in aqueous solution (18). However, many peptides, which do not adopt a regular structure in aqueous solution, do so upon addition of an organic solvent. A common technique to stabilize latent secondary structures in peptides is to add a strong hydrogen bond donor such as trifluoroethanol. Therefore, spectra recorded in a mixture of 50% trifluoroethanol and 50% acetate buffer (pH 5) were subtracted from spectra in 100% acetate buffer (pH 5) and difference spectra are presented.

**Fourier Transform-Infrared (FTIR) Spectroscopic Analysis**

FTIR spectra for deslorelin, blank PLGA (50:50) microparticles and deslorelin–PLGA (50:50) microparticles before and after SC $CO_2$ treatment (1200 psi, 33°C, for 30 min) were obtained using a Nicolet FTIR spectrometer with a DTGS detector. Crystalline KBr (FTIR grade, 99%), obtained from Aldrich Chemical Co. (San Francisco, CA, USA) and dried overnight at 120°C was used. The samples for analysis were mixed with dry KBr at 1:100 (sample:KBR) ratios and compressed to form KBr pellets. For each sample 256 scans were collected at a resolution of 2 $cm^{-1}$ over the wavenumber region of 4000–400 $cm^{-1}$. All samples were measured three times and spectra were corrected for background.

**Mass Spectrometry for Deslorelin**

Deslorelin released over 24 h into 1 mL of aqueous medium from deslorelin–PLGA (50:50) microparticles before and after SC $CO_2$ treatment (1200 psi, 33°C, for 30 min) was lyophilized and subjected to electrospray mass spectrometry employing fast atom bombardment (Nebraska Center for Mass Spectrometry, Lincoln, NE, USA). Pure deslorelin and deslorelin treated with SC $CO_2$ (1200 psi, 33°C, for 30 min) were used as controls. Three individual batches of particles were used and samples were analyzed in duplicate from each batch.

**Residual Methylene Chloride Determination Using Gas Chromatography**

Residual methylene chloride in the untreated and SC $CO_2$ treated deslorelin–PLGA microparticles was measured using headspace gas chromatography. A Varian Chrompack CP 3380 was used with a DB-5MS column. Ultra high purity helium at a flow rate of 30 mL/min was used as the carrier gas. Detection was done with a flame ionization detector. Approximately 20–25 mg of particles were dispersed in 500 μL of ethyl acetate, which was used as an internal standard. The samples placed in a 4 ml glass vial equipped with a teflon septum closure were incubated at 60°C in an oven for 10 min. Fifty microliters of the headspace was then injected onto the GC column. The column temperature was initially equilibrated to 40°C for 1 min and ramped from 40°C to 75°C at 10°C/min. The peak areas of methylene chloride and ethyl acetate were determined and a ratio of their responses was taken. The amount of residual methylene chloride in the microparticles was determined from a linear calibration curve constructed using mixtures of methylene chloride and ethyl acetate as standards.

**Cellular Accumulation of Deslorelin–PLGA Microparticles**

Calu-3, A549 and rat alveolar macrophages (NR8383) were purchased from the American Type Culture Collection (Manassas, VA, USA). Calu-3 and A549 are two human cell lines derived from the respiratory epithelium. The A549 cell line possesses properties characteristic of Type II pulmonary epithelial cells whereas the Calu-3 cell line has properties representative of the bronchial or airway epithelium (19). Calu-3 (passage 28–36), A549 (passage 89–95) and rat alveolar macrophages were cultured in 6 well culture plates (Costar, Fisher Scientific; culture area 9.6 $cm^2$) in an atmosphere

of 5% $CO_2$/95% $O_2$ at 37°C. Confluent monolayers of A549 and Calu-3 cells (days 8–10) and sub-confluent (day 9) cells of alveolar macrophages were used for the uptake study. Deslorelin, untreated deslorelin PLGA 50:50 microparticles, and SCF treated deslorelin–PLGA 50:50 particles, each at a concentration of 100 μg of deslorelin in 2.5 ml of assay buffer (pH 7.4) were placed on the apical surface of the cells after the culture medium was aspirated. At the end of 6 h, the supernatant was removed and cells were washed five times with cold assay buffer. Cells were then lysed in 400 μl of 0.5% Triton-X solution for 2 h. For estimating the particle uptake into cells, lysates were vortexed with twice the volume of methylene chloride (nonaqueous) for 1 h. The aqueous layer was then collected, dried under nitrogen, reconstituted in assay buffer, centrifuged at 8000 g for 10 min and the deslorelin in this supernatant was estimated using HPLC.

### Data Analysis

In all the above studies, three different batches of microparticles were assessed. In the results, representative plots are provided for CD, DSC, X-ray diffraction, and FTIR studies. Throughout the manuscript, data is expressed as mean ± SD for n = 3 batches of particles or 4 monolayers of cells. Statistical comparisons between different groups were made using a paired *t* test and differences were considered significant at $p < 0.05$.

## RESULTS

### Polymeric Microparticles

Initially we studied the feasibility of preparing large porous polymeric microparticles using a homopolymer of L-lactic acid (L-PLA) and copolymers of lactic and glycolic acid (PLGA) of lactide:glycolide ratios 50:50 (96 kDa), 50:50 modified with acid end groups (23.2 kDa), 65:35 (10.05 kDa), and 75:25 (97.1 kDa). We prepared blank polymeric particles, treated them with SC $CO_2$, and assessed their morphology using SEM. Also, we examined the thermal properties of the polymers and the effect of SC $CO_2$ on the same using differential scanning calorimetry. Table I summarizes the polymer properties used in our preliminary studies and our observations using DSC and SEM.

