Preparation and evaluation of double-walled microparticles prepared with a modified water-in-oil-in-oil-in-water (w₁/o/o/w₃) method

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ORIGINAL ARTICLE

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Abstract

In this study, a modified water-in-oil-in-oil-in-water (w₁/o/o/w₃) method was developed to prepare double-walled microparticles containing ovalbumin (OVA). The microparticles were characterized with respect to their morphology, particle size, encapsulation efficiency, production yield, thermal properties and *in vitro* drug release. Microscopy observations clearly showed that microparticles have spherical shape and smooth surface. These microparticles were characterized to have double-walled structure, with a cavity in the centre. By using w₁/o/o/w₃ method, a significant decrease in mean particle size and a significant increase in encapsulation efficiency were obtained. The mean particle size and the encapsulation efficiency of double-walled microparticles were also affected by the changing amount of OVA and mass ratio of polymers. Microparticles prepared with two polymers exhibited a significantly lower initial burst release followed by sustained release compared to microparticles made from poly(D,L-lactide-co-glycolide) 50/50 only. It can be concluded that these microparticles can be a potential delivery system for therapeutic proteins.

Introduction

Development of new delivery systems that better control the amount, rate and location of drugs goes on to be an important and popular area of research. Biodegradable polymer devices that encapsulate drug and release it over a prolonged time, by either diffusion through the matrix or matrix degradation, form an important class of delivery methods (Okada and Toguchi, 1995; Jain, 2000; Berkland et al., 2004; Malik et al., 2007). Poly(D,L-lactide-co-glycolide) (PLGA) has been widely used for the encapsulation and sustained delivery of drugs in the past several years because it is biocompatible, biodegradable, nontoxic and has been approved for several products. PLGA particles are made by many different techniques and have been used to encapsulate, release and deliver various types of therapeutic agents, from low-molecular-weight drugs to macromolecular one (Jeon et al., 2000; Aubert-Pouessel et al., 2004; Barakat and Radwan, 2006; Mao et al., 2007; Chen et al., 2009). The two main release control mechanisms associated with drug release from PLGA-based drug delivery systems are diffusion and degradation/erosion. The release rate is often controlled by diffusion initially and degradation/erosion during the final stage of the release period (Mollo and Corrigan, 2003; D'Souza et al., 2005). The duration of drug release can be varied from hours to several months (Fredenberg et al., 2011).

The technique of microencapsulation by solvent evaporation/extraction is widely applied to obtain the controlled release of drug. There are different methods to use microencapsulation by solvent evaporation/extraction technique. The choice of the method that will give rise to an efficient drug encapsulation depends on the hydrophilicity or the hydrophobicity of drug. While oil-in-water emulsion solvent evaporation/extraction had been widely used to fabricate microparticles containing insoluble or poorly water-soluble drugs, hydrophilic drugs including proteins and peptides possessed very limited loading capacity due to the long-term contact with aqueous solution during solvent evaporation/extraction (Giunchedi et al., 1998; Li et al., 2008a, 2008b). The double-emulsion (water-in-oil-in-water) solvent evaporation/extraction technique, originally described and patented in 1970, has been successfully adapted to the encapsulation of highly water-soluble drugs (Blanco and Alonso, 1997; Ruan et al., 2002; Yeh et al., 2004; Li et al., 2008a, 2008b; Devrim et al., 2011). However, these microparticles prepared with a single polymer will give out a very large initial burst caused by the release of the drug trapped on the surface during the encapsulation process. For more potent peptides, proteins and nucleic acids with narrow therapeutic ranges or high toxicity, excessive initial release rates could result in drug levels close to or exceeding toxic threshold levels. Another problem associated with microparticles made of a single polymer is lack of sustained release for periods suitable for periodic therapy especially with hydrophilic drugs (Pekarek et al., 1994). While some formulation optimization can be used to increase the efficacy of single polymer microparticles for drug delivery, double-walled microparticles offer an alternative for sustained drug delivery. Double-walled microparticles present several advantages as compared to more conventional monolithic microparticles. Drug encapsulated in the core of double-walled microparticles may overcome the problem of high initial burst release commonly encountered in microparticles (Lee et al., 2002; Rahman and Mathiowitz, 2004; Tan et al., 2005; Zheng, 2009). Higher drug loads with improved drug stability may be achieved by using materials in the core phase (Berkland et al., 2004). Drug release rates may be

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controlled by controlling the shell material or thickness (Pollauf et al., 2005). Finally, drugs can be released either in a sequential or simultaneous manner by selectively loading them into the core or shell phase, thereby potentially enhancing drug efficacy (Choi et al., 2010; Nie et al., 2010; Lee et al., 2011).

There are several techniques of making microparticles with a two-layered structure from polymer blends. One technique is to simply encapsulate a therapeutic agent in microparticles using a conventional microencapsulation technique and then to coat the microparticles with a second polymer. This coating acts as an additional diffusion barrier and reduces the burst effect. However, dip or pan coating processes yield microparticles with unequal or incomplete polymer coatings, and each coating adds an additional step to the manufacturing process and further decreases the yield (Ichikawa et al., 1997). Air suspension coating methods generally produce a more evenly coated product, but are limited by the size of the particles that can be suspended in a fluidized bed; particles less than 100 μm are difficult to coat by this method (Ichikawa et al., 1997; Mathiowitz and Kreitz, 1999). A simple coating method involves dipping the microparticles into a polymer solution in which the microparticles are not soluble. A microsphere with reduced initial burst and controlled drug release from the PLGA microspheres was obtained by post-coating with different chitosans (Jaganathan and Vyas, 2006; Manca et al., 2008). However, due to non-biodegradable coating used, these microspheres are not suitable for parenteral use. To overcome the limitation of these techniques, Zheng (2009) developed a water-in-oil-in-oil-in-water ($w_1/o/o/w_2$) technique to produce double-walled microparticles. In this technique, aqueous solutions of hydrophilic drugs or proteins were first emulsified with one appropriate polymer solution, which were subsequently emulsified with another polymer solution. Then, this emulsion was dripped into the non-solvent bath containing a surfactant to form microparticles. The main advantages of this technique include the following: (1) drugs and therapeutic proteins can be loaded into microparticles in solution form; therefore, no further drying and dispersion work for drugs are needed. This also benefits uniform distribution of drugs inside polymer matrix in comparison to dispersion of solid drug particles in polymers, and thus improving drug release profiles. Furthermore, proteins can be loaded together with excipients in solutions, thereby reducing the risk of losing protein stability in the process of protein drying and dispersing; (2) the initial burst release of double-walled PLGA microparticles prepared in this way is very low; and (3) the cumulative release profiles showed reduced initial burst and prolonged release.

In this study, we made some modifications in the $w_1/o/o/w_2$ emulsion technique. In previous technique, $w_1/o/o$ emulsion was injected into a 100-mL aqueous solution of poly(vinyl alcohol) (PVA) to create $w_1/o/o/w_2$ emulsion. The emulsion was then continuously stirred using a mechanical stirrer or a homogenizer to allow solvent evaporation. Differently, the obtained $w_1/o/o/w_2$ emulsion was transferred in 300 mL of PVA solution (w_3) and stirred with a mechanical stirrer to extract the organic solvent in our study. Thus the solvent diffusion rate was decreased by dividing the double emulsification into two steps and solidifying the droplets step by step. This modification ensured smaller particle size and higher encapsulation efficiency values (Devrim et al., 2011). We used polymer combination of PLGA 50/50 with another PLGA polymer has different lactic-to-glycolic acid ratio to prepare microparticles in contrast to the previous study performed by Zheng (2009). Furthermore, we investigated the effects of mass ratio of polymers and initial weight of protein dissolved in the inner aqueous phase on the physicochemical properties of the double-walled microparticles. In this study, ovalbumin (OVA) was used as a hydrophilic model protein.

Materials and methods

Materials

PLGA 50/50 (Resomer® RG 502) and PLGA 75/25 (Resomer® RG 755) were purchased from Boehringer Ingelheim (Ingelheim, Germany). PLGA 85/15 and PLGA 65/35 (Medisorb®) were obtained from Alkermes (Wilmington, OH). Poly(D,L-lactide) (PDLLA) was obtained from Aldrich (Seelze, Germany). OVA, PVA (87–89 mol% hydrolysed, Mw = 30 000–70 000) and dichloromethane (DCM) (99.9%, HPLC grade) were from Sigma (Deutschland, Germany). Micro bicinchoninic acid (micro BCA) protein assay reagent kit was obtained from Thermo Scientific Pierce (Rockford, IL). All the other chemicals used were of analytical grade.

