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RESEARCH PAPER

Modulating Protein Release Profiles by Incorporating Hyaluronic Acid into PLGA Microparticles Via a Spray Dryer Equipped with a 3-Fluid Nozzle

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ABSTRACT

Purpose The purpose of this study was to modulate the release profiles of the model protein drug from spray dried poly(DL-lactic-co-glycolic acid) (PLGA) microparticles by incorporating hyaluronic acid (HA) in the formulation.

Methods Bovine serum albumin (BSA)-loaded PLGA microparticles with or without HA were prepared using a spray dryer equipped with a 3-fluid nozzle. The effects of HA on the surface tension and the rheological behavior of the inner feed solution were investigated. The physicochemical properties of the resulting microparticles were characterized using scanning electron microscopy (SEM), laser diffraction (LD), confocal laser scanning microscopy (CLSM) and X-ray photoelectron spectroscopy (XPS). Circular dischoism (CD) was used to characterize conformational integrity of BSA released from the microparticles.

Results Spherical microparticles with D_{50} of 5–10 μm were obtained. Addition of HA in inner feed solutions increased the feed viscosity, but with no influence on the surface tension. All inner feed solutions showed non-Newtonian shear thinning behavior and the rheological properties were not time dependent. The CLSM and XPS analyses suggested a core-shell like structure of the microparticles when HA was incorporated. The release profiles of BSA were extended and the initial burst releases were suppressed with an increase in HA in the microparticle formulations. In addition, HA seemed to protect BSA from degradation upon the spray-drying process.

Conclusions The present work demonstrates the potential of HA to modulate protein release profile from PLGA microparticle formulations produced *via* spray drying using 3-fluid nozzle.

KEY WORDS controlled release · hyaluronic acid · microencapsulation · PLGA microparticles · protein · spray drying

INTRODUCTION

Injectable poly(DL-lactic-co-glycolic acid) (PLGA) microparticles represents an effective approach to ensure the sustained release of proteins, which in turn can enable a reduction of the frequency of dosing in a therapeutic schedule (1–3). Although huge efforts have been made during the past two decades, a number of challenges still remain within the product development of injectable PLGA microparticles for sustained protein release (4). Some significant hurdles include complex production procedure, high cost and the lack of feasibility of industrial scale production for the currently available encapsulation processes (5). The drop off of the production of Nutropin Depot (the first and only FDA approved injectable protein depot) further suggests the importance of easy, simple microencapsulation techniques (6).

In our previous study, we investigated a fast one step production method to prepare protein loaded PLGA microparticles using a spray dryer equipped with a 3-fluid nozzle, which demonstrated the potential for simplifying the production process and reducing the manufacturing costs (7). However, it was observed that the protein surface enrichment of the dry PLGA microparticles was high due to the migration of protein molecules during the spray drying process (8,9), which resulted in the high burst release in the initial stage. In clinic, the initial burst release could pose a serious toxicity threat, thus, control of the initial burst release would be of

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great importance for efficacious and safe therapy (10). The purpose of the present study was to explore how to reduce the protein surface enrichment and suppress the initial burst release. Two hypotheses for achieving this were formulated: i) the protein surface enrichment can be reduced by inhibiting the migration of protein molecules upon the drying process with an increase in the viscosity of the inner feed solution; ii) the initial burst release can be suppressed by affecting the diffusion of BSA in PLGA matrix.

Hyaluronic acid (HA) is an anionic biopolymer composed of alternating disaccharide units of D-glucuronic acid and N-acetyl-Dglucosamine connected *via* β(1→3) and β(1→4) glycosidic bonds. It is biodegradable, biocompatible, non-toxic, non-immunogenic and non-inflammatory (11). HA has been widely investigated within the areas of drug delivery (12–15), gene delivery (11,16), and tissue engineering (17,18). In addition, the use of HA as an excipient for spray-dried formulations has increased over the past years (13,19). For example, it had an impact on the morphology, structure and supramolecular organization of microparticles prepared by spray drying (20,21). Furthermore, HA has also been demonstrated to form a stable matrix that can be processed to entrap bioactive molecules, and modulate drug release (21–23). Herein, HA was used to co-encapsulate with a model protein drug in PLGA microparticles due to: i) the relatively high viscosity of HA even in very low concentration, which has the potential to reduce the immigration rate of protein molecules in the drying process; ii) the intermolecular entanglement formed among HA molecules and PLGA molecules in the spray-dried microparticles would retard the release of the entrapped protein molecules during the release process (22). In this study, bovine serum albumin (BSA) was selected as model protein due to its well-known surface activity (24,25), which often resulted in high enrichment at the surface of the microparticles prepared by spray drying (26). The rheological properties of the inner feed solution were investigated using a dynamic rotational rheometer. The distribution of BSA in the PLGA microparticles were visualized using confocal laser scanning microscopy (CLSM) and BSA surface enrichment was characterized by X-ray photoelectron spectroscopy (XPS). Finally, *in vitro* BSA release profiles were fitted to mathematical model to investigate the BSA release mechanism and the impact of co-encapsulation of the hydrophilic polymer into PLGA microparticles on the protein release kinetics.

MATERIALS AND METHODS

Materials

PLGA (lactide:glycolide molar ratio of 75:25, molecular weight: 20 kDa) was purchased from Wako Pure Chemical Industries, Ltd. (Wako, Germany). Bovine serum albumin (BSA, Lot# 120M1887V) and fluorescein isothiocyanate-labeled BSA (FITC-BSA) were purchased from Sigma-Aldrich (Poole, UK). Hyaluronic acid (Sodium Hyaluronate, HA, molecular weight: 0.9 MDa, Batch No. 850S-0089) was donated by Novozymes A/S (Bagsvaerd, Denmark). Acetone (ACE, 99.9% HPLC grade) and dichloromethane (DCM, 99.9% HPLC grade) were obtained from Sigma-Aldrich (Poole, UK). BSA and HA were stored at 4°C. The deionized water was used for all experiments.

