

Preparation and *In vitro* Evaluation of Biodegradable Microspheres with Narrow Size Distribution for Pulmonary Delivery

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Zhao, et al: Biodegradable Microspheres for Pulmonary Delivery

This study was performed to evaluate the feasibility of double emulsion technique combined with membrane emulsification to prepare microspheres for pulmonary delivery. An attempt was made to counter the shortcomings of microspheres; unsuitable size diameters and broad size distribution. Three formulation variables were optimized in order to achieve desired particle size and narrow size distribution. Compared with microspheres prepared by conventional double emulsion technique, the microsphere morphology, aerodynamic particle size, drug encapsulation efficiency and *in vitro* release were characterized in detail. The particles prepared by double emulsion technique combined with membrane emulsification showed the advantage on controlling the particle size to meet the requirement of respiration, as well as on encapsulation efficiency. Overall, this strategy could be promising for the application of pulmonary delivery.

Key words: Protein drug, pulmonary delivery, mPEG-PLGA microspheres, membrane emulsification, narrow size distribution

In the past several years, pulmonary drug delivery has attracted much attention as a non-invasive means of drug administration^[1]. Due to the characteristic features of the lungs such as sufficient surface area (approximately 100 m²), ample blood supply, avoidance of hepatic first-pass metabolism and low enzymatic metabolism^[2], pulmonary drug delivery is used as the route for local as well as systemic diseases^[3-5]. However, the vigorously mucociliary clearance in the upper airways and regular respiration could lead to the loss of sustained drug absorption in the lung, which hinders the development of pulmonary drug delivery. It is reported that particles with aerodynamic diameter between 1 and 5 μ m could deposit and accumulate in the deep lungs^[6]. Therefore, the strategy to control the size of particles could be one of effective ways to circumvent the mentioned problem for pulmonary delivery.

In this paper, membrane emulsification combined with double emulsion technique is developed to manufacture the microspheres for pulmonary delivery. Due to the linear relationship between the diameter of the microsphere and the pore size of the membrane, the size of microspheres could be controlled accurately by using the membrane with desired pore size. Compared

with conventional processes to prepare microspheres such as homogenization^[7-10], or spray technique^[11-13], the size distribution of microspheres produced by membrane emulsification technology is relatively narrow, and the particle size is easy to control. This could increase the particle performance and drug delivery efficiency, and contribute to study the release behaviour of drug^[14,15].

Until now, polylactic acid (PLA) and poly lactide-co-glycolide (PLGA) microspheres have been widely investigated as the polymer materials to encapsulate the drugs for sustained release. However, the hydrophobic drawback of PLA and PLGA can lead to some common problems, including a high initial burst, protein denaturation caused by the acidic microenvironment followed with degradation of PLA or PLGA^[16,17], and adverse reactions, such as inflammation^[18]. It is generally known that introduction of hydrophilic

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blocks could improve the performance of the hydrophobic polymers. Methoxy polyethyleneglycol (mPEG), has been considered as one of the most promising polymers to improve the biocompatibility of the blood contacting materials^[19]. Due to the surface modifier of hydrophobic PLA/PLGA, mPEG could improve the stability of the interfacial layer between the oil and water interface^[20]. Its amphipathic property could reduce the possibility of aggregation of the encapsulated protein during encapsulation and release^[21-23]. Moreover, low-molecular weight mPEG is also a nontoxic, water-soluble polymer, which could be easily excreted from human body. Li *et al.*^[24] exhibited the degradation behaviour and drug release of PLGA-mPEG microparticles. Therefore, mPEG-PLGA as the biodegradable material could be used in preparing microspheres.

In this work, mPEG-PLGA microspheres prepared by a double emulsion method followed with a membrane emulsification technique were exploited. In order to obtain microspheres with narrow size distribution and proper aerodynamic diameter, the factors including volume ratio of W1/O/W2, PVA concentration were optimized. For comparison, mPEG-PLGA microspheres fabricated by conventional double emulsion method were also prepared. Then, the formulations were characterized in terms of morphology, encapsulation efficiency (EE), aerodynamic properties and *in vitro* drug release profiles of the microspheres in order to evaluate the feasibility of this method. Nevertheless, the scope of this study focuses on fabrication, optimization and *in vitro* characterization of the particles only. The degree to which this microsphere may influence its bioactivity and *in vivo* performance will be subjects of our future investigations.

MATERIALS AND METHODS

mPEG-PLGA with a molecular weight of 38 000 Da were purchased from the Dai Gang Company (Shandong, China), in which the mPEG block had a molecular weight of 2000 Da. Polyvinyl alcohol (PVA, degree of polymerization 1700, degree of hydrolysis 87.0-89.0 %) was purchased from Aladdin company. Bovine serum albumin (BSA, Mw 66 kDa) was from Wako (Japan). SPG membrane (with pore size of 5.3 microns) was provided by SPG Technology Co. Ltd. (Japan). Fast membrane emulsification equipment (FMEM-500M) was provided by National Engineering Research Center for Biotechnology (Beijing).

Homogenizer was purchased from IKA (Germany). All other reagents were of analytical grade.

