Insulin-loaded poly-L-lactide porous microspheres prepared in supercritical CO2 for pulmonary drug delivery

Ai-Zheng Chen, Na Tang, Shi-Bin Wang, Yong-Qiang Kang, Hu-Fan Song

College of Chemical Engineering, Huaqiao University, Xiamen 361021, China
Institute of Biomaterials and Tissue Engineering, Huaqiao University, Xiamen 361021, China

1. Introduction

Pulmonary drug delivery by dry powder inhalation is a considerable alternative for delivery of therapeutic drugs such as peptides, proteins, gene and vaccine [1–4], which should otherwise be injected. The pulmonary drug delivery possesses non-invasiveness and remarkable bioavailability when compared with other administration routes. These are attributed to the physiological features of the lung, which displays a more than 100 m² of surface area, high vascularization, thin blood-alveolar barrier, inferior enzyme activities and the absence of hepatic first-pass effect [5–7]. Since the Exubera®, the insulin-based dry powder approved by FDA via pulmonary delivery for diabetes treatment, was withdrawn from the market, the other insulin preparations via pulmonary route have been extensively studied [8–11].

The large porous particle for pulmonary drug delivery was first proposed by Edwards in 1997 [12]. The large porous particle with a low value of aerodynamic diameter (Dₐ) as well as a reasonably high value of geometric mean diameter (Dₙₐ) could not only achieve an effective lung deposition [13,14] and prolong the residence time in lung, but also improve the stability and sustained-release effect of the macromolecular drugs [15–17]. The representative strategies for preparation of large porous particles can be summarized as spray-drying [18–20] and double emulsion-solvent evaporation [21–23]. The spray-drying method is not suitable for temperature-sensitive drugs and the resulting porosity is relatively low [24]. The double emulsion-solvent evaporation method needs appropriate pore-forming agents and further treatments must be carried out to reduce the organic solvent residue of the product. Using the cycloexdrin as a porogen, Ungaro et al. [15] produced the inhalable insulin-loaded PLGA large porous particles by a double emulsion method; the resulting particles displayed a fine aerodynamic property and a considerable hypoglycemic activity in the diabetic rats.

Notably, supercritical CO2 has been exhibiting great advantages in the field of drug carrier preparations due to its low organic solvent residue, moderate operating condition and environmental friendliness [25–28]. Falco et al. [29] used the continuous supercritical emulsions extraction process to prepare the insulin-loaded PLGA microspheres with a low residual solvent; and the process
was appropriate for the preparation of the thermolabile compounds. For the large porous particle fabrication, supercritical CO₂ can be used as drying agent for polymer gel [30,31], gas foaming agent in polymer microspheres [32] or porogen in polymerization reaction system [33,34]. Nevertheless, these methods generally suffered a low porosity and difficulty in loading drugs into carriers by one-step process, or required a high temperature for some foaming processes, which are not suitable for producing proteins-loaded large porous particles for pulmonary drug delivery, Dhanda et al. [33] used a solvent evaporation technique to produce the celecoxib loaded PLGA microspheres, followed by a supercritical CO₂ based pressure-quench technology to develop the large porous structures for pulmonary drug delivery; the resulting porous microspheres displayed a low porosity and slight residual solvents.

Herein, we attempted to employ an emulsion-combined precipitation of compressed CO₂ antisolvent (PCA) process with ammonium bicarbonate (AB) as a porogen for the preparation of protein-loaded porous microspheres for pulmonary drug delivery [35,36], which would avoid the drawbacks of organic solvent residue and low porosity caused by the conventional methods. In this study, we chose insulin as a protein drug model to prepare insulin-loaded poly-L-lactide porous microspheres (INS-PLLA PMs) for treatment of diabetes via pulmonary delivery. The relating properties including morphology, particle size, aerodynamic properties, physicochemical properties, hypoglycemic activity, drug loads, encapsulation efficiencies and drug release profiles were characterized.

2. Materials and methods

2.1. PLLA (MW 50,000, 1.5 dL/g) was purchased from the Jinan Daigang Co., Ltd. (Jinan, China). Pluronic F-127 (PF-127) and AB were purchased from Sigma-Aldrich (USA). Insulin from bovine pancreas (>27 IU/mg, 98% purity) was purchased from Dalian Meilun Biological Technology Co., Ltd. (Dalian, China). Dichloromethane (DCM, 99.8% purity) was purchased from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). CO₂ of 99.99% purity was supplied by the Rihong Air Products Co., Ltd. (Xiamen, China). Fluorescein isothiocyanate (FITC) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Bicinechonic Acid (BCA) Kit was purchased from Appylen Technologies Inc. (Beijing, China). All other compounds were of analytical purity.

