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Abstract

Biodegradable poly (lactic-co-glycolic acid) (PLGA) microparticles are an effective way to achieve sustained drug release. In this study, we investigated a sustained release model of PLGA microparticles with incorporated protein via either emulsion or coaxial electrospray techniques. PLGA (75:25) was used as the carrier, and bovine serum albumin as a model protein. Coaxial electrospray resulted in a type of core–shell structure with mean diameters of $2.41 \pm 0.60\,\mu m$ and a centralised protein distribution within the core. Emulsion electrospray formed bigger microparticles with mean diameters of $22.75 \pm 8.05\,\mu m$ and a heterogeneous protein distribution throughout the microparticles. The coaxial electrospray microparticles presented a much slighter burst release than the emulsion electrospray microparticles. Loading efficiency was significantly higher ($p < 0.05$) in the coaxial group than emulsion group. This indicated that both emulsion and coaxial electrospray could produce protein-loaded microparticles with sustained release behaviour, but the former revealed a superior approach for drug delivery.

Keywords: microparticles, electrospray, controlled release, drug delivery

Introduction

The concept of using polymer-based sustained-release delivery systems to maintain therapeutic drug concentrations for extended periods has been accepted for decades. Drug carriers like microspheres can deliver molecules of interest over longer times than by standard bolus injection, while maintain therapeutic concentrations at target sites and reducing potential toxicity (Whittlesey and Shea, 2004). Consequently, the attempts to produce sustained-release microparticles were highlighted. Different ways, e.g. $W_1/O/W_2$ double emulsion, coacervation and spray, have been studied intensively (Gupta et al., 1998; Singh and O'Hagan, 1998; Sinha and Trehan, 2003; Dai et al., 2005; Freitas et al., 2005; Tamber et al., 2005). But, some of those techniques had numerous shortcomings,

including modest encapsulation efficiency, batch-nature of the process, difficulty to scale-up, poor control of the particle size distribution, difficulty to generate sufficiently small particles ($< 100\,nm$), poor repeatability and limitations with respect to encapsulation of hydrophilic agents (Jain, 2000; Almeria et al., 2011; Enlow et al., 2011). Recently, electrospray has attracted much attention in generating drug-loaded polymeric particle (Ding et al., 2005; Ciach, 2006; Xie et al., 2006). It utilises electrostatic forces to eject polymer solutions or melts into droplets, producing particles with diameters ranging from nanometres to micrometres after solvent evaporation. Electrosprayed microparticles have been regarded as effective drug carriers (Loscertales et al., 2002; Almeria et al., 2010).

Protein-loaded microparticles can generally be achieved by two different electrospray approaches: emulsion or

coaxial electrospray. Emulsion electrospray involves mixing of aqueous solution containing proteins with immiscible polymeric solution by ultrasonication (Xu and Hanna, 2006; Xie and Wang, 2007). Compared to other conventional producing methods, the second emulsion or high temperature is omitted in emulsion electrospray. It increases the drug-loading efficiency and is suited for encapsulation of thermosensitive bioactive compounds. However, it still involves the step of atomisation of protein aqueous solution in organic polymer solution, which causes bioactivity loss during ultrasonication (van de Weert et al., 2000). Besides, emulsion stability is also a crucial parameter for the process, together with other parameters as concentration, voltage and flow rate.

Coaxial electrospray was first reported by Loscertales et al. (2002). Two immiscible solutions are coaxially and simultaneously electrosprayed through two separate feeding channels into one nozzle. The eventual jet, by which the outer polymeric solution encapsulates the inner proteinaceous liquid, breaks into droplets to generate microparticles with core–shell structure. This technique is preferred for preparing protein-loaded microcapsules, because it totally eliminates the emulsion step that is basically unsuitable for sensitive biomacromolecules (Xie et al., 2008; Wu et al., 2009). Xie et al. (2008) have used poly (lactide-co-glycolide) (PLGA) dichloroform solution coating protein aqueous solution via coaxial electrospray and encapsulating bovine serum albumin (BSA) or lysozyme inside microparticles. Poly (ethyl glycol) was included in the core to modify the electrospray process and drug release behaviour. Coaxial electrospray is envisioned a promising approach to prepare biomacromolecule-loaded microcapsules for controlled drug delivery applications. Because of its complexity, however, coaxial electrospray is not popular as single needle electrospray.