Preparation of microspheres:

mPEG-PLGA microspheres loading BSA were prepared by water-in-oil-in-water (W1/O/W2) double emulsion-membrane emulsification method. The internal aqueous phase (W1, IAP) containing BSA ($20 \text{ mg} \times \text{ml}^{-1}$) was first emulsified in dichloromethane (organic phase, O) containing $50 \text{ mg} \times \text{ml}^{-1}$ of mPEG-PLGA by homogenizing for 60 s to form a primary emulsion (W1/O). The W1/O was further poured into the external aqueous phase (W2, EAP) containing 1 % w/v PVA and 0.9 % w/v NaCl to prepare coarse double emulsions. These coarse double emulsions were then extruded through the SPG membrane under a high pressure of 150 kPa to achieve the double emulsions with smaller and relatively uniform size (fig. 1a).

mPEG-PLGA microspheres prepared by only double emulsion method was obtained according to the literature^[25]. The same procedure was carried out to form the primary emulsion (W1/O) as mentioned above. Then, this emulsion was injected into the external aqueous phase consisting of 1 % w/v PVA and 0.9 % w/v NaCl and homogenized for 5 min to form the secondary emulsion (W1/O/W2, fig. 1b).

These two kinds of resulting double emulsions were stirred for 4 h at room temperature for evaporation of O and hardening of the microspheres. The polymeric microspheres were then washed thrice with water and lyophilized for 48 h to get the powder formulations. Each formulation was prepared in triplicate and stored at 4° for further studies.

EE:

The EE of the formulations was determined by micro bicinchoninic acid (micro-BCA) assay (Pierce,

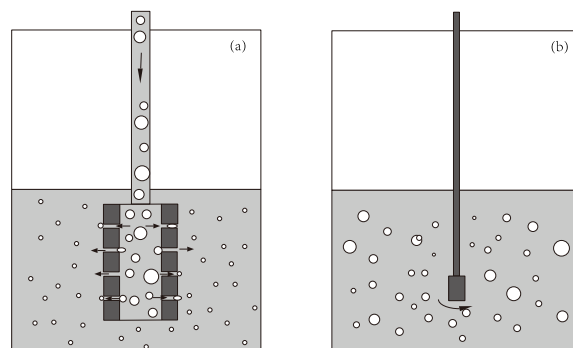


Fig. 1: Schematic illustration of the formation of droplets prepared with different emulsification technologies
(a) Membrane emulsifying; (b) homogenizing. ○ Droplet of W1/O; ■ external water phase

USA)^[26]. Briefly, the freeze-dried microspheres (10 mg) was added into 1 ml of 1 M NaOH. The BSA concentration in the aqueous phase was measured using a UV/Vis spectrophotometer (Sunrise™, Tecan, Switzerland) at 526 nm. The EE was calculated by the following Eqn. 1: $EE (\%) = m/m_0 \times 100$, where m_0 was the theoretical amount of BSA added in the microspheres and m was the amount of BSA loaded in the microspheres.

Differential scanning calorimetry (DSC):

DSC experiments were carried out using a DSC 2910 from TA Company (Boston, Massachusetts, USA). The samples were stabilized at 25° in the calorimeter before heating up to 120° with a scanning rate of 5° min⁻¹.

Characterization of microspheres for pulmonary delivery:

The microspheres were characterized for their morphology, diameter, the size distribution and the densities. The morphology of the microspheres was examined under Jeol JSM 7401F (Japan) scanning electron microscope (SEM). The volume-mean diameter and particle size distribution of the microspheres was measured by laser scattering using a Mastersizer 3000 (Malvern, UK). The span value (SP) was calculated using Eqn. 2^[27]: $span = D_{90} \% - D_{10} \% / D_{50} \%$, where $D_N \%$ (N=10, 50 or 90) was the volume percentage of microspheres with diameters. Smaller SP indicated narrower size distribution.

The tapped densities of the particles were determined according to the procedure described previously^[28]. An aliquot of particles from each formulation was put to a 10 (±0.05) ml graduated cylinder. The tapped density (ρ_t) was calculated as the ratio of sample weight (g) and the volume (ml) occupied after tapping until no further change in volume was observed. The theoretical aerodynamic diameter (d_a) was calculated based on the following Eqn. 3^[29]: $d_a = d_p \sqrt{\rho_t / \rho_0}$, where d_p was the geometric mean diameters of the particle, ρ_0 was the standard particle density (1 g×ml⁻¹), ρ_t was the tapped density.

Flow property test:

The Carr's compressibility index and the Hausner's ratio were calculated to provide a measure of the flow properties. The Hausner's ratio and Carr's index was determined as follows, Eqn. 4: Hausner's ratio = ρ_t / ρ_b , Eqn. 5: Carr's index = $\rho_t - \rho_b / \rho_t$, where ρ_t and ρ_b were the tapped density and bulk density of the microspheres, respectively. Angle of repose method^[30] was also

measured by falling microspheres through a funnel on the horizontal surface. The angle of repose was calculated as follows, Eqn. 6: $\theta = \tan^{-1}(h/r)$, where θ was angle of repose, h and r were the height and radius of the heap, respectively.