Methods

Rheological Study of the Inner Feed Solutions

The rheological characterization of the inner feed solutions were conducted on an AR-G2 rheometer (TA Instruments, Newcastle, USA) equipped with a cone-plate geometry (cone diameter 40 mm, angle 1.04°) at 25°C (27). The apparent viscosity of the inner feed solutions were measured *via* steady state flow test with two consecutive up-down shear scans from 1 to 1,000 s^{-1} followed by a descent from 1,000 to 1 s^{-1}. Three consecutive tests were conducted with a tolerance of 5%. The maximum measuring time for each shear rate was set to 2 min. Measurements not reaching equilibrium within the 2 min period were not taken into account. The sample solution was applied on the plate and the cone was lowered to the measuring position. The free surface of the sample was covered with a thin layer of low viscosity silicone oil to prevent evaporation of the solvents from the samples.

Interfacial Tensions Measurement 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 (28). 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).

Preparation of BSA Loaded PLGA Microparticles

All microparticle formulations were prepared *via* Büchi B-290 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) equipped with a 3-fluid nozzle, as described in our previous study (7). Briefly, BSA and HA were dissolved in deionized water as the inner feed solution. PLGA was dissolved in DCM or ACE as the outer feed solution (Table I). Then, the aqueous solution and the organic solution were pumped into the spray dryer at constant total feeding rate of 4 mL/min with the

Table I Composition of the Feed Solutions and Characterization of the Resulting BSA-Loaded PLGA Microparticles ($n = 3$)

Batch no.	Composition of feed solution					Characterization	
	Outer feed solution		Inner feed solution			Residual moisture (%)	Actual BSA loading (%)
	PLGA (mg)	Solvent/volume (mL)	BSA (mg)	HA (mg)	Solvent/volume (mL)		
M10F10HA0ACE	500	ACE/10	50	0	H_2O/1	1.1 ± 0.6	5.4 ± 0.7
M10F10HA0.2ACE	500	ACE/10	50	2	H_2O/1	1.0 ± 0.2	4.5 ± 0.3
M10F10HA0.4ACE	500	ACE/10	50	4	H_2O/1	1.2 ± 0.3	4.4 ± 0.2
M10F10HA0DCM	500	DCM/10	50	0	H_2O/1	1.4 ± 0.4	7.1 ± 0.6
M10F10HA0.2DCM	500	DCM/10	50	2	H_2O/1	1.6 ± 0.3	6.0 ± 0.4
M10F10HA0.4DCM	500	DCM/10	50	4	H_2O/1	2.1 ± 0.3	5.2 ± 03

p.s. In the term of Sample ID, "M" refers to the mass ratio of PLGA to protein, e. g. M10 represents the mass ratio of PLGA to BSA is 10:1; "F" refers to the feeding rate ratio of the outer PLGA-containing feed solution to inner BSA-containing feed solution, e. g. F10 represents feeding rate ratio of outer feed solution to inner feed solution is 10:1; HA0, HA0.2 and HA0.4 represents HA concentration in inner feed solution is 0%, 0.2% and 0.4%, respectively; ACE and DCM represent that BSA-loaded PLGA microparticles was prepared by using acetone and dichloromethane as the organic solvent, respectively

feeding ratio of the outer feed solution to the inner feed solution at 10:1. Identical drying conditions (inlet temperature 60°C; outlet temperature 40–45°C; drying air flow rate about 37 m^3/h and atomization air flow rate 742 L/h) were used for all samples. The spray-dried microparticles obtained were collected in glass vials sealed with Parafilm and stored in a desiccator at room temperature until further characterization.

Scanning Electron Microscopy (SEM)

The morphology of the spray-dried particles was visually examined using a scanning electron microscope (SEM; JSM-5200, JEOL Ltd., Tokyo, Japan). Samples were transferred onto carbon sticky tape and mounted in metal stubs, followed by sputter coating with a thin layer of gold–palladium for 120 s with an E5200 Auto Sputter Coater (BIO-RAD, Polaron Equipment Ltd., Watford, England) under an Argon gas purge (Air Liquide, Taastrup, Denmark). The specimens were then imaged at an accelerating voltage of 25 kV energy at magnification of ×3500.

Particle Size and Size Distribution

The particle size distribution of the powders was measured by laser diffraction (Malvern Mastersizer 2000, Malvern Instruments Ltd., U.K.) equipped with a wet dispersion unit (Hydro 2000S). Approximately 10 mg of the resulting microparticles was dispersed in 3 mL of water with the aid of sonication in a water bath for 10 min. The average particle size distribution was determined from 3 replicates of each sample. The polydispersity of the powder was expressed by the span.

$$\text{Span} = D_{80} - D_{20}/D_{50}$$

where D_{20}, D_{50}, D_{80}, are the equivalent volume diameters at 20%, 50%, and 80% cumulative volume, respectively.

Thermogravimetric Analysis (TGA)

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

Confocal Laser Scanning Microscopy (CLSM)

The distribution of BSA in the PLGA microparticles was investigated by using confocal laser scanning microscopy (CLSM). FITC-BSA (0.5%, w/w, relative to the mass of BSA) was added in the formulations and processed using the same spray drying process to produce PLGA microparticles. The samples were placed between glass slides and observed using a Zeiss LSM 710 confocal laser scanning microscopy (Carl Zeiss, Germany) equipped with a 1 mW Helium/Neon laser and an Argon laser, using a Plan-Apochromat 63× objective (NA1.40, oil immersion) upon both Photomultiplier (PMT) and transmitted light Photomultiplier (T-PMT). The green fluorescence was observed with a 505–550 nm band-pass emission filter and excitation at 488 nm upon PMT. The pinhole diameter was set at 54 μm. Stacks of images were collected every 0.4 μm along the z axis.

X-ray Photoelectron Spectroscopy (XPS)

The chemical compositions on the surface of the PLGA microparticles were analyzed by XPS using a K-Alpha (Thermo Scientific, Denmark) equipped with a monochromated AlKα

X-ray source with an energy of 1486.6 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 BSA contents (in weight %) on the particle surface was determined as the detected amount of nitrogen in the sample relative to the theoretical nitrogen content in BSA.