In this study, we fabricated BSA-loaded core–shell microcapsules by coaxial electrospray. To modify the release of hydrophilic protein, the inner liquid was prepared by mixing BSA/H_2O solution into PLGA/trifluoroethanol solution, to obtain a kind of matrix-type delivery system in the core and a wholly reservoir-type with the PLGA shell. Emulsion electrospray was also performed. Both systems were compared in terms of the processing set-up and microparticles characterisation as well as the release kinetics.

Materials and methods

Materials

PLGA with a lactide–glycolide molar ratio of 75:25 (MW, 10 kDa) was obtained from Birmingham Polymers (Birmingham, AL). BSA (purity 98%), fluorescein isothiocyanate-conjugated BSA (FITC–BSA) (MW 67 kDa) was purchased from Beijing Biosynthesis Biotechnology Co. Ltd (Beijing, China). Rhodamine B (purity 80%) was purchased from Sigma–Aldrich (St. Louis, MO). Organic solvents 2,2,2-trifluoroethanol (TFE) (purity 99.9%) and trichloromethane (THM) (purity 99.0%) were supplied by Sinapharm Chemical Reagent Beijing Co. Ltd (Beijing, China) and used directly. PBS (PH7.4) was purchased from Beijing Zoman Biotechnology Co. Ltd (Beijing, China).

Coaxial electrospray

BSA was dissolved in deionised water at a specified concentration and the aqueous solution was mixed into PLGA/TFE solution to obtain the core solution. The shell solution was set at 3% w/v PLGA/TFE and the concentration of inner solution was tried several times before the most appropriate concentration was determined. The two solution was electrosprayed from a 20 mL syringe with a steel needle (inner diameter of 0.5 and 0.8 mm, respectively) both at a rate of 0.8 mL/h continuously with a programmable syringe pump (Top 5300, Japan). A voltage (15 kV) was applied to the tip of the needle by the use of a high-voltage supply (DW-P303-1AC, China) with a collection distance of 20 cm. All the formulas used in this study are presented in Table 1. Electrosprayed microparticles were collected on a plate covered with aluminium foil. They were freeze dried for 1 day before further characterisation.

Emulsion electrospray

PLGA/THM solutions were prepared by dissolving PLGA in trichloroform as the continuous oil phase, with BSA dissolved in deionised water as the inner aqueous phase.

Table 1. Preparation parameters of coaxial electrospray used in this study.

Sample	Theoretical drug loading (w/w%)	Compositions		Shell solution	Diameter (μm)	Loading efficiency (%)
		Core solution (v/v = 1/5)				
		BSA/DI water (w/v%)	PLGA/TFE (w/v%)	PLGA/TFE (w/v%)		
a-0% PLGAin	5	0.15	0	3	–	83.15 ± 2.78
b-6% PLGAin		4.65	6	3	2.25 ± 0.56	82.04 ± 3.46
c-5% PLGAin		4.15	5	3	2.37 ± 0.56	82.57 ± 3.02
d-4% PLGAin		3.65	4	3	2.41 ± 0.60	82.35 ± 3.08
e-4% PLGAin	3	2.19	4	3	2.39 ± 0.33	87.51 ± 3.53
f-4% PLGAin	1	0.73	4	3	2.39 ± 0.56	88.93 ± 3.77
g-4% PLGAin	0	0	4	3	2.37 ± 0.60	0

Table 2. Preparation parameters of emulsion electrospray used in this study.