In vitro aerodynamic study:

In vitro aerodynamic study was performed using an eight-stage Marc-II Andersen Cascade Impactor (Seak-Mar Environmental Instrument Ltd., Beijing, China). Typically, a 15-20 mg powder formulation was emitted into the impactor using a HandiHaler® (Pfizer) at a flow rate of 28.3 l/min. The aerodynamic process was conducted in triplicate. The amount of powder deposited on each stage was determined by calculating the weight changes of the glass fiber filter paper before and after passing the particle through the impactor. The mass median aerodynamic diameter (MMAD) was determined at the 50th percentile on the percent cumulative amount of particles (probability scale) versus logarithmic effective cut-off diameter (μm) plot.

The emitted fraction (E_f %) and the fine particle fraction (FP_f %) were calculated according to the following relationships^[31], Eqn. 7: $E_f \% = (m_{full} - m_{empty}) / m_t \times 100$; $FP_f \% = m(d_a < 4.7 \mu m) / m_{stages}$, where m_{full} and m_{empty} were the weights (mg) of capsule before and after simulating inhalation. $m(d_a < 4.7 \mu m)$, m_t and m_{stages} were the amount of particles with aerodynamic diameters less than 4.7 μm, total amount of particles and the summation of particle amounts collected from stages, respectively.

In vitro protein release studies:

To study the *in vitro* release pattern of encapsulated BSA from the microspheres, 100 mg freeze-dried microspheres was suspended in 20 ml simulated lung fluid (SLF), prepared according to Moss formula plus 0.1 % Tween 80 at pH 7.4 and stirred at 60 rpm at 37±1 °C^[25,32,33]. An aliquot of samples (800 μl) was withdrawn following centrifugation over a period of 28 d at various time intervals. The amount of drug released from the microspheres was measured using micro-BCA assay method as described above.

Calculations and statistics:

Results are depicted as mean±SD from at least three different batches of microspheres prepared at the specified conditions. Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Since microsphere morphology was important for pulmonary delivery, the formulation variables that could significantly influence the morphology of microspheres were investigated. We first studied the effect of volume ratio of W1/O (1:2.5, 1:5, 1:7.5, 1:10, 1:12.5) while keeping O/W2 ratio (v/v) constant at 1:10 and the concentration of PVA at 1 % (w/v). As shown in Table 1, the volume-based mean size diameters decreased from $2.68 \pm 0.04 \mu\text{m}$ (W1/O, 1:5) to $2.32 \pm 0.02 \mu\text{m}$ (W1/O, 1:7.5) and subsequently increased to $2.51 \pm 0.02 \mu\text{m}$ (W1/O, 1:12.5). Similarly, SP could be observed with a trend that reduced first and then increased. The minimum SP (0.55 ± 0.02) was achieved at W1/O ratio of 1:7.5. Furthermore, SEM photograph in fig. 2 showed that microspheres with

the W1/O ratio at 1:7.5 and 1:10 possessed relatively smooth surface.

In terms of O/W2 ratio, increasing the ratio from 1:5 to 1:15 at fixed PVA concentration (1 %, w/v) and W1/O ratio (1:7.5) could slightly affect both the particle size diameters and their size distributions. The size diameters were in the range of 2.21 ± 0.01 and $2.51 \pm 0.02 \mu\text{m}$, and narrowest size distribution (0.67 ± 0.03) was found at O/W2 ratio of 1:10 (Table 1). Besides, the SEM photographs (fig. 3) of these formulations showed that the microspheres were spherical geometry. Particles at O/W2 ratio of 1:10 exhibited more regular shape without any aggregate between particles.

The effect of concentration of PVA was shown in Table 1. Both the size diameters and SP were slightly

TABLE 1: SIZE AND SIZE DISTRIBUTION OF BSA-LOADED MICROSPHERES

| W ₁ /O | Volume-based mean diameter ($\mu\text{m} \pm \text{SD}$) | Span value ($\pm \text{SD}$) | O/W ₂ | Volume-based mean diameter ($\mu\text{m} \pm \text{SD}$) | Span value ($\pm \text{SD}$) | Content of PVA (%) | Volume-based mean diameter ($\mu\text{m} \pm \text{SD}$) | Span value ($\pm \text{SD}$) |
|-------------------|--|--------------------------------|------------------|--|--------------------------------|--------------------|--|--------------------------------|
| 01:02.5 | 2.68 ± 0.04 | 0.65 ± 0.03 | 01:05 | 2.21 ± 0.01 | 0.93 ± 0.02 | 0.25 | 3.10 ± 0.04 | 1.73 ± 0.05 |
| 01:05 | 2.32 ± 0.02 | 0.58 ± 0.02 | 01:07.5 | 2.28 ± 0.03 | 0.69 ± 0.02 | 0.5 | 2.95 ± 0.3 | 1.42 ± 0.03 |
| 01:07.5 | 2.32 ± 0.02 | 0.55 ± 0.02 | 01:10 | 2.35 ± 0.03 | 0.67 ± 0.04 | 1 | 2.35 ± 0.03 | 0.67 ± 0.04 |
| 01:10 | 2.35 ± 0.03 | 0.68 ± 0.04 | 01:12.5 | 2.4 ± 0.04 | 0.68 ± 0.03 | 1.5 | 2.36 ± 0.02 | 0.70 ± 0.04 |
| 01:12.5 | 2.51 ± 0.02 | 1.05 ± 0.04 | 01:15 | 2.51 ± 0.02 | 0.98 ± 0.07 | 2 | 2.36 ± 0.03 | 0.71 ± 0.05 |

