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RESEARCH PAPER

One-Step Production of Protein-Loaded PLGA Microparticles via Spray Drying Using 3-Fluid Nozzle

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ABSTRACT

Purpose The aim of this study was to investigate the potential of using a spray-dryer equipped with a 3-fluid nozzle to microencapsulate protein drugs into polymeric microparticles.

Methods Lysozyme and PLGA were used as a model protein and a model polymer, respectively. The effects of process and formulation variables, such as i) the type of organic solvent, ii) the feeding rate ratio of the outer PLGA-containing feed solution to inner lysozyme-containing feed solution, and iii) the mass ratio of PLGA to protein, on the properties (morphology, internal structure, protein surface enrichment and release profiles) of the spray dried microparticles were investigated to understand protein microencapsulation and particle formation mechanisms.

Results The spherical, condensed microparticles were obtained with D_{50} of 1.07–1.60 μm and Span in the range of 0.82–1.23. The lysozyme surface content decreased upon different organic solvents used as follows: acetonitrile > acetone > dichloromethane. Additionally, the lysozyme surface enrichment decreased slightly when increasing the feeding rate ratio of the outer feed solution to the inner feed solution from 4:1 to 10:1. Furthermore, it was observed that there was a correlation between the degree of burst release and the lysozyme surface enrichment, whereas the lysozyme loading content had no substantial impact on the release kinetics.

Conclusions The present work demonstrates the potential of spray dryer equipped with a 3-fluid nozzle in microencapsulation of proteins into PLGA matrices with different characteristics by varying process and formulation parameters.

KEY WORDS 3-fluid nozzle · PLGA microparticles · protein microencapsulation · spray drying

INTRODUCTION

With the introduction of recombinant DNA technology, the number of protein therapeutics has dramatically increased. However, due to their short half-lives *in vivo*, susceptibility to enzymatic degradation and poor membrane passage, administration of protein drugs have been limited to the parenteral route, and do most often call for frequent injections (1–3). Hence, the effective sustained release protein delivery systems are very attractive for improving their stability against enzymatic degradation, prolonging the therapeutic effect and increasing patient compliance and convenience. Formulating protein drugs into injectable biodegradable polymer microspheres may represent the most commonly used method to obtain sustained release of biopharmaceuticals to overcome the aforementioned problems. Injectable poly (DL-lactic-co-glycolic acid) (PLGA) microspheres have been studied intensively for this purpose, and sustained release profiles for protein drugs over weeks or months have been reported in a number of studies (4–6).

Generally, proteins are encapsulated in PLGA matrices by using water-in-oil-in-water (w/o/w) double emulsion solvent evaporation methods (4,7,8), s/o/w single emulsion solvent evaporation methods (6,9) and coacervation (10). However, these formulation processes are strongly limited by the risk of

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protein denaturation and aggregation due to the exposure to the interfaces existing between the water and oil phases (2,11,12). Hence, a number of efforts have been made to overcome these problems during the last decades, such as co-incorporation of stabilizing excipients with the biopharmaceuticals in the inner water phase to minimize the interfacial effects (13,14). However, these traditional encapsulation methods are still facing a number of challenged related to the industrial large-scale production (4).

Compared with the aforementioned microencapsulation techniques, spray drying is a well-established, fast, one-step process with a potential for operation in a continuous mode. It has been widely used in various industries, including also the pharmaceutical industry (15–18). It has been commonly used to fabricate sophisticated and functionalized microparticles for drug delivery (19,20). Although there is always a stability concern for spray drying of peptide and protein drugs, many reports have shown that thermal denaturation of peptides and proteins during the spray drying process is usually not observed (19,21,22), and the atomization shear stress seems not to influence stability (23,24). However, microencapsulation of protein drugs using the conventional spray drying technique with a 2-fluid nozzle is not optimal due to highly diverse solvent requirements when dissolving hydrophobic polymers and hydrophilic protein drugs. Hence, preprocessing is needed prior to spray drying, such as the poly(ethylene glycol) (PEG)-assisted protein solubilization technique (5), which not only increases the risk of protein denaturation, but also make the production process much more complicated, in turn, leading to the commercial failure of the formulation. In addition, from industrial production of view, the parenteral microparticles in general are manufactured by an aseptic process in order to assure sterility of products (25), however, the multisteps would also increase the difficulty and cost for sterilization of products. All these challenges involved in spray drying of protein injectable formulations may result in so far unsuccessful commercialization of proteins loaded injectable microparticles obtained by the spray drying process.

In recent years, advances in microencapsulation technologies have been made by applying the interfacial solvent exchange technique (1,26) and the dual-capillary electrospray technique (27–29). However, the overall complexity associated with the traditional encapsulation methods still remains the same. Hence, new microencapsulation methods are needed to overcome the manufacturing process complexity. In the present study, a one-step spray drying process with a 3-fluid nozzle (two liquid and one gas channels) was used to prepare protein-loaded PLGA microparticles. By feeding the aqueous protein solution and the organic polymer solution in separate channels, protein-loaded PLGA microparticles were prepared in a one-step process. Although 4-fluid nozzle (with two liquid and two gas channels) and 3-fluid nozzle (with two liquid and one gas channels) have been utilized to design functionalized microparticles (30–32) and microencapsulate fish oil and small molecular drugs (33–35), the potential application of these nozzles for microencapsulation of proteins and peptides has not been studied. In addition most reports have been focused on the characterization of the final products without the efforts on addressing the potential influence of the process parameters (such as the solvent properties, the feeding rate ratio of the outer feed solution to inner feed solution, and the critical properties of the feed solution) on the physicochemical properties of the resulting particles. To the best of our knowledge, for the first time, we investigated the potential of using a spray-dryer equipped with a 3-fluid nozzle to microencapsulate protein drugs into PLGA microparticles in one-step with an emphasis on understanding the protein microencapsulation mechanism and the particle formation process.