The DSC curves for untreated PLGA 50:50, PLGA 50:50 with acid end groups, PLGA 65:35, and PLGA 75:25 microparticles prepared using the emulsion solvent evaporation method indicated peak glass transition temperatures ($T_g$) of 52.7, 52.0, 52.8 and 49.3°C, respectively. Upon SC $CO_2$ treatment, these Tg values reduced to 50.2, 48.1, 51.1, and 48°C, respectively. Thus, SC $CO_2$ had a plasticizing effect on all of the PLGA polymers. Also, SEM studies indicated a change in the morphology of these particles with the appearance of pores and surface irregularities upon SC $CO_2$ treatment. Because L-PLA is a crystalline polymer, the DSC curve for L-PLA microparticles exhibited a sharp melting peak corresponding to a $T_m$ of 174.4°C. Upon exposure to SC $CO_2$ (1350 psi, 37°C for 15 min) the melting curve narrowed and the melting enthalpy decreased from 39.9 to 31.7 Joules/g. However, the $T_m$ (175.2°C) did not decrease significantly. Indeed, such a decrease in melting enthalpy might occur either due to a decrease in polymer crystallinity or due to an increase in polymer purity. An examination of the powder X-ray diffraction pattern revealed that the polymer crystallinity was unchanged (Fig. 2a). Furthermore, SEM studies indicated that SC $CO_2$ treatment did not affect L-PLA particle morphology (Fig. 2b and c). Based on these studies, PLGA 50:50 with acid end groups ($M_w$ 23.2 kda, i.v. 0.26) was chosen for the preparation of polymeric deslorelin particles.

**Table I.** Effect of Supercritical Carbon Dioxide (SC $CO_2$) Treatment on Morphology and Thermal Properties of Different Blank Polymeric Particles

| Polymer | Molecular weight (Mw, kDa) | SC $CO_2$ treatment (SC $CO_2$ pressure in psi/ temperature in °C/ treatment duration in min) | Scanning electron microscopy | DSC Onset, peak, and endset temperatures (°C) | | |
|---|---|---|---|---|---|---|
| | | | | Control | SC $CO_2$ | ΔTg[a] |
| PLGA 50:50[b] | 96 kDa | 1100/37/15 | Change in morphology. Appearance of surface irregularities and foam like structures | 50.0, 52.7, 55.1 | 47.8, 50.2, 53.8 | 2.5 |
| PLGA 50:50 with acid end groups | 23.2 kDa | 1100/37/15 | Change in morphology. Expansion and visible surface pores. | 48.8, 52, 53.7 | 46.6, 48.1, 51.7 | 3.9 |
| PLGA 75:25 | 97.1 kDa | 1100/37/15 | Change in morphology. Surface irregularities and peeling and agglomerated structures. | 50.1, 52.8 54.7 | 48.2, 51.1, 53.7 | 1.7 |
| PLGA 65:35 | 10.05 kDa | 1100/37/15 | Change in morphology. Appearance of irregular, broken egg shell like structures. | 46.6, 49.3, 52.3 | 44.8, 48, 52.2 | 1.3 |
| | | | | Tm (°C) | | |
| L-PLA | 160 kDa | 1350/37/15 | No change in morphology (Fig. 2) | 174.8 | 175.2 | |

[a] Difference in peak Tg temperatures (°C) between control and SC $CO_2$-treated polymers.
[b] PLGA, poly-lactide-co-glycolide.

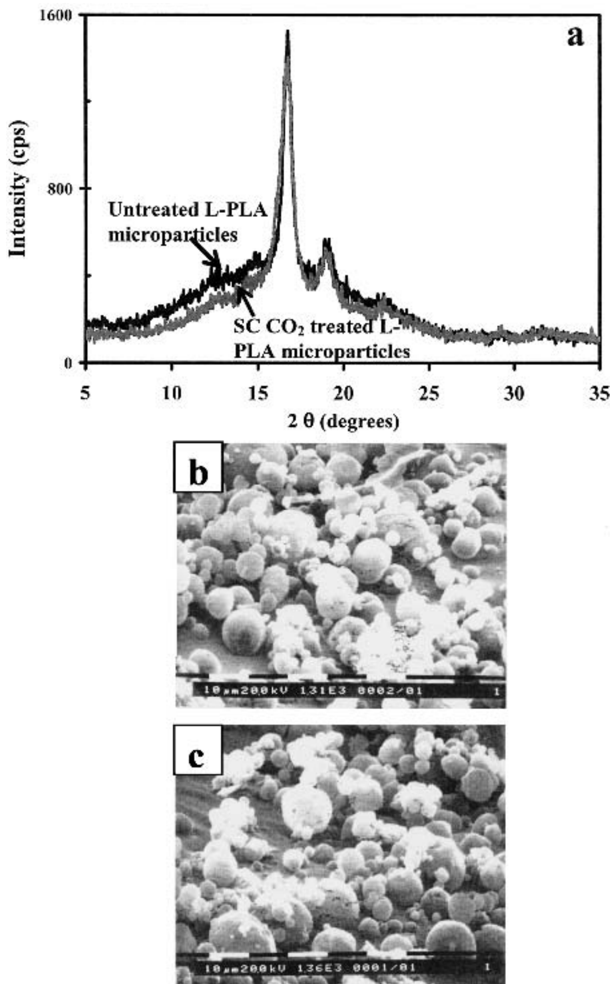


**Fig. 2.** a, X-ray diffraction patterns of untreated and supercritical carbon dioxide treated (1350 psi at 37°C for 15 min) L-PLA microparticles. Scanning electron microscopy pictures of L-PLA microparticles before (b) and after (c) supercritical carbon dioxide treatment.