Protein Loading of the PLGA Microparticles and Release Behavior In Vitro

The protein loading of the PLGA microparticles was determined using a previously reported method (3). Briefly, about 10 mg of PLGA microparticles 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 BSA content in the resulting solution was quantified using a UV spectrophotometer (Evolution 300, Thermo scientific, Madison, WI, USA) at 280 nm (29). The *in vitro* release of protein from the microparticles was examined under mild agitation conditions. About 50 mg of PLGA microparticles were placed in 1 mL phosphate buffered saline (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 NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, Denmark) to calculate the cumulative release of protein from the microparticles (30).

The initial burst release profile within 24 h was fitted to evaluate the diffusion coefficient of BSA in the different PLGA matrices by using the Eq. 1 (31).

$$\frac{M_t}{M_\infty} = 6\left(\frac{Dt}{\pi R^2}\right)^{\frac{1}{2}} - \frac{3Dt}{R^2} \tag{1}$$

where t is time and D is the diffusion coefficient. M_t, M_∞, and R are the released drug amount at time t, the releasable drug amount at infinite time (*i.e.* the total drug in the microparticles), and the microparticle radius, respectively.

Circular Dichroism (CD)

The secondary structure of BSA after the release study was characterized using CD to investigate conformational integrity of the protein after the spray drying process. The BSA as received was dissolved in phosphate buffered saline (PBS, pH 7.4) as reference native sample. The BSA released from PLGA microparticles were obtained at 1 day and 4 days under the same condition as the release study. Far ultraviolet CD spectra (190–265 nm) of these BSA 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 BSA concentration in the liquid samples was approximately 0.15 mg/mL.

Statistics

All 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

Characterization of the Inner Feed Solutions

The investigated inner feed solutions showed non-Newtonian shear thinning behavior in the investigated range of shear rate from 1 to 1,000 s^{-1} (Fig. 1). It was observed that 0.4% HA solution had the highest apparent viscosity and a plateau can be observed at the lower region of shear rate from 1 to 50 s^{-1}. 5% BSA (w/v) solution had the lowest apparent viscosity and the plateau appeared at the higher region of shear rate. With an increase in the content of HA in the BSA solution, the apparent viscosity of the mixed solution increased (Fig. 1). Additionally, a hysteresis curve indicated that rheological properties of the inner feed solutions are not time dependent (data not shown), suggesting that the solutions would have the same viscosity as at the beginning of the test after the completion of the rotational test (32). Moreover, all the solutions showed shear thinning behavior with the 5% BSA (w/v) solution possessing the strongest shear thinning behavior. The shear thinning behavior also decreased with an increase in concentration of HA in the solution.

Before studying the surface tension of mixed solution of HA with BSA, each component was investigated separately (Fig. 2a). The dynamic surface tension profile of the 0.4% HA (w/v) solution was very stable in the investigated time range with an equilibrium surface tension of around 72.5 mN/m. In addition, the surface tension of the HA solution appeared to be non-concentration dependent (Fig. 2a). However, 5% BSA (w/v) solution showed a sharp decrease in the surface tension at the initial stage (from 0 to 50 s) (28). The equilibrium surface tension was less than 50 mN/m. Furthermore, the shape of the dynamic surface tension profile of the BSA solution was not changed with an

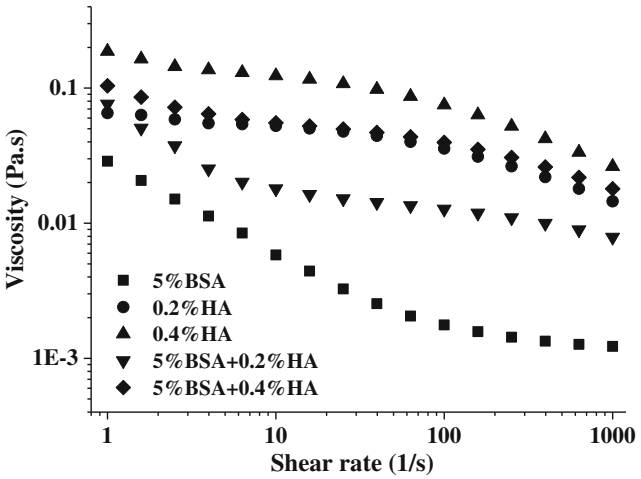

Fig. 1 The flow curve of inner feed solutions in the shear rate range from 1 to 1,000 s^{-1}.

increase in the concentration of HA in the solution, suggesting that HA has no influence on the surface tension of BSA solution in the investigated concentration range (Fig. 2b).

Characterization of BSA-Loaded PLGA Microparticles Prepared Using the Spray Drying Process

The actual protein loading for all microparticle formulations was in the range of 4.4–7.1% (w/w) with a rather low residual moisture content of less than 1.5% (w/w) (Table I). As observed from the SEM images (Fig. 3), spherical particles with a smooth surface were obtained independently of the formulation compositions. The D_{50} of the resulting microparticles was around 5–10 μm with a span of 1.0–1.75 (Table II). It seemed that the particle size and size distribution of the microparticles prepared using DCM was not significantly influenced by co-encapsulating HA, however, an increase in the HA concentration of the inner feed solution was likely to significantly increase the particle size and size distribution of the PLGA microparticles prepared using ACE (Table II).

As seen from the CLSM images, the spatial distribution of BSA in the PLGA matrix was highly influenced by HA and organic solvent applied. The cross section images of FITC labelled BSA (in green) loaded PLGA microparticles prepared by using ACE without HA in the inner feed solution showed the fluorescence intensity decreased sharply from the surface to the core. It indicated that more BSA molecules located on the surface of the PLGA microparticles (Fig. 4 A1). The fluorescence intensity on the surface or in the superficial layer decreased when co-encapsulating HA in the PLGA matrix and several fluorescence clusters appeared in the core of the PLGA matrix (Fig. 4 A2). When DCM was used to prepare PLGA microparticles, the BSA fluorescence was apparently distributed much more evenly in the PLGA matrix, as compared to the PLGA microparticles prepared using ACE (Fig. 4 A3–4). Additionally, for the BSA-loaded PLGA