Sample	Theoretical drug loading (w/w%)	Compositions of emulsion (W/O = 1/10 in v/v)		Diameter (μm)	Loading efficiency (%)
		BSA/H$_2$O (w/v%)	PLGA/THM (w/v%)		
h-2% PLGA	5	1	2	22.26 ± 5.40	74.65 ± 4.32
i-4% PLGA		2	4	22.12 ± 5.25	73.97 ± 3.98
j-6% PLGA		3	6	22.75 ± 8.05	74.34 ± 4.02
k-8% PLGA		4	8	23.12 ± 4.73	74.39 ± 3.24
l-6% PLGA	3	1.8	6	22.82 ± 4.95	75.83 ± 4.36
m-6% PLGA	1	0.6	6	22.34 ± 4.68	82.31 ± 4.34
n-6% PLGA	0	0	6	22.77 ± 4.79	0

The two immiscible solutions were mixed and emulsified by ultrasonication for 30 s, and then electrosprayed from a 20 mL syringe with a steel needle (inner diameter of 0.6 mm) at a rate of 1.8 mL/h continuously with a programmable syringe pump (Top 5300, Japan). A voltage of 10 kV was applied to the tip of the needle by the use of a high-voltage supply (DW-P303-1AC, China) with a collection distance of 20 mm. All the formulas used for emulsion electrospray in this study are presented in Table 2.

Morphological characterisation of electrospray microparticles

Scanning electron microscopy
Electrosprayed microparticles, mounted on metal stubs using conductive double-sided tape, were sputter coated with gold under an argon atmosphere. Microparticle morphology was examined by scanning electron microscope (SEM, S-450; Hitachi Global, Japan) at an accelerating voltage of 10 kV. Microparticle diameters were analysed with Image J software (National Institutes of Health, Bethesda, MD). Approximately 150 counts for each type of microparticles were used to calculate the diameter.

Transmission electron microscopy
The microstructure of BSA-loaded microparticles was examined using an H-7650B (Hitachi Global) transmission electron microscope (TEM) equipped with a CCD camera, and operated at 80 kV. The samples for TEM observation were prepared by direct deposition of electrosprayed microparticles onto copper grids.

Laser scanning confocal microscopy
To visualise the presence and distribution of the proteins in the electrosprayed microparticles, FITC–BSA was used instead and fluorescent rhodamine B (50 μg/mL) was added into PLGA/THM or PLGA/TFE shell solutions. Fluorescent-stained microparticles were emulsion and coaxial electrosprayed as described above, collected on glass slides, and then observed by laser scanning confocal microscopy (LSCM; ZEISS LSM Exciter 5 System, Carl Zeiss, Germany). The excitation wavelengths for rhodamine B and FITC–BSA were 559 and 488 nm, respectively, and multi-track images were captured with a 63 × /1.40 NA objective.

Determination of protein loading efficiency

The total protein content was determined according to a reported method (Sah, 1997) with some modification. Briefly, microparticles ($n = 8$, 50 mg for each) were incubated in 3 mL DMSO for 1 h, then 4 mL 0.2 M NaOH solution containing 0.5% SDS was added for a further 1 h incubation at room temperature. The protein concentration in the solution was measured by the Micro-BCA^{TM} assay (Pierce, Rockford, IL). Results are presented as loading efficiency values, which indicated the percentage of protein loaded in the microparticles with respect to the total amount of protein used in the process. Blank microspheres were used as reference.

In vitro *release study*

The *in vitro* release study was performed in PBS (pH 7.4), and samples were incubated with shaking at 65 rpm at 37°C. Initially, 3 mL 0.01 M PBS water was added to a sealed vial containing 50 mg BSA-loaded microparticles, and the system was maintained at the pre-set conditions for different times. At each time point, all the liquid was taken out by centrifugation and the vial was refilled with 3 mL fresh PBS. The BSA concentration in the collection supernatant was analysed by the Micro-BCA^{TM} assay (Pierce). The results for the release test are presented as cumulative release as a function of time: cumulative release (%) = Mt/Ml, where Mt is the amount of BSA released at time t and Ml is the total amount of BSA loaded in the microparticles determined by protein loading efficiency. For each group, the assay samples were taken in triplicate ($n = 3$) at each time interval.

