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Fabrication of uniform enzyme-immobilized carbohydrate microparticles with high enzymatic activity and stability via spray drying and spray freeze drying

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ABSTRACT

Enzyme-immobilized particles with high enzymatic activities are fundamentally and practically important for many areas, such as pharmaceuticals, medicine, and biocatalysis. In this study, by selecting trypsin and trehalose as the representative enzyme and excipient, two techniques, spray drying (SD) and spray freeze drying (SFD), have been utilized to generate enzyme-immobilized particles and comparatively studied. In both methods, uniform enzyme-immobilized microparticles are successfully obtained by using a micro-fluidic aerosol nozzle (MFAN) as the monodisperse droplet generator. The particle morphology, size and inner structure are distinctly different between the SD- and SFD-derived trypsin/trehalose composite microparticles. The former shows crumpled morphology, smaller sizes and dense inner structure while the latter shows spherical and open porous morphology with larger particle sizes. The particle formation processes in both methods are discussed. The more surface-active and large-sized trypsin molecules tend to be accumulated at the air-liquid interface of drying droplets, leading to particle buckling in SD and the formation of thin surface trypsin-enriched layer in SFD. The trypsin enzymatic activity is highly related to the presence of trehalose and the processing method. For the pure trypsin microparticles, SFD leads to a better activity preservation than SD does due to the much higher temperature adopted in SD. The presence of trehalose can significantly protect the enzymatic activity of trypsin, reaching 97.7 ± 2.6% and 97.3 ± 1.6% activity preservation with the optimal trypsin/trehalose mass ratio of 1:1 for both the SD- and SFD-derived microparticles, respectively. The protection of the enzymatic activity originates from the hydrogen bonding formation between trypsin and trehalose and the formation of highly amorphous glass matrices, which decrease enzyme unfolding and aggregation. In terms of process operability, SD offers a rather simple and economic means to produce enzymatic microparticles of high activity with the appropriate dosage of trehalose.

1. Introduction

Manufacturing solid dosage in powder form of therapeutic agents with high biological activity has attracted numerous research & design (R&D) efforts in pharmaceutical research and industries [1–4]. Dry formulation is generally preferable for product transportation and storage by reducing structural alternations of active molecules [5,6]; whereas liquid formulation can impose mechanical stresses on biological substances at air-liquid interfaces during handling, likely leading to structural damage and loss of activity [7–9]. Dehydration processes for solidification mainly include spray drying (SD) [10–12], freeze drying (FD) [13,14], and spray freeze drying (SFD) [15,16]. SD is an efficient and up-scalable technology to produce particulate biologics, which evaporates droplets containing active ingredients atomized from precursor liquid. As SD regularly adopts hot air over 100 °C for drying, the molecular conformational stability of biologics could be easily undermined. Comparatively, FD offers a milder, but more expensive and time-consuming means to manufacture solid biological formulation. In addition, in FD processing, further steps, e.g. grinding and milling, are required to form powdered products [17]. Comparatively, SFD consolidates some of the advantages of SD and FD by spraying a precursor liquid into cryogenic liquid or pre-cooled chamber to obtain frozen microparticles, followed by sublimation by using a vacuum freeze dryer [18]. However, freezing can also cause denaturation of cold liable biological molecules due to structural unfolding and irreversible aggregation [19]. A general route to avoiding the inactivation of dried bioproducts is the addition of various excipients to the precursors prior to drying, such as polypeptides [20], metal ions [21], carbohydrates, disaccharides and etc. [22,23].

Furthermore, the applicability of therapeutic-agent-loaded microparticles for a large variety of therapeutic purposes depends on particle size. A narrow particle size distribution is an essential prerequisite for different administration routes, such as particles with aerodynamic diameter

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ranging from 1–5 μm for pulmonary [24], 4.8–23 μm for nasal [25], and 40–70 μm for needle-free powder epidermal injection [26]. Apart from the particle size, the uniformity in particle shape, morphology, and density also play important roles for functioning. Individual product batches with high controllability and reproducibility can optimize the dose efficiency by avoiding undesirable burst release of active molecules from pharmaceutical microparticles.

To overcome the possible inverse influences of non-uniformity in particle property on particle quality and function, fabrication of uniform microparticles is critical. One-step enzyme immobilization with well-preserved enzymatic activity is highly demanded. Moreover, elucidation of the influences of using the two different processing techniques, i.e. SD and SFD, on the properties and biological activities of the resultant uniform microparticles can provide valuable information. In this study, trypsin, a soluble globular protease which plays an important role in the digestion into the pancreas [27,28] is selected as the model enzyme. Trehalose, a natural disaccharide which is present quite widely in the biological world, is adopted as the model excipient [29,30], respectively. SD and SFD techniques, both equipped with a specially designed micro-fluidic aerosol nozzle (MFAN) capable of generating monodisperse and uniform droplets, have been used to directly fabricate uniform trypsin-immobilized carbohydrate microparticles. The effects of precursor formula and manufacturing means on particle size, morphology, density, structure and enzymatic activity have been comparatively investigated. It is found that SD produces crumpled dense microparticles while SFD produces light microspheres with open macropores. The presence of the excipient is essential for protecting the enzyme from activity loss during drying. Possible mechanisms for the protection of enzyme are discussed. Under optimal formulations, both SD and SFD are robust to generate highly active enzyme-immobilized microparticles. In particular, it is verified that enzymatic activity can be well preserved in the presence of a protecting excipient even using the SD technique at high temperatures.