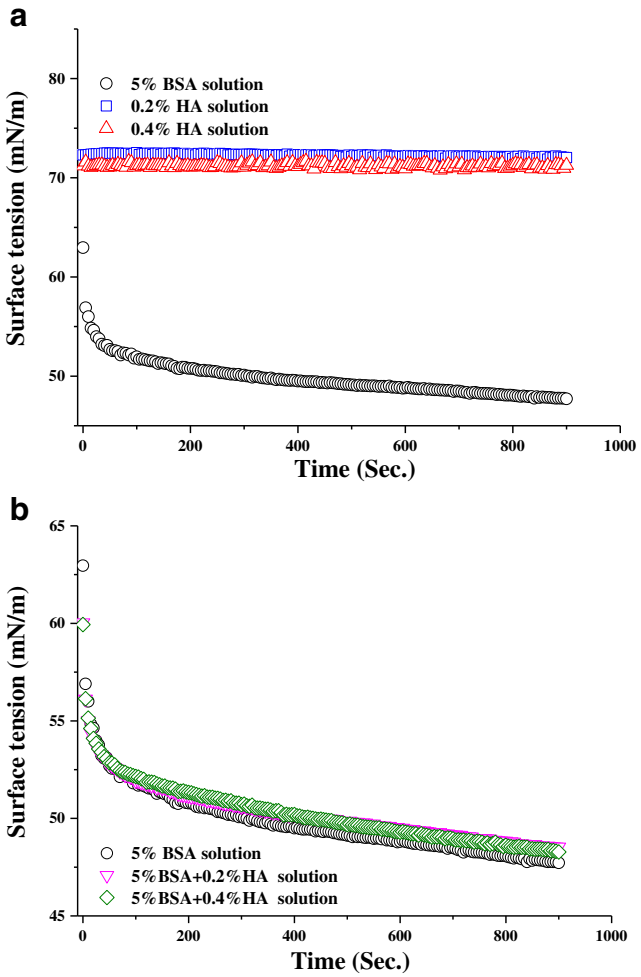

Fig. 2 Dynamic surface tension profiles of inner feed solutions applied for preparation of the BSA-loaded PLGA microparticles.

microparticles prepared using DCM, a core-shell like structure was clearly observed (Fig. 4b). These observations were supported by the fluorescence intensity profiles (in green) (Fig. 4c). As seen in Fig. 4C-1, the fluorescence intensity of BSA on the particle surface or superficial layer was about 2–2.5 times higher than that of the center of the PLGA matrix. However, the fluorescence intensity distribution of BSA showed a nearly Gaussian distribution fashion for the microparticle formulations prepared by co-encapsulating HA into the PLGA matrix (Fig. 4 C2, C4).

As apparent from the XPS data, the chemical composition of the particle surface was also dependent on the organic solvents applied and on the HA content in the PLGA matrix (Fig. 5a). The tendency of the N/C ratio complied with that of the nitrogen content (Fig. 5a) suggested a good reproducibility of the sample preparation process in XPS study and little influence induced by the "blank glass area" of the samples (7). The nitrogen content on the microparticle surface was decreased significantly from approximately 1.0% to approximately 0.5% for the microparticles prepared using ACE and

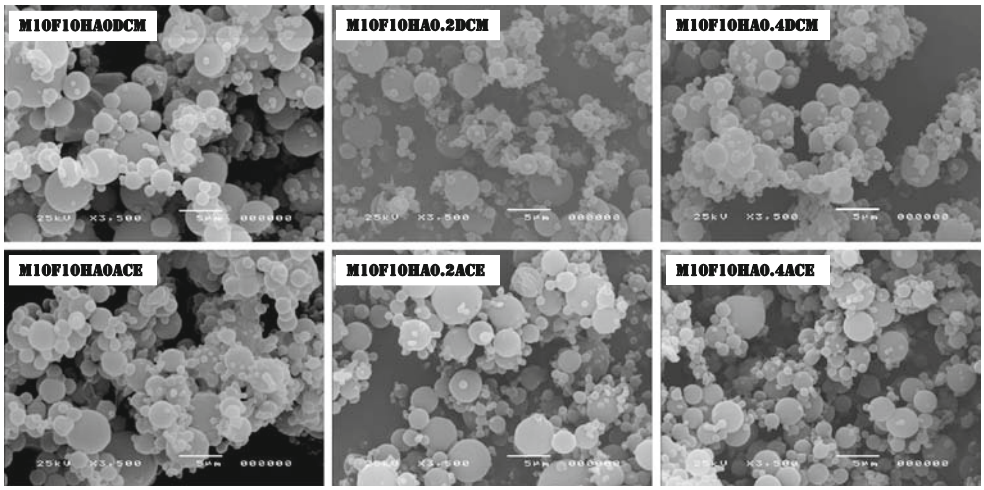

Fig. 3 SEM images of BSA-loaded PLGA microparticles prepared using a spray dryer equipped with a 3-fluid nozzle (Scale bar 5 μm). Sample ID referring to Table I.

from approximately 0.6% to approximately 0.3% for the microparticles prepared using DCM by co-encapsulating HA with BSA into the PLGA matrices. Furthermore, when comparing the surface content of BSA and the actual loading content of BSA, it is apparent that the deviation between them increased with an increase in the HA content co-encapsulated into the PLGA matrix, irrespectively of the applied organic solvents (Fig. 5b). This suggests that the BSA surface enrichment was suppressed and the BSA distribution in PLGA microparticles may exhibit a core-shell like pattern.

Characterization of the BSA Release from the PLGA Microparticles

The BSA release profiles of the microparticles were characterized by a pronounced initial burst release within the first 24 h, followed by a slow, continuous release up to at least 15 days (Fig. 6a & b). The microparticles prepared by using DCM and ACE exhibited the initial burst release of approximately 20–40% and 30–55%, respectively. Further, the initial burst release within the first 24 h decreased significantly by co-encapsulating HA with BSA in the PLGA matrix, irrespectively of the applied organic solvent. On the other hand, the microparticles prepared by using DCM showed a subsequent slow release pattern up to 15 days with a cumulative release of approximately 45–65%, and the release rates at this stage were around 1.8%, 1.7%, and 1.6% per day for microparticles with a HA content of 0%, 0.2%, and 0.4%, respectively. However, the release rate in the subsequent stage for the microparticles prepared using ACE was slower as compared to that of microparticles prepared using DCM. Additionally, the BSA recovery experiments were conducted to extract the unreleased BSA from the microparticles after the release study and showed that the total amount of BSA measured in both the release study and the recovery study reached up to 90–105%.