Preparation of microparticles

Double-walled microparticles were prepared using $w_1/o/o/w_3$ method. A schematic representation of microparticles is presented in Figure 1. Briefly, 1 mL DCM solution of PLGA 50/50 was emulsified with 0.2 mL OVA aqueous solution (w_1) using a homogenizer (Ultra Turrax® T-25, Ika, Staufen, Germany). The resultant emulsion was then emulsified with 1 mL DCM solution of PLGA 65/35, PLGA 75/25, PLGA 85/15 or PDLLA. This emulsion was subsequently injected into a 100 mL aqueous solution of 2.5% (w/v) PVA (w_2), which was stirred using the same homogenizer. Then, the obtained $w_1/o/o/w_2$ emulsion was transferred in 300 mL of 0.5% (w/v) PVA solution (w_3) and stirred with a mechanical stirrer (Eurostar Power Control-Visc 6000, Ika, Staufen, Germany) at 700 rpm for 5 h to extract the organic solvent and subsequent particle hardening at room temperature, which was controlled at $25 \pm 2 \,°C$. Finally, the microparticles were collected by centrifugation (Sigma 2-16P) at 5000 rpm for 15 min, washed with ultrapure water (MilliQ water) three times and lyophilized to obtain free flowing powder. Compositions used for microparticles are listed in Table 1.

Characterization of microparticles

Morphology of microparticles

To investigate the shape and morphology of microparticles, they were dispersed in a droplet of water, directly on a slide and observed under an optical microscope (Leica, Model DM 4000B, Wetzlar, Germany).

The surface morphologies of microparticles were also observed using field-emission scanning electron microscope (FE-SEM) (FEI Quanta Model 400F, Tokyo, Japan). For the sample preparation, a small aliquot of the microparticles were mounted onto metal stubs using double-sided adhesive tape. After being vacuum-coated with a thin layer (100–150 A°) of gold, the microparticles were examined by FE-SEM operated at 10–30 kV accelerating voltage. The photomicrographs were then taken at a magnification of 800–6000.

Composition of microparticles

To determine the composition of the core and shell polymer of microparticles prepared with PLGA 50/50 and PDLLA, we used the dissolution method based on the different solubility of the polymer pair PLGA and PDLLA in ethyl acetate (Tan et al., 2005). A double-walled microparticle was first immersed in ethyl acetate for 10 min. The remnant was then collected for optical observation. PLGA 50/50 is soluble in ethyl acetate, while PDLLA is not. Keeping this idea, we can determine the core–shell composition by examining whether the remnant has a solid core or hollow shell structure. The former would mean a double-walled microparticle with PLGA
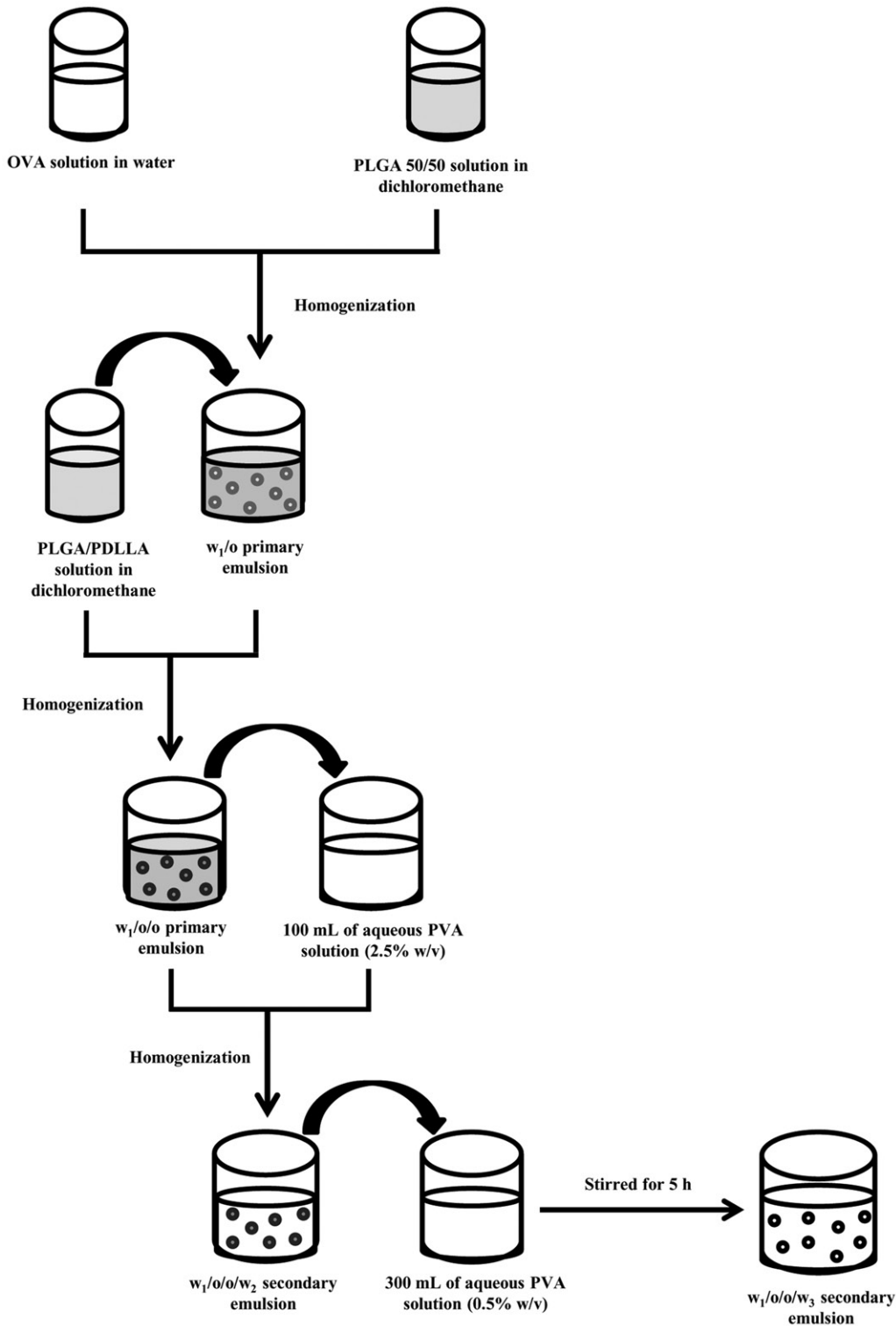
Figure 1. Schematic representation of the $w_1/o/o/w_3$ emulsion technique.

shell, and the latter would be derived from a microparticle with a PLGA core.

Particle size analysis

Particle size and size distribution of microparticles were measured with a laser diffraction particle size analyzer (Sympatec Helos Model H0849, Clausthal-Zellerfeld, Germany). Briefly, 5 mg of dry microparticles was mixed with distilled water containing 0.1% Tween 20 and suspended completely for several minutes using an ultrasonic bath. The suspension was then placed in the laser particle counter. The size is measured at $25 \pm 2\,^\circ\text{C}$. Each sample was measured in triplicate. The medium particle size (D_{50}, the particle size when cumulative value is 50% by volume in the particle size cumulative distribution profile) and particle size distribution were measured. The size distribution was evaluated by the span value defined using Equation (1) (Vladisavljevi'c and Schubert, 2003):

$$\text{Span} = \frac{D_{90\%} - D_{10\%}}{D_{50\%}} \qquad (1)$$

Table 1. Compositions used for microparticles.