MATERIALS AND METHODS

Materials

PLGA (lactide:glycolide molar ratio of 75:25, Mw: 20 kDa, purchased from Wako Pure Chemical Industries, Ltd. (Wako, Germany)) was selected to prepare lysozyme-loaded PLGA microparticles because of its lower degradation rate compared to PLGA with lactide:glycolide molar ratio of 50:50, which can consequently lead to the prolonged release of active substances (7,36,37). Chicken egg-white lysozyme crystalline powder (Product No. 62971) was purchased from Fluka Analytical (Sigma-Aldrich, Poole, UK). Trehalose (VWR International Ltd., Poole, UK) was used as protein stabilizer in the spray drying process (38). Acetone (ACE, 99.9% HPLC grade), acetonitrile (ACN, 99.9% HPLC grade) and dichloromethane (DCM, 99.9% HPLC grade) were purchased from Sigma-Aldrich (Poole, UK). Lysozyme was stored at 4°C and trehalose powders were stored in airtight, light resistant containers at room temperature until use. The deionized water was used for the whole experiment.

Methods

Preparation of Lysozyme-Loaded PLGA Microparticles

All formulations in the study were prepared via spray drying using a Büchi B-290 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) equipped with a 3-fluid nozzle (Buchi 046555, Three fluid nozzle, Büchi Labortechnik AG, Flawil, Switzerland), an inert loop B-295 (Büchi Labortechnik AG) and a dehumidifier (Büchi Labortechnik AG). A schematic diagram of the spray drying process with a 3-fluid nozzle is presented in Fig. 1. Lysozyme and trehalose were dissolved in deionized water as the inner feed solution and PLGA was dissolved in variety of organic solvents and used as the outer

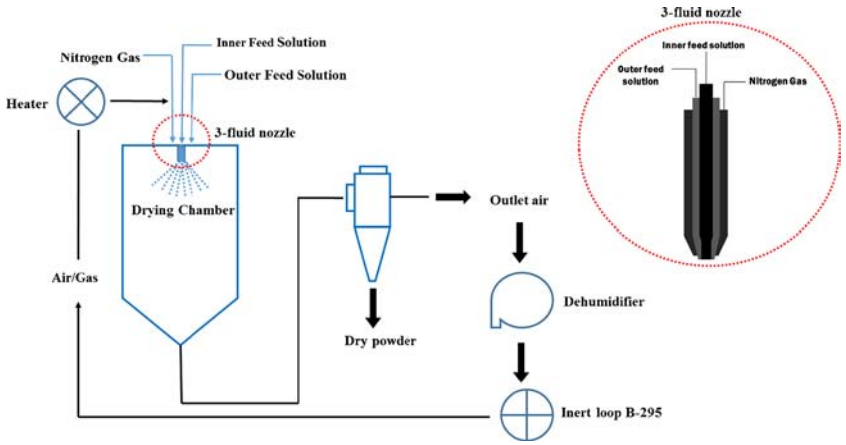

Fig. 1 Schematic diagram of the spray drying process with a 3-fluid nozzle.

feed solution. The composition of the feed solutions is listed in Table I. Then, the aqueous solution and the organic solution were pumped into the spray dryer at constant total feeding rate. Identical drying conditions (total feed rate 3 mL/min; inlet temperature 60°C; outlet temperature 40–45°C; drying air flow rate 37.5 m^3/h and atomization air flow rate 742 L/h) were used for all samples. The feeding ratio of the outer feed solution to the inner feed solution (4:1 or 10:1) was varied to investigate the effect of the solvent composition on the particle properties. The dried powder was collected and stored in a desiccator at room temperature for further characterization.

Particle Morphology

The morphology of the spray-dried particles was visually examined using a Zeiss Ultra55 scanning electron microscope (SEM, Carl Zeiss, Germany). The samples were transferred onto sticky carbon tape and mounted on metallic stubs, followed by sputter coating with a thick layer of gold to make the surfaces conductive. The specimens were then imaged at an accelerating voltage of 3 kV with a 30 μm aperture. The particle sizes and distributions were measured by conventional image analysis using a software (Image J, NIH, USA): The Martin's diameter of 100–150 randomly selected particles was determined in a fixed direction (left to right) from the field of view of the SEM images (39). The size data were expressed as D_{20}, D_{50}, D_{80}, which are diameters at 20%, 50%, and 80% cumulative number, respectively. The broadness of the size distribution, known as the span, equals $(D_{80} - D_{20})/D_{50}$.

Focused ion Beam-Scanning Electron Microscopy (FIB-SEM)

A Zeiss 1,540 XB Cross Beam SEM (Carl Zeiss, Germany) equipped with a 30 kV Ga^+ focused ion beam (FIB)-gun was used to investigate the inner structure of the spray-dried microparticles. Briefly, an automated FIB-SEM slice-and-view procedure was used for observation. The ion beam removes thin sections of the particles, pausing the milling

Table I Composition of the Feed Solutions and Characterization of The Resulted Lysozyme Loaded PLGA Microparticles ($n = 3$)