In addition, the initial part (within first 24 h) of the BSA release profile was fitted to mathematical model (Eq. 1, $R^2 > 0.9$) to understand the BSA release kinetics. As illustrated in Fig. 7, the diffusion coefficient of BSA from the PLGA matrix decreased significantly with incorporating HA into PLGA matrix, irrespectively of the applied organic solvent. Furthermore, the diffusion coefficient of BSA from the PLGA matrix prepared by using ACE is much higher than the coefficient from the PLGA matrix prepared using DCM, correspondingly.

Table II D_{20}, D_{50}, D_{80} and Span of the Resulting BSA-Loaded PLGA Microparticles

	D_{20} (μm)	D_{50} (μm)	D_{80} (μm)	Span
M10F10HA0ACE	3.1 ± 0.1	6.1 ± 0.1	10.1 ± 0.4	1.1 ± 0.1
M10F10HA0.2ACE	$3.5 \pm 0.2^{*}$	$7.7 \pm 0.4^{**}$	$15.5 \pm 1.8^{**}$	$1.5 \pm 0.1^{**}$
M10F10HA0.4ACE	$4.6 \pm 0.3^{**}$	$10.7 \pm 0.8^{**}$	$23.2 \pm 3.3^{**}$	$1.7 \pm 0.2^{**}$
M10F10HA0DCM	2.9 ± 0.2	5.9 ± 0.4	10.7 ± 1.9	1.3 ± 0.2
M10F10HA0.2DCM	2.9 ± 0.4	5.3 ± 0.8	8.3 ± 0.7	1.0 ± 0.1
M10F10HA0.4DCM	$4.3 \pm 0.3^{\#\#}$	$8.9 \pm 0.4^{\#\#}$	$16.3 \pm 0.6^{\#\#}$	1.3 ± 0.1

p.s. * means $p < 0.05$ referred to M10F10HA0ACE; ** means $p < 0.02$ referred to M10F10HA0ACE; ## means $p < 0.02$ referred to M10F10HA0DCM; Sample ID referring to Table I

DISCUSSION

As evident from the CLSM images (Fig. 4) and the XPS results (Fig. 5), the BSA distribution within the PLGA matrix could be modified by co-encapsulating HA into the PLGA microparticles. In short, the BSA distribution within the PLGA microparticles was changed from a surface-enriched pattern to a core-enriched pattern when HA was introduced into the PLGA matrix. To elucidate the reason, the protein microencapsulation mechanism and the drying process need to be considered. As discussed in our previous study (7), the protein microencapsulation efficiency is highly dependent on the

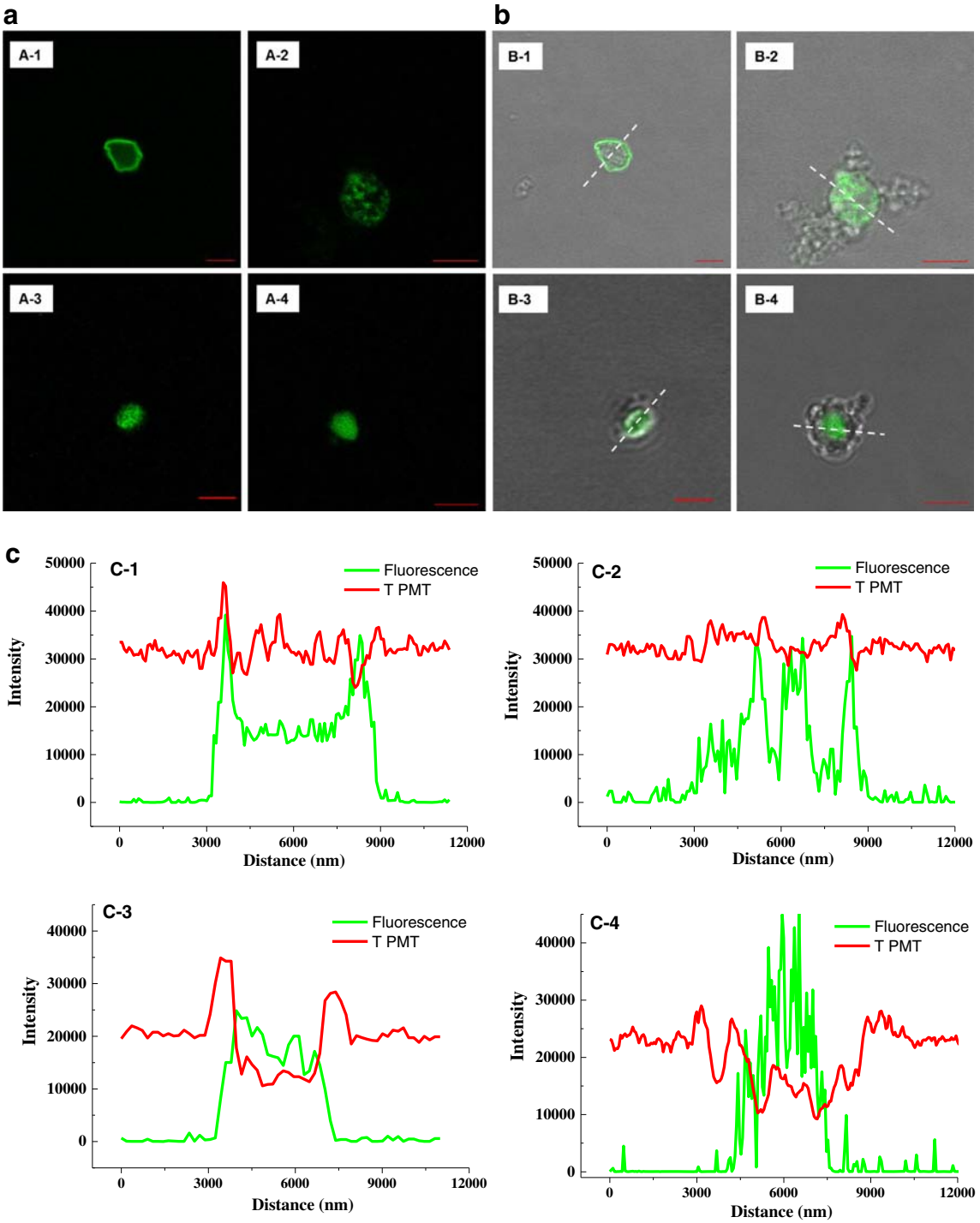

Fig. 4 Representative CLSM images of BSA-loaded PLGA microparticles prepared using a spray dryer equipped with a 3-fluid nozzle (Scale bar 5 μm): Cross section images of FITC labelled BSA loaded PLGA microparticles under fluorescence channel (**a**); Cross section images of FITC labelled BSA-loaded PLGA microparticles under combined fluorescence channel with T-PMT channel (**b**); Intensity profiles along the line (in *B1-B4*) for FITC labelled BSA-loaded PLGA microparticles (**c**). Sample ID referring to Table I (1: M10F10HA0ACE; 2: M10F10HA0.4ACE; 3: M10F10HA0DCM; 4: M10F10HA0.4DCM).