Formulation code	Polymer composition	Initial weight of OVA dissolved in inner aqueous phase (mg)
F1	PLGA 50/50	5
F2	PLGA 85/15: PLGA 50/50 (1:1)	5
F3	PLGA 85/15: PLGA 50/50 (2:1)	5
F4	PLGA 85/15: PLGA 50/50 (3:1)	5
F5	PLGA 85/15: PLGA 50/50 (2:1)	7.5
F6	PLGA 85/15: PLGA 50/50 (2:1)	10
F7	PLGA 65/35: PLGA 50/50 (2:1)	5
F8	PLGA 75/25: PLGA 50/50 (2:1)	5
F9	PDLLA: PLGA 50/50 (2:1)	5

Encapsulation efficiency

The OVA content of microparticles was determined by digestion method (Spiers et al., 1999; Feng et al., 2006; Florindo et al., 2009). This method involves alkaline hydrolysis of the microparticles and determination of the OVA recovered. Briefly, a determined quantity (10 mg) of microparticles was suspended in 1 mL of 0.1 M NaOH containing 2.0% (w/v) sodium dodecyl sulfate (SDS) for 24 h at room temperature. After incubation, the solution was centrifuged (Sigma 2-16P) at 4000 rpm for 5 min. The supernatant was neutralized with 0.1 M HCl (Meng et al., 2003). The amount of OVA was determined using micro BCA protein assay reagent kit according to the instructions of the manufacturer. All experiments were performed in triplicate. Background readings were corrected to use the supernatant of empty microparticles of a corresponding batch. The actual drug loading and encapsulation efficiency (%) were calculated using Equations (2) and (3), respectively:

$$\text{Actual drug loading (\%)} = \frac{\text{Drug mass in microparticles}}{\text{Mass of microparticles}} \times 100 \quad (2)$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Calculated drug concentration}}{\text{Theoretical drug concentration}} \times 100 \quad (3)$$

Production yield

Microparticles recovered at the end of preparation were weighed and the production yield (%) was calculated using Equation (4) (Leonardi et al., 2009).

$$\text{Production yield (\%)} = \frac{\text{Total microparticle amount (mg)}}{\text{Total solid material amount (mg)}} \times 100 \quad (4)$$

Thermal analysis using the differential scanning calorimeter

Thermal analysis of the microparticles was performed using a differential scanning calorimeter (DSC) (Shimadzu DSC-60, Kyoto, Japan). The samples (about 6.0 mg) were placed in sealed aluminium pans and subjected to heating from 20 to 200 °C at a rate of 10 °C/min. All data obtained were processed on TA 60 universal analyzer software and glass transition temperature (T_g) was determined. The calorimetric enthalpy (ΔH) was also determined by integrating the area under the transition peak.

Fourier transform infrared spectroscopy

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrums of OVA, PLGA, empty microparticles and OVA-loaded microparticles were obtained by a Perkin Elmer spectrometer (Perkin Elmer Spectrum 100 FT-IR system, Boston, MA). The spectra were recorded in the IR range from 650 to 4000 cm^{-1}, with a resolution of 1 cm^{-1}. All measurements were taken at room temperature.

In vitro release studies

The release of OVA from the microparticles was studied as follows. In 1.5-mL Eppendorf® Protein LoBind tubes (Eppendorf, Westbury, NY), 40 mg of OVA containing microparticles were reconstituted with 1.0 mL of pH = 7.4 phosphate buffer solution containing 0.02% (w/v) NaN$_3$ as a bacteriostatic agent and 0.1% (w/v) SDS and then shaken horizontally in a water bath rotary shaker (Gesellschaft fur Labortechnik Shaking Water Bath Model 1083, Burgwedel, Germany) at 100 rpm and 37 ± 2 °C. At specific time points (at 0.5, 1, 2, 4, 8 h, thereafter, 1, 2, 4, 7, 14, 21, 28, 35, 42, 49, 56 and 63 days), the samples were centrifuged (Sigma 2-16P) at 4000 rpm for 5 min. The supernatants were removed and fresh buffer was added. The microparticles were vortexed and returned to the shaker. Supernatants for each of the sets of microparticles were frozen and stored at −40 °C for subsequent analysis by micro BCA for total protein released. All experiments were performed in triplicate for each of the samples. Empty (without protein) microparticles were treated similarly, and the absorbance from their supernatant was subtracted in all measurements. The percentage of protein released from the microparticles was defined as the mass of protein released divided by the total mass of protein in the microparticles.

Kinetics of drug release

The *in vitro* release data of microparticles were evaluated kinetically by different kinetic models such as zero-order $F(t) = kt$, first-order $\ln [1 - M_t/M_\infty] = -kt$, Higuchi $M_t/M_\infty = kt^{1/2}$ and Hixson–Crowell $[M_\infty/M_t]^{1/3} = kt$. The ideal kinetic model was determined using SPSS for Windows statistical software version 11.5 (SPSS Inc., Chicago, IL).

Statistical analysis

Results were expressed as mean ± standard deviation (SD) from at least three separate measurements. A one-way analysis of variance followed by *post hoc* Turkey's multiple comparison test was used to assess statistical difference. All analyses were performed by SPSS for Windows statistical software version 11.5. Significance was established when the *p* value was less than 0.05.

Results and discussion

Preparation of microparticles

In the w$_1$/o/o/w$_2$ emulsion technique developed by Zheng (2009), aqueous solutions of hydrophilic drugs or proteins were emulsified with one appropriate polymer solution in the first emulsification step. In the next step, this mixture was further emulsified with another polymer solution to form w$_1$/o/o emulsion, which was evident from the change of the clear polymeric solution to a translucent and milky solution after this emulsification. This solution was subsequently injected into the non-solvent bath containing PVA as a stabilizer, leading to DCM evaporation and an increase in polymer concentration. As the polymer concentration reaches and exceeds the critical concentration, phase separation between two polymers will occur (Pekarek et al., 1994; Rahman and Mathiowitz, 2004), and double-walled structure will be formed. However, when the w$_1$/o/o emulsion was added into all of the external aqueous phase (w$_2$), droplets are rapidly solidified followed by rapid solvent diffusion

before being divided into smaller ones during the double emulsion step, which leads to the formation of larger particle size and wider particle size distribution. If shear homogenization remained until the division of the large solidified particles into micro-sized fragments without the protection of liquid oil phase, then the encapsulated protein aspirated into external aqueous phase and a small particle but low encapsulation efficiency was obtained (Zhao et al., 2007). In order to overcome the requirements of both small particle size and high encapsulation efficiency, this study developed a modified $w_1/o/o/w_3$ emulsion method, which included five steps (Figure 1). In this method, the solvent diffusion rate was decreased by dividing the double emulsification into two steps and solidifying the droplets step by step: the first double emulsification ($w_1/o/o/w_2$) in which the $w_1/o/o$ emulsion was emulsified into the first partial external aqueous phase (w_2) to form a $w_1/o/o/w_2$ double emulsion, the less volume of external aqueous phase possessed of less solubility of diffused solvent and subsequently most of the solvent stayed in the oil phase of the droplets; the second double emulsification ($w_1/o/o/w_3$) and pre-solidification in which $w_1/o/o/w_2$ double emulsion was further emulsified into the external aqueous phase (w_3) to form a $w_1/o/o/w_3$ double emulsion. After the second double emulsion was performed, most of solvent diffused into the external aqueous phase and pre-solidified droplets were formed.

Characteristics of microparticles

Morphology of microparticles

Figure 2 shows optical observation of microparticles produced with a single polymer and polymer mixtures. It is seen that these microparticles have spherical shape. Microparticles prepared with PDLLA/PLGA 50/50 polymer combination (F9) were characterized to have double-walled structure, with a cavity in the centre. Similar morphologies for double-walled microparticles have also been reported by others (Leach et al., 1999; Rahman and Mathiowitz, 2004; Zheng, 2009; Kokai et al., 2010).

The surface morphologies of microparticles were also analyzed using a FE-SEM. As shown in Figure 3, these microparticles have spherical, smooth and non-porous morphology. However, as microparticles are too small to cross-sectioning, the internal structure of microparticles could not be investigated by FE-SEM.

Composition of microparticles

The morphological changes of double-walled microparticles before and after dissolution in ethyl acetate were observed under the optical microscope. Figures 4(A) and (B) show a microparticle prepared with PDLLA and PLGA in the mass ratio of 2:1 before and after dissolution in ethyl acetate, respectively. When tested using ethyl acetate dissolution, the shell of microparticles disappeared. Based on the known solubility of PLGA and insolubility of PDLLA in ethyl acetate, the core and shell were identified as PDLLA and PLGA (Lee et al., 2002; Tan et al., 2005).

Particle size and size distribution of microparticles

The particle size and size distribution of microparticles were determined by laser diffraction method. Span values as an index of the polydispersity of microparticles are shown in Table 2. While the span value of the microparticles prepared with a single polymer (F1) was 1.92, significant decrease of span value ($p < 0.05$) caused by using two polymers to prepare microparticles. These results demonstrated that the microparticles prepared with two polymers were more homogenous than that

prepared with a single polymer. Although previously reported microparticles prepared using o/o/w method were often large ($\sim$150–170 μm) and polydispersed (Lee et al., 2002; Rahman and Mathiowitz, 2004; Tan et al., 2005), $w_1/o/o/w_3$ method can create monodispersed double-walled microparticles of a specified diameter within a size range easily administered by injection through a narrow-gauge needle ($<150\,\mu$m) in this study. This is important for efficient administration of the double-walled microparticles.

As shown in Table 2, the mean particle size of microparticles prepared with two polymers significantly increased compared to that of microparticles made from a single polymer (PLGA 50/50) ($p < 0.05$).

