International Journal of Pharmaceutics 433 (2012) 79–88

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Encapsulation of azithromycin into polymeric microspheres by reduced pressure-solvent evaporation method

Xiujuan Li[a], Si Chang[a], Guangsheng Du[a], Yi Li[a], Junbo Gong[a], Mingshi Yang[b], Zhenping Wei[a,c,*]

[a] Department of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
[b] Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
[c] Tianjin Key Laboratory for Modern Drug Delivery & High-Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China

ARTICLE INFO

Article history:
Received 28 January 2012
Received in revised form 14 April 2012
Accepted 30 April 2012
Available online 11 May 2012

Keywords:
Azithromycin
Reduced-pressure solvent evaporation
Burst release
Crystallinity
Antoine equation

ABSTRACT

Azithromycin loaded microspheres with blends of poly-L-lactide and ploy-D,L-lactide-co-glycolide as matrices were prepared by the atmosphere-solvent evaporation (ASE) and reduced pressure-solvent evaporation (RSE) method. Both the X-ray diffraction spectra and DSC thermographs demonstrated that poly-L-lactide existed in a crystalline form in the ASE microspheres, while an amorphous form was present in the RSE formulations. Besides, solvent removal at atmosphere gave microspheres of porous and rough surfaces, but smooth surfaces appeared in the RSE microspheres. The incorporation efficiency as well as the burst release (cumulative release in the first 24 h) in the ASE formulations was 39.94 ± 1.18% and 23.96 ± 2.01% respectively, yet the encapsulation efficiency of the microspheres fabricated under 385 mmHg was high up to 57.19 ± 3.81% and the burst release was 4.12 ± 0.15%. The in vitro drug release studies indicated that the ASE microspheres presented a zero-order profile; while the RSE formulations followed first-order kinetics. Other factors including solidification time, temperature, drug to polymer ratio and pH value of the continuous phase could also influence the physicochemical characteristics and release profiles of microspheres. In conclusion, the overall improvement of microspheres in appearance, encapsulation efficiency and controlled drug release through the RSE method could be easily fulfilled under optimal preparation conditions.

1. Introduction

Biodegradable polymer microspheres for controlled drug delivery have got much attention due to their outstanding clinical benefits: reducing dosing frequency, improving convenience and acceptance for patients, as well as drug targeting to specific locations (Berkland et al., 2002; Freiberg and Zhu, 2004; Li et al., 2008).

Emulsion-solvent extraction/evaporation method is commonly used among the various microsphere preparation techniques. In the solidification process, microspheres can be formed by extraction or evaporation of the organic solvent from the dispersed oil droplets containing both polymer and drug. The art of this preparation technology has significant impacts on the characteristics of drug loaded microspheres such as the surface morphology, particle size, encapsulation efficiency and in vitro release profiles (Igartua et al., 2008; Ye et al., 2010; Angadi et al., 2011; Chaturvedi et al., 2011; Oh et al., 2011).

For the solvent extraction method, isopropanol is usually added to extract the methylene dichloride (DCM) which is used as the organic solvent (Igartua et al., 2008). Another way is to use ethyl acetate (EA) as the organic solvent due to its partial miscibility with water (8.7 wt.% EA in water). In this case, a pre-emulsification step into a smaller volume of external aqueous phase is necessary in order to avoid premature polymer precipitation and to obtain microparticles (Freytag et al., 2000).

Most of the time, solvent evaporation technique is applied in microencapsulation for its simplicity in operation. The evaporation process is often conducted under atmospheric pressure and at room temperature, which is a time-consuming procedure. Besides, long duration of solidification may be detrimental to the physicochemical characteristics of microspheres and drug properties. As is well known, the contact between protein and hydrophobic interface has been identified as one of the major causes of protein denaturation (Mundargi et al., 2008). Meng et al. (2004) reported that bovine hemoglobin microspheres prepared within shorter time could retain bio-activity of encapsulated BHb to a great extent. The solvent evaporation rate can be accelerated either by increasing the temperature of the continuous phase or by reducing the pressure in the reactor (Li et al., 2008). The microspheres fabricated at 38 °C yielded the highest encapsulation efficiency and lowest initial drug

* Corresponding author at: Department of Chemical Engineering and Technology, Tianjin University, 92 Weijin Road, Nankai District, Tianjin 300072, China. Tel.: +86 22 27890336.
E-mail address: zpwei2000@sina.com (Z. Wei).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2012.04.081

release, while those produced at 22 °C had the lowest encapsulation efficiency and highest initial burst in the work of Yang et al. (2000). While using the temperature gradient (Tmp) technique, microspheres with a hollow internal core and a porous wall were obtained and the specific surface area was higher and bulk density was lower (Jeyanthi et al., 1996). The results were heavily depended on the process parameters. In general, temperature-rising method has its limitations: it cannot be applied for thermosensitive drugs; most of the cases, obtained microspheres are filled with pores and the product recovery decreases (Miyazaki et al., 2006).