surface tension gradient between the protein-loaded aqueous droplet and the PLGA organic droplet (33), as well as the diffusional motion of protein molecules inside a droplet in the drying process (8,9). In the present study, it was observed that the surface tension of the inner feed solution was not influenced by adding HA into the BSA solution (Fig. 2), indicating that the surface tension gradient cannot explain the changed BSA distribution in the PLGA microparticles. On the other

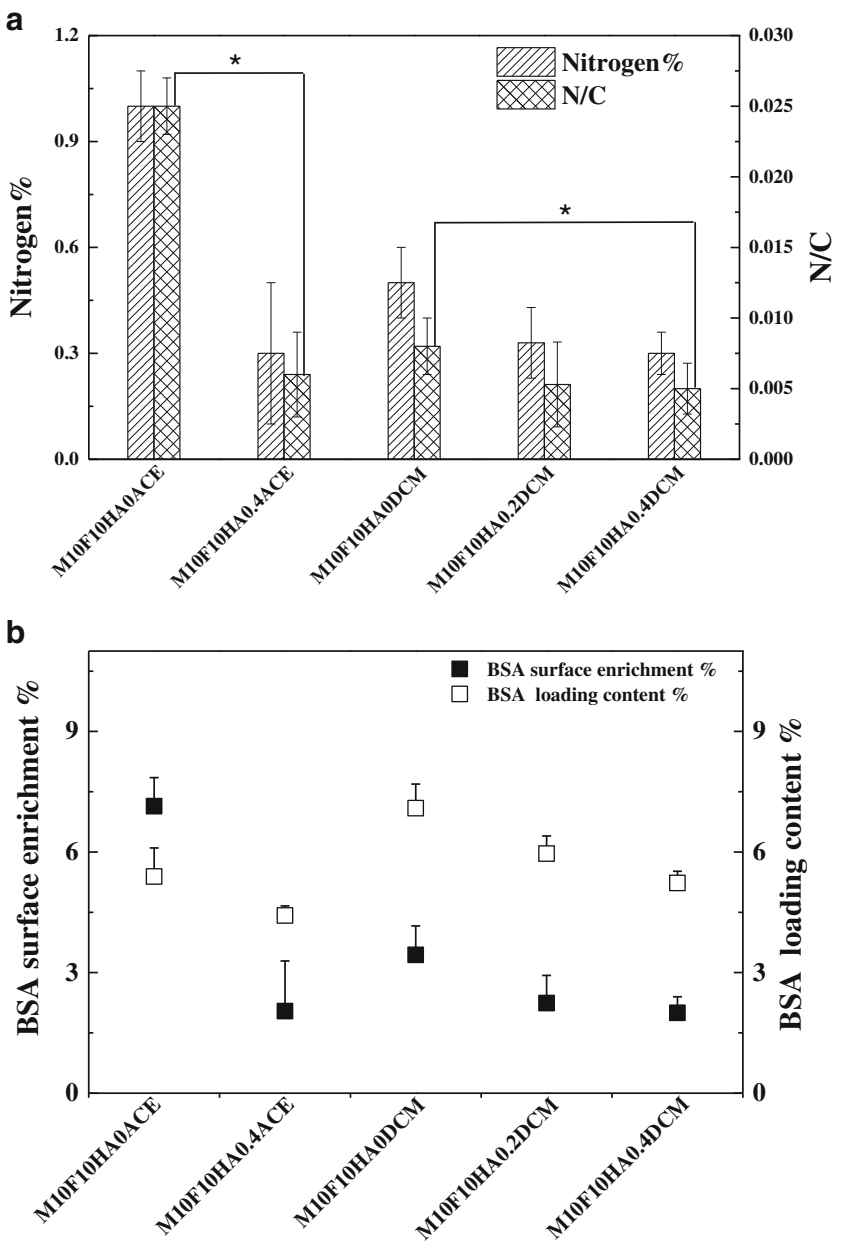

Fig. 5 Nitrogen content and N/C ratio at the surface of the BSA-loaded PLGA microparticles (**a**) and Comparison of actual BSA loading content and BSA surface enrichment (**b**) ($n \geq 3$). * means $p < 0.05$; Sample ID referring to Table I.

hand, the viscosity of the inner feed solution increased dramatically by adding HA (Fig. 1) and the rheological behavior was shear thinning, and not time dependent (Fig. 1). This suggests that the inner feed solution becomes less viscous upon the high atomization air flow rate, however, the viscosity of the inner droplet will recover after the completion of atomization (32). The high viscosity of the core droplet may suppress the migration rate of BSA from the inner to the outer layer during the drying process. Additionally, the entanglements formed among HA molecules and PLGA molecules in the drying process may also hinder the migration of BSA molecules (22). Although the network formed by the entanglements among the polymer molecules would be influenced by the concentration of the polymer (34), no obvious differences in the nitrogen content and the BSA surface enrichment were observed between the microparticle formulations prepared by using DCM with different HA content. It may result from the similar viscosity between these two inner feed solutions (Fig. 1), implying that the viscosity of the inner feed solution is the major factor influencing the BSA distribution in the PLGA microparticles.