The mean particle diameter of microparticles, prepared from PLGA 85/15 and PLGA 50/50 in the mass ratio of 1:1 is $30.74 \pm 0.34\,\mu$m. The increase in mass ratio of PLGA 85/15 to PLGA 50/50 from 1:1 to 2:1 and 3:1 resulted in slight larger mean particle diameter of $33.95 \pm 0.26\,\mu$m and $34.07 \pm 0.44\,\mu$m, respectively. This statistically important increase in particle size ($p < 0.05$) is believed to be due to the increase in the viscosity of polymer solution depending on increasing mass ratio of PLGA 85/15 to PLGA 50/50. Similarly, Zheng (2009) reports that increase of mass ratio of PLGA 80/20 to PLGA 75/25 from 1:1 to 2:1 resulted in larger mean particle diameter.

The effect of OVA loading on the particle size of the microparticles is shown in Table 2. Increasing the initial weight of OVA dissolved in the inner aqueous phase caused a small increase in mean particle size of microparticles ($p < 0.05$).

When the microparticles prepared with PLGA 65/35:PLGA 50/50 polymer combination was compared with microparticles prepared with PLGA 85/15:PLGA 50/50 polymer combination, a significant decrease was observed in particle size ($p < 0.05$) (Table 2). Similarly, the use of PDLLA as a second polymer caused a significant decrease in particle size of microparticles ($p < 0.05$).

Estimation of drug content

A successful drug delivery system may be the one which has a high loading capacity to reduce the quantity of the carrier required for administration (Soppimath et al., 2001). High encapsulation efficiency is an advantage for the industrial process. Whereas the fabrication using the $w_1/o/o/w_2$ emulsion method was able to produce encapsulation of approximately 86%, using the $w_1/o/o/w_3$ emulsion method provided to obtain higher encapsulation efficiency values (up to 100%) in this study. The encapsulation efficiency of double-walled microparticles made of PLGA 85/15 and PLGA 50/50 in mass ratio of 2:1 is 100.90 ± 2.76%. Decreasing mass ratio of PLGA 85/15 to PLGA 50/50 from 2:1 to 1:1 caused a significant decrease in encapsulation efficiency ($p < 0.05$). One explanation could be that PLGA 85/15 concentration in mass ratio of 1:1 was not enough to create a barrier preventing leakage of OVA from droplets during double emulsification step. However, increasing the mass ratio of PLGA 85/15 to PLGA 50/50 from 2:1 to 3:1 did not cause a significant change in encapsulation efficiency ($p > 0.05$) (Table 2).

Table 2 shows that initial weight of OVA dissolved in the inner aqueous phase affected encapsulation efficiency of microparticles. Increasing the amount of OVA from 5 mg to 7.5 mg caused a slight decrease in the encapsulation efficiency of microparticles ($p < 0.05$). However, the encapsulation efficiency decreased from 100.90 ± 2.76% to 75.48 ± 3.22% with increase in the initial weight of OVA dissolved in the inner aqueous phase from 5 mg to 10 mg. It was clear that increasing the OVA concentration in the emulsion droplets increased the concentration gradient of OVA between the internal water phase and the external water phase,
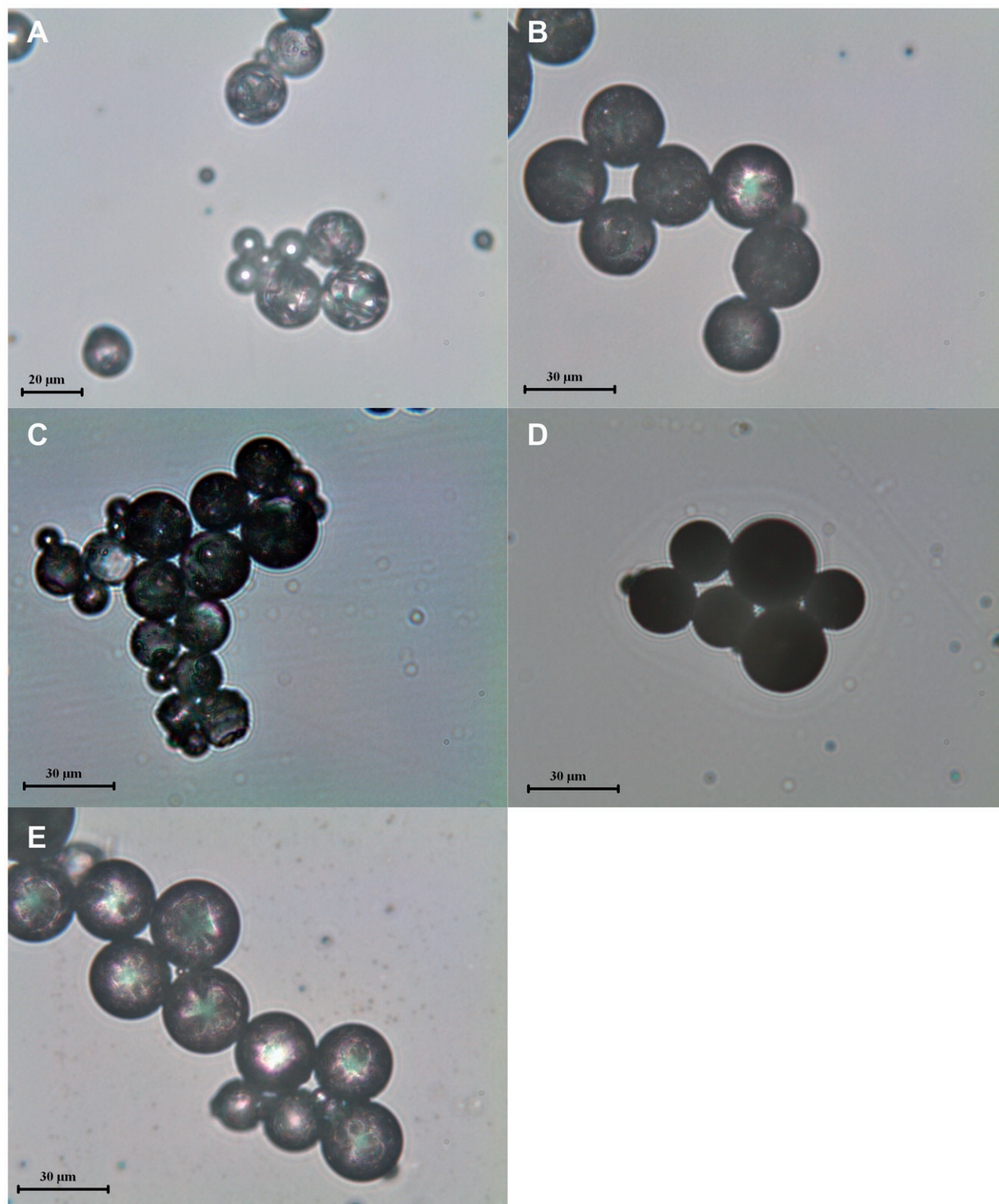
Figure 2. Optical micrographs of microparticles. (A) F1; (B) F3; (C) F7; (D) F8 and (E) F9.

and the drug diffusion from the emulsion droplets into the external water phase was promoted (Cui et al., 2005).

Very high encapsulation efficiencies were also provided with those microparticles made of PLGA 65/35:PLGA 50/50 and PLGA 75/25:PLGA 50/50 in mass ratio of 2:1 (Table 2).

When the microparticles prepared with PDLLA and PLGA 50/50 combination were compared with microparticles prepared by PLGA combinations, it was observed that the use of PDLLA as a second polymer caused a slight decrease in encapsulation efficiency of microparticles (93.98 ± 3.27 %). The reason might be that increasing the lactic acid content of the polymer (PDLLA)

caused a increase in solubility of polymer in organic solvent (DCM). Due to high solubility of polymer in organic solvent, encapsulation efficiency decreased with the time period of the polymer phase before solidification (Yeo and Park, 2004; Devrim et al., 2011).