Pressure reduction is another way to promote the evaporation of the organic solvent. Progesterone loaded poly-L-lactide (PLLA) microspheres produced under reduced pressure (200 mmHg) had higher encapsulation efficiency, smaller surface area and slower drug release rate compared with those manufactured at atmospheric pressure (760 mmHg) (Izumikawa et al., 1991). Besides, atmospheric pressure-solvent evaporation (ASE) yielded microspheres of crystalline polymer matrices, while reduced pressure-solvent evaporation (RSE) gave microspheres of amorphous polymer matrices (Izumikawa et al., 1991). Higher encapsulation efficiency was also observed in Bovine hemoglobin loaded microspheres prepared by RSE method (Meng et al., 2004). However, in the work of Chung et al. (2001), both lidocaine loaded poly-L-lactide (PLLA) and poly-D,L-lactide (PDLLA) microspheres fabricated under reduced pressure showed lower drug encapsulation efficiencies than those prepared in ASE process. Compared with progesterone-loaded microspheres, converse results might be due to the different emulsification methods which affected the particles size. Until now, most of the studies focus on the comparison between ASE and RSE method in terms of the properties of microspheres produced. For the RSE method, there is less work has been done to investigate the effect of process parameters on the physicochemical characteristics and drug release profiles of the microspheres. In this work, the influence of process variables, i.e. preparation pressure, solidification time, temperature of the continuous phase and the ratio of drug to polymer on the physicochemical properties of microspheres were systematically investigated.

Microspheres consisting of a single polymer have several inherent problems, including high initial burst and low encapsulation efficiency (Pekarek et al., 1996; Tan and Ye, 2008). Therefore, the blends of PLGA/PLLA with a mass ratio at 1:1 were used as the polymer matrix due to their properties of good biocompatibility and biodegradability as well as the sustained release (Liu et al., 2006; Ye et al., 2010). Azithromycin (AZI), which has a broad antibacterial spectrum and high activity against gram-negative organisms (Girard et al., 1987; Ballow and Amsden, 1992), was selected as a model drug to prepare antibiotic loaded microspheres for the purpose of sustained and controlled drug releasing. The O/W emulsion-solvent evaporation method was used in the preparation process with polyvinyl alcohol (PVA) as an emulsifier.

2. Materials and methods

2.1. Materials

Azithromycin was obtained from CSPC OUYI Pharmaceutical Co., Ltd. (Shijiazhuang, China). Poly-D,L-lactide-co-glycolide copolymer (PLGA, lactide: glycolide molar ratio was 50/50 and molecular weight was 30,000 Da) was supplied by Shandong Institute of Medical Instrument (Jinan, China). Poly-L-lactide (PLLA, molecular weight was 24,000 Da) was purchased from Jinan Dai Gang Biotechnology Co., Ltd. (Jinan, China). Polyvinyl alcohol EG-30 (PVA EG-30, the viscosity was in a range of 27.0–33.0 mPa s and 88% hydrolyzed) was provided by Japanese Synthetic Chemical Industry Co., Ltd. (Guang Dong, China). All other reagents were of analytical grade.

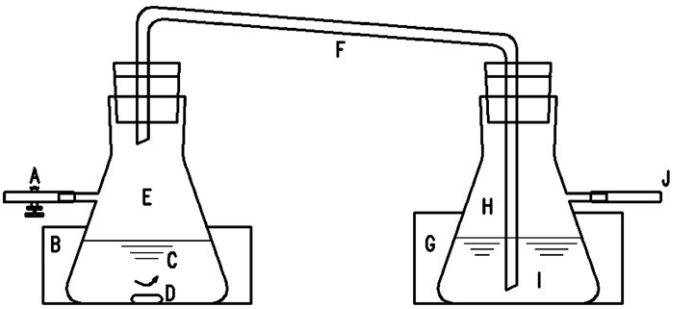

Fig. 1. The schematic diagram of the experimental apparatus: (A) vacuum control valve; (B) magnetic stirrer with thermostatic water-bath; (C) continuous phase; (D) magnetic stirring bar; (E) reaction flask; (F) condensation tube; (G) Ice bath; (H) DCM collection bottle; (I) low foaming surfactant solution; (J) to vacuum pump.

2.2. Preparation of AZI microspheres

All microspheres were obtained by the O/W emulsion solvent evaporation method using DCM as the organic solvent. In brief, 40 mg of AZI, 100 mg of PLLA and 100 mg of PLGA were dissolved in 1.5 ml DCM, and then added dropwise into 6 ml of PVA solution (1%, w/v). This process was carried out under 4 °C by a homogenizer (Ultra Turrax®, IKA T25, Germany) at 5000 rpm for 1 min to form an O/W emulsion. The resulting O/W emulsion was quickly poured into 80 ml of PVA aqueous solution (0.2%, w/v) and stirred magnetically at 300 rpm at 25 °C for another 3 h under atmospheric pressure or 0.5 h under reduced pressure. Besides, NaCl solution (2%, w/v) was also added to modulate the osmotic pressure. In the RSE method, the solvent evaporation process was accomplished in a decompression device, as depicted in Fig. 1. The solidified microspheres were collected by centrifugation, washed three times with distilled water and then lyophilized (Christ Alpha 1–2, Martin Christ Co., Germany) overnight to obtain a free flowing water dispersible powder. The final dry powder was taken out for physicochemical analysis and in vitro release study. Single factor tests were designed to investigate the process parameters such as preparation pressure, solidification time, temperature and pH value of the continuous phase as well as the ratio of drug to polymer on the physicochemical characteristics and drug release profiles of the microspheres. In this study, the drug–polymer ratio was modulated by varying the amount of the drug with a constant quantity of polymer.