BSA-loaded microspheres were prepared by double emulsion method combined with membrane emulsification at different conditions. Volume-based mean size diameters decreased from $2.68 \pm 0.04 \mu\text{m}$ (W1/O, 1:5) to $2.32 \pm 0.02 \mu\text{m}$ (W1/O, 1:7.5) and subsequently increased to $2.51 \pm 0.02 \mu\text{m}$ (W1/O, 1:12.5)

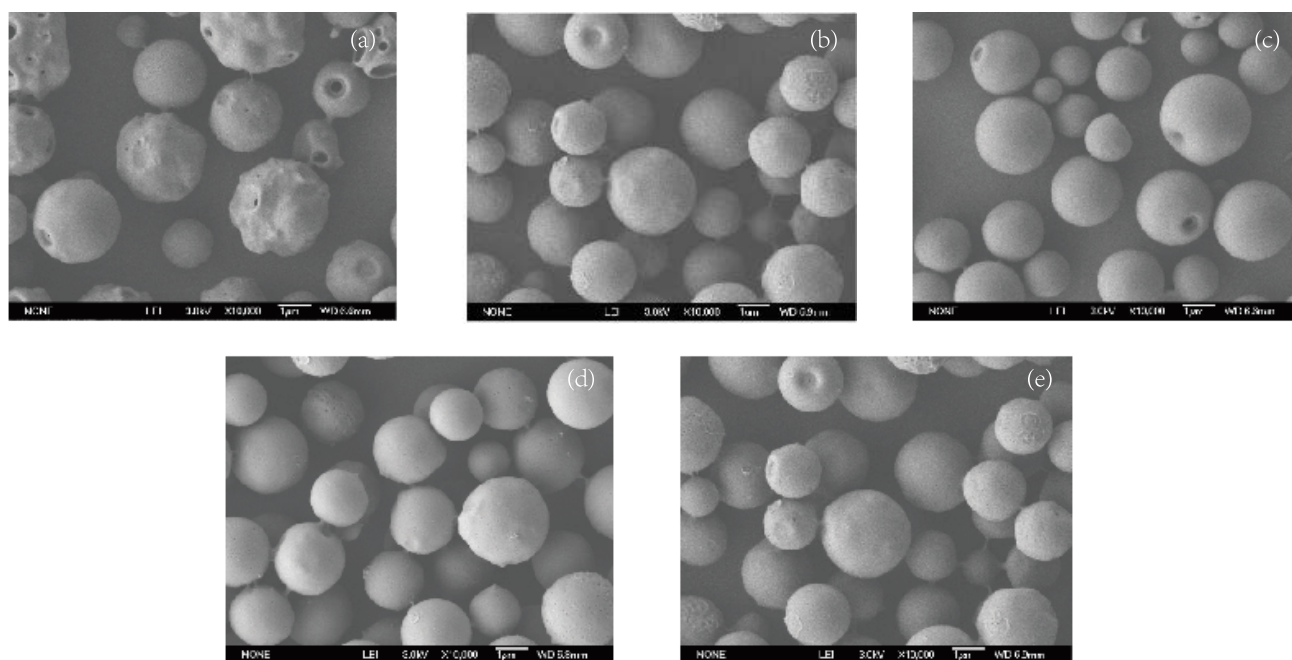


Fig. 2: Surface morphology of microspheres
Microspheres prepared at W1/O ratio (a) 1:2.5; (b) 1: 5; (c) 1:7.5; (d) 1:10; (e) 1:12.5. Microspheres with the W1/O ratio at 1:7.5 and 1:10 possessed relatively smooth surface

enhanced when increasing PVA concentration from 1.5 to 2 %. While the diameters of microspheres were significantly decreased with the concentration of PVA increased from 0.25 to 1 %. Interestingly, it was noted that spherical microspheres with smooth surface were observed when PVA content was below 1 %. But the

microspheres surface became porous when the PVA concentration was between 1 and 2 % (fig. 4). It was reported that the porous of microspheres was formed during the evaporation of organic solvent^[34]. When the PVA concentration in the external water phase was higher, the evaporation rate of solvent in the oil