Production yield of microparticles

A production yield of range 66.75–76.35% in the microparticle formulations was obtained (Table 2). In the F9 coded formulation prepared with PDLLA and PLGA 50/50 combination, some
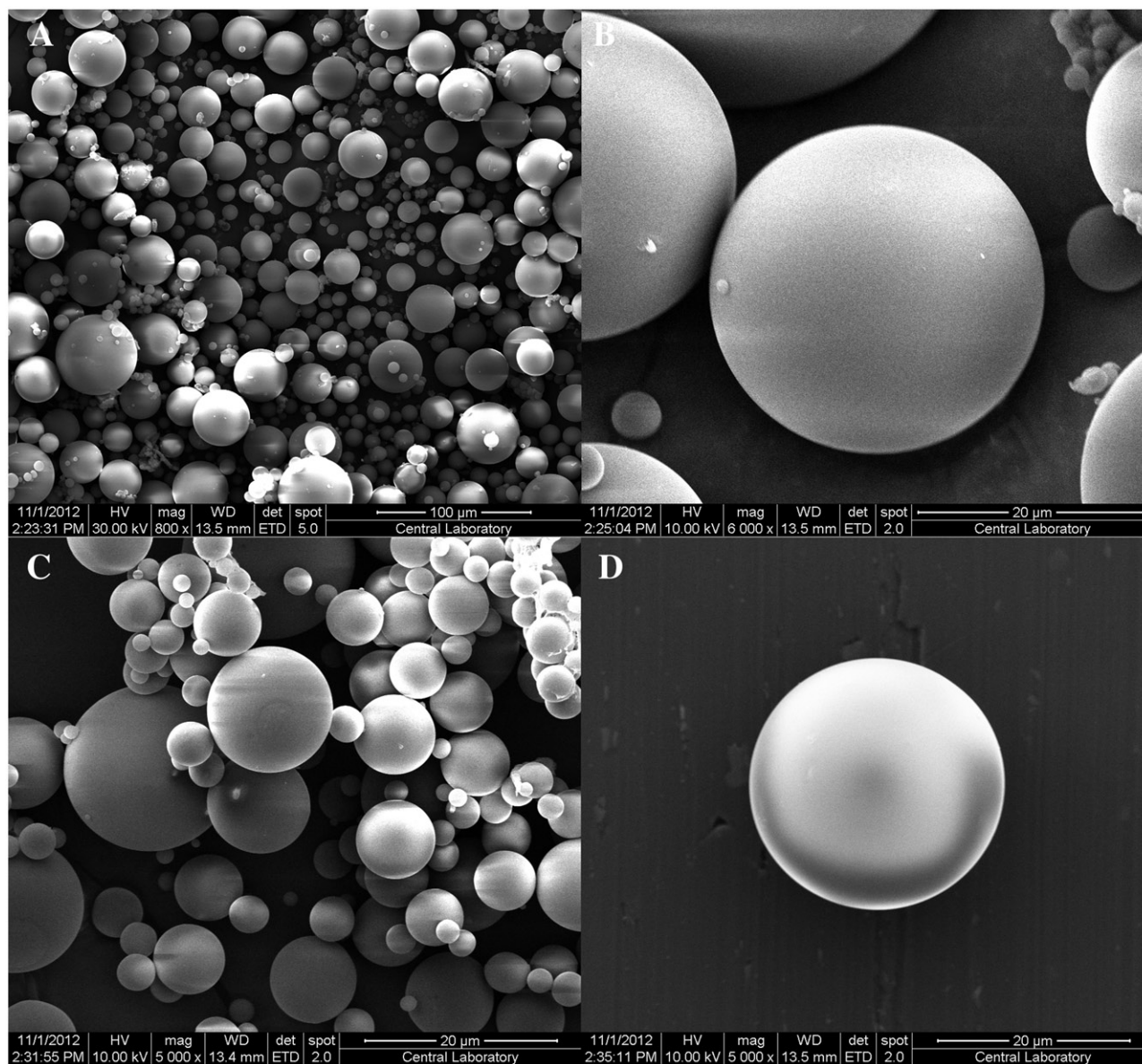
Figure 3. Low and high magnification scanning electron images of microparticles. (A) and (B) F1; (C) and (D) F9.
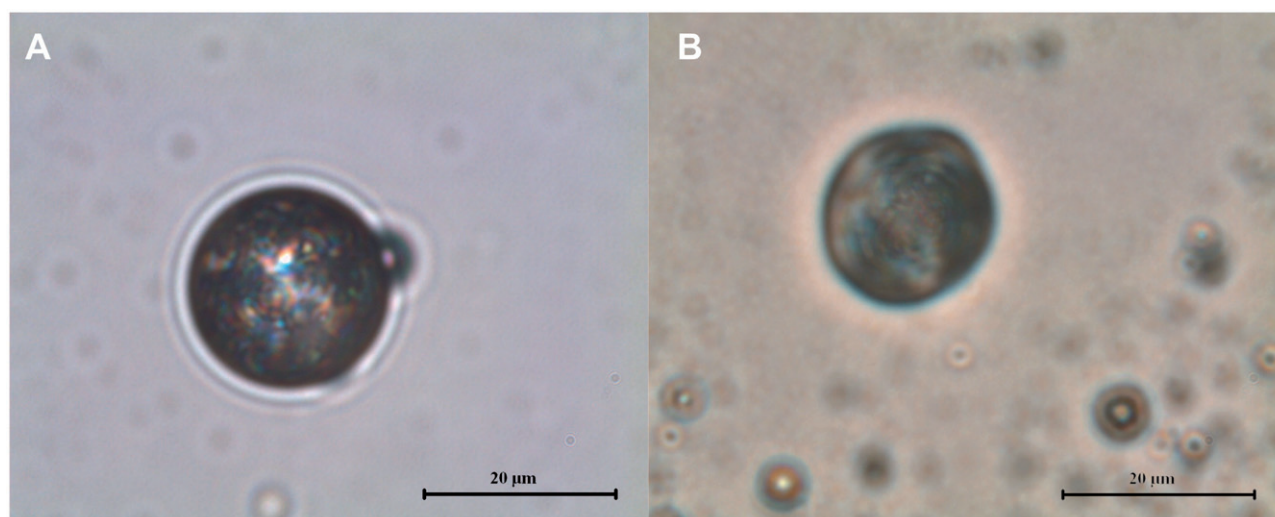

Figure 4. Optical micrographs of double-walled microparticles before and after dissolution with ethyl acetate. (A) F9 before dissolution; (B) F9 after dissolution.

Table 2. Characterizations of microparticles prepared at different conditions.

Formulation code	Mean particle size (µm ± SD)	Span ± SD	Yield of production (%)	Actual drug loading (% ± SD)	Encapsulation efficiency (% ± SD)
F1	19.68 ± 0.51	1.92 ± 0.27	76.35	0.80 ± 0.02	64.11 ± 3.84
F2	30.74 ± 0.34	1.43 ± 0.03	71.70	0.97 ± 0.01	77.95 ± 2.75
F3	33.95 ± 0.26	1.07 ± 0.04	72.80	1.26 ± 0.01	100.90 ± 2.76
F4	34.07 ± 0.44	1.40 ± 0.03	70.85	1.22 ± 0.04	97.84 ± 8.54
F5	29.28 ± 0.45	1.55 ± 0.01	71.90	1.73 ± 0.03	92.13 ± 5.06
F6	31.97 ± 0.30	1.52 ± 0.03	72.40	1.89 ± 0.02	75.48 ± 3.22
F7	24.38 ± 0.09	1.49 ± 0.02	73.25	1.26 ± 0.02	101.11 ± 4.77
F8	34.47 ± 0.11	1.08 ± 0.01	72.20	1.27 ± 0.01	101.28 ± 2.84
F9	16.59 ± 0.03	1.31 ± 0.01	66.75	1.17 ± 0.02	93.98 ± 3.27

SD = Standard deviation, n = 3.

decrease in production yield was observed due to the increase in the polymer solution viscosity compared to other formulations. As reported in our previous study (Devrim et al., 2011), the increase in viscosity of polymer solution causes an increase in the amount of polymer adhering to the container and, thus, decreases the production yield.

Thermal analysis

PLGA and PDLLA are completely amorphous polymers. Amorphous polymers are thermally characterized by the presence of the transition temperature (T_g), which is the transition point between a highly viscous brittle structure called glassy state and a less viscous, more mobile, rubbery state (Blasi et al., 2007). As shown in Figure 5(A), the pure PLGAs and PDLLA exhibit an endothermic event (41–55 °C) referring to the relaxation peak that follow T_g. No melting point was observed, because PLGAs and PDLLA appear amorphous in nature (Mainardes et al., 2006). Since certain proteins have strong emulsifying activity (Rouse et al., 2007), it is reasonable to ask what consequence encapsulation of protein has on the thermomechanical properties of PLGAs and PDLLA. The OVA presence shifted the transition of PLGAs and PDLLA to slightly lower temperature in comparison to that of the free polymers (Figure 5B and Table 3). Also, ΔH values of microparticles were slightly lower than that of the free polymers (data not shown).