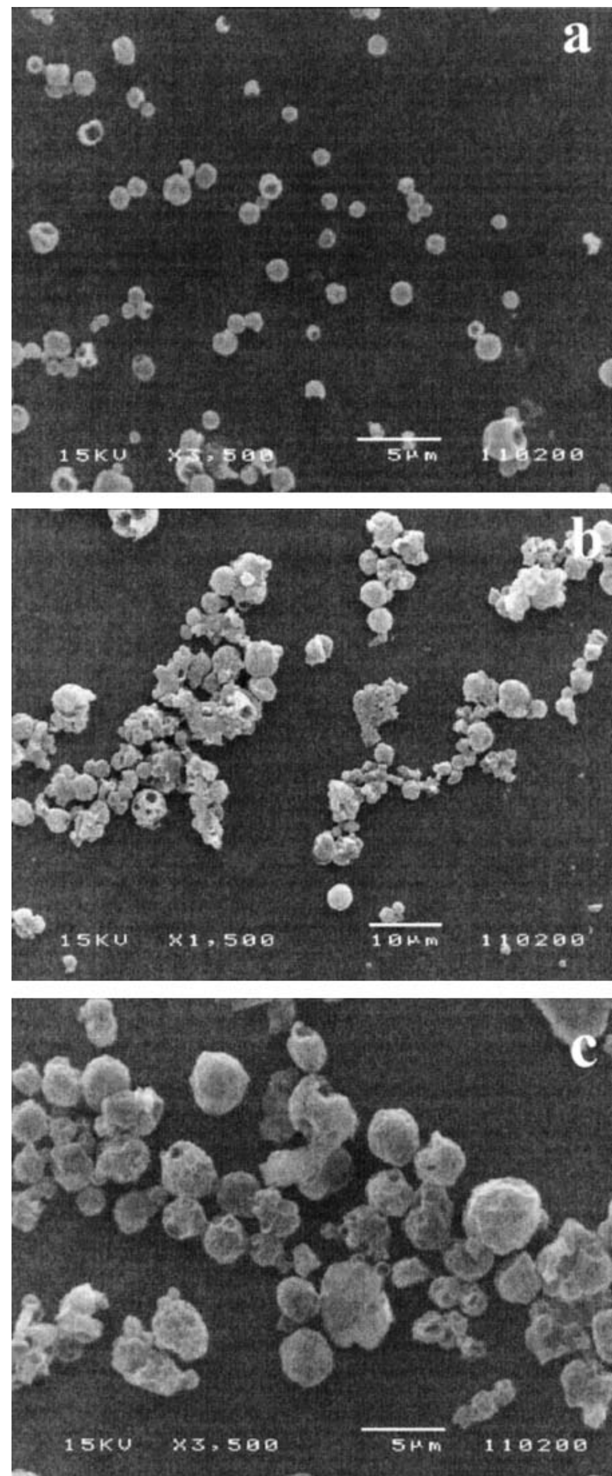


**Fig. 3.** Scanning electron microscopy pictures of deslorelin-PLGA microparticles before (a) and after (b and c) supercritical carbon dioxide treatment at 1200 psi and 33°C for 30 min.

### Deslorelin–PLGA 50:50 Microparticles Morphology

SEM studies of microparticles of deslorelin–PLGA 50:50 with acid end groups before and after SC $CO_2$ treatment at 1200 psi and 33°C revealed a distinct change in particle morphology upon SC $CO_2$ treatment. SEM pictures of particles of three different batches were obtained and representative pictures are presented. The particles prepared using the conventional emulsion-solvent evaporation method were of 1–2 μm diameter (Fig. 3a). Upon SC $CO_2$ exposure, the particle size increased to 10–15 μm and in some cases a few aggregates could be observed (Fig. 3b). Also, there was the presence of visible pores on the particle surface (Fig. 3c) and it appears that the particle surface was rendered irregular. Indeed, it was shown that particles with irregular surfaces have improved dispersion as the insertion of spaces between point charges within a particle results in the reduction of van der Waals forces between particles (9).

### Polymer Properties

The DSC curves for deslorelin and deslorelin–PLGA microparticles before and after SC $CO_2$ treatment are shown in Fig. 4. The DSC curve for deslorelin exhibited no transitions in the temperature range tested. The DSC curve for the microparticles of deslorelin–PLGA 50:50 with acid end groups prepared using the emulsion-solvent evaporation method exhibited an endothermic peak temperature at 52°C (onset and endset temperatures were 48.94 and 53.27°C, respectively), corresponding to the glass transition of PLGA 50:50 with acid

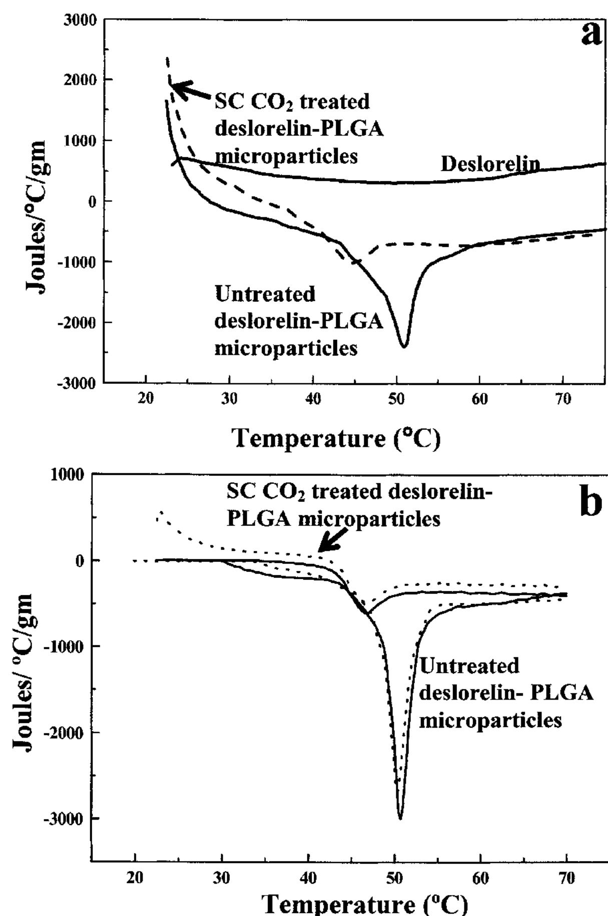


**Fig. 4.** a, Differential scanning calorimetry thermograms of pure as received deslorelin, deslorelin–PLGA microparticles prepared by the emulsion solvent evaporation method, and supercritical carbon dioxide-treated (1200 psi at 33°C for 30 min) deslorelin–PLGA microparticles. b, Differential scanning calorimetry thermograms demonstrating reversibility of glass transitions for untreated and supercritical carbon dioxide-treated deslorelin–PLGA microparticles. Particles heated up to 70°C (scan, one-solid lines) were recooled to 20°C and rescanned (scan two-broken lines).