2.3. X-ray powder diffraction

An X-ray diffractometer (X'Pert Pro, PANalytical B.V., Netherlands) with Co-Kα radiation of wavelength 1.5406 Å (40 kV, 40 mA) was employed to study the crystalline form of the polymer and drug in the microspheres. The samples were then analyzed over a 2θ range of 4–50° with a scanning rate of 5°/min and step size of 0.02.

2.4. Differential scanning calorimetry (DSC)

Thermal analysis on the samples was carried out using a differential scanning calorimeter (DSC200 F3 Maia®, Netzsch Instrument Co., Germany). Samples in sealed aluminum pans were scanned from 30 to 200 °C at a heating rate of 10 °C/min under a dry nitrogen purge.

2.5. Morphology and particle size analysis

The surface morphology of microspheres, fabricated under different preparation conditions, was observed by Scanning Electronic Microscope (SEM) (S-4800, Hitachi Co., Japan). Microspheres were mounted onto metal stubs using a double-side adhesive tape. After vacuum-coated with gold, the microspheres were examined by SEM at 3 kV or 5 kV.

To assess the particle size, microspheres were dispersed in 1% (W/V) of carboxymethyl cellulose and the average diameter was measured by a laser particle size analyzer (BT-2002, Dandong Better Instrument Co., Ltd, China). All the samples were treated in triplicates.

2.6. AZI loading and encapsulation efficiency

The amount of AZI encapsulated in the microspheres was determined in triplicate by solvent extraction method. Briefly, 10 mg of microspheres were dissolved in 0.5 ml DCM and then mixed with 4.5 ml of Ethanol to have the polymer precipitated out. After centrifugation at 14,500 rpm for 10 min, the supernatant was drawn and diluted 10 times by PBS (pH 6.8, 50 mM). The drug concentration was determined by a modified spectrophotometric method (Gao et al., 2006; Sultana et al., 2006): Four milliliters of the diluted solution and 4 ml sulfuric acid solution (75 ml sulfuric acid was added to 100 ml distilled water exactly) were combined and allowed to react for 30 min to produce a color, then the solution was analyzed with spectrophotometer (UV/vis 765, Shanghai Stech Electronics Co., Ltd, China) at 482 nm to determine the drug concentration. The drug loading efficiency (D.L.) and encapsulation efficiency (E.E.) were calculated with the following two equations (He et al., 2006).

$$\text{D.L. } (\%) = \frac{D_t}{M_t} \times 100 \quad (1)$$

$$\text{E.E.} (\%) = \frac{L_a}{L_t} \times 100 \quad (2)$$

where D_t was the amount of AZI encapsulated in microspheres and M_t was the amount of microspheres; L_a was the actual drug content and L_t was the theoretical drug content.

2.7. In vitro drug release studies

Ten mg of the dried microspheres were placed in test tubes and resuspended in 6 ml release medium, which consisted of PBS (50 Mm, pH 6.8), sodium azide (0.05%, w/v) and Tween 80 (0.2%, w/v). Then the suspension was incubated in an incubator at 37 °C with a shaking speed of 100 rpm. At appropriate intervals, the samples were centrifuged and 4 ml of supernatant was withdrawn. At the same time, 4 ml of fresh medium was added to the tubes. The drug concentration was determined by the same method as the estimation of the D.L. All the samples were treated in triplicate.

Besides, the similarity factor (f_2) method (Moore and Flanner, 1996; Shah et al., 1998) was applied in this study to assess the similarity of two dissolution profiles.

$$f_2 = 50 \times \lg \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{i=1}^{n} (T_i - R_i)^2 \right]^{-1/2} \times 100 \right\} \quad (3)$$

where R_i and T_i represented the cumulative drug dissolution of the reference formulation and the test formulation at the time point of i respectively, and n was the number of sampling. When the value of f_2 was between 50 and 100, the dissolution curves were considered to be similar; while if the value was less than 50, they were considered to have noticeable difference.