Sample ID	Composition of feed solution					Characterization	
	Outer feed solution		Inner feed solution			Residual moisture (%)	Actual lysozyme Loading (%)
	PLGA (mg)	Solvent/Volume (mL)	Lysozyme (mg)	Trehalose (mg)	Solvent/Volume (mL)		
M50F10ACN	500	ACN/20	10	20	H_2O/2	0.9 ± 0.1%	2.0 ± 0.2%
M50F10ACE	500	ACE/20	10	20	H_2O/2	0.8 ± 0.2%	2.0 ± 0.2%
M50F10DCM	500	DCM/20	10	20	H_2O/2	0.5 ± 0.3%	1.5 ± 0.5%
M50F4ACE	500	ACE/20	10	20	H_2O/5	1.2 ± 0.1%	2.1 ± 0.2%
M50F4DCM	500	DCM/20	10	20	H_2O/5	1.0 ± 0.3%	1.7 ± 0.4%
M50F2DCM	500	DCM/20	10	20	H_2O/10	1.1 ± 0.3%	1.7 ± 0.3%
M20F10DCM	500	DCM/20	25	20	H_2O/2	1.0 ± 0.4%	4.2 ± 0.7%
M10F10DCM	500	DCM/20	50	20	H_2O/2	1.2 ± 0.2%	7.4 ± 0.7%

p.s. In the term of Sample ID, "M" refers to the mass ratio of PLGA to protein, e. g. M50 represents the mass ratio of PLGA to protein is 50:1; "F" refers to the feeding rate ratio of the outer PLGA-containing feed solution to inner lysozyme-containing feed solution, e. g. F10 represents feeding rate ratio of outer feed solution to inner feed solution is 10:1; ACN, ACE and DCM represent that lysozyme-loaded PLGA microparticles was prepared by using acetonitrile, acetone and dichloromethane as the organic solvent, respectively

process at predetermined time intervals, and an image of the newly exposed area is saved. The slice-and-view process continued through the particles, exposing the internal structure. The resulting image stack was post processed in Image J (NIH, USA) using StackReg for alignment (translation in x, y only).

Thermogravimetric Analysis (TGA)

The residual moisture of the bulk samples was analyzed using a TGA 7 (Perkin Elmer, Waltham, Massachusetts, USA) under a nitrogen purge of 20 mL/min. Samples (2–5 mg) were loaded onto an open platinum pan and heated from 20 to 120°C at a scan rate of 10°C/min.

X-ray Photoelectron Spectroscopy (XPS)

Lysozyme surface enrichment of microparticles was analyzed by using XPS. Briefly, the particles were transferred to a glass slide and manually placed "shoulder by shoulder" under a light microscope to obtain a homogeneous coverage of the particles on the glass sides. Then, the microparticles on glass slides were analyzed by XPS using a K-Alpha (Thermo Scientific, Copenhagen, Denmark) equipped with a monochromated AlKα X-ray source with an energy of 1,486.6 eV. Wide energy survey scans (0–1,350 eV binding energy) were acquired with a pass energy of 200 eV and a step size of 1.0 eV. Additional high resolution spectra of carbon (C1s), oxygen (O1s) and silicon (Si2p) were acquired with a pass energy of 25 eV and a step size of 0.1 eV. The angle between the sample surface and the analyzer (take-off angle) was 90°. Charge compensation was accomplished with a dual beam flood gun. An X-ray spot size of 200 μm was used. The lysozyme content (in weight %) on the particle surface was determined as the detected amount of nitrogen in the sample relative to the theoretical nitrogen content in lysozyme.

Interfacial Tension Measurements by Drop Profile Analysis

A drop shape analysis system (DSA100, KRÜSS, Hamburg, Germany) was employed for characterization of the interfacial tension of the feed solutions used in the present study by using the pendant droplet model. Briefly, the shape of a pendant drop was recorded using a live video model. Then, the interfacial tension was obtained by comparing to its theoretical profile, which was calculated from the Gauss-Laplace equation by using the software (DSA1, KRÜSS, Hamburg, Germany). All measurements were performed at room temperature (around 20 ~ 22°C).

Lysozyme Loading of Microparticles and Release Behavior In Vitro

The loading of lysozyme in the microparticles was determined following reconstitution of lysozyme in PBS (pH 7.4) after dissolving the polymer in acetone (40). Briefly, lysozyme-loaded PLGA microparticles were dissolved in acetone. After 0.5 h, they were centrifuged (10,000*g*, 10 min) and the organic phase was removed. The precipitates were then dispersed with freshly added acetone to eliminate the excess PLGA. Rinsing was carried out three times. After evaporation of acetone, the collected protein fraction was dissolved in PBS (pH 7.4) for protein content measurement.

The *in vitro* release profile of the protein from the microparticles was determined under mild agitation conditions (45 rpm/min) (36). An amount of 50 mg lysozyme-loaded microspheres was placed in 1 mL in PBS (pH 7.4) and incubated at 37°C. At predetermined time intervals, the release medium was replaced. The concentration of protein in the release medium was then measured by using a UV spectrometer (Evolution 300, Thermo scientific, Madison, WI, USA) at 280 nm to calculate the cumulative release profiles of lysozyme from the microparticles, as reported previously (41,42).

Lysozyme Recovery After Release Study

After 48 h of release, the lysozyme recovery was determined by extracting the unreleased lysozyme from the residual microparticles using a previously reported method (5). Briefly, the residual PLGA microparticles after 48 h release study were dissolved in 1 mL of 1 N NaOH under stirring at 37°C for 4–5 h until a clear solution was obtained. The amount of lysozyme recovered was also determined by using a UV spectrometer (Evolution 300, Thermo scientific, Madison, WI, USA).

Circular Dichroism (CD)

The microparticle formulation prepared by DCM (M10F10DCM) was selected as a reference for CD measurement because DCM seems to be an optimal solvent to improve lysozyme microencapsulation efficiency, and render prolonged release profile for the model protein drug. The lysozyme as received was dissolved in PBS (pH 7.4) as reference native sample. The extracted lysozyme from the spray dried microparticles was obtained by using the procedure used in the section of determination of lysozyme loading. The released lysozyme from PLGA microparticles were obtained at 1 day, 2 day under the same condition as the release study. Far ultraviolet CD spectra (190–265 nm) of these lysozyme solutions were recorded with a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA) at 20°C with a step size of 0.5 nm. The cell compartment was purged with nitrogen at a flow rate of 10 L/min. A quartz cell (with $L = 1$ mm) was used. The lysozyme concentration in the liquid samples was approximately 0.2 mg/mL (22,27,28).