Statistical analysis

Statistical analysis was conducted using one-way ANOVA by SPSS software. Data were displayed as mean ± SD and statistical significance was set at $p < 0.05$.
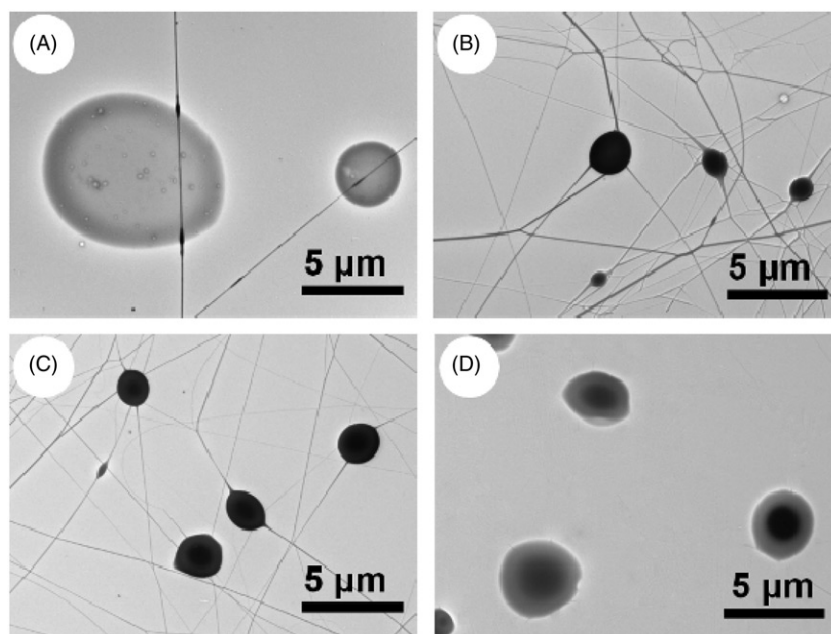
Figure 1. TEM images of microparticles prepared by coaxial electrospray technique. (A) Formula *a*-0% PLGAin; (B) formula *b*-6% PLGAin; (C) formula *c*-5% PLGAin and (D) formula *d*-4% PLGAin.
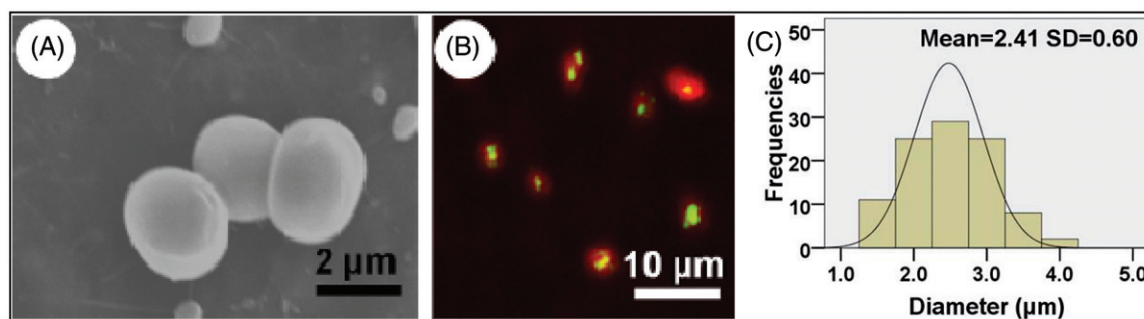

Figure 2. SEM image (A) and LCSM image (B) and the diameter distributions (C) of microparticles prepared by coaxial electrospray technique of formula *d*-4% PLGAin.