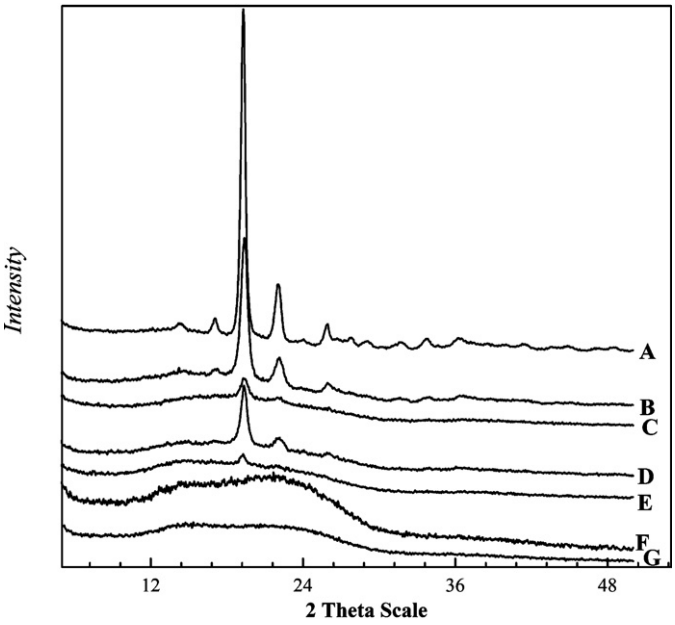

Fig. 2. X-ray powder diffractograms of (A) PLLA powder, (B) PLLA blank microspheres prepared by ASE method, (C) PLLA blank microspheres prepared by RSE method, (D) blank microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by ASE method, (E) blank microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by RSE method, (F) PLGA blank microspheres prepared by ASE method and (G) PLGA blank microspheres prepared by RSE method.

2.8. Statistic analysis

Statistic analysis was made with SPSS 16.0 software for windows® (SPSS®, Chicago, USA). One-way analysis of variance (ANOVA) was performed to assess statistical significance among different formulations of the microspheres. Results were considered statistically significant if $P < 0.05$.

3. Results and discussions

3.1. X-ray powder diffraction

The X-ray powder diffraction diagrams were shown in Figs. 2 and 3. PLLA blank microspheres prepared by the ASE method exhibited diffraction peaks at the 2θ of 17°, 19° and 22° (Fig. 2B) which coincided with those observed in PLLA powder (Fig. 2A). However, PLLA blank microspheres prepared by the RSE method showed nearly no peaks except for a small peak at the 2θ of 19° (Fig. 2C), suggesting that PLLA was mainly present in an amorphous state after the RSE process. Similar results were reported in the study of progesterone-loaded PLLA microspheres by Izumikawa et al. (1991). Poly(ε-caprolactone) (PCL), which was a semicrystalline polymer, was used to fabricate BSA loaded microspheres, and the DSC results were also showed that accelerating the solvent evaporation rate significantly reduced the crystallinity of this polymer (Lin and Yu, 2002). While for the amorphous polymer matrix of PLGA, there was no difference in XRD pattern between the ASE and RSE method (Fig. 2F and G). Using blends of PLGA/PLLA (1:1, w/w) as the preparation materials, the X-ray diffraction diagrams were similar to those of pure PLLA microspheres when the same solvent evaporation method was employed (Fig. 2D and E). It was suggested that the rate of solvent evaporation would be an important factor to determine the crystallinity of polymers in the solidification process

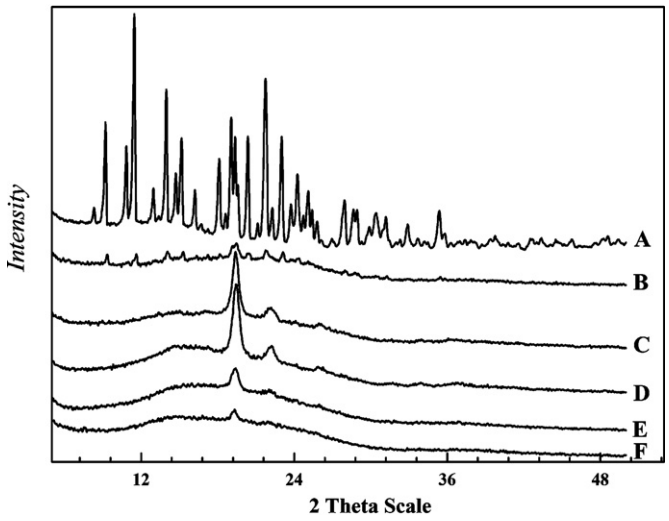

Fig. 3. X-ray powder diffractograms of (A) crystalline azithromycin, (B) physical mixtures of AZI (A) and blank microspheres (F) with a mass ratio at 1:11, (C) blank microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by ASE method, (D) 8.33 wt.% of AZI-loaded microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by ASE method, (E) 8.33 wt.% of AZI-loaded microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by RSE method and (F) blank microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by RSE method.

of emulsion solvent evaporation method (Izumikawa et al., 1991; Chung et al., 2001).

Sharp diffraction peaks at the 2θ of 11.48°, 13.98°, 15.14°, 19.08°, and 21.74° were present in the X-ray powder diffraction pattern of pure AZI (Fig. 3A). The same X-ray powder diffraction patterns could also be obtained from the physical mixtures of pure AZI and blank microspheres (Fig. 3B). In the case of ASE method, AZI-loaded microspheres showed no drug diffraction peaks compared with blank microspheres, and the visible peaks were attributed to the crystalline PLLA (Fig. 3C and D). Similarly, no X-ray powder diffraction of drug could be seen in AZI-loaded microspheres prepared by the RSE method (Fig. 3E and F), suggesting that AZI was changed into amorphous form during the microencapsulation process. The transformation might be due to the impediment effect of polymer chains on the drug crystallization process. The same results could also be found in the study of azithromycin-loaded PLGA nanoparticles (Mohammadi et al., 2010) and roxithromycin-loaded Eudragit S100 microspheres (Gao et al., 2006).