Adult male Kunming (KM) mice weighed of 30~35 g were purchased from the Slac Laboratory Animal Co., Ltd. (Shanghai, China). Food and water were available ad libitum. Mice were kept in a facility under a 12 h light/dark cycle.

2.2. Methods

2.2.1. Preparation of INS-PLLA PMs by emulsion-combined PCA process

The INS-PLLA PMs were prepared by an emulsion-combined PCA process as previously reported [35,36]. Briefly, 306.7 mg of PLLA and 153.3 mg of PF-127 were dissolved in 20 mL of DCM as the oil phase. The water phase consisted of insulin and 2 mL of AB aqueous solution at a concentration of 250 mg/mL. The water phase was added into the oil phase, followed by an ultrasonic emulsification at 200 W for 1 min. The finally-prepared emulsion was then subjected to the PCA process. As shown in Fig. 1, the apparatus consists of a CO₂ supply system, a solution delivery system and an autoclave with a volume of 500 mL. In a PCA process, the CO₂ was cooled down to around 0 °C by using a cooler, followed by delivery using a high pressure meter pump. Then a heat exchanger was used to preheat the liquefied CO₂. When the operating parameters reached at 8 MPa and 30 °C, we adjusted the frequency of CO₂ pump to maintain a steady flow of CO₂ (1000 L/h). The emulsion was fed into the autoclave through a nozzle at a flow rate of 4.0 mL/min. When the emulsion was completely injected into the autoclave, a washing process was performed by the compressed CO₂ for about 30 min under the same conditions described in above. This was to remove the residual organic solvent. Next, the autoclave was slowly depressurized and the microspheres containing AB could be obtained. The resulting microspheres were dried using vacuum drying at 35 °C for 2 h to decompose AB. Finally, INS-PLLA PMs were harvested for further characterizations.

2.2.2. Morphology and particle size characterizations

Surface morphological examinations were carried out by a field-emission scanning electron microscope (FE-SEM S-4800, Hitachi, Japan). The samples were absorbed onto the conducting resin and then sprayed with gold under vacuum conditions.

The D₅₀ and particle size distribution were investigated by Laser particle size analyzer (LS13-320, Beckman Coulter, USA). For the aerodynamic property, a lower mass median aerodynamic diameter (MMAD) was adopted as D₅₀, which was measured by an eight-stage Andersen Mark II cascade impactor (ACI 20-810, Thermo Scientific, USA) to calculate the practical deposition into the lung [17,37]. The capsule containing 10 mg of the INS-PLLA PMs was placed in a dry powder inhaler device and a hole was made so that the powder could be released. Then the samples were absorbed into the ACI at a flow rate of 28.3 L/min. Finally, the weight of powder at each stage was recorded for the calculation of the D₅₀ and the fine particle fraction (FPF).

2.2.3. Aerosolization properties analysis

To study the aerosolization of INS-PLLA PMs, photos were captured at 0.04 s intervals after actuation using a digital camera (D3100, Nikon, Japan) [22]. The aerosolization efficiencies by an insufflator device (DP-4) and an air pump (AP-1) (Penn-Century, Inc., Philadelphia, PA) were researched. The air volume was set at 2.0 mL for a single actuation. Data are presented as means ± SDs of three independent.

2.2.4. Fourier transform infrared (FTIR) analysis

For FTIR analysis, samples were separately mixed with KBr and measured by FTIR analyzer (8400S, Shimadzu, Japan) in the transmission mode, with the wavenumber ranging from 4000 cm⁻¹ to 400 cm⁻¹.

2.2.5. Gas chromatography (GC) analysis of organic solvent residue

GC (6890N, Agilent Technologies Inc., USA) was used to detect the residual organic solvent in the INS-PLLA PMs. Approximately 500 mg of sample was accurately weighed, and the analysis was performed by the static head-space method.

2.2.6. Circular dichroism (CD) spectroscopy analysis

For CD spectroscopy analysis, a 0.1 mm path cell for far-UV CD was used. The parameters were as follows: a scanning wavenumber of 190~250 nm, a scanning speed of 100 nm/min, a flow rate of N₂ at 5~10 L/min and sensitivity of 5 mdeg/cm. CD spectrum of the protein analysis was removed from the reference set and the secondary structure fractions were determined using computer programs CDSTR in CDP pro software [38]. Each sample was carried out in independent triplicate.