Results

Characterisation of coaxial electrosprayed microparticles

Different formulas were attempted using coaxial electro-spray to obtain core–shell structure microparticles. The outer flow was set 3wt% PLGA/TFE solution. When the inner flow was completely BSA aqueous solution, no apparent core–shell structured microparticles could be detected (formula *a*-0% PLGA in, Figure 1A). Only nanopores were scattered inside the electrosprayed microspheres. To increase the viscosity of the inner flow, PLGA/TFE solutions with different concentrations were applied. By dropping BSA aqueous solution into PLGA/TFE solutions, BSA nano-powders suspended in PLGA solutions were prepared and supplied as inner flow with PLGA/TFE outer solution, and coaxial electrosprayed. The viscosity of the inner solution had a significant effect on the morphology of coaxial electrosprayed microparticles. As shown in Figure 1(B)–(D),

distinct core–shell structured microparticles formed in all the three cases. However, fibrils could not be avoided completely, if the concentration of the inner PLGA/TFE solution was above 5wt% (formula *b*-6% PLGAin and *c*-5% PLGAin). Satisfactory core–shell structured microparticles (formula *d*-4% PLGAin) resulted when the concentration of inner PLGA/TFE solution was 4wt%.

SEM showed that microparticles made from formula *d*-4% PLGAin had a relatively uniform spherical morphology, with an average diameter of $2.41 \pm 0.60\,\mu m$ analysed by Image J (Figure 2A). To visualise the protein distribution within the coaxially electrosprayed microparticles, $50\,\mu g/mL$ fluorescent rhodamine B was introduced into the outer solution and FITC–BSA was used instead. The microparticles were prepared under exactly the same conditions as above and observed on LSCM. The red stain was attributed to rhodamine B, representing the shell region, whereas the green stain relating to FITC–BSA confirmed the encapsulation of protein inside the core area of the
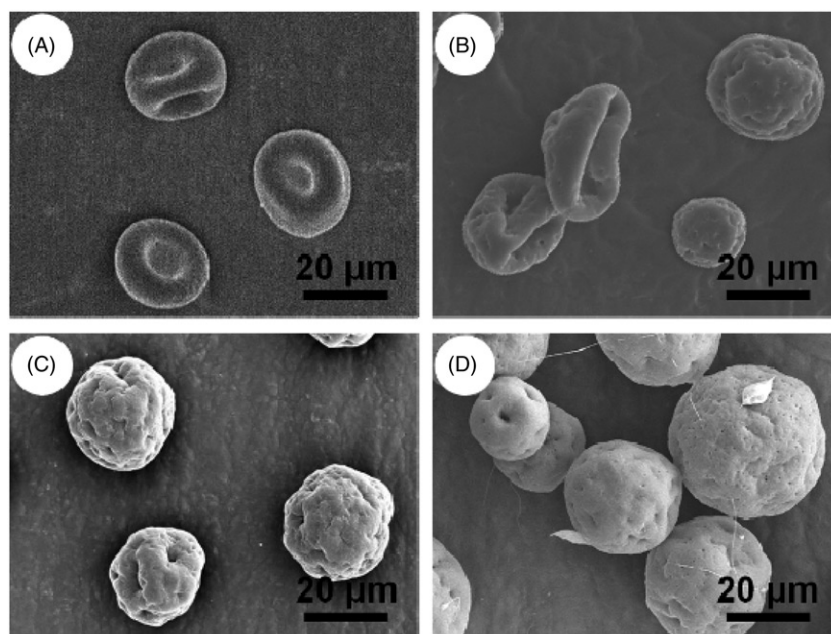
Figure 3. SEM images of microparticles prepared by emulsion electrospray technique. (A) formula *h*-2% PLGA; (B) formula *i*-4% PLGA; (C) formula *j*-6% PLGA and (D) formula *k*-8% PLGA.
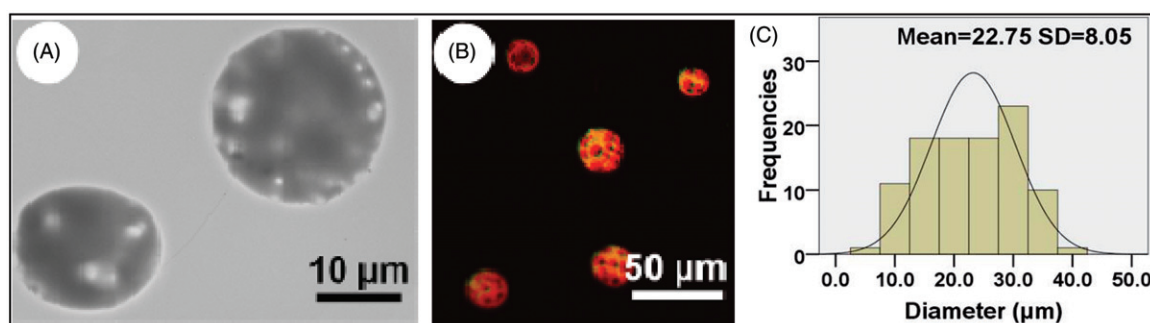