3.2. Differential scanning calorimetry (DSC)

Fig. 4 showed DSC thermographs of the drug and different formulations of microspheres. Both the ASE and RSE microspheres exhibited endothermic peaks around 170 °C due to the melting of PLLA (Fig. 4A and B). Glass transition in the PLLA blank microspheres prepared by the ASE method presented a step-like form (Fig. 4A). However, by the RSE method, it appeared as an endothermic peak (Fig. 4B). In addition, an exothermic peak derived from crystallization of amorphous PLLA could also be observed around 90 °C (Fig. 4B), which was in accordance with the result of Izumikawa et al. (1991), indicating that PLLA existed in an amorphous state in the RSE microspheres. For the PLGA microspheres, the polymer remained in an amorphous state in the microspheres prepared by both ASE and RSE methods (Fig. 4C and D). Using the blends of PLLA and PLGA (1:1, w/w) as the microencapsulation material, the DSC thermographs presented the properties of PLLA and PLGA microspheres when the same solvent evaporation method was applied. In addition, there were two glass transition temperatures in Fig. 4E and F respectively, implying that the two kinds of polymers became immiscible as the solvent evaporated which was in agreement with the report of Tan et al. (2005). Polymer–polymer immiscibility occurred in the solvent removal process had been investigated in the research of double-walled microsphere fabrication (Matsumoto et al., 1997; Pollauf and Pack, 2006). Both studies showed that, in the

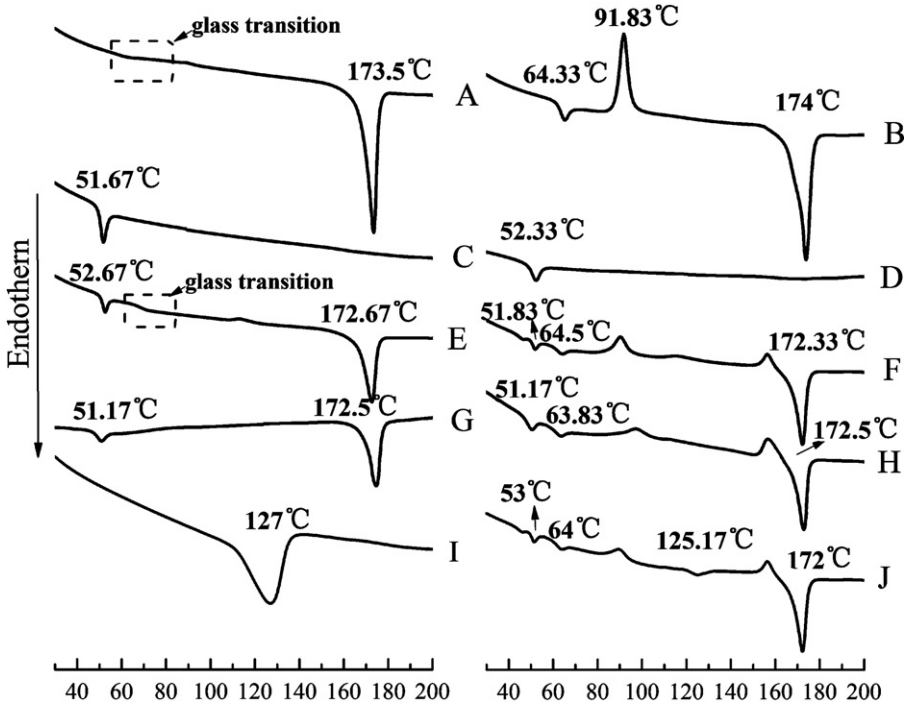

Fig. 4. DSC thermographs of drug and microspheres: (A, C, E, and G) microspheres prepared by atmosphere-solvent evaporation method; (B, D, F, and H) microspheres prepared by reduced pressure-solvent evaporation method. (A and B) PLLA blank microspheres; (C and D) PLGA blank microspheres; (E and F) blank microspheres with a PLLA/PLGA mass ratio 1:1; (G and H) 8.33 wt.% of AZI-loaded microspheres with a PLLA/PLGA mass ratio 1:1; (I) crystalline azithromycin; (J) physical mixtures of AZI (I) and blank microspheres (F) with a mass ratio 1:11.

polymer–polymer–organic solvent ternary system, the immiscible phenomenon could only come up when the total polymer concentration exceeded a critical level as the solvent evaporated and distinctive phase separation resulted in the formation of double-walled microspheres. No drug melting peaks were observed in the AZI-loaded microspheres prepared by either ASE or RSE method (Fig. 4G and H), indicating that the drug dispersed uniformly in the microspheres in an amorphous state. The above results coincided with that of X-ray diffraction.