2.2.7. Bioactivity verification test

For the bioactivity verification test, KM mice were randomly divided into three groups. The mice were fasting for 12 h and
given subcutaneous injection of different samples (saline, insulin in INS-PLLA PMs and raw insulin) at the insulin dose of 0.6 IU/kg. At the scheduled time intervals, a drop of blood was collected from the mice tail vein and blood glucose levels were determined using a blood glucose meter (ACCU-CHEK Performa, Roche Diagnostics Corp., USA). Blood glucose levels are expressed as mg/dL and reported as mean ± SDs of three independent. Data analysis was carried out using SPSS for one-way ANOVA. P values less than 0.05 were considered as significant.

2.2.8. Determination of drug loads, encapsulation efficiencies and release profiles

For the drug loads and encapsulation efficiencies, 10 mg of INS-PLLA PMs was dissolved in 5 mL of DCM, and then 10 mL of phosphate buffered saline (PBS) was added and stirred by a magnetic force stirrer to volatilize the DCM. The amount of insulin was analyzed by BCA Kit after the resulting solution was filtered through a 0.22 μm membrane. The drug load and encapsulation efficiency were calculated by Eqs. (1) and (2), respectively.

\[
\text{Drug load} = \frac{W_1}{W_2} \times 100\% \quad (1)
\]

\[
\text{Encapsulation efficiency} = \frac{W_1}{W_3} \times 100\% \quad (2)
\]

where \(W_1\), \(W_2\) and \(W_3\) represented the amount of insulin in INS-PLLA PMs, the gross weight of INS-PLLA PMs, and the weight of initial insulin, respectively. Each experiment was carried out in independent triplicate.

For drug release profiles, different amounts of samples (raw insulin, 5.0%, 7.5%, 10.0%, corresponding to insulin amount of 3 mg) were placed in the dialysis bags; the bags were immersed in a bottle with 10 mL of simulated lung fluid (SLF, pH 7.4) and incubated in a shaking water-bath at 37 °C, 60 rpm. A sample of 1 mL of solution was periodically removed and 1 mL of fresh SLF was subsequently added. The amount of insulin was analyzed by BCA Kit. Drug release profiles were calculated in terms of cumulative release percentages of insulin (% w/w) with incubation time. Each experiment was carried out in independent triplicate.

3. Results

3.1. Morphology and particle size characterizations

As shown in Fig. 1, the PCA process combined with AB for a lower residual organic solvent and a better pore-forming effect was adopted to develop the INS-PLLA PMs. According to Fig. 2(a), the resulting microspheres exhibited a rough and porous shape owed to the decomposition of AB into NH₃, CO₂ and H₂O [39]. Remarkably, the microspheres revealed a nanoparticle-aggregated surface in Fig. 2(b), which could be explained by the nucleation of the polymer and the mass transfer property of fabrication process as recorded in previous studies [35,40]. Fig. 2(c) reveals that the insulin was uniformly distributed in the matrix of polymer microspheres. The INS-PLLA PMs displayed a \(D_v\) of 15.62 ± 1.32 μm and a uniform size distribution (Fig. 2(d)), and this is favorable to achieve a better drug delivery efficiency than those microspheres with a \(D_v\) less than 10 μm, which could be easier to be uptaken by alveolar macrophage [41]. Furthermore, the INS-PLLA PMs possessed a \(D_a\) of 4.31 ± 0.23 μm and a FPF of 65.57 ± 1.81%, which meet the requirements for pulmonary drug delivery [42].

3.2. Aerosolization property

For the aerosolization property, the initial aerosolization of INS-PLLA PMs shown in Fig. 3 was maintained for 0.12 s after actuation, and aerosol particles exhibited good mobility as well as good aerosolization characteristics. The aerosolization efficiency was 90.95 ± 2.18% with the single actuation for 3 mg of INS-PLLA PMs.