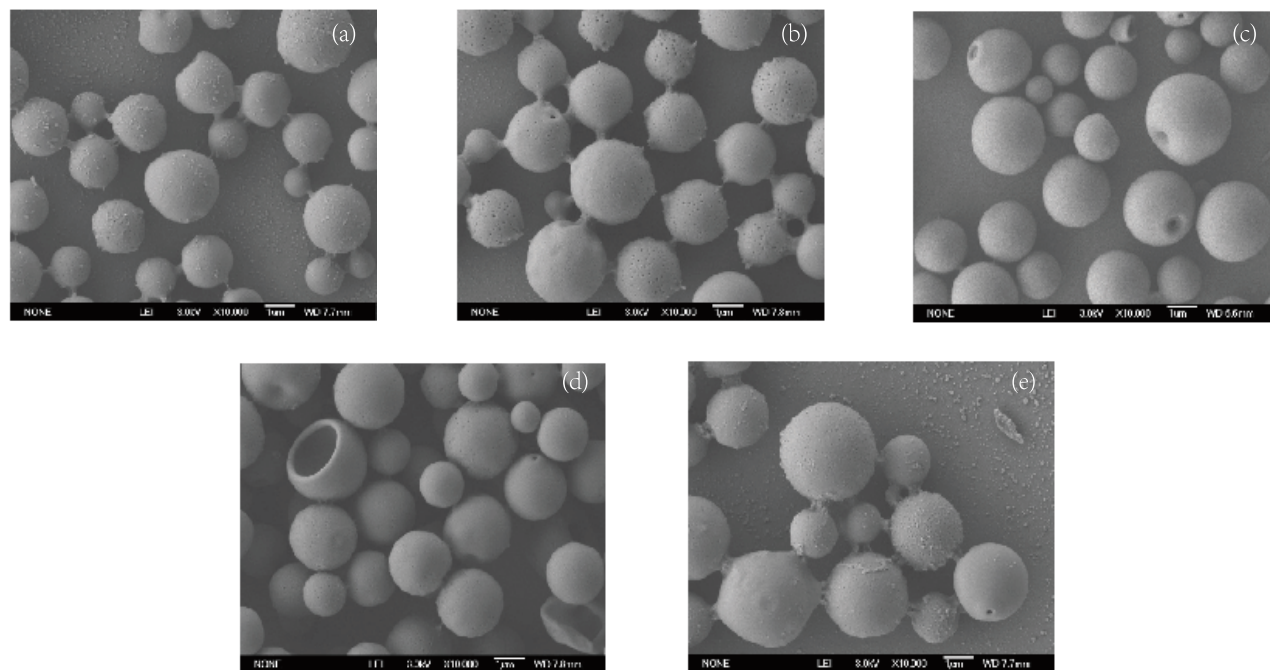


Fig. 3: Surface morphology of microspheres

Microspheres prepared at O/W2 ratio (a) 1:5; (b) 1:7.5; (c) 1:10; (d) 1:12.5; (e) 1:15. Microspheres were in spherical geometry. Particles at O/W2 ratio of 1:10 exhibited more regular shape without any aggregate between particles

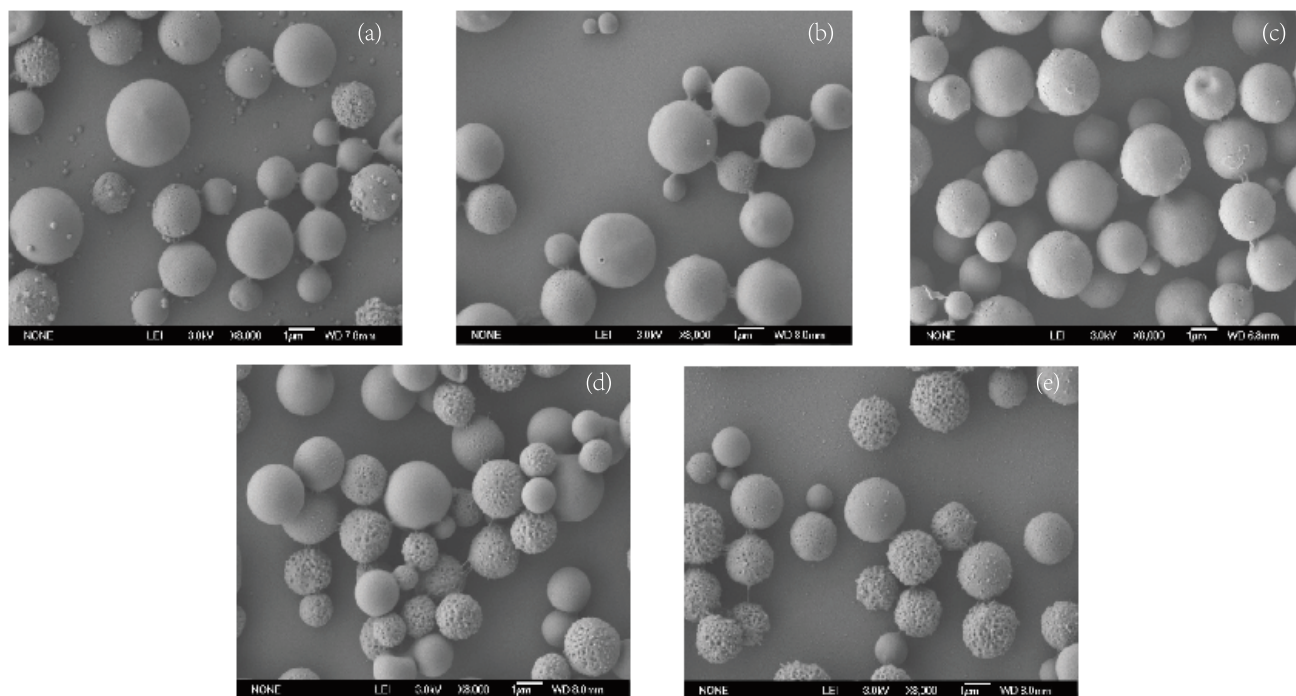


Fig. 4: Morphology of microspheres

Microspheres prepared with different PVA content of (a) 0.25 %; (b) 0.5 %; (c) 1.0 %; (d) 1.5 %; (e) 2.0 %. Spherical microspheres with smooth surface were observed when PVA content was below 1 %. But the microspheres surface became porous when the PVA concentration was between 1 and 2 %