end groups. Upon SC $CO_2$ treatment, the intensity of this glass transition peak decreased and the onset and enset temperatures were 42 and 47.8°C and the peak glass transition temperature decreased to 45°C. Also, the shape of the endotherm has changed after SC $CO_2$ treatment and the transition enthalpies decreased from 6.46 ± 0.76 J/g to 2.8 ± 0.34 J/g.

### Particle Properties

The particle properties including porosity, pore volume, pore size distribution, particle size distribution, and bulk density were determined (Porous Materials Inc., Ithaca, NY, USA) for the deslorelin–PLGA microparticles before and after SC $CO_2$ exposure at 1200 psi and 33°C for 30 min. Mercury intrusion porosimetry, dynamic light scattering, and tap bulk density measurements were used for obtaining the porosity, particle size, and density parameters, respectively. Table II presents the mean summarized parameters for three individual batches of both these particle formulations. The intrusion volume of deslorelin–PLGA microparticles significantly ($p = 0.032$) increased from 4.65 ± 0.55 to 10.74 ± 1.98 cc/g upon SC $CO_2$ treatment. The mean porosity increased from 39 ± 4.2 to 92.38 ± 2.96% ($p = 0.02$) and mean pore diameter increased from 90 ± 12.4 to 190 ± 30 nm ($p = 0.036$) upon SC $CO_2$ treatment. Furthermore, there was an increase in the, surface area, minimum pore diameter and median pore diameter. Also, upon SC $CO_2$ treatment, the mean particle size increased from 2.2 to 13.8 μm ($p = 0.03$) and the mean bulk density reduced significantly ($p = 0.01$) from 0. 7 to 0.082 g/cc. Thus, SC $CO_2$ treatment (1200 psi, 33°C), increased particle size, particle porosity and surface area, while decreasing the bulk density of the particles.

### Deslorelin Conformation

The far UV circular dichroism spectrum of deslorelin in 50% TFE (Fig. 5a) displayed a strong negative band at 227–230 nm, a strong positive band at ≈190 nm, and a weak negative broad band at 200–210 nm. Such a CD spectrum, termed as a class B spectrum (20), is indicative of the formation of a Type 1 β turn. We assessed the effect of SC $CO_2$ on pure deslorelin conformation. Upon SC $CO_2$ treatment, the shape

**Table II.** Comparison of Properties of Deslorelin Lactide-co-Glycolide PLGA (50:50) Microparticles Before and After Supercritical Carbon Dioxide (SC $CO_2$) Treatment (1200 psi, 33°C)

| Property/parameter | Untreated deslorelin–PLGA microparticles[a] | SC $CO_2$-treated deslorelin–PLGA microparticles[a,b] |
|---|---|---|
| Particle size | 2.2 ± 0.8 μm | 13.8 ± 1.3 μm |
| Bulk density | 0.7 ± 0.2 g/cc | 0.082 ± 0.00084 g/cc |
| Porosity | 39 ± 4.2% | 92.38 ± 2.96% |
| Total intrusion volume | 4.65 ± 0.55 cc/g | 10.74 ± 1.98 cc/g |
| Median pore diameter based on volume | 4.76 ± 0.9 nm | 9.8 ± 0.82 nm |
| Average pore diameter | 90 ± 12.4 nm | 190 ± 30 nm |
| Minimum pore diameter | 1.77 ± 0.1 nm | 3.7 ± 0.6 nm |
| Surface area | 0.454 ± 0.07 nm | 4.84 ± 0.83 nm |
| Residual solvent (methylene chloride) | 4500 ± 215 ppm | ND (<25ppm) |

[a] Data are expressed as mean ± standard deviation for three individual batches of particles and all parameters between the untreated and SC $CO_2$-treated groups were significantly different with $p < 0.05$.
[b] Deslorelin–PLGA microparticles prepared by the emulsion solvent evaporation method were exposed to SC $CO_2$ at 1200 psi and 33°C for 30 min.