Statistics

Measurements were performed in triplicate, unless otherwise stated. Values are given as means ± SD. Statistically significant differences were assessed by an analysis of variance (ANOVA) at a 0.05 significance level, followed by the *t*-test (Excel, Microsoft, USA).

RESULTS AND DISCUSSION

Physicochemical Properties of Lysozyme-Loaded PLGA Microparticles Prepared by Using a 3-Fluid Nozzle

D_{50} of 1.07–1.60 μm and sizes distributions with Span in the range of 0.82–1.23 were observed for the resulted lysozyme loaded PLGA microparticles. A decrease in the feeding rate ratio of the outer feed solution to the inner feed solution was likely to increase D_{50}. The effect of the organic solvents applied in this study on D_{50} and Span seemed to be subtle (Table II & Fig. 2). However, it was observed that the particle shape was influenced by the type of organic solvents used and the feeding rate ratio of the outer feed solution to the inner feed solution. Although particles with a smooth surface were obtained by applying the different organic solvents, yet irregular microparticle aggregates (Fig. 2, arrows) were observed when acetone was used to prepare the lysozyme-loaded PLGA microparticles. Furthermore, the internal structure of the microparticles, characterized by FIB-SEM, was dense, irrespectively of the organic solvents applied. However, the irregular aggregates showed a porous inner structure (Fig. 3).

In order to explain the observation, it is necessary to understand the micro-droplet formation process and the microencapsulation mechanism. Two theories might be used to describe the particle formation and microencapsulation process via spray drying with a 3-fluid nozzle. One is that the micro-droplets can be formed by midair collision between the liquid drops of individual feeds (inner feed and outer feed) after atomization by the high speed air flow, as described by Park *et al.* in the application of two ink-jet nozzles and a coaxial ultrasonic atomizer (1,26). Subsequently, the aqueous droplets are encapsulated into the polymer organic droplets driven by the surface tension gradient between the two droplets. Another potential theory is that the two liquids would form an "emulsion mixture" upon atomization. When the miscible solvents are applied in the 3-fluid nozzle, the two feeds (inner feed and outer feed) mix intimately to make a "transient film", which then break up into micro-droplets. However, it may differ when dichloromethane (immiscible with water) is used as the organic solvent. In this case, the two liquids are more likely to form "transient w/o emulsion droplets" upon the atomization by the high speed air flow (1).

According to both theories, particle formation is initiated by the formation of a transient liquid PLGA film at the interface between the aqueous and the organic feed, which is a result of mass transfer between the organic solvent and water (i.e., solvent exchange) leading to a decrease in the solubility of the polymer in the solvents (43). In the case that the transitional liquid PLGA film appears instantaneously once the organic PLGA solution comes into contact with the aqueous protein solution, it forms irregular PLGA aggregates in the subsequent drying process. The time required for the formation of the transitional liquid PLGA film is highly dependent on the solvent properties, such as the surface tension of the organic solvent and the miscibility of the organic solvent with water (44). It is well-known that the use of organic solvents with a low surface tension and a certain degree of miscibility with water causes the instant phase separation of the PLGA film (45). In the present study, the organic solvent dichloromethane is immiscible with water, hence aggregates are unlikely to form when the dichloromethane is used. Although the organic solvents acetone and acetonitrile are miscible with water (Table III), the application of acetone resulted in more irregular PLGA aggregates due to its lower surface tension, leading to the faster spreading rate of acetone on the surface of the aqueous droplets (46). On the other hand, a turbidity study of an organic PLGA solution mixed with water was performed and it showed that an acetonitrile solution of PLGA had a higher miscibility with water compared to the acetone solution of PLGA (data not shown), which can further explain the observed phenomenon.

Surface Composition of Lysozyme-Loaded PLGA Microparticles

The chemical components on the particle surface were studied using XPS. XPS is a surface chemical analysis technique used to analyze the elemental composition on the topmost 5–10 nm of the particle surface (47,48). The lysozyme content (in weight %) on the surface of the microparticles was determined by taking

Table II D_{20}, D_{50}, D_{80} and Span of the Resulted Lysozyme Loaded PLGA Microparticles

	M50F10ACN	M50F10ACE	M50F10DCM	M50F4ACE	M50F4DCM
D_{20}	0.68 ± 0.02	0.93 ± 0.07	0.59 ± 0.10	1.03 ± 0.09	0.98 ± 0.06
D_{50}	1.15 ± 0.02	1.39 ± 0.06	1.07 ± 0.11	1.60 ± 0.07	1.59 ± 0.04
D_{80}	1.95 ± 0.04	2.07 ± 0.06	1.91 ± 0.08	2.49 ± 0.14	2.59 ± 0.07
Span	1.10	0.82	1.23	0.91	1.01