phase was getting lower. Due to the osmotic pressure between the IAP and EAP, the internal water tended to diffuse through the oil layer to the external water phase. Therefore porous spheres were formed. Overall, the results suggested that optimized formulation was 1 % w/v PVA concentration, 1:7.5:75 ratio of W1/O/W2 for the following experiments.

It was well known that EE was an important parameter that should be taken into account. Besides microspheres (A) prepared by double emulsion technique combined with membrane emulsification technique, microspheres (B) fabricated by only double emulsion method were also fabricated for comparison. Both of these formulations were prepared with W1/O/W2 volume ratio of 1:7.5:75 and 1 % PVA. Higher EE was achieved from microspheres (A). A possible reason was that the uneven pores existed on the surface of the microspheres B (fig. 5b). As a result, the drug located near the microsphere surface could diffuse towards EAP through the channels and pores during the solidification and washing process.

For pulmonary delivery, the aerodynamic property of microspheres is a key factor, which is influenced significantly by the shape, density, size and size

distribution. Therefore, the morphologies of these microspheres prepared by a double emulsion method followed with a membrane emulsification technique (A) and only double emulsion method (B) were recorded first. As shown in fig. 5a and b, microspheres (A) formed regular sphericity with smooth surface. By contrast, the uneven pores and wrinkles on the surface could be observed from microspheres (B). A possible reason was that the microspheres (A) were formed by extrusion through membrane pores. The water in IAP was stably coated by oil phase in the whole emulsification and solidification process. While microspheres (B) was emulsified by violent homogenization, it could increase the opportunity of collision between the droplets and then coalesced to form the new droplets. Hence, the water in IAP rearranged in the oil layer, and increased the possibility of IAP diffusing towards EAP. It happened during the polymer precipitation and therefore led to the particles with non-uniform pores and grooves on the surface. Microspheres (B) with this surface structure were not desired. It could probably lead to high initial burst release, which brought about surplus drug concentration *in vivo* at the beginning and decreased drug efficacy in later period.

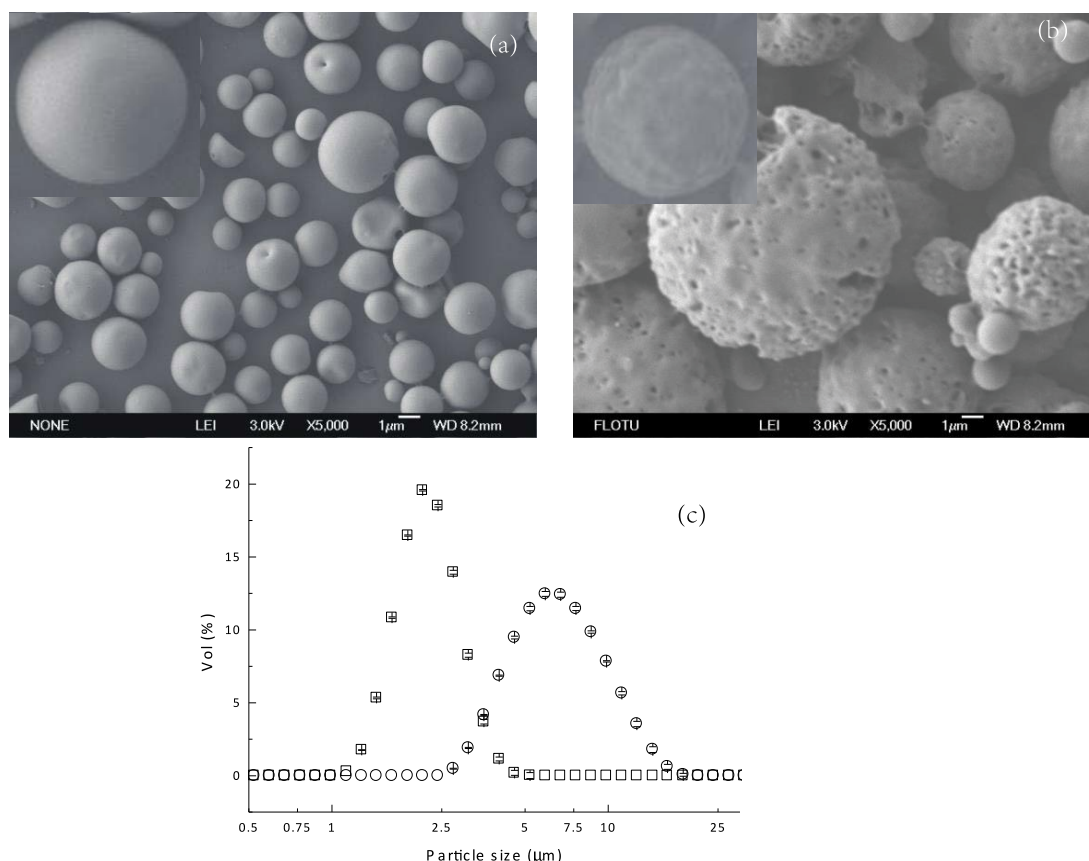


Fig. 5: Scanning electron microphotographs and size distributions
(a) Microspheres A; (b) microspheres B; (c) microspheres C. n=3, mean±SD