3.3. FTIR analysis

Fig. 4 shows the FTIR spectra of raw insulin, raw PLLA, PF-127 and INS-PLLA PMs. From Fig. 4, the major peaks at 1655 cm⁻¹ and 1539 cm⁻¹ in INS-PLLA PMs were ascribed to the stretching vibration of C=O and bending vibration of N–H, respectively [11], and this result is coincident with raw insulin. When compared with the peaks at 3312 cm⁻¹ and 1242 cm⁻¹ in raw insulin, there was no apparent peaks in INS-PLLA PMs or the peaks were overlapped.
Fig. 2. Photographs of INS-PLLA PMs and their particle size distributions (a) SEM image of INS-PLLA PMs, (b) the nanoparticle-aggregated surface, (c) fluorescent image of INS-PLLA PMs and (d) particle size distributions, respectively (INS-PLLA PMs: insulin-loaded poly-ß-lactide porous microspheres).

by the other materials of microspheres [43], which indicates that minor structural changes might have occurred on a molecular level in insulin after supercritical processing. The characteristic peaks at 1759 cm⁻¹ of the PLLA [35] and 2889 cm⁻¹ of the PF-127 [44] were clearly observed in INS-PLLA PMs, which suggests no chemical changes happened in matrix of polymer microspheres. The PLLA and PF-127 precipitated with entrapped insulin by supercritical CO₂ antisolvent, thus forming the INS-PLLA PMs.

Fig. 3. The aerosolization of insulin-loaded poly-ß-lactide porous microspheres by dry powder sprayer after the actuation (a) 0.00 s, (b) 0.04 s, (c) 0.08 s and (d) 0.12 s.
3.4. Organic solvent residue study

DCM was used as the solvent of PLLA and PF-127 in this study. It is essential to determine the organic solvent residue since DCM is a limited solvent under the International Conference on Harmonisation (ICH) guidelines. As illustrated in Fig. S1 of the Supplementary Material, the results of GC analysis reveal that the DCM residue in INS-PLLA PMs was too low to be detected, namely under the detection limits of 41 ppm, which is far less than the maximum acceptable limit of the Pharmacopeia of People’s Republic of China (2010) (max. 600 ppm). This implies that the INS-PLLA PMs with very little organic residue could promote the safety and suitability of protein-loaded carriers for pulmonary drug delivery.

3.5. CD analysis

For the secondary structure, the far-UV CD spectra of insulin were available in Fig. S2 of the Supplementary Material. The results show that the α-helix characteristic peaks at 208 nm and 223 nm of insulin released from INS-PLLA PMs were identical with the raw insulin [15]. The β-sheet characteristic peak at 217 nm of insulin in microspheres was parallel with raw insulin. These results demonstrate that no obvious changes occurred on the secondary structure of insulin. For a quantitative analysis, the ratio between the mean residue ellipticity at 208 and 223 nm was calculated for the associated reaction of insulin [45], being \[ \frac{\theta_{208}}{\theta_{223}} = 1.44 \] for raw insulin, \[ \frac{\theta_{208}}{\theta_{223}} = 1.48 \] for insulin in INS-PLLA PMs, respectively. The proportions of secondary structure in insulin are shown in Table 1. The major secondary structure in insulin is α-helix, which is the most stable and important structure. Compared with raw insulin, the proportions of α-helix and unordered were reduced by 5% and 1.5%, respectively. This indicates there was a minor structural change on the secondary structure of insulin after supercritical processing.

3.6. Hypoglycemic activity analysis

The hypoglycemic activity measurement was conducted with subcutaneous injection of insulin in KM mice, the result is shown in Fig. 5. After treatments with insulin from microspheres and raw insulin for 1 h, the fasting blood glucose level of KM mice declined from initial value of 70.06 mg/mL to 37.20 mg/mL and from 65.72 mg/mL to 31.00 mg/mL, respectively; the corresponding declined percentages were 47.3% and 52.8%, respectively. Then the blood glucose level got rebounded after 2 h. However, there is no statistically significant difference \( (P > 0.05) \) between the insulin from INS-PLLA PMs and raw insulin on hypoglycemic activity, which is corresponding to the results of CD analysis. This means that the supercritical process is a mild method to produce insulin-loaded carriers with very little or no damage on hypoglycemic activity of insulin.

3.7. Drug loads, encapsulation efficiencies and drug release profiles

Fig. 6 illustrates the drug loads and encapsulation efficiencies of INS-PLLA PMs prepared by PCA process. When theoretical drug loads were set at 5.0%, 7.5% and 10.0%, the corresponding drug loads were 4.85%, 6.70% and 6.90%, respectively, and their encapsulation efficiencies were 97.02%, 89.40% and 68.97%, respectively. It is obviously that the drug load increased and encapsulation efficiency decreased with the increase in drug dose.