Drug release from the PLGA microparticles is a rather complex process involving in a series of physicochemical processes (*e.g.* diffusion of water into the system, drug dissolution, drug diffusion out of the delivery system, polymer swelling, matrix erosion, *etc.*) that influence the release rate (35). For PLGA-based controlled release systems, diffusion and release resulting from degradation/erosion of the polymer are

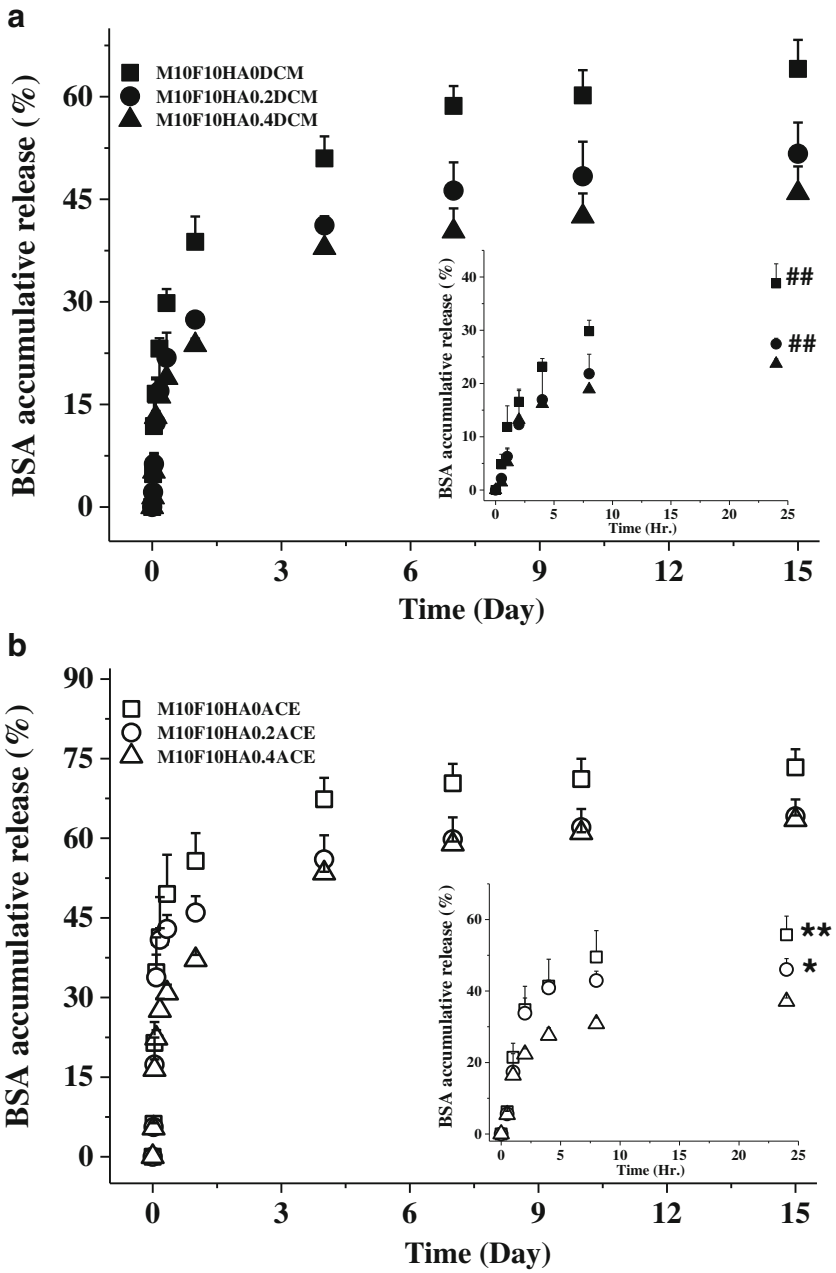

Fig. 6 *In vitro* BSA release profiles of microparticle formulations prepared by using DCM (**a**) and ACE (**b**) ($n = 3$). * means $p < 0.05$ referred to M10F10HA0ACE; ** means $p < 0.02$ referred to M10F10HA0ACE; ## means $p < 0.02$ referred to M10F10HA0DCM; Sample ID referring to Table I.

considered to be two main release mechanisms. The release rate is often considered to be diffusion-controlled during the initial phase and degradation/erosion controlled during the final stage of the release period (35,36). In the present study, we hypothesize that the co-encapsulation of HA might modulate the BSA release behavior by reducing the protein surface enrichment and affecting the diffusion of BSA molecules within PLGA matrix.

In fact, the observations in the release study supported the hypothesis. As observed, the initial burst release is suppressed with a decrease in BSA surface enrichment after co-encapsulation of HA into PLGA matrix, irrespectively of the applied organic solvent (Figs. 5 and 6). Furthermore, as comparison of the release amounts from the 4 h–15days and the 24 h–15days, it is found that the difference in the release behavior among the formulations mainly occurs in the time range of 4–24 h. As it is widely believed that the initial burst release results from the rapid escape of drug from the surface or/and the superficial layer of the particles (10), it is rationally speculated that the main factor inducing the difference in the release behavior among the formulations is the drug release from the superficial layer of microparticles, which is affected significantly with co-encapsulating HA into PLGA matrix. In contrary, there is no obvious difference in the release profiles after 3 days, implying the addition of HA in PLGA matrix cannot adjust the kinetics of PLGA degradation and erosion.

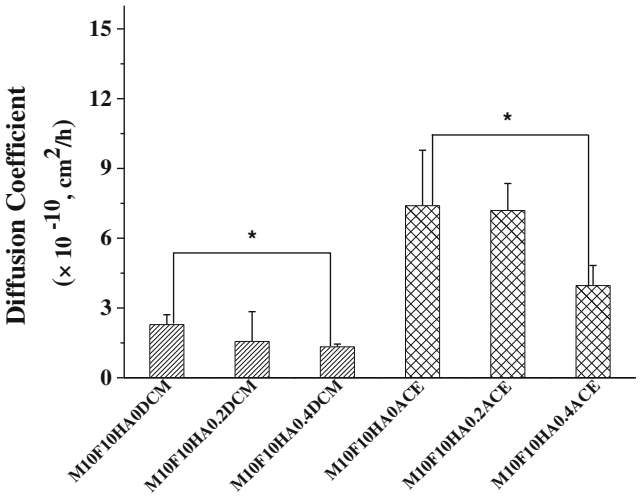

Fig. 7 Diffusion coefficient of BSA in PLGA matrices calculated by curve-fitting of initial burst release profiles using Eq. 1 ($n = 3$). * means $p < 0.05$; Sample ID referring to Table I