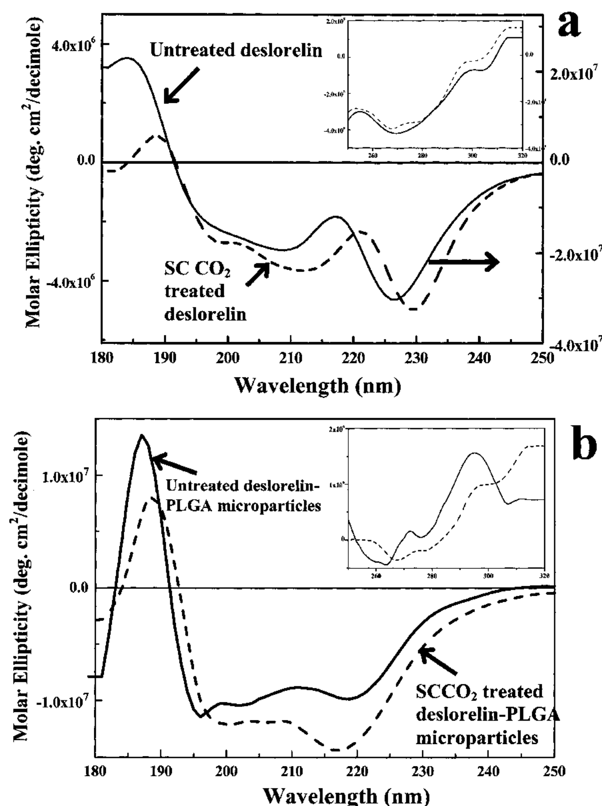


**Fig. 5.** Far ultraviolet circular dichroism spectra of deslorelin before (a) and after supercritical carbon dioxide treatment (1200 psi at 33°C for 30 min) and deslorelin released from deslorelin–PLGA particles prepared using emulsion solvent evaporation method (b) before and after supercritical carbon dioxide treatment (1200 psi at 33°C for 30 min). Insets show the corresponding near UV CD (280-320 nm) spectra of the various formulations.

of the spectrum changed and the intensity of the negative bands at ~≈230 and 200 nm increased from $4.9 \times 10^6$ to $3.4 \times 10^7$ and $2.6 \times 10^6$ to $2.5 \times 10^7$ deg $cm^2$/decimole, respectively (Fig. 5a). This indicates a considerable increase in the conformational rigidity and β-turn content of deslorelin. Indeed, this change upon SC $CO_2$ treatment could be a result of decreased conformational mobility or an increase in optical purity. The far UV CD spectrum of deslorelin released from the SC $CO_2$ treated particles indicated a β-turn conformation with a negative band centered around 218 nm, a negative shoulder at 200 nm, and a positive band at around 190 nm. However, in the CD spectrum of deslorelin released (Fig 5b) from untreated particles, the negative shoulder at 200 nm was distorted and intensified to a negative band and was shifted to lower wavelengths (≈195 nm). Also, the intensity of the negative band at 218 nm decreased. Indeed, such an intensification of negative band and loss of shoulder at 200 nm and a decrease in intensity at 218 nm were observed when LHRH was incubated at 65.5°C (21), consistent with a loss of intermolecular hydrogen bonds characteristic of β-turns. Also, the intensities of the positive band at 185 nm and the negative band at 180 nm increased. Thus, it appears that deslorelin, when encapsulated in PLGA microparticles, exhibits a conformational instability resulting in lower β-turn content. However, this change appears to be reversible because the deslorelin released from SC $CO_2$ treated particles exhibited positive band, negative band, and negative shoulder intensities comparable to pure deslorelin. Also, we examined the near UV CD (250-320 nm) spectra of deslorelin, SC $CO_2$ treated deslorelin, and deslorelin released from untreated and SC $CO_2$ treated deslorelin–PLGA particles. The near UV CD spectrum of deslorelin appeared as a single negative maximum centered at ≈270 nm (Fig. 5a, inset). Upon SC $CO_2$ treatment, there was no significant distortion of band shape or intensity, indicating lack of aggregation (Fig. 5a, inset). With deslorelin–PLGA microparticles, the CD spectrum of deslorelin from untreated particles was slightly distorted with two negative maxima appearing at 265 and 275 nm (Fig 5b, inset). Such a type of near UV CD pattern indicates a change in the local environment around the aromatic groups. Interestingly, for SC $CO_2$ treated particles, the spectral pattern reverts and is similar to the untreated deslorelin (Fig. 5a, inset), indicating that this change is reversible.

## FTIR Spectroscopy

To determine the structure of deslorelin encapsulated within the PLGA particles and the effect of SC $CO_2$ on the same, FTIR spectra of untreated and SC $CO_2$ treated deslorelin, deslorelin–PLGA particles, and blank PLGA particles were collected. FTIR spectra of deslorelin indicated a broad band at 1600-1700 $cm^{-1}$ with the peak centered at ≈1650 $cm^{-1}$, indicative of the amide I vibrational stretching of deslorelin. Upon SC $CO_2$ treatment (1200 psi, 33°C, 30 min), no broadening of band or shift in peak was observed indicating no significant structural perturbation (Fig. 6). In the case of deslorelin–PLGA particles, a C = O stretching vibration corresponding to the PLGA was seen at 1750 $cm^{-1}$ and also the amide I stretching was seen at ≈1650 $cm^{-1}$ in both untreated and SC $CO_2$ treated deslorelin–PLGA particles. A comparison of the FTIR spectra of deslorelin and deslorelin–PLGA microparticles indicated a change in the shape of the band suggesting a conformational perturbation compared to the native peptide.

## Mass Spectrometry

The mass spectrometric data for deslorelin released from the deslorelin–PLGA particles indicated a m/z value of 1282.5 for the MH+ ions in the electrospray ionization mass spectra. The monoisotopic mass calculated from this is 1281.5, which corresponds to the molecular weight of the intact deslorelin. The abundance of this peak was ~90-100%. The deslorelin released from the SC $CO_2$ treated (1200 psi, 33°C, for 30 min) particles as well as pure deslorelin showed the same peak, with ~90-100% abundance. These data indicate that deslorelin was intact following large-porous particle formation and no significant degradation occurred.