Figure 4. TEM image (A) and LCSM image (B) of microparticles prepared by emulsion electrospray technique and the diameter distributions of formula *j*-6% PLGA (C).

microparticles (Figure 2B). The green fluorescence turned out to be yellowish because it blended with the red stain of rhodamine B in the shell.

Characterisation of emulsion electrospray microparticles

In addition to coaxial electrospray, microparticles can be obtained from a wide range of polymeric solution concentrations using emulsion electrospray. As illustrated in Figure 3, as the concentration of PLGA/TFE solution increases from 2 to 8wt%, almost no fibre structures were formed. However, the morphology of emulsion electrosprayed microparticles changed significantly depending on polymeric solution concentration. At low solution concentration (formula *h*-2% PLGA and *i*-4% PLGA), the microparticles were shrunk and collapsed (Figure 3A and B). By increasing the concentration to 6wt%, fine-structured microspheres resulted with relatively uniform size (formula *j*-6% PLGA, Figure 3C), which was analysed as $22.75 \pm 8.05 \,\mu m$ (Figure 4C). 8wt% seemed a little

concentrated for emulsion electrospray, because particle sized deviated to a certain extent and fibrils began to show up (formula *k*-8% PLGA, Figure 3D).

Emulsion electrosprayed microparticles demonstrated a completely different interior structure from those coaxially electrosprayed. BSA aqueous solution was dispersed throughout the microparticles, as confirmed by Figure 4(A) and (B). TEM observation showed the porous structure and yellowish stain (a combination of red stain of rhodamine B and green stain of FITC–BSA) distributed over the microparticles. Some BSA was close to the surface of the microparticles with strong green staining at the peripheral region. There was some strong green (FITC–BSA) staining near the surface of the microparticles (Figure 2B).

Protein loading efficiency and in vitro release

Protein loading efficiencies under different formulas were measured, and those data are listed in Tables 1 and 2. On average, the loading efficiencies of emulsion electrospray
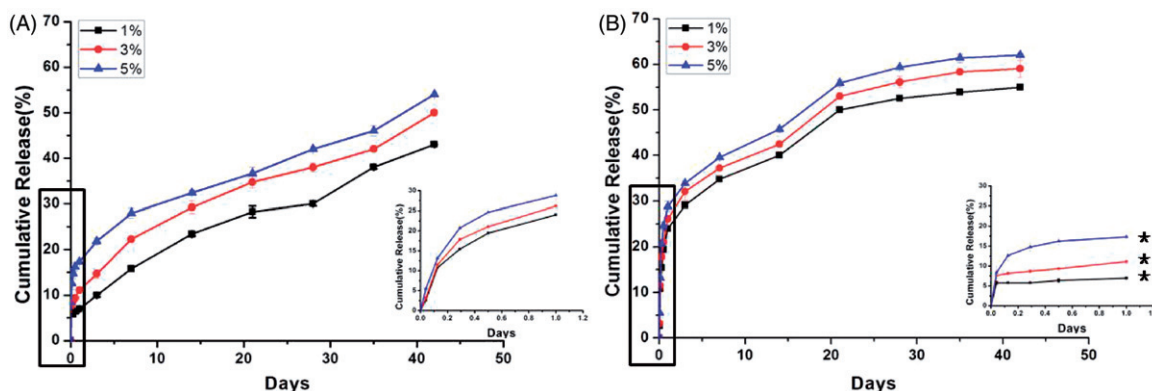
Figure 5. Protein release profiles for the electrospray microparticles. (A) Protein release profiles for the emulsion electrospray microparticles theoretically loaded with 1wt%, 3wt% & 5wt% BSA. (B) Protein release profiles for the coaxial electrospray microparticles theoretically loaded with 1wt%, 3wt% and 5wt% BSA. (*$p < 0.05$).