2. Material and methods

2.1. Chemicals and precursor solutions

The raw materials, including trypsin (from porcine pancreas lyophilized powder, 1000–2000 units/mg dry solid), D (+) trehalose dihydrate, *N* α benzoyl Larginine ethyl ester (BAEE) and phosphate buffered saline (PBS) were purchased form Sigma-Aldrich (Sigma-Aldrich Co., USA). Hydrochloric acid (HCl, 36–38 wt%) was purchased from Chinasun Specialty Products Co., Ltd. (China). Potassium bromide (KBr) was purchased from Sigma (China). Distilled water (18.2–18.3 MΩ·cm) was used to prepare the aqueous solutions.

Pure trypsin, pure trehalose and mixtures of trypsin and trehalose with various mass ratios (from 3:1 to 1:9) were dissolved in distilled water. The total solid content (the total mass percentage of trypsin and trehalose of the precursor solutions) was fixed at 5.00 wt% (Table S1).

2.2. Generation of monodisperse droplets

A microfluidic aerosol nozzle (MFAN; Fig. 1a) was used to generate monodisperse droplets from the precursor solutions. The operating principle of the MFAN system can be found in a previous paper [31]. A certain trypsin/trehalose precursor solution was kept in a reservoir which is connected to the microfluidic aerosol nozzle. A liquid jet was forced out of the nozzle through an orifice with a diameter of 75 μm. The liquid jet was broken up into monodisperse liquid droplets with the assistance of periodic vibrations at 8000 Hz, which is generated by a piezoceramic material surrounding the nozzle. Monodisperse droplets with nearly identical droplet size (~170 μm) for each precursor solution could be achieved (Fig. 1b). The solution flow rate is kept constantly at ~1.5 ml/min by controlling the applied air pressure to the feed reservoir. Then, the liquid droplets were dispersed into the drying chamber for drying, or sprayed into liquid N_2 for freezing and subsequent drying. The droplet formation process was monitored by photographs taken by using a digital SLR camera (Nikon, D3200).

2.3. Fabrication of enzyme-immobilized carbohydrate microparticles

2.3.1. Spray drying process

A micro-fluidic jet spray dryer (MFJSD; Fig. 1c) equipped with the MFAN was used to produce uniform microparticles. The MFJSD design has been previously reported [32]. In this study, the dryer inlet and outlet temperatures were kept at 180 ± 3 °C and 90 ± 3 °C, respectively, with a hot air flow rate of 260 ± 5 l/min. The yield of the collected dried microparticles is about 70 wt% after the spray drying process. The dried microparticles were collected and stored in a desiccator at 4 °C for further characterizations [33].

2.3.2. Spray freeze drying process

The precursor solutions were atomized by using the MFAN at 10 cm above the surface of a stainless steel plate containing liquid N_2 (Fig. 1d). The suspended frozen droplets in the liquid N_2 were transferred to a vacuum freeze drier (Four-Ring Science Instrument Plant Beijing Co., Ltd., China) with a pre-cooled shelf temperature of −50 °C. After evaporation of liquid N_2, vacuum freeze drying was performed for 72 h. The yield of the collected dried microparticles after the spray freeze drying process is about 90 wt%. Finally, the dried microparticles were collected and stored in a desiccator at 4 °C for further characterizations.

2.4. Particle characterizations

A field-emission scanning electron microscope (FE-SEM, S-4700, Hitachi High Technologies Corporation, Japan) was used to obtain the information on particle size and morphology. Particle size distribution was acquired by analyzing SEM images containing over 500 particles by using Shineso (SHINESO, Hangzhou, China). The microparticles flowability was characterized by Carr's index (CI) calculated from Eq. (1), where ρ_{bulk} is the bulk density measured by measuring the volume of microparticles of 0.1–2.0 g in a 5 ml measuring cylinder without tapping; ρ_{tap} is the tap density determined by tapping the above volume of microparticles of a known mass in a 5 ml measuring cylinder [34]. The densities were measured three times and averaged for each sample.

$$CI = \left[\left(\rho_{tap} - \rho_{bulk}\right)/\rho_{tap}\right] \times 100\% \quad (1)$$

Powder X-ray diffraction (XRD) patterns were collected by using a desktop diffractometer (D2 PHASER, Bruker, Germany) with Cu-Kα (λ = 1.54056 A) as the radiation source and operated at 30 kV and 10 mA. Samples were ground into powders, fed into sample holder and were subjected to manual compaction. The scanning range of was 10°–90° (2θ basis) with a step size of 0.03° and 0.5 s for each step. The thermostability of the microparticles was tested by using a Mettler Toledo DSC 3+ instrument. Samples of ~5 mg were loaded onto a zirconia pan with a 1-hole lid and heated from 25 to 250 °C at a heating rate of 10 °C/min, and nitrogen was used as the purge gas at 10 ml/min. Fourier transform infrared (FTIR) spectra were obtained to estimate the protein secondary structure (β-sheet, β-turn and α-helix) by using a Bruker tensor 27 spectrometer (Bruker, Germany) with a resolution of 2 cm^{-1} with the wavenumber range of 400 to 4000 cm^{-1} using KBr pallets at room temperature. Second-derivative spectra were calculated using Nicolet Omnic software (Thermo Nicolet corporation, USA) and smoothed with a nine-point function to remove white noises as described elsewhere [35].