## Residual Methylene Chloride Content

The residual methylene chloride associated with the deslorelin–PLGA microparticles before and after SC $CO_2$ treatment at 1200 psi, 33°C for 30 min was determined using headspace gas chromatography. Methylene chloride content in the untreated deslorelin–PLGA microparticles was 4500 ± 215 ppm (n = 3). After SC $CO_2$ treatment, the residual methylene chloride levels were undetectable (< 25 ppm). Thus, possibly due to its ability to efficiently extract organic solvents

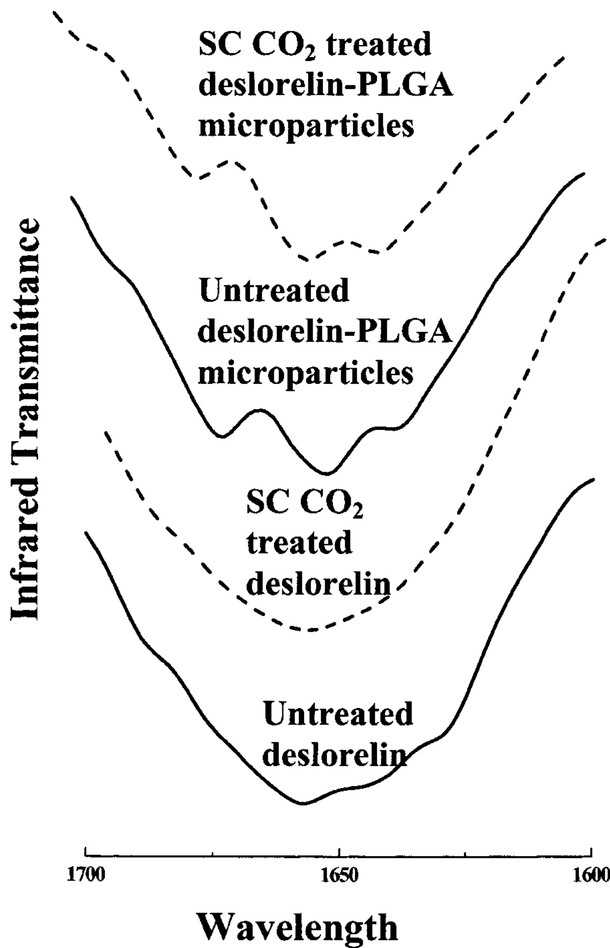


**Fig. 6.** Amide 1 Fourier-transform infrared spectra of deslorelin and deslorelin within the deslorelin–PLGA microparticles before and after supercritical carbon dioxide treatment (1200 psi at 33°C for 30 min).

(13,17), SC $CO_2$ decreased the residual methylene chloride content in deslorelin–PLGA microparticles.

### Deslorelin Release

The actual deslorelin drug loading was 5.45 ± 0.56% w/w (n = 3) in the conventionally prepared deslorelin–PLGA microparticles, corresponding to an encapsulation efficiency of 62.5 ± 0.36% (n = 3). Upon SC $CO_2$ treatment at 33°C and 1200 psi for 30 min, the drug loading was 5.16 ± 0.93% (n = 3), which was not significantly different from the conventionally prepared particles, indicating that no drug was being extracted away by the SC $CO_2$.

To characterize the release profile of deslorelin from deslorelin–PLGA microparticles and the effect of SC $CO_2$ treatment on the same, the *in vitro* deslorelin release was monitored in acetate buffer (pH 5) at 37°C. The release of deslorelin from deslorelin–PLGA microparticles was characterized by an initial burst of 7% followed by a sustained release for the 7 day study period (Fig. 7). At the end of 7 days, 40 ± 5.5% (n = 3) of deslorelin was released from the untreated deslorelin–PLGA particles. In the SC $CO_2$ treated particles, the percent deslorelin released was 26 ± 5.86 (n = 3) at the end of 7 days. Thus, SC $CO_2$ treatment at 33°C and 1200 psi did not accelerate deslorelin release from deslorelin–PLGA microparticles. However, SC $CO_2$ exposure at higher pressure (2000 psi) and temperature (37°C) accelerated deslorelin release and 97 ± 2.3% of the drug was released at the end of 7 days.

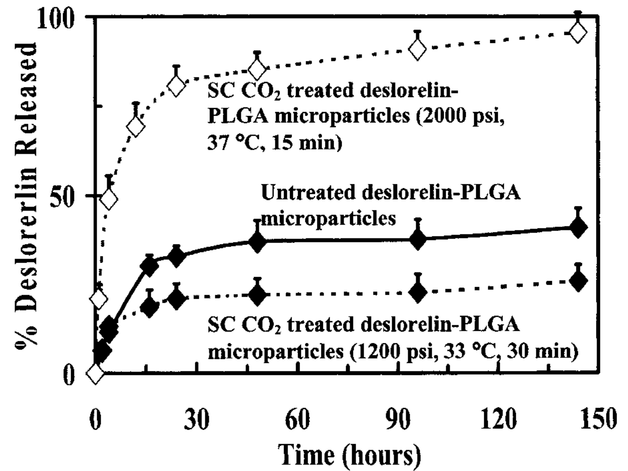


**Fig. 7.** Deslorelin release profiles from deslorelin–PLGA microparticles before and after supercritical carbon dioxide treatments at 1200 psi at 33°C for 30 min and at 200 psi at 37°C for 15 min. Data are expressed as mean ± SD for n = 3.

### Cellular Uptake of Particles

The uptake of deslorelin from solution form into Calu-3, A549, and rat alveolar macrophages was 35.8 ± 4.6, 55 ± 8, and 333.5 ± 51 ng/mg protein (n = 4), respectively (Fig. 8). With the untreated deslorelin–PLGA 50:50 microparticles,