3.3. Morphologic characteristics

The surface morphology of the microspheres was shown in Fig. 5. When the temperature was fixed at 25 °C, drug-loaded microspheres prepared by the ASE method had a porous surface with a few cracks (Fig. 5A), while smooth surface of drug-loaded microspheres could be seen with the RSE method (Fig. 5B and C). Similar results were also obtained in the report of Chung et al. (2001) when PLLA and PDLLA microspheres were prepared by both kinds of methods. Izumikawa et al. (1991) used the nitrogen adsorption/desorption method to determine the surface area of microspheres and further confirmed the above results. One possible reason for the surface difference was the crystallinity of the polymer matrix which was closely correlated with the morphology (Izumikawa et al., 1991). The microspheres with amorphous polymer matrices seemed to have smooth surfaces. Another major reason might be the fast surface solidification of emulsified droplets caused by the reduced pressure impeded the formation of "water channels" which always appeared in the microspheres with the emulsion solvent evaporation method. However, when the pressure was fixed at 460 mmHg, differences in surface morphology were observed by varying the temperature of the continuous phase. Pinholes existed on the surface of microspheres could be seen when the preparation temperature increased to 35 °C (Fig. 5D). In preparing peptide-loaded PLGA microspheres by the temperature gradient (Tmp) technique, microspheres with a hollow internal core and a porous wall were also obtained with a rapid ramp of temperature or a gradual temperature rise, which was coincided with the above phenomenon (Jeyanthi et al., 1996).

3.4. The choice of a suitable range of pressure at a given temperature

Theoretically, lower pressure could result in a quicker volatilization rate. But once the actual preparation pressure was much lower than the saturated vapor pressure of the solvent at a given temperature, the solvent began to boil and the formation of bubbles could destroy the emulsified droplets (Li et al., 2008). Therefore, the key point in this study was the choice of a suitable range of pressure at a fixed temperature. Antoine equation, which was widely used in engineering as an experienced equation due to its good agreement with the experimental data and simple expression, was applied here to firstly determine the values of pressure and temperature. The form of the equation was as follows:

$$\log_{10}(P) = A - \left(\frac{B}{T + C}\right) \quad (4)$$

where P was vapor pressure (bar) and T represented temperature (K); A, B and C were three constants that correlated to the properties of certain substance. Here, the methylene dichloride (DCM) was taken as an example and the values of A, B and C were 4.53691, 1327.016 and −20.474 respectively when the temperature was from 253 K to 313 K (data from National Institute of Standards and Technology (NIST)).

Besides, from the results of our study, the preparation pressure should not be lower than 385 mmHg at any given temperature to resist the formation of foams. The reason was that PVA, as an emulsifying agent and surfactant, could lower the surface tension of aqueous solution and gas bubbles were easily generated in the case of lower pressure. However, the pressure, which was slightly lower than the saturated vapor pressure, seemed to have no bad effect on the characteristics of microspheres.

3.5. Effects of process parameters on the physicochemical characteristics of the AZI-loaded microspheres

3.5.1. Solvent evaporation pressure

The solvent evaporation pressure on the physicochemical properties of the AZI-loaded microspheres was shown in Table 1. Microspheres prepared under the different pressures had the similar average particle size as reported by Meng et al. (2004). However, it was not in agreement with the study of Chung et al. (2001), in which the microspheres prepared by the RSE method had a smaller size than those prepared by the ASE method. The different results between the two studies might be due to the different droplet forming methods. In this study, emusified droplets were obtained by a homogenizer instead of the ultrasonic method applied by Chung et al. (2001). The influence of pressure on the size of the microspheres was not clear yet. One-way analysis of variance (ANOVA) method was used here to analyze the effect of the different pressures on the encapsulation efficiency of drug-loaded microspheres. Statistical analysis showed that there was a significant difference between the ASE and RSE method ($P < 0.05$) but the difference among the various pressures with the RSE method was not noticeable. The longer duration of the evaporation process was considered as the main cause for more drug loss in the ASE method (Izumikawa et al., 1991). From the results of single factor analysis of variance within groups in the RSE method, it could be concluded that the drug loss mainly happened in the stage of emulsification. Similar results were reported in the work of Ye et al. (2010) and Liu et al. (2006) that the mechanical breakage of the incipient microspheres was one major factor for the drug loss.

The AZI-loaded microspheres prepared by the ASE method had the highest "initial burst" due to the porous surface (Fig. 5A and B). Nevertheless, the microspheres with smooth surface produced at 610 mmHg also presented a relatively high "initial release". The possible reason was that short solidification time of 30 min resulted in a high residual solvent within the microspheres. The AZI molecules might migrate to the surface of microspheres in the process of centrifugation and lyophilization. As shown in Table 1 and Fig. 7, the highest encapsulation efficiency, lowest "initial burst" and slowest release were obtained when the microspheres were prepared at 385 mmHg. The results of drug release-curve fitting except for the "initial release" indicated that microspheres prepared by the ASE method presented a zero-order profile with a R^2 value of 0.9939; while those microspheres fabricated under reduced pressure mainly followed first-order kinetics except for F2 formulation which was fit well by Ritger–Peppas equation ($R^2 = 0.9942$). The R^2 values of F3, F4 and F5 formulations were 0.9920, 0.9958 and 0.9923 respectively. As an example, the SEM images of the microspheres underwent 45-day release study were presented in Fig. 6. After the release of the AZI molecules, many poles left behind on the microspheres, whereas the matrix of microspheres remained intact indicating the main release mechanism was diffusion.