Moreover, the effect of HA on the diffusion of BSA molecules within PLGA matrix can be learnt from the distinct diffusion coefficients of BSA in the PLGA matrix calculated by curve-fitting of the initial burst release profiles using Eq. 1 (Fig. 7). Equation 1 is widely used to predict drug release behavior from a monolithic system (*i.e.* the drug is not physically separated from the release rate controlling barrier, but more or less homogeneously distributed within the latter) (31). In the present study, although the BSA distribution showed a core-shell like structure in the PLGA microparticles with HA, it is not completely physically separated from the PLGA shell like for microcapsules or coating. Hence, it is rational to apply Eq. 1 to fit the BSA initial burst release profiles. As seen in Fig. 7, the diffusion coefficient of BSA from the PLGA matrix with HA is significantly lower than the coefficient from the PLGA matrix without HA, irrespectively of the applied organic solvent. It probably results from the formation of a viscous microenvironment and/or an intermolecular entanglements of polymers by co-encapsulating HA into the PLGA matrix (22,37–39). Additionally, it is observed that the diffusion coefficient of BSA from the PLGA matrix prepared by using ACE is much higher than the coefficient from the PLGA matrix prepared using DCM, correspondingly. It is probably due to: i) a higher BSA surface enrichment generates more water-filled pores after the release of surface-enriched BSA, thus, accelerates the subsequent release of BSA (10); ii) a distinct microscopic connectivity and diffusivity of the PLGA matrix generated by the different PLGA molecular behavior in ACE and DCM, respectively, and their distinct concomitant microstructural changes of the PLGA matrices upon the release process (40).

The evaluation of the stability of protein upon the spray drying process is often critical for such formulations. An obvious alteration in CD spectra of BSA released from the PLGA microparticles prepared using ACE without HA was observed as compared to native reference samples, suggesting that the secondary structure of BSA was affected (related to the reduction of α-helix content) by the spray-drying process (Fig. 8a). However, the BSA secondary structure was preserved when co-encapsulating HA with BSA. The mechanism is not clear at present, but it may be explained by the fact that incorporating HA with BSA in the inner feed solution decreases the mixing of the two miscible solvents, *i.e.* water and ACE during the drying process due to increased viscosity of the inner feed, thus, minimizing the negative influence of the organic solvent on the protein integrity. In contrast, the CD spectra of BSA released from the PLGA microparticles prepared using DCM suggested that BSA retained its secondary structure regardless of adding HA in the formulation or not (Fig. 8b). It may be due to the least migration of BSA into DCM, which is an immiscible phase for the protein solution. This result may support the explanation for the findings in Fig. 8a, *i.e.* diminishing the mixing of protein solution and organic solvent could prevent protein from degradation upon drying, suggesting a rational selection of the composition of

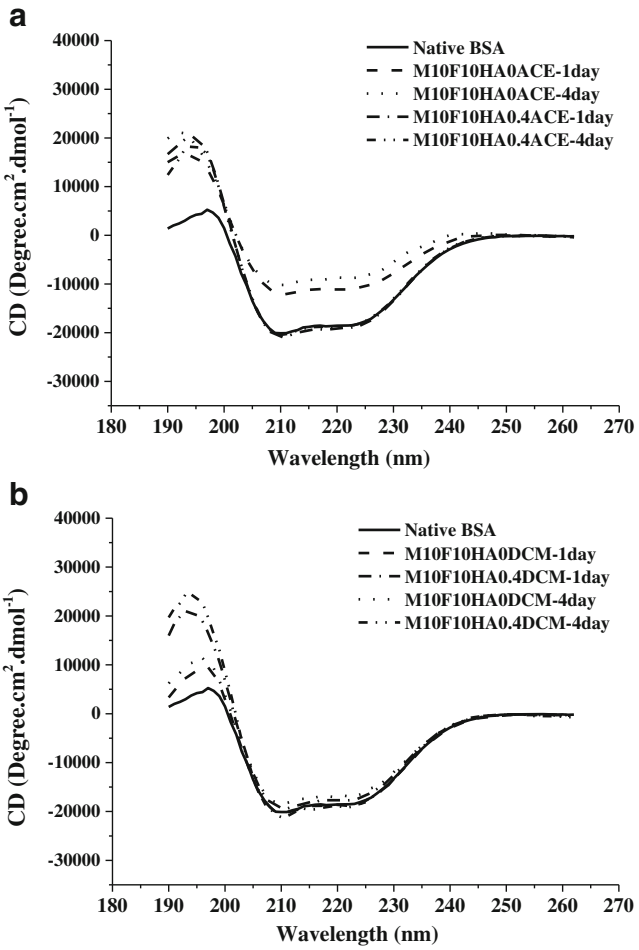

Fig. 8 Representative CD spectra of native BSA and BSA released from PLGA microparticles formulations prepared by using ACE (**a**) and DCM (**b**) at specific intervals. Sample ID referring to Table I.

inner feed solution and outer feed solvent may prevent protein from degradation in the spray drying process.

CONCLUSIONS

The present study demonstrates that the release profiles of BSA, a model protein drug, from spray-dried PLGA microparticles can be modulated by incorporating HA, a hydrophilic polymer, into the formulations produced using a spray dryer equipped with a 3-fluid nozzle. The initial burst release of the model protein from the PLGA microparticles was suppressed with an increase in the amount of incorporated HA in the PLGA matrix. It may be due to a decrease in the diffusion of protein molecules to the superficial layers of the PLGA microparticles upon the spray drying process, thus resulting in a decrease in the protein surface enrichment. The release rate of the protein can be decreased with an increase in the amount of hyaluronic acid incorporated in the PLGA matrix, which can be explained by reducing the diffusion rate of BSA in PLGA matrix. In addition, protein degradation upon the spray drying process was diminished by addition of hyaluronic acid in the formulations. The present study implies the potential of HA incorporated into the PLGA matrix in suppressing the protein surface enrichment and moderating the release profile, as well as protecting protein integrity during the spray drying process.

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