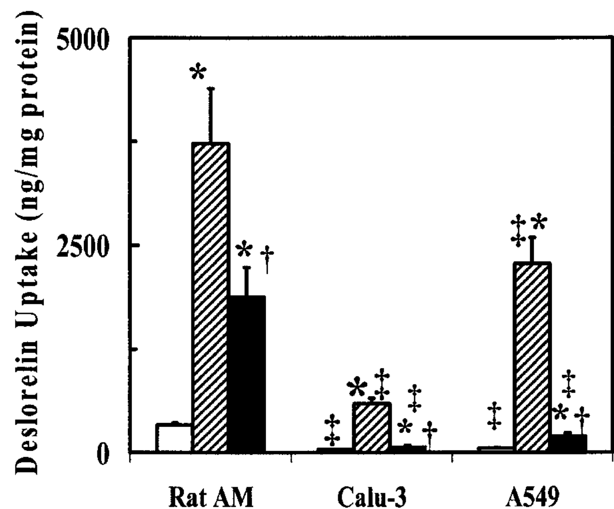


**Fig. 8.** Uptake of deslorelin from solution (open bars), nonporous deslorelin–PLGA 50:50 microparticles (hatched bars), and supercritical carbon dioxide-treated (1200 psi at 33°C for 30 min) porous deslorelin–PLGA 50:50 microparticles (closed bars) into rat alveolar macrophages, Calu-3, and A549 cell monolayers. Data are expressed at mean ± SD for n = 4. *$p < 0.05$ compared with deslorelin solution within each cell type; †$p < 0.05$ compared with nonporous deslorelin–PLGA 50:50 microparticles within each cell type; ‡$p < 0.05$ compared with uptake in rat alveolar macrophages for the same formulation.

the amount of deslorelin uptake into Calu-3, A549, and rat alveolar macrophages was 592 ± 66, 2276 ± 310, and 3724 ± 661 ng/mg protein (n = 4), respectively. The amount of deslorelin uptake from SC $CO_2$ treated particles was 74 ± 16.4, 202 ± 31.6, and 1881 ± 353 ng/mL (n = 4) into Calu-3, A549 and rat alveolar macrophages, respectively. Thus, compared with untreated particles the uptake of SC $CO_2$ treated particles into Calu-3, A549, and rat alveolar macrophages was reduced by 87, 91, and 50% respectively. Because of the phagocytic activity, the particle uptake was the highest in rat alveolar macrophages. Drug accumulation into cells from both particle types was significantly higher than that from solution. This increased accumulation may be either due to enhanced entry of the particulate formulations into the cells or the reduced degradation of deslorelin encapsulated within the PLGA particles.

## DISCUSSION

A modified emulsion solvent evaporation technique with a combination of solvents has been previously used to prepare porous polymeric peptide particles (22). However, the residual methylene chloride associated with such methods can be as high as 3000–30,000 ppm, which is above the United States Pharmacoepia (USP) limit (500 ppm) for these solvents (23). Organic solvents impose a harsh environment, which often denatures macromolecules (24). Removal of this solvent requires an additional drying step. In the current study, we used supercritical carbon dioxide under mild processing conditions to increase porosity, minimize residual solvent content, and reduce macrophage uptake of preformed polymer-peptide microparticles.

It has been suggested that physicochemical properties of polymers such as backbone composition, molecular weight, and crystallinity affect their processing by supercritical carbon dioxide (25). In our preliminary pressure-quench studies with SC $CO_2$, we observed that amorphous PLGA polymers with lower molecular weight were more amenable to plasticization compared to the crystalline L-PLA (Table I). This is because amorphous polymers are less ordered and exhibit high polymer chain mobility, which enables the diffusion and interaction of $CO_2$, a Lewis acid, with basic carbonyl groups within the polymer (26). This reduces inter-chain polymer interactions and results in enhanced segmental and chain mobility within the polymer. Upon pressure quench, $CO_2$ within the polymer rapidly expands and escapes. This results in particle expansion and pore formation. Once the gas escapes, the porous expanded structure vitrifies (Fig. 1b). Consistent with this, PLGA (50:50) (Fig. 3b) but not L-PLA formed large porous particles (Fig. 2b). Another reason for the amenability of the lower molecular weight amorphous polymeric particles to expand could be the coalescence of smaller particles as a result of decreased glass transition. Based on these results, we selected PLGA 50:50 (i.v. 0.26), an amorphous polymer of low molecular weight (23.2 kDa), high glycolide content, and better plasticizability by SC $CO_2$ for forming deslorelin containing large porous microparticles.

We have successfully transformed preformed deslorelin–PLGA 50:50 microparticles to large porous particles using SC $CO_2$ pressure-quench method (Table II). Upon SC $CO_2$ treatment, the particle size increased 10-fold and the bulk density reduced 5.34-fold. Previous studies have prepared large porous particles for deep lung delivery (10,27) but did not report the porosity of these particles. Porosity, pore volume and pore diameter, summarized in Table II, are important parameters influencing the aerodynamic diameter as well as the settling velocity and distribution of the particles in the lung (28). The theoretical mass median aerodynamic diameter (MMAD) of the porous particles prepared in our study is 3.7 μm, when estimated as per the method used by Vanbever *et al.* (28). It has been previously demonstrated that the theoretical MMAD closely correlates to that measured using the Aerosizer™ (28). Indeed, particles of similar diameter (10 μm) and densities (0.080 g/cc) had an aerodynamic size near 3 μm and were deposited with greater than 60% efficiency in the deep lungs (29). Also, particles possessing high porosity and large size with theoretical MMAD between 1–3 μm aerosolized much better and had 96% emitted dose and the respirable fractions ranging up to 49% or 92% depending on the measurement technique (28).

Apart from forming large porous microparticles with SC $CO_2$ treatment, we were able to reduce the residual methylene chloride content in these particles to below 25 ppm, which is within the USP limits. Methylene chloride, the most commonly used solvent in particle preparation, is a carcinogen and has been shown to induce other toxicities including neurotoxicity or teratogenicity, especially following inhalation. Indeed, mice treated with 1000 ppm inhaled methylene chloride showed an increased DNA synthesis and cell proliferation in lungs (30). Therefore, there is a need to reduce the residual methylene chloride within particles prepared for inhalation. The USP limits the amount of methylene chloride in pharmaceuticals to 500 ppm. Interestingly, with the conventional methods of particle preparation, the residual methylene chloride levels are 3000–30,000 ppm (23,31). We have similar results in our studies wherein the untreated deslorelin–PLGA microparticles contained 4500 ppm of methylene chloride.