In general, a miscible blend of two polymers will have properties of both two unblended polymers. That is, the existence of two T_gs, each representative of the original polymers, is the evidence of a phase-separated blend. The existence of a single T_g, between the T_g's of the original polymers, indicates a miscible blend (Rahman and Mathiowitz, 2004). Figure 5(B) and Table 3 showed that the DSC thermograms of microparticles prepared with PLGA 65/35:PLGA 50/50 and PLGA 75/25:PLGA 50/50 combinations indicated a single peak, whereas two peaks were obtained with the PLGA 85/15:PLGA 50/50 and PDLLA:PLGA 50/50 polymer combinations. Therefore, phase separation was observed in combinations of polymers such as PLGA 85/15:PLGA 50/50 and PDLLA:PLGA 50/50, whereas two other combinations did not show any phase separation. The DSC thermograms of the pure polymers were also plotted as a control.

Figure 5(C) illustrates the thermal characteristics of the OVA, empty microparticles and a physical blend of OVA and polymer (PLGA 50/50) in the same ratio as the loading of protein in microparticles (F1). OVA exhibits a broad endotherm with a glass transition of 79.79 °C. The glass transition temperatures for empty microparticles and physical blend of OVA and polymer were 43.54 and 44.90 °C, respectively. There was no evidence of a broad endotherm related to OVA in the protein–polymer blend possibly because of the relatively low amount of protein relative to polymer (<2%, w/w). Similar results were obtained with double-walled microparticles (data not shown).

FTIR spectroscopy studies

The ATR-FTIR spectra of PLGA 50/50, empty microparticles, OVA and OVA-loaded microparticles (F1) are shown in Figure 6. The FTIR spectrum of PLGA shows the peaks contributed by its functional groups such as –CH, –CH$_2$ and –CH$_3$ stretching in the region of 2860–2940 cm^{-1}, carbonyl –C=O stretching vibrations in the region of 1700–1800 cm^{-1} and C–O stretching in the region of 1050–1250 cm^{-1} (Kumar et al., 2012; Patel et al., 2012; Sharma et al., 2013). The empty microparticles showed the same peaks contributed by the functional groups of PLGA. This indicates that the microencapsulation process did not significantly modify polymer characteristics. Additionally, the spectra of the OVA-loaded microparticles show no characteristic differences than those of control formulations; this may be the result of the relatively low ratio of drug to polymer (Patel et al., 2012; Sharma et al., 2013).

In vitro *OVA release from microparticles*

The cumulative percentage of OVA release from the microparticles were examined *in vitro* in phosphate buffer solution (pH = 7.4). The *in vitro* cumulative release profiles of double-walled microparticles showed reduced initial burst and prolonged sustained release of OVA over 2 months compared to monolithic microparticles (Figure 7). A significant reduction in initial burst was observed for double-walled microparticles compared with OVA release from microparticles composed of pure PLGA. As reported in previous studies (Lee et al., 2002; Rahman and Mathiowitz, 2004; Tan et al., 2005; Kokai et al., 2010), double-walled microparticles provided effective suppression of the undesirable initial burst of drug release. This fulfilled one of the key motivations for a double-walled system intended for application with highly water-soluble drugs such as peptides and proteins.

The initial burst release of microparticles made of two polymers in the mass ratio of 1:1 was typically 20.35 ± 0.96%, for PLGA 85/15:PLGA 50/50 combination (Figure 7A and Table 4). Increasing the mass ratio of PLGA 85/15 to PLGA 50/50 up to 2:1 and 3:1 further reduced the initial burst release to 12.42 ± 0.95% and 10.54 ± 1.01%, respectively. In the previous study (Zheng, 2009), increasing the mass ratio of PLGA 80/20 to PLGA 75/25 up to 2:1 reduced the initial burst release. This result proven that immediate diffusion of hydrophilic drug from drug-loaded inner polymer layer can be retarded by an enhanced non-drug-loaded outer layer. Matsumoto et al. (2006) also reported that the addition of high molecular weight PLA into outer layer provided the drug release depending on PLGA/PLA ratio.

Figure 5. DSC thermograms of (A) pure polymers; (B) OVA-loaded microparticles and (C) PLGA 50/50, empty microparticles, and physical blend of OVA and PLGA 50/50.
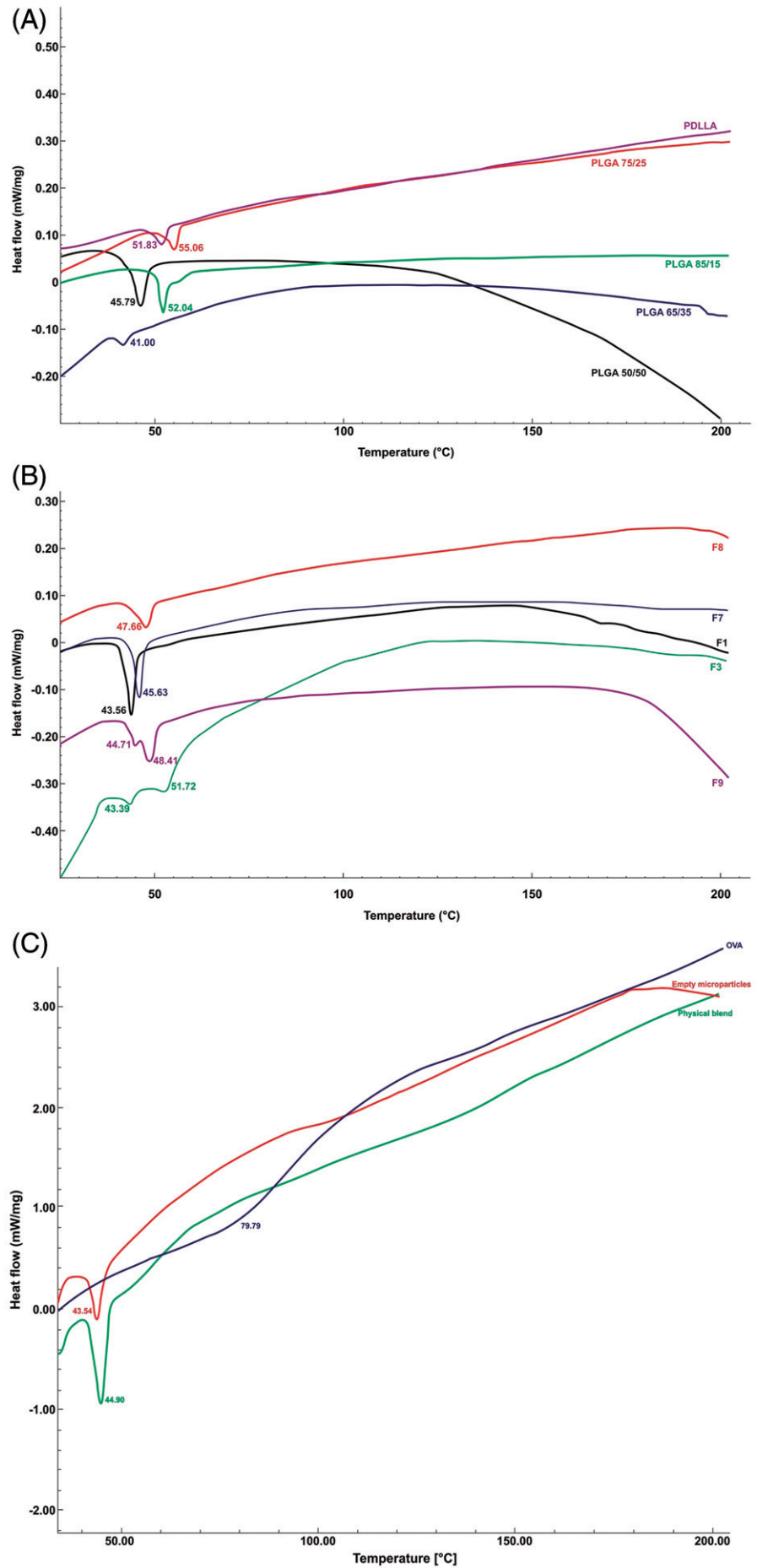
The change in second polymer combined with PLGA 50/50 affected the initial burst release little as seen from Figure 7(B) and Table 4. Using the PLGA 65/35 or PLGA 75/25 instead of PLGA 85/15 as a second polymer caused a slight increase in the initial burst release. Our previous study suggested that high glycolic acid content allows for a more hydrophilic property and a high rate of water uptake from the release medium to the microparticles results in a high release rate (Devrim et al., 2011). The initial burst release of OVA is the lowest with microparticles made of PDLLA and PLGA 50/50, due to the slow degradation rate of PDLLA polymer that has high lactide ratio.