p.s. Sample ID referring to Table I

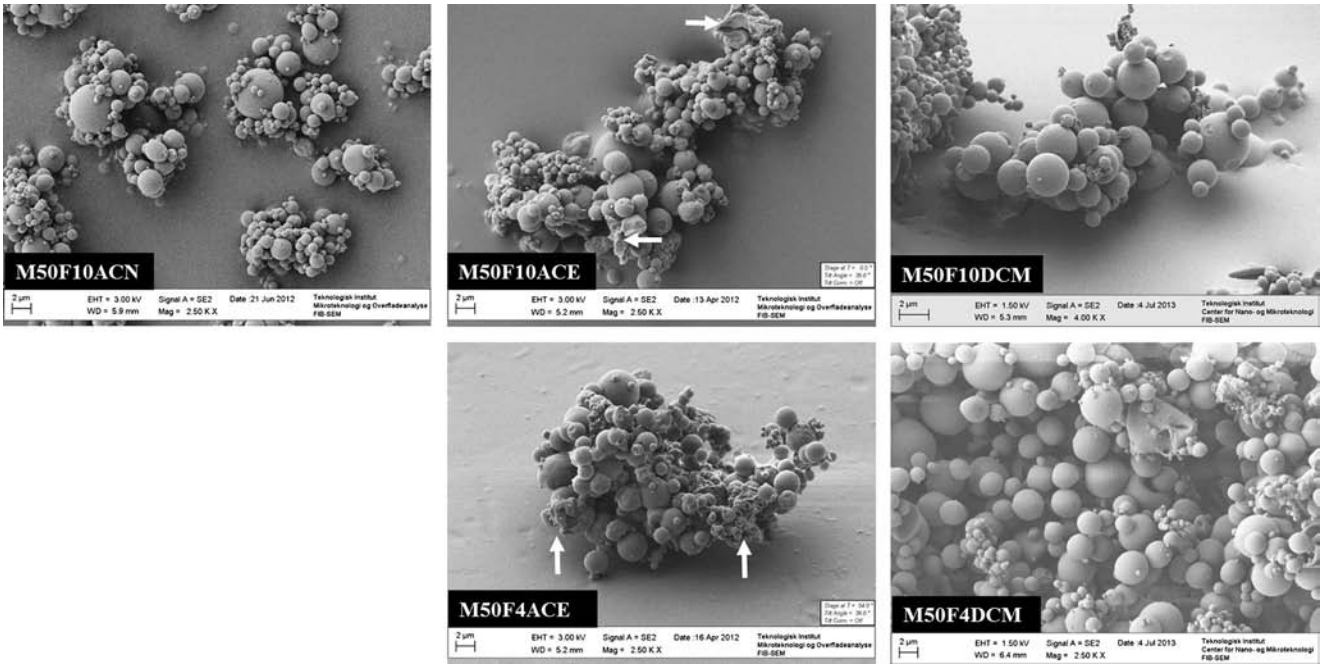

Fig. 2 SEM images of lysozyme-loaded PLGA microparticles prepared via spray drying with a 3-fluid nozzle (scale bar 2 μm). Sample ID referring to Table I.

the ratio of the detected amount of nitrogen in the sample to the amount of nitrogen in pure lysozyme. In addition, to validate the measurement, the nitrogen-to-carbon (N/C) ratio was also adopted to inspect whether there was any interruption from the "blank glass area" produced during sample preparation (Fig. 4a).

It was observed that the nitrogen content on the surface was highly dependent on the applied organic solvent and on the feeding rate ratio of the outer feed solution to the inner feed solution, which was also seen from the N/C ratio, suggesting a good reproducibility of the sample preparation process and the little influence induced by the "blank glass area" of the different samples. First, the nitrogen content on the microparticle surface was decreased significantly by applying the organic solvents in the following order: acetonitrile > acetone > dichloromethane (Fig. 4a). For example, when acetonitrile was used, the highest

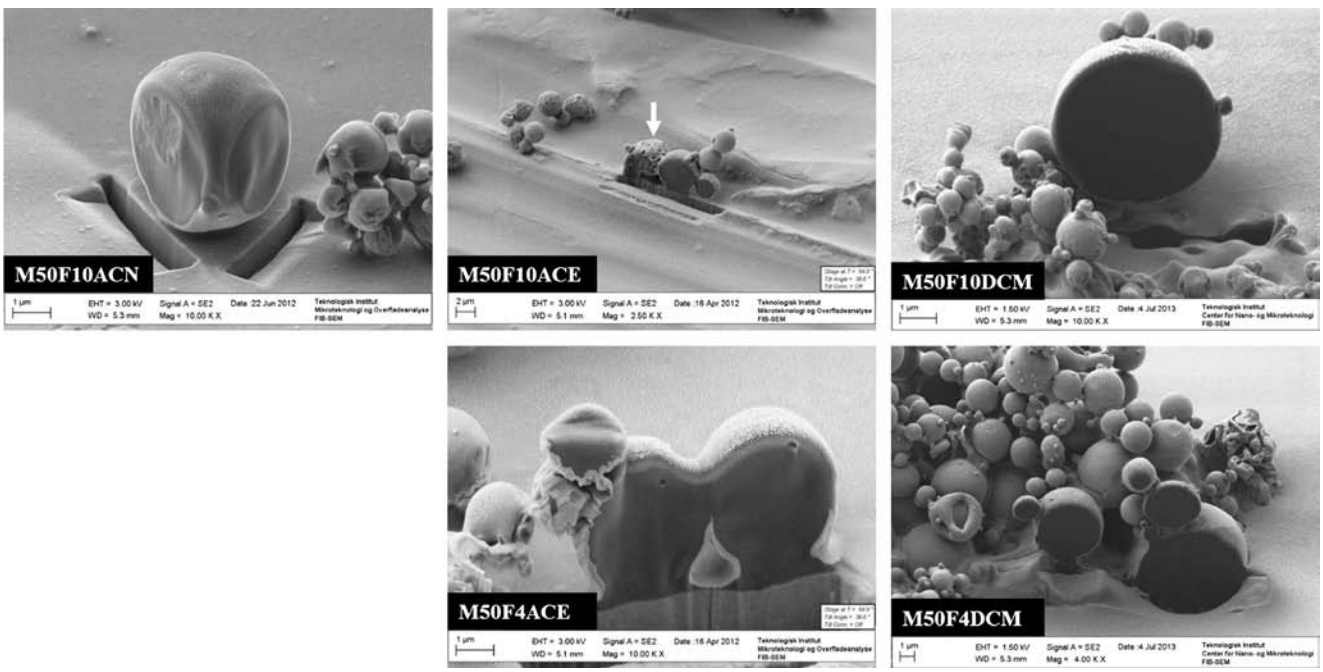

Fig. 3 FIB-SEM images of lysozyme-loaded PLGA microparticles prepared via spray drying with a 3-fluid nozzle (scale bar 2 μm for the upper middle image and 1 μm for the other images). Sample ID referring to Table I.