The deslorelin primary structure was intact after SC $CO_2$ processing as indicated by our mass spectrometric data. The analysis of secondary structure of deslorelin using CD spectrum (Fig. 5a) indicated a Type-1 β-turn typical of LHRH type peptides. With SC $CO_2$ treatment we observed more deslorelin residues in the β-turn conformation (Fig. 5a). The β-turn in short polypeptides is frequently associated with the polypeptide chain folding upon itself and in deslorelin, this turn is most probably centered at the W3-DW6 (tryptophan3-D-tryptophan6) position. This assumption is reasonable as type I turns are favorable with Serine in the i+1 position and a D-amino acid at the i+3 position (32). Interestingly, studies with LHRH and its active analogs have indicated that a β-turn structure is essential for maintenance of proper spatial arrangement of N- and C-termini for function at the receptor level (33). Furthermore, loss of β-turn structure in LHRH analogs correlated with poor receptor binding and decreased activity (34). Thus, increasing β-turn conformation may have a beneficial effect in maintaining peptide activity. However, the mechanisms by which SC $CO_2$ increases and stabilizes a β-turn conformation remain to be investigated.

For deslorelin released from the untreated deslorelin–PLGA microparticles, the characteristic β-turn feature was lost as observed by the distortions in the far UV CD spectrum (Fig 5b). Such changes, which are often reversible, are observed in LHRH agonist peptides under thermal or pH

stresses and/or when the peptide is exposed to an organic solvent (35). Interestingly, these changes could be partially reversed upon SC $CO_2$ treatment (Fig. 5b). Our near UV-CD data also indicate a similar reversal effect with SC $CO_2$ treatment. One possible reason for the observed structural perturbation in deslorelin could be the high residual solvent associated with the conventionally prepared particles. Indeed, the deteriorated secondary structures of proteins trypsin and lysozyme upon dissolution in DMSO was previously reported to be reversed after extraction of the solvent by supercritical carbon dioxide (36).

Our FTIR studies indicated perturbation of deslorelin conformation within the PLGA microparticles (Fig. 6). This perturbation may be caused by the presence of a conformationally restrained form of deslorelin within the hydrophobic PLGA environment. This may also be due to the pH microenvironment within the particles. This change is not necessarily a reflection of the biological activity or conformation of released drug, which may be different. Indeed, although amide I band Raman spectra indicated structural perturbation in solid state for lysozyme and trypsin microparticles, these proteins exhibited native conformation and 95–100% biological activity upon reconstitution in a buffer (37).

The drug polymeric particles need to avoid uptake and clearance by the alveolar macrophages, because macrophage clearance limits the duration of drug delivery (10). Another mechanism by which particles deposited in the lungs are cleared is by uptake into the epithelial cells, wherein they are sequestered within cells and lost with cell turnover or enter the lymphatic circulation and are carried away to pleural, hilar, and more distant lymph nodes (38). Thus, epithelial uptake of drug particles would likely accelerate their degradation and shorten their effect. In our studies, the ratio of deslorelin uptake from porous particles to nonporous deslorelin–PLGA particles was in the order: rat alveolar macrophages (0.505) > A549 (0.124) > Calu-3 (0.093). This order is consistent with the phagocytic nature of these cells because macrophages are more phagocytic compared to A549 and Calu-3 cells. Also, consistent with our observations, A549 cells have previously been shown to have a higher phagocytic activity compared to Calu-3 cells (19). Furthermore, the efficiency of particle uptake by all these cells (macrophages, A549 and Cau-3) decreases with an increase in particle size (19,39). In human alveolar macrophages, particles having geometric diameters of 1–2 μm were taken up in quantities of six particles/cell. When the particle size increased above 5 μm, the uptake was reduced and only one to two particles were taken up per cell (39). For A549 cells, and Calu-3 cells, the internalization reduced dramatically beyond a particle size of 3 and 2 μm, respectively (19).

One would expect that upon rendering the deslorelin–PLGA microparticle porous the increased surface area and the surface pores would cause an acceleration of release. However, at the conditions used (1200 psi and 33°C) for the preparation of deslorelin–PLGA particles characterized in this study, we observed an increase in mean porosity without an increase in drug release (Fig. 7). Indeed, the drug release was slightly decreased. Therefore, although there is an increase in mean pore sizes of the particles upon SC $CO_2$ treatment, this increase in porosity may be compensated for by an increase in particle size, tortuosity, and path lengths. The decrease in deslorelin release with increased porosity can also be explained if particles have closed pores with large interior voids interconnected with smaller passages (40).

The dose requirements for humans and animals are very low for LHRH agonists. A total dose of 30 mg in leupron depot is sufficient for a 4-month effect in case of prostate cancer (41). Previously we have prepared short-term deslorelin pluronic gel formulations and evaluated these in cattle for their ability to delay the pre-ovulatory LH surge (42). We observed therapeutic effects for deslorelin in cattle with a total dose as low as 100 μg. With the pluronic systems, a 2-h delay in the pre-ovulatory LH surge was observed and increasing this delay is beneficial in increasing the number of ovulations due to better follicular development (42). Indeed, the deslorelin particles prepared in this study may also be used in cattle or mares to induce ovulation as in this case a 7- to 10-day release system would suffice.

## CONCLUSIONS

Using a novel supercritical fluid process we were able to modify pre-made microparticles and produce large porous deslorelin–PLGA microparticles. These particles produced with mild processing conditions (33°C and 1200 psi), exhibited little or no residual methylene chloride and reduced macrophage and epithelial cellular uptake. This process can be potentially applied to produce macromolecule particles for sustained delivery via the respiratory tract.

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