microparticles were a little lower than those of coaxial electrospray microparticles. The loading efficiencies in both cases decreased as the drug loading increased. The *in vitro* release behaviour of these BSA-loaded microparticles was compared. For the emulsion electrospray microparticles (Figure 5A), a significant initial burst release was detected. Within 1 day, >30% of the loaded BSA had been released. Afterwards, the release rate slowed down but was sustainable, and the cumulative BSA release was 50–60% at the end of 6 weeks. The coaxial electrospray microparticles showed better release behaviour than the emulsion electrospray microparticles (Figure 5B). The initial burst release was minor. The BSA release remained at a steady rate within the experimental period. About 40–50% BSA was released up to day 42. For all three emulsion groups, a burst release was observed within 24 h, which was 24.02% ± 0.27%, 26.15% ± 0.35% and 28.80% ± 1.14% for the 1%-BSA, 3%-BSA and 5%-BSA microparticles, respectively. These values were significantly different ($p > 0.05$) from the coaxial groups when compared between the same theoretical loading efficiency (*d*-4% PLGAin and *j*-6% PLGA, *e*-4% PLGAin and *l*-6% PLGA, *f*-4% PLGAin and *m*-6% PLGA) groups at 24 h time point. In both cases, one thing in common was that the BSA release rates were accelerated as drug loading increased.

Discussion

Most clinical diseases require long-term treatment with bioactive factors. Sustained release from polymeric matrixes has been proven as the simplest and most effective strategy to achieve that goal (Malathi and Balasubramanian, 2011). Tablets, films, gels, microparticles and nanoparticles are all possible pharmaceutical preparations. Microparticles and nanoparticles are preferred over other methods as a result of their flexibility in preparation and use (Xu et al., 2009). Electrosprayed microparticles have attractive features in narrow particle size distribution and minor loss of protein bioactivity in comparison with the conventional double-emulsion method,

which has made them attractive to researchers (Scholten et al., 2011). Many kinds of materials can be used to produce microparticles with drug or protein incorporated in by electrospray technique. Biodegradable alginate had been electrosprayed into micro size and with BSA encapsulated (Suksamran et al., 2009).

Emulsion electrospray was investigated in the present study. In this case, protein could be entrapped in the electrosprayed microparticles by dissolving in water and dispersing in polymeric inorganic solution by ultrasonication. Theoretically, the proteins should be distributed evenly throughout the electrosprayed microparticles, however, they were scattered throughout the microparticles in various sizes in most cases. As shown in Figure 4, BSA in emulsion electrosprayed PLGA microparticles was unevenly loaded. Especially at the edge of the microparticles, stronger BSA signals were detected, as confirmed by the green stain. This distribution pattern is closely related to the structure and stability of water-in-oil emulsions (Yang et al., 2011). The aqueous phase composed of BSA dissolved in deionised water was dispersed into the organic phase composed of PLGA/THM by ultrasonication. The scattered aqueous droplets might merge into drops of various sizes during emulsion electrospray. Therefore, emulsion electrospray microparticles had a clear initial burst release owing to the rapid diffusion of water into the microparticles surface and the rapid dissolution of BSA near the surface into the release medium (Figure 5A). The burst release would be more significant if the drug loading were increased.