Table III Critical Properties of Solvents Applied in this Study

Solvents	Boiling point (°C) [a]	Solubility in water (g/L at 20°C) [a]	Evaporation rate (BuAc = 1) [a, b]
Acetone	56.0	Miscible	5.6
Acetonitrile	82.0	Miscible	2
1,2-dichloroethane	39.8	13	6.5
Water	100.0	–	0.3

a: values are from the solvent handbook; b: relative to the evaporation rate of Butyl Acetate

amount of elemental nitrogen (~1.2% w/w) was detected, which corresponds to approximately 8.7% w/w of the lysozyme molecules (calculated by assuming approximately 15% w/w of elemental nitrogen per lysozyme molecule) located on the surface of the microparticles (Fig. 4b). When dichloromethane and acetone were used, about 3.5% w/w and 4.2% w/w of the lysozyme content was detected on the microparticle surface, respectively (Fig. 4b). On the other hand, it was found that the amount of elemental nitrogen, as well as the lysozyme content, on the microparticle surface were decreased slightly (not statistically significant) with an increase in the feeding rate ratio from 4:1 to 10:1 (Fig. 4a&b). Furthermore, when comparing the surface content of lysozyme and the actual lysozyme loading content, the larger deviations between them were observed for the microparticles prepared by using acetonitrile at the feeding rate ratio of 10:1 and for the microparticles prepared by using DCM and ACE at the feeding rate ratio of 4:1 (Fig. 4b), indicating higher degree of lysozyme surface enrichment of the corresponding microparticles.

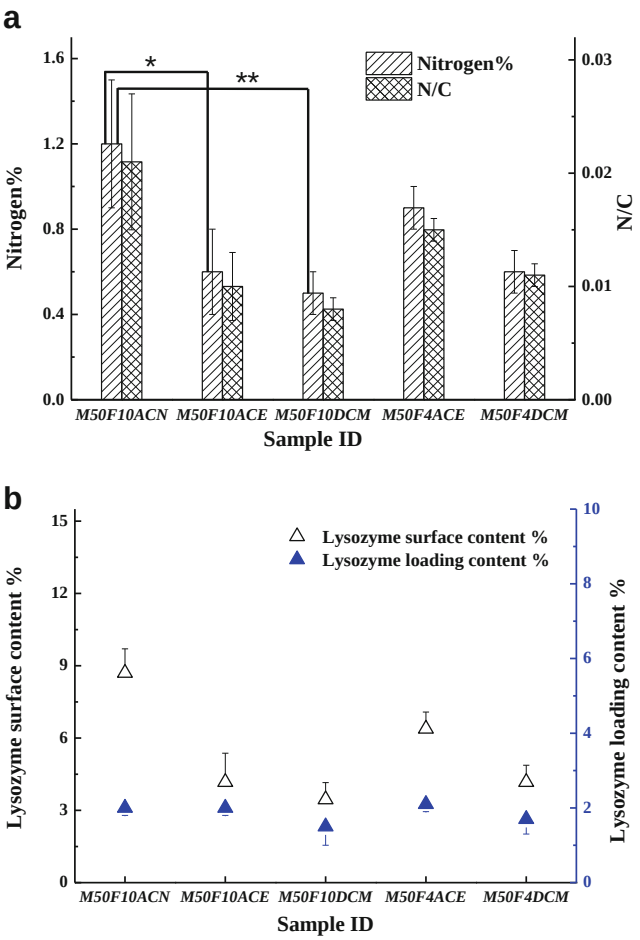

Fig. 4 Nitrogen content and N/C ratio at the surface of the lysozyme loaded PLGA microparticles prepared via 3-fluid nozzle spray drying measured by using XPS (**a**) ($n \geq 3$); Comparison of actual lysozyme loading content and amount of lysozyme surface content (**b**) ($n \geq 3$); * means $p < 0.05$; ** means $p < 0.02$. Sample ID referring to Table I.

To elucidate the surface enrichment of lysozyme on the microparticles, both the protein microencapsulation mechanism and the drying kinetics must be considered. It has been reported that the microencapsulation efficiency is highly dependent on the surface tension gradient between the lysozyme aqueous droplet and the PLGA organic droplet (49). As shown in Table IV, PLGA acetonitrile solution has a higher interfacial tension, as compared to PLGA solutions prepared with acetone and dichloromethane, suggesting the surface tension gradient between the lysozyme aqueous droplet and PLGA acetonitrile droplet is smaller. The smaller surface tension gradient and the higher interfacial tension would result in reduced spreading efficiency of the outer fluid (PLGA acetonitrile solution) on the inner fluid (lysozyme aqueous solution), which negatively influences the microencapsulation efficiency of lysozyme in the process of droplet formation (46). Furthermore, it is well known that the diffusional motion inside a droplet plays an important role in the resulting radial distribution of the individual components (16,48). In the present study, a binary solvent system was used in the droplets composed of water and an organic solvent. According to the previous studies of the drying kinetics of a binary solvent mixture (50,51), one can expect that the solvent with lower boiling point and faster evaporation rate (Table III) dictates the drying kinetics in the initial phase of the drying process, followed by a secondary drying phase, where the drying kinetics is predominated by the evaporation rate of the solvent with lower evaporation rate, i.e. water in this study. The faster evaporation of organic solvent would lead to the formation of PLGA shell around the droplet in the initial phase of the drying process. The PLGA shell would get thicker and denser

Table IV Interfacial Tensions of the Feed Solutions Applied in this Study ($n \geq 3$)

Feed solutions	Interfacial tension (mN/m)
2.5% PLGA acetonitrile solution	35.7 ± 0.2
2.5% PLGA acetone solution	27.4 ± 0.5
2.5% PLGA dichloromethane solution	17.2 ± 0.1
0.2% lysozyme aqueous solution	69.8 ± 1.8
0.5% lysozyme aqueous solution	66.6 ± 2.4

upon drying, however, it is still possible for the mass transport and diffusional flux with the evaporation of inner water during the certain period in the drying process (48,52). Hence, it is rational to suggest that the migration of lysozyme molecules towards to the droplet surface with the evaporation of water results in the surface enrichment of lysozyme. The fact that the surface enrichment of lysozyme was decreased upon an increase in the feeding rate ratio of outer feed solution to inner feed solution and using the organic solvent with faster evaporation rate could support the hypothesis.