The size distributions of microspheres (A) and (B) were shown in fig. 5c. The volume-based mean diameters (D_v) of microspheres A and B were 2.34 ± 0.02 and 6.95 ± 0.02 μm , respectively. Microspheres A exhibited narrow size distribution, and the SP was 0.58 ± 0.03 . However, microspheres B had a broad size distribution with the SP 1.05 ± 0.02 . Therefore, a double emulsion method combined with membrane emulsification technique showed the advantage in preparing uniform microspheres for pulmonary delivery. Though the geometric mean diameters of the formulation B were larger than 5 μm , it could not conclude that microsphere B was not suitable for pulmonary delivery. Therefore, flowability and *in vitro* aerodynamic study were necessary for further discussion. Flow properties of the two formulations were also evaluated in terms of Carr' index, Hausner's ratio and angle of repose (Table 2). It was observed that microspheres A had better rheological properties than microspheres B.

The aerodynamic characteristics of the microspheres prepared by double emulsion method combined with membrane emulsification (A) and only double emulsion method (B) were assessed *in vitro* using a dry powder inhaler device and Andersen Cascade Impactor. As shown in Table 3, the MMADs of the formulation A and B were in the range of 1 – 5 μm , which was desirable for targeting the respiratory region of the human lung. While the MMAD of microspheres (A) was 1.15 μm , it was preferable aerodynamic diameter (1 – 3 μm) for lung deposited. Moreover, it should be noted that the theoretical primary aerodynamic diameter was not the same as the MMAD determined by Andersen Cascade Impactor for both kinds of microspheres. Hence, the MMAD as the important index for particle aerodynamic study should be investigated, and could not be absolutely taken place by theoretical calculation method.

TABLE 2: FLOW PROPERTIES OF THE TWO FORMULATIONS

| Formulation | Carr's index (\pm SD) | Hausner's ratio (\pm SD) | Angle of repose (\pm SD) |
|-------------|--------------------------|-----------------------------|-----------------------------|
| A | 25.00 ± 0.17 | 1.33 ± 0.03 | $41.3^\circ \pm 1^\circ$ |
| B | 33.33 ± 0.28 | 1.50 ± 0.04 | $43.2^\circ \pm 1^\circ$ |

TABLE 3: *IN VITRO* AERODYNAMIC PROPERTIES OF MICROSPHERES A AND B

| Encapsulation efficiency ($\% \pm$ SD) | Theoretical aerodynamic diameter ($\mu\text{m} \pm$ SD) | MMAD ($\mu\text{m} \pm$ SD) | E_f ($\% \pm$ SD) | FP_f ($\% \pm$ SD) |
|---|--|------------------------------|----------------------|-----------------------|
| A 79.60 ± 5.47 | 1.26 ± 0.04 | 1.15 ± 0.01 | 95.3 ± 0.7 | 83.7 ± 0.7 |
| B 65.28 ± 4.48 | 4.28 ± 0.07 | 3.94 ± 0.03 | 93.1 ± 0.7 | 33.0 ± 1.1 |

MMAD is the mass median aerodynamic diameter. E_f is the emitted fraction and FP_f is the fine particle fraction. The MMADs of the formulation A and B were in the range of 1 – 5 μm , which was desirable for targeting the respiratory region of the human lung. Data are represented as mean \pm SD.

The particle fraction (%) collected on inhaler device, capsule and each stage of Anderson Cascade Impactor was plotted in fig. 6. Between stage 3 and stage 7, a higher percentage of drug deposition was observed from Andersen cascade impactor of microspheres A. This result suggested that more microspheres A were able to effectively deposit in bronchioles and alveoli when a certain amount of powder was inhaled.

Moreover, the microspheres A presented a higher emitted fraction, E_f % (95.3 %) than microspheres B (Table 2). Similarly, the fine respirable fraction (FP_f %) of microspheres A were found to be higher than that of microspheres B, those were 82.0 ± 2.5 % and 33.0 ± 1.1 %, respectively. It was well known that FR_f % was the important parameter, which were promising to evaluate most of the powder deposition in the deep lung. The powders with the higher FP_f could achieve better performance in pulmonary delivery. Therefore, the microspheres prepared by double emulsion combined with membrane emulsification technique may have the potential for sustained pulmonary drug delivery in terms of *in vitro* aerodynamic studies.