The similarity factors (f_2) of the release profiles of the AZI-loaded microspheres prepared under different preparation pressures were presented in Table 2. Except for the microspheres prepared under 610 mmHg (which was the most close to atmospheric pressure),

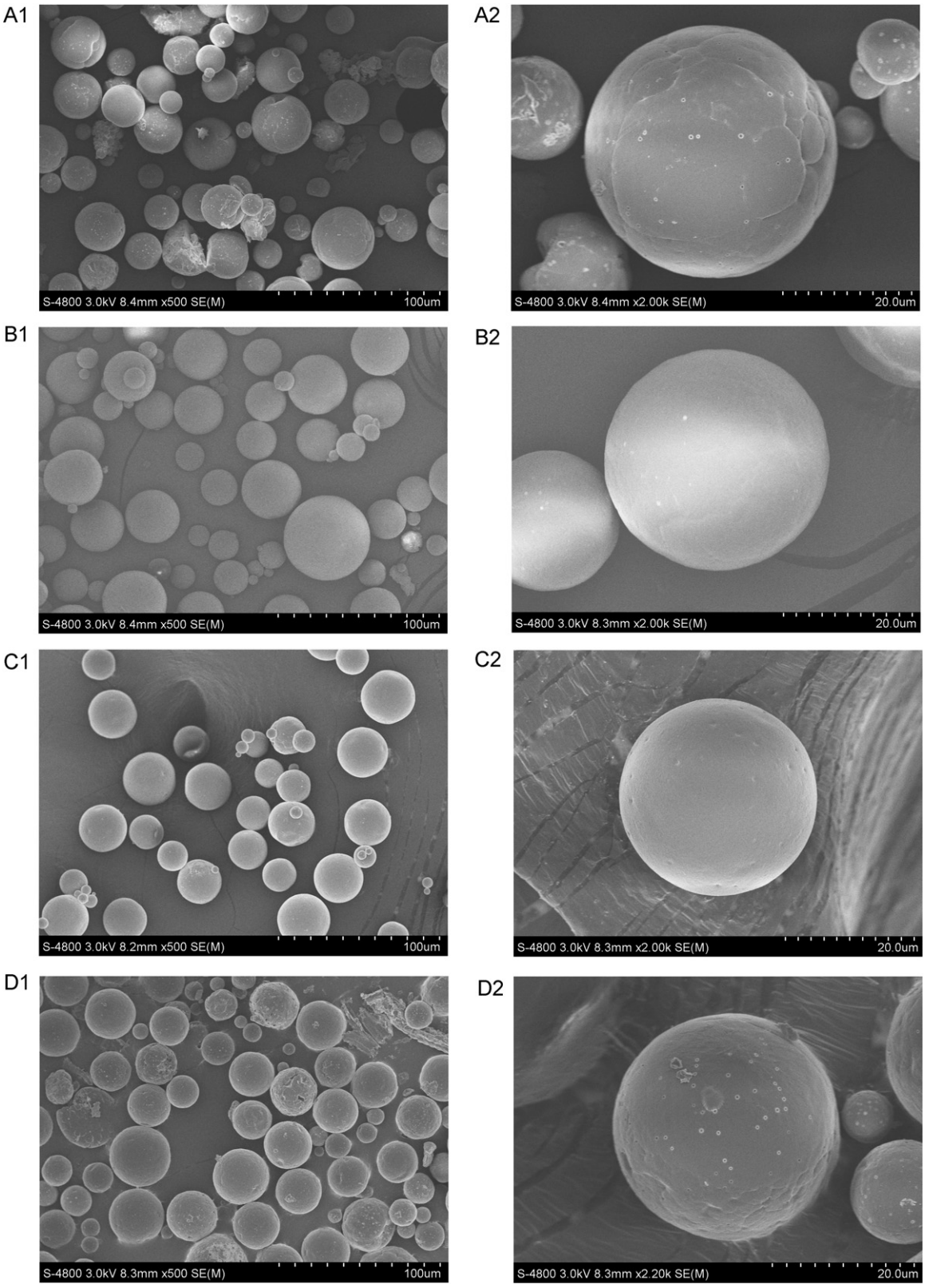

Fig. 5. SEM images of AZI-loaded microspheres with a PLLA/PLGA mass ratio at 1:1 prepared by the ASE and RSE method: (A) AZI-loaded microspheres prepared under atmospheric pressure at 25 °C; (B and C) AZI-loaded microspheres prepared at 25 °C under reduced pressure at 610 mmHg and 460 mmHg respectively; (D) AZI-loaded microspheres prepared at 35 °C with the pressure of 460 mmHg. A1, B1, C1 and D1 represented the micrographs taken at 500×, and the size of the bar was 100 μm; A2, B2, C2 and D2 represented the micrographs taken at 2000×, and the size of the bar was 20 μm.