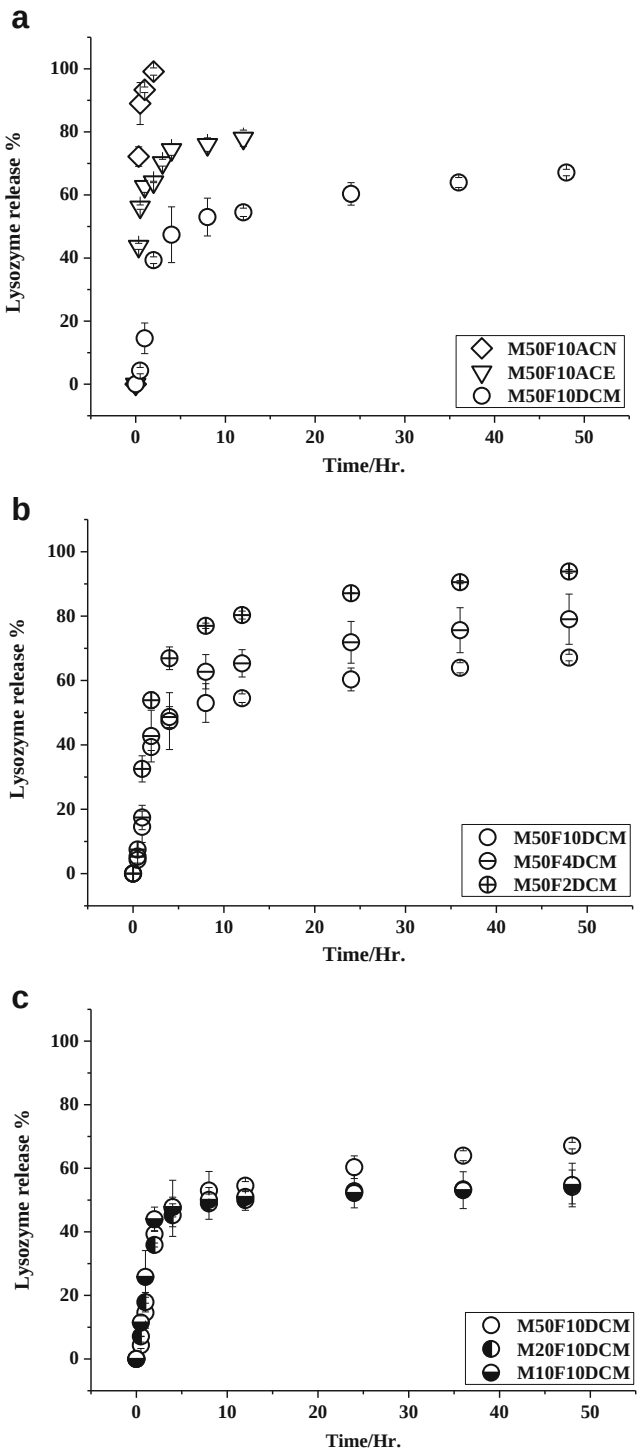

Fig. 5 *In vitro* drug release profiles of lysozyme loaded PLGA microparticles ($n=3$), (**a**) effect of organic solvents; (**b**) effect of feed rate ratio of outer feed solution to inner feed solution; (**c**) effect of protein loading. Sample ID referring to Table I.

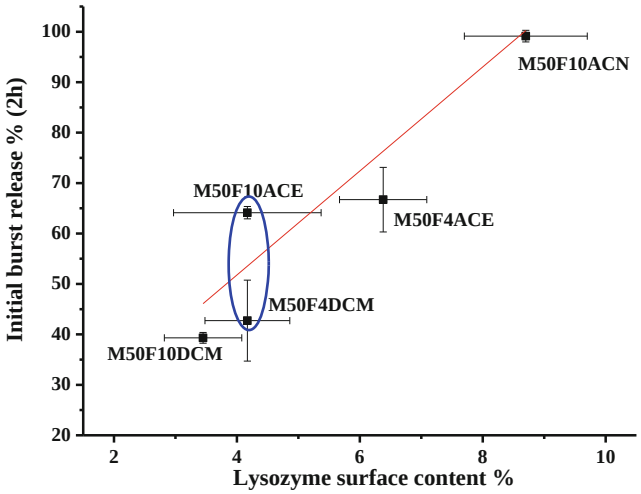

Fig. 6 Correlation between lysozyme surface content and initial burst release of lysozyme from PLGA microparticles prepared by spray drying with a 3-fluid nozzle. Sample ID referring to Table I.