The *in vitro* release profile of the BSA from microspheres prepared by double emulsion technique combined with membrane emulsification showed a typical trend: initial burst release followed by cumulative drug release, as depicted in fig. 7. In the first 1 h, an initial burst release was 11.43 ± 0.62 %. The burst release was encountered

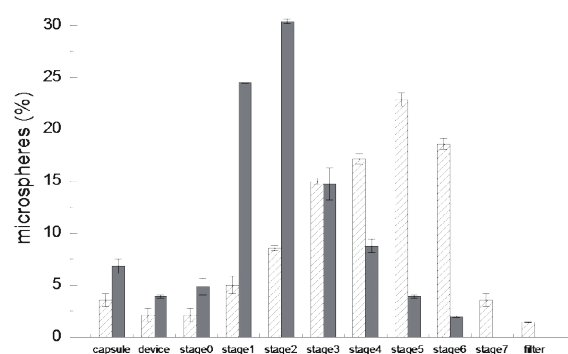


Fig. 6: *In vitro* lung model Andersen cascade impactor of microspheres A and B

/// Microspheres A; ■ microspheres B

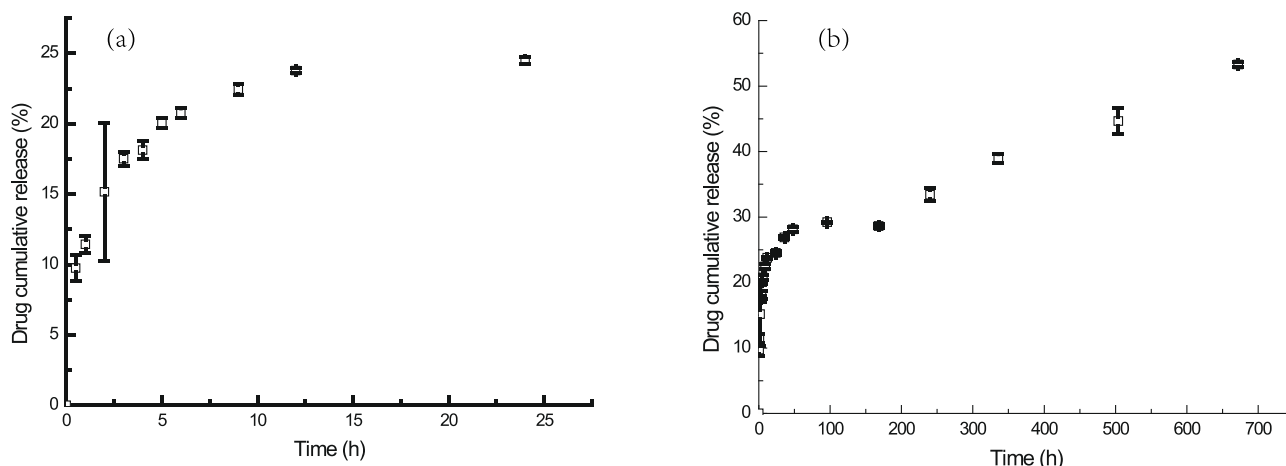


Fig. 7: *In vitro* drug release profiles of microspheres

(a) *In vitro* drug release profile within the first 24 h; (b) *in vitro* drug release profile in 28 d. mean±SD, n=3

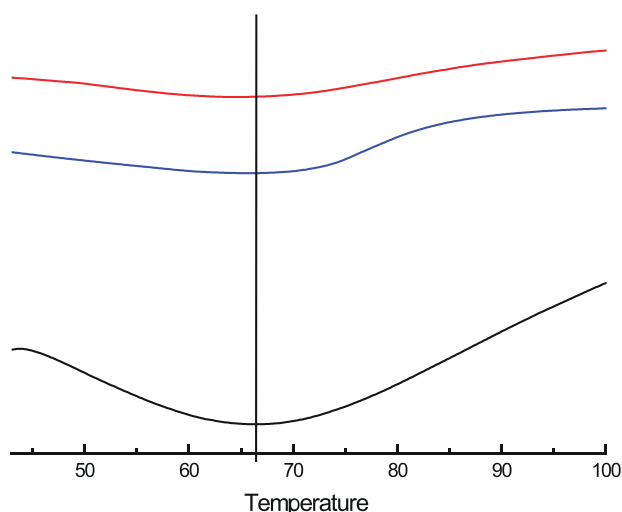


Fig. 8: DSC thermograms of BSA released from mPEG-PLGA microspheres at different times

— 0 week; — 2nd week; — native BSA

for many drugs encapsulated in PLA and PLA-based microsphere preparations, but there is no evidence that the molecular weight and size of the drugs play a role in causing the burst release^[35-38]. It was reported that the burst effect was predominantly attributed to precipitating drugs on the surface of microspheres, during thermodynamically transient phases of nascent microspheres^[38]. The cumulative release was performed for 35 d, and about 53.29 ± 0.45 % of BSA was released from microspheres. In order to confirm the structural integrity of the BSA released from the mPEG-PLGA microspheres, DSC test was employed. It can be seen from fig. 8 that the native BSA and BSA released from microspheres showed the same thermal transition temperature. It was indicated that aggregation or hydrolysis did not occur during the preparation and release process.

Conflicts of interest:

There are no conflicts of interest.

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