Drug loading was also another factor influencing drug release. Figure 7(C) showed the effect of different degrees of OVA loading on the initial burst release of microparticles made with combination of PLGA 85/15 and PLGA 50/50 in the mass ratio of 2:1. When the initial weight of OVA dissolved in the inner aqueous phase from 5 mg to 7.5 mg or 10 mg, the initial burst release of microparticles increased from $12.42 \pm 0.95\%$ to $17.42 \pm 0.95\%$ or $19.13 \pm 1.32\%$. It was obvious that a higher concentration of OVA embedded in the microparticles structured in the PLGA matrix led to a higher concentration gradient between the microparticles and dissolution medium. In addition, at a high loading level, more OVA may be distributed over the surface of the microparticles, so a higher drug loading always leads to a greater initial burst release (Homayoun et al., 2003).

Drug release kinetics were analyzed by plotting the cumulative release data to time by fitting to an exponential equation; zero-order kinetics, first-order kinetics, Hixson–Crowell's cube-root equation and Higuchi's square root of time equation. The r^2 value

Table 3. Thermal properties of pure polymers and microparticles obtained from DSC thermograms.

T_g (°C)			
Pure polymer		Formulation code	
PLGA 50/50	45.79	F1	43.56
PLGA 85/15	52.04	F3	43.39–51.72*
PLGA 65/35	41.00	F7	45.63
PLGA 75/25	55.06	F8	47.66
PDLLA	51.83	F9	44.71–48.41†

*The two glass transition temperatures refer to those of PLGA 50/50 and PLGA 85/15, respectively, in the composite.
†The two glass transition temperatures refer to those of PLGA 50/50 and PDLLA, respectively, in the composite.

is an empirical parameter characterizing the release mechanism, and whereas F1 coded formulation prepared with PLGA 50/50 only has been fitted with first-order kinetic, other formulations have been fitted with Higuchi kinetic according to r^2 values (Table 4).

To describe the drug release mechanism from microparticles, the semi-empirical Equation (5) proposed by Korsmeyer and Peppas was used (Hickey et al., 2002; Ferrero et al., 2010; Song et al., 2012):

$$M_t/M_\infty = kt^n \tag{5}$$

Where M_t/M_∞ is the fractional drug release at time t, k is the kinetic constant related to the structural and geometric characteristic of the formulation used to measure the release rate and n is a diffusional exponent, indicative of the drug release mechanism. Three different release mechanisms can be inferred from the value of n. For drug release from spherical particles, the values below 0.43 indicate for purely Fickian diffusion, values between 0.43 and 0.85 indicate for anomalous (non-Fickian) transport and n above 0.85 for pure non-Fickian mechanisms (case II transport).

The values of n for microparticle formulations except F4 and F9 coded formulations were <0.43. These results indicate that the drug release took place by diffusion from these microparticles. Nevertheless, the values of n for F4 and F9 coded formulations were between 0.43 and 0.85 indicating that the release was due to both diffusion through the matrix and degradation of the polymer (Table 5).

The morphological changes of formulations in release tests were also evaluated. The optical microscopic photographs regarding the morphological changes of the formulations are shown in Figure 8. At the end of *in vitro* release study, on the 63rd day, while F1 formulation prepared with PLGA 50/50 only had disintegrated and lost their spherical integrity, F8 and F9 formulations prepared with PLGA 85/15:PLGA 50/50 and PDLLA:PLGA 50/50, respectively, retained spherical structure. The biodegradation rate of polyesters is dependent on several factors, such as the molar ratio of the lactic and glycolic acids in the copolymer chain, the molecular weight of the polymer, the degree of crystallinity and the T_g of the polymer (Gopferich, 1996; Chen and Ooi, 2008). The glycolic acid units are more hydrophilic than the lactic acid units. It is also known that the ester bonds of PLGA copolymers linked with the glycolic acid unit (glycolic–glycolic or glycolic–lactic) may be preferentially cleaved, as compared with those of lactic–lactic acid linked ester (Yang et al., 2001; Wang et al., 2002; Kepmen et al., 2004).
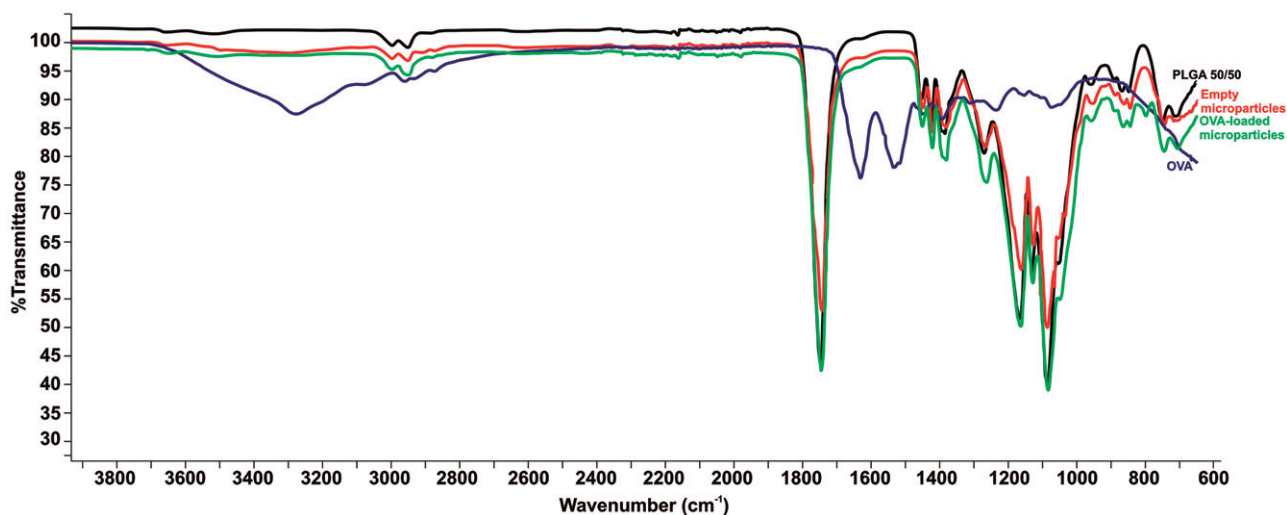
Figure 6. ATR-FTIR spectra of PLGA 50/50, empty microparticles, OVA, and OVA-loaded microparticles.