Characterization of Initial Release of Lysozyme from PLGA Microparticles

It is well known that the initial burst release poses a serious toxicity threat and is a major challenge for developing a PLGA-based microparticle product. Hence, the present study focused on how the formulation and manufacturing variables influence

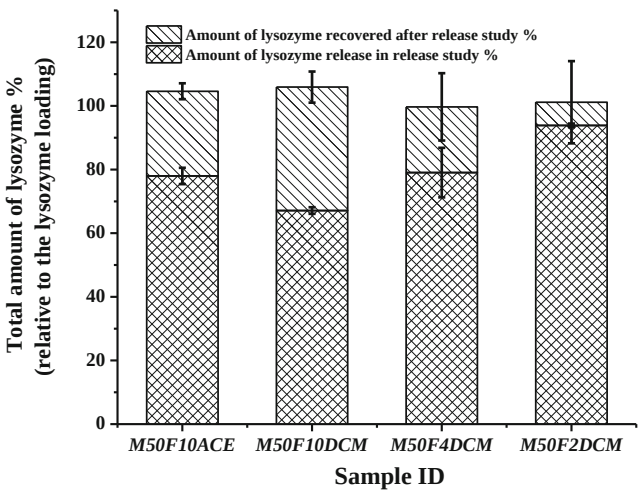

Fig. 7 Total amount of lysozyme measured in both release study and recovery study ($n=3$). p.s. For M50F10ACE, the release study lasted 8 h, other microparticles formations, the release study lasted 48 h. Sample ID referring to Table I.

the initial release (48 h) of lysozyme from PLGA microparticles prepared via spray drying with a 3-fluid nozzle. It is not unexpected that the lysozyme release behavior is significantly influenced by the organic solvent applied (Fig. 5a). For example, the lysozyme-loaded PLGA microparticles prepared by using acetonitrile have the fastest burst release rate and reached the complete release within 4 h. The lysozyme-loaded PLGA microparticles prepared by using dichloromethane presented a release profile of around 50% burst release in the first 8 h followed by a linear release up to 48 h with a release rate of 0.38% per hour (Fig. 5a). On the other hand, it was found that the burst release could be reduced with an increase in the feeding rate ratio of the outer feed solution and the inner feed solution. However, the following release stage (8 to 48 h) demonstrated the independence of the feeding rate ratio (Fig. 5b). Moreover, the changes in the lysozyme loading in the microparticles did not result in the obvious impact on the release profiles *in vitro* (Fig. 5c).

It is widely believed that the initial burst release is a result of the rapid release of drug molecules from the surface of the microparticles (8). In the present study, it was found that there was a correlation between the burst release and the lysozyme surface enrichment (Fig. 6). The burst release was suppressed when decreasing the lysozyme surface enrichment by applying different organic solvents or by varying the feeding rate ratio of the outer feed solution to the inner feed solution. For the microparticles prepared by using acetonitrile, as discussed in "Surface composition of lysozyme-loaded PLGA microparticles", there was approximately 9% lysozyme on the surface (5–10 nm) of the microparticles, which can be correlated to the very high burst release. Then, the water-filled pores or channels generated during the burst release stage would accelerate the release at the following release stage due to the increased diffusivity and connectivity of the PLGA microparticles (12,53). Interestingly, the burst release of lysozyme from microparticles formulations of M50F10ACE and M50F4DCM deviated, though both of them exhibited a similar lysozyme surface enrichment (Fig. 6). It could be a result of distinct microscopic connectivity and diffusivity of PLGA matrices generated by the different PLGA molecular behavior and their distinct concomitant microstructural changes of the PLGA matrices upon the release process (54). As dichloromethane is a better solvent for PLGA, as compared to acetone (ACE), the meso-structre of the PLGA matrices prepared by DCM would be much denser and has a better ability to resist the collapse of PLGA matrices in the release process.

It showed that the total amount of lysozyme measured in both the release study and the lysozyme recovery study reached up to about 100% (Fig. 7). Additionally, the burst release of lysozyme was observed for all the microparticle formulations, which could be due to the lysozyme surface enrichment and the existence of a large amount of submicro particles (Fig. 2). Hence, in order to sustain the protein release up to several weeks or months, approaches to suppress the diffusion of protein molecules during solidification of the atomized droplets should be attempted to improve the microencapsulation efficiency of the protein and increase the particle size with a narrow size distribution. The CD results suggested that lysozyme retained secondary structure upon the spray-drying process, as well as during the release study, indicating the encapsulation process did not compromise the protein integrity or the degradation of protein was negligible if any (Fig. 8).

CONCLUSIONS

In the present study, we introduced a one-step method to prepare protein loaded PLGA microparticles via a spray drying process using a 3-fluid nozzle and investigated the effects of solvent selection and feeding rate on the physicochemical properties of the spray dried microparticles. The spherical microparticles with a condensed internal structure were obtained. It seemed that the organic solvents had no obvious impact on particle size, which was likely to be adjusted by varying the feeding rate ratio of the outer feed solution to the inner feed solution. In addition, the lysozyme microencapsulation efficiency, represented by the decrease in lysozyme surface enrichment, was highly influenced by the solvent properties including the miscibility with water, volatility, and interfacial tension. On the other hand, the feeding rate ratio of outer feed solution to inner feed solution also plays a role for the encapsulating of lysozyme in PLGA microparticles by tuning the drying kinetics of the droplets during the spray drying process. The present study implied the potential of encapsulation of protein in polymeric microparticles via spray drying with a 3-fluid nozzle.

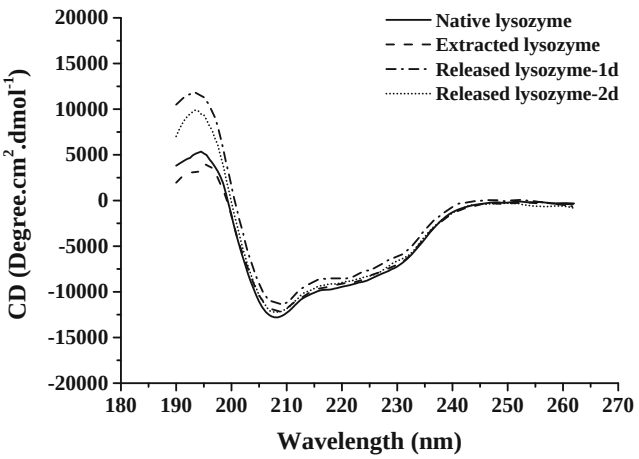

Fig. 8 Representative CD spectra of native lysozyme, extracted lysozyme and released lysozyme from PLGA microparticles formulation (M10F10DCM) at specific intervals. Sample ID referring to Table I.

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