As shown in Fig. 7, the percentages of insulin released in the first half hour from the INS-PLLA PMs with different theoretical drug loads were 18.13%, 33.17% and 50.69%, respectively. Only the INS-PLLA PMs with drug dosage of 5.0% showed no burst release effect and displayed a sustained-release effect for 24 h. INS-PLLA PMs with drug dosage of 7.5% and 10.0% exhibited a nearly complete release in 12 h accompanied with a burst release.

4. Discussion

Taking AB as the porogen, an emulsion-combined PCA process for producing INS-PLLA PMs was successfully developed. According to the previous studies [22,35], the particle in a low value of \( D_0 \) (1–5 μm) with a reasonably high value of \( D_0 \) (10–30 μm) and strongly developed specific surface area would achieve an
effective lung deposition as well as a reduced uptake by alveolar macrophages. The resulting microspheres exhibited a \( D_{90} < 4.7 \, \mu m \) and \( D_{95} > 15 \, \mu m \). Furthermore, the rough and porous surface demonstrated a better FPF because of the coarse structure, which could be explained by reducing effective contact areas and cohesion among particles for a better dispersity [4]. Eventually, the INS-PLLA PMs displayed a good aerosolization characteristic, which meets the requirements for pulmonary drug delivery.

The physicochemical characterizations of INS-PLLA PMs consisted of FTIR, DCM residue, CD and bioactivity analyses. The results of FTIR and CD show that no chemical changes occurred on INS-PLLA PMs, while there was a slight change from \( \alpha \)-helix and unordered to \( \beta \)-sheet as well as \( \beta \)-turn in the secondary structure, which revealed a slight decrease in stability of insulin. This is consistent with the decrease in the content of \( \alpha \)-helix structure since \( \alpha \)-helix is the most stable structure in insulin. Jiang [46] and Liu [47] reported that the decrease of \( \alpha \)-helix would generate a slight loss of activity in insulin and proinsulin. However, the result of bioactivity test shows that the insulin after supercritical processing retained a good hypoglycemic activity, which displays no statistically significant difference with raw insulin. This is coincident with previous study that supercritical process showed little damage on proteins [36,48]. Notably, the DCM residue in INS-PLLA PMs is far less than the maximum acceptable limit, which mainly owes to the PCA process. This presents that the supercritical process could avoid further heavy downstream processing for the removal of organic solvent residue, which would be potential for producing protein-loaded carriers, especially in the large-scale production. In summary, the supercritical process is a typical physical process to produce drug-loaded polymer carriers without alternating their properties.

For the drug loading behavior, insulin was encapsulated in the matrix of polymer microspheres and displayed a homogenous distribution. There was a tendency to raise drug load and reduce encapsulation efficiency when the drug quantity increased. The phenomenon could be explained by the exceeded load capacity of the microspheres, which could not achieve a good encapsulation of the redundant insulin [49]. The initial release of INS-PLLA PMs with different drug loads was mainly attributed to the insulin on the surface of microspheres or contacted loosely with microspheres. Thus the burst release in 0.5 h of INS-PLLA PMs with drug dosages of 7.5% and 10.0% was caused by the external insulin contents of 41.85% and 56.52%, respectively. After that, the insulin from the matrix of microspheres was released slowly. We believe that the INS-PLLA PMs prepared by PCA process would be potential in sustained-release of insulin for pulmonary drug delivery.

### 5. Conclusion

The INS-PLLA PMs were successfully fabricated by an emulsion-combined PCA process with AB as a porogen. The resulting microspheres with a porous morphology, a low value of \( D_{90} \) and a reasonably high value of \( D_{95} \) and strongly developed rough surface, conformed to the requirements for pulmonary drug delivery. With almost no organic solvent residue, the supercritical process is a typical physical process to produce INS-PLLA PMs without the devitalization of insulin. Furthermore, the INS-PLLA PMs could realize a sustained-release of insulin for 24 h. This study indicates that the supercritical process is an environmental protection technology for the development of protein-loaded drug delivery system, and the INS-PLLA PMs would have potential to be applied in the treatment of diabetes by pulmonary drug delivery.

### Acknowledgements

Financial supports from Natural Science Foundation of China (31470927 and 31170939), Natural Science Foundation of Fujian Province of China (2014J01128), Public Science and Technology Research Funds Projects of Ocean (201505029) and Promotion Program for Young and Middle-aged Teacher in Science and Technology Research of Huaqiao University (ZQN-PY107) are gratefully acknowledged.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.supflu.2015.03.010.

### References