Figure 7. Effect of the (A) mass ratio of
PLGA 85/15 to PLGA 50/50; (B) polymer
type combined with PLGA 50/50 and (C)
initial weight of OVA dissolved in the inner
aqueous phase on *in vitro* release profiles of
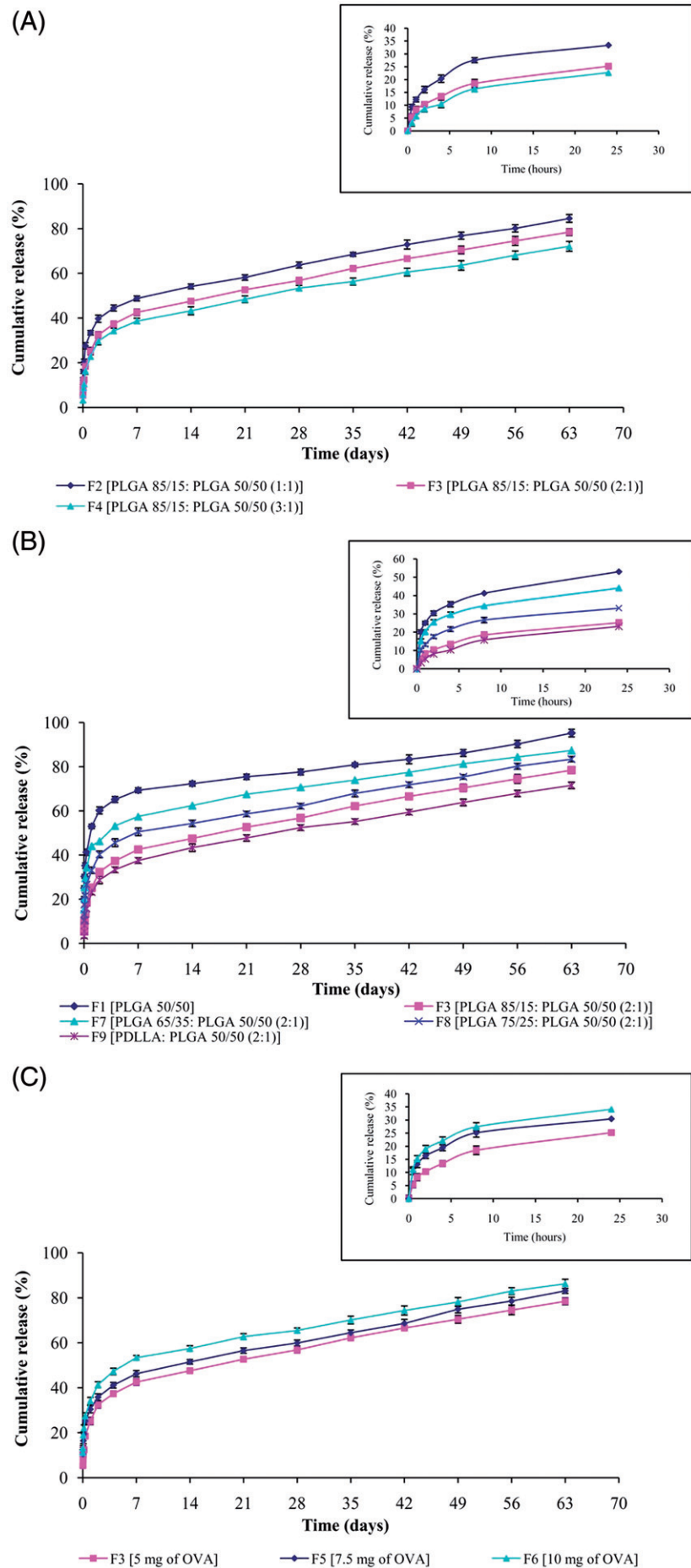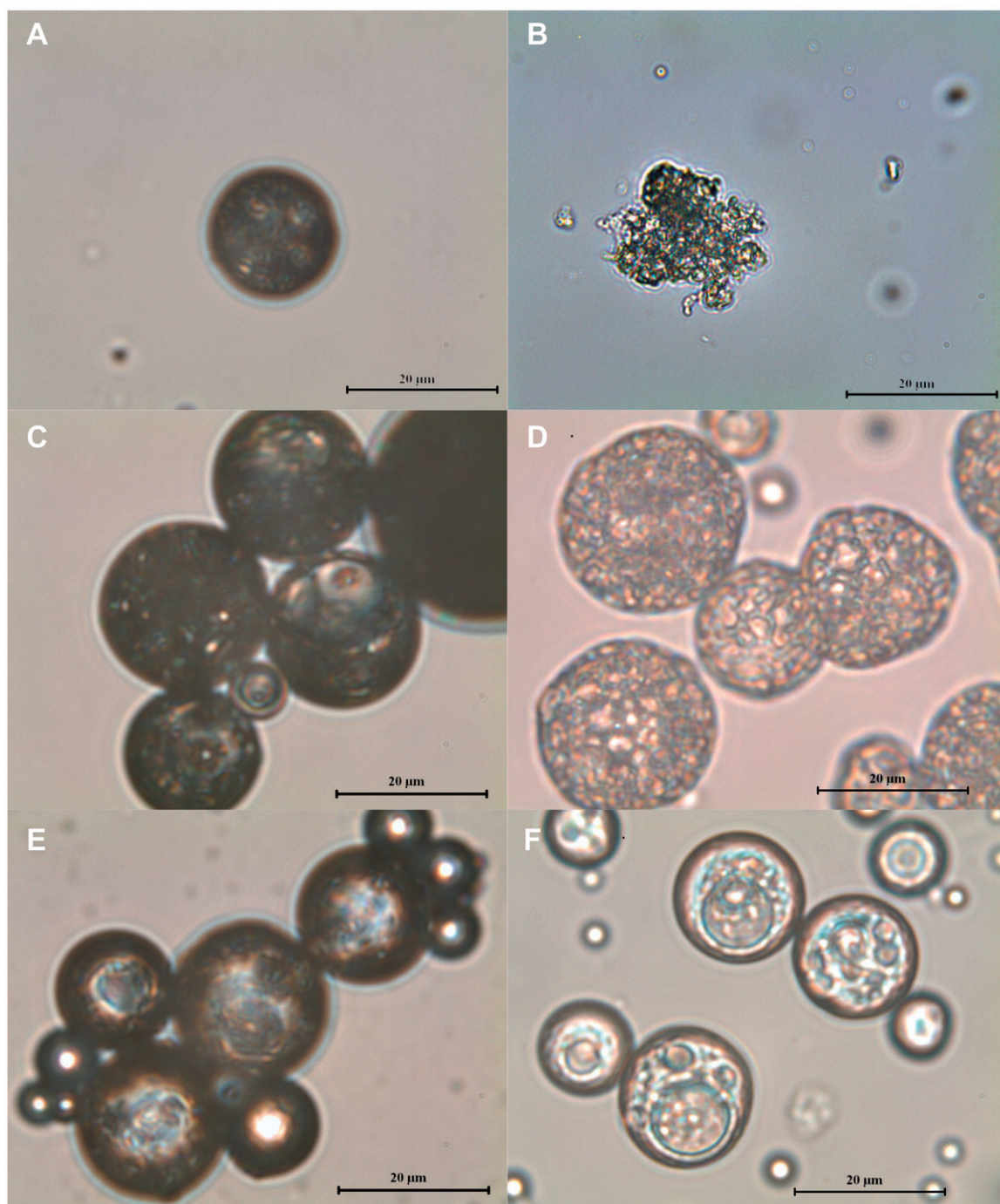the microparticles.

Table 4. Initial burst release and release kinetic values (r^2) of OVA encapsulated microparticles.

Formulation code	Initial burst release (% ± SD)	Zero order (r^2)	First order (r^2)	Higuchi (r^2)	Hixson Crowell (r^2)
F1	44.11 ± 0.96	0.794	0.917	0.909	0.909
F2	20.35 ± 0.96	0.901	0.960	0.963	0.959
F3	12.42 ± 0.95	0.893	0.963	0.975	0.953
F4	10.54 ± 1.01	0.887	0.959	0.973	0.941
F5	17.42 ± 0.95	0.917	0.975	0.982	0.966
F6	19.13 ± 1.32	0.877	0.968	0.981	0.950
F7	25.56 ± 1.32	0.848	0.964	0.956	0.937
F8	20.65 ± 1.21	0.886	0.968	0.979	0.952
F9	10.36 ± 1.01	0.895	0.963	0.987	0.946

SD = Standard deviation, $n = 3$.

Table 5. Result of drug release mechanism of OVA encapsulated microparticles.

Formulation code	Regression equation	Correlation coefficient	Drug transport mechanisms
F1	$M_t/M_\infty = 54.075t^{0.245}$	0.995	Fickian diffusion
F2	$M_t/M_\infty = 36.475t^{0.340}$	0.983	Fickian diffusion
F3	$M_t/M_\infty = 26.853t^{0.392}$	0.988	Fickian diffusion
F4	$M_t/M_\infty = 25.704t^{0.493}$	0.970	Anomalous transport
F5	$M_t/M_\infty = 32.285t^{0.290}$	0.985	Fickian diffusion
F6	$M_t/M_\infty = 36.559t^{0.299}$	0.978	Fickian diffusion
F7	$M_t/M_\infty = 46.026t^{0.266}$	0.982	Fickian diffusion
F8	$M_t/M_\infty = 35.727t^{0.308}$	0.982	Fickian diffusion
F9	$M_t/M_\infty = 25.293t^{0.495}$	0.987	Anomalous transport

Figure 8. Morphological changes of monolithic and double-walled microparticles during *in vitro* release test. (A) F1 initial; (B) F1 63 days; (C) F3 initial; (D) F3 63 days; (E) F9 initial and (F) F9 63 days.

Thus, more glycolic acid content makes it easier for water to penetrate into the polymer matrix. PLGA polymers containing a 50:50 ratio of lactic and glycolic acids have been reported to hydrolyze much faster than those containing higher proportion of either of the two monomers (Gopferich, 1996). Thus F1 coded microparticle formulation prepared with PLGA 50/50 only degraded the fastest compared to the other microparticle formulations.

Conclusions

In this work, double-walled microparticles were successfully produced using a $w_1/o/o/w_3$ emulsion technique. In this method, the solvent diffusion rate was decreased by dividing the double emulsification into two steps and solidifying the droplets step by step. This modification ensured smaller particle size and higher encapsulation efficiency values. The initial burst from the microparticles prepared with two polymers was significantly decreased compared to that of microparticles prepared with a single polymer. The initial burst could be controlled by the formulation parameters such as type and mass ratio of polymers combined with PLGA 50/50 and drug loading. The initial burst release was followed by sustained release over 2 months. Thus, these microparticles provide great versatility as drug delivery platforms, providing decreased initial burst and sustained delivery of therapeutic agents that might have a low therapeutic index.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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