In contrast, the development of coaxial electrospray has provided an efficient way to ameliorate the protein release behaviour. Through coaxial jets, a type of core–shell nanoparticles are generated by electrohydrodynamic forces, in which proteins are usually embedded in the core region (Loscertales et al., 2002; Hwang et al., 2008; Xie et al., 2008). As illustrated in Figure 2, BSA could be only found in the centre of coaxially electrosprayed microparticles with no hint of existence in the PLGA shell. These core–shell structured coaxial microparticles provide a barrier membrane that controls the protein diffusion rate

(Lee et al., 2010), thus ensuring that BSA is released at a constant rate with an insignificant initial burst release (Figure 5B), which was more welcomed by practical application for the fine control of release behaviour. A research showed that PVA coated PLGA microparticles not only presented a decrease in the drug release rate during the initial burst, but also the percentage of the drug released by diffusion is substantially reduced, from 77% (TFE) to 52% (TFE–DMSO) compared to uncoated ones (Almeria et al., 2011). The DMSO helped PLGA dissolve and swell in TFE, which made the protein released from the microparticles more easily and thoroughly. Those coating PVA served as a barrier that slow down the drug release rate. And the shell PLGA in our coaxial electrosprayed experiment just played the same role in drug release. The hydrophilic BSA dissolution into water was the key step during the drug release process. Apart from the surface location, the porous structure (Figure 4A) allowed the water entered inside of the emulsion microparticles faster. However, the hydrophobic PLGA shell (Figure 1D) of coaxial microparticles slowed down the water contact with BSA in the core, thus significantly reduced burst release.

Microparticle morphology might be another factor to consider for practical application. Microparticles from emulsion electrospray usually demonstrated a shrunken and wrinkled appearance, owing to the evaporation of a large amount of solvent in electrosprayed drops (Figure 3). In fact, this was a common phenomenon in microparticle formation using electrospray, which remarkably depended on concentrations of polymeric solutions. This damage to particle morphology could be weakened by increasing the concentration of the polymeric solution, however, it could not be too high for allowing fibre structure to occur (Enayati et al., 2009). Nevertheless, the coaxially electrosprayed microparticles resulted in much better morphology. As shown in Figure 2(A), the core–shell structured microparticles had a relatively smooth surface with small pinholes. The morphology difference in the two groups of electrospray microparticles suggested an additional reason for the rapid initial release from emulsion electrospray microparticles. Water molecules penetrated into and BSA molecules diffused out of the microparticles rapidly through the micropores and microducts on the surface of the particles.

Additionally, there were other two notable differences existing between the emulsion and coaxial electrosprayed microparticles in this study. Firstly, the mean diameter of the emulsion electrosprayed microparticles was much bigger than that of the coaxial electrosprayed ones. As is known to all, the diameter of electrosprayed microparticles could be affected by factors such as flow rate, polymer concentration, voltage, conductivity of the solution and so on (Xie et al., 2006). To produce microparticles by emulsion electrospray, THM was used to form the W/O emulsion. However, TFE was applied in the coaxial electrospray to ensure the stable formation of core-shell microparticles. The big difference in the polarity and conductivity between THM and TFE had significantly affected the forming and splitting of the droplets. On the other hand, the coaxial

electrospray group had an apparently higher BSA loading efficiency than the emulsion electrospray group (Tables 1 and 2). One of the reasons for this was that some protein denatured during the ultrasonication used for emulsion preparation (Wang et al., 2004). Besides, BSA encapsulated in emulsion microparticles had a direct contact with the air, which may cause more protein loss during the electrospun process. Moreover, the drug loading could be increased by increasing inner protein aqueous solution flow rate or increasing inner protein concentration. But the inner solution flow had a great impact on microparticles formation (Xie et al., 2008). Coaxial electrospray had avoided the shortcomings of emulsion electrospray, these features had made coaxial electrospray further favourable for preparing protein-loaded microparticles in comparison to emulsion preparation.

Conclusion

In conclusion, both emulsion and coaxial electrospray techniques can produce protein-loaded microparticles to achieve sustained drug release. The core–shell structure of the coaxially electrosprayed microparticles provided them with a greater ability to control release behaviour and guaranteed higher drug loading efficiencies in comparison with emulsion electrosprayed microparticles. This study indicated that the coaxial electrospray might be a superior approach to achieve sustained drug release with a minor initial burst release, which is preferable for clinical use.

Declaration of interest

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