Table 1
The effect of preparation pressure on the properties of AZI-loaded microspheres ($n = 3$, $\bar{x} \pm$ S.D.).

Formulation code	Pressure[a] (mmHg)	Mean particle size (μm)	Actual loading (%)	E.E. (%)	Burst release (%)
F1	760 (ASE)	14.11 ± 0.78	6.67 ± 0.20	39.94 ± 1.18*	23.96 ± 2.01
F2	610 (RSE)	14.17 ± 0.67	8.76 ± 0.30	52.61 ± 1.78	15.47 ± 0.75
F3	535 (RSE)	14.06 ± 0.58	9.12 ± 0.55	54.60 ± 3.27	9.02 ± 1.01
F4	460 (RSE)	14.45 ± 0.57	8.33 ± 0.33	49.97 ± 1.98	6.62 ± 0.71
F5	385 (RSE)	15.29 ± 0.67	9.55 ± 0.64	57.19 ± 3.81	4.12 ± 0.15

[a] The relative pressure within the reaction bottle.
* $P < 0.05$

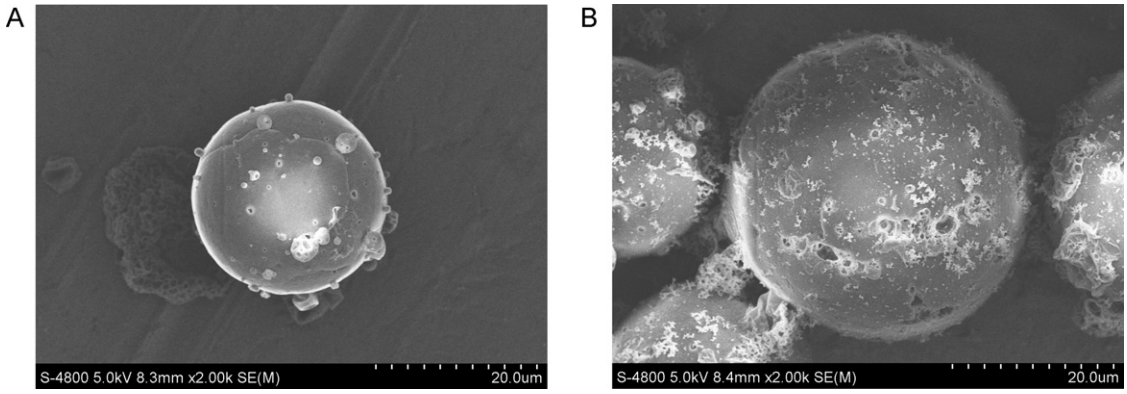

Fig. 6. Scanning electron micrographs of AZI-loaded microspheres underwent 45-day release study: (A) AZI-loaded microspheres prepared under atmospheric pressure; (B) AZI-loaded microspheres prepared at 460 mmHg.

Table 2
Comparison of similarity factors (f_2) of AZI-loaded microspheres with different preparation pressures.

Code	F1 (760 mmHg)	F2 (610 mmHg)	F3 (535 mmHg)	F4 (460 mmHg)	F5 (385 mmHg)
F1	100[a]	78	50	44	37
F2		100[a]	53	46	37
F3			100[a]	72	52
F4				100[a]	62
F5					100[a]

[a] Reference formulation.

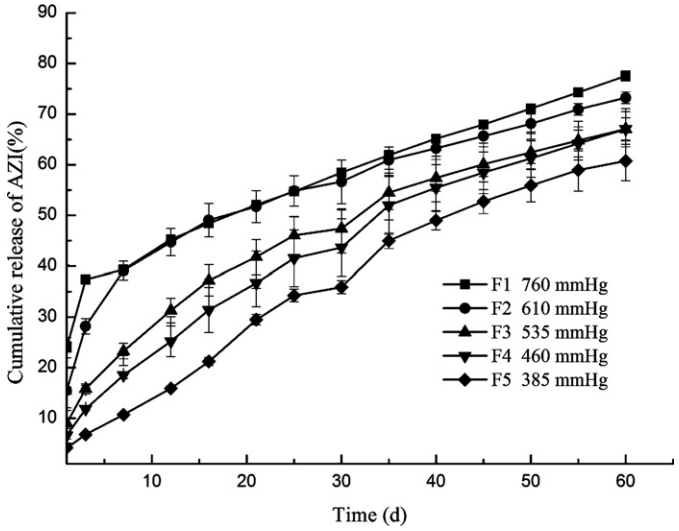

Fig. 7. Release profiles of AZI from microspheres prepared under different pressures ($n = 3$) (F1: ASE (760 mmHg); F2: 610 mmHg; F3: 535 mmHg; F4: 460 mmHg; F5: 385 mmHg).

the release profiles of the microspheres prepared with the reduced pressures were obviously different from the microspheres prepared under atmospheric pressure. It was implied that the solvent removal rate had significant effects on the in vitro drug release behaviors of the polymeric microspheres.

3.5.2. Duration of solidification

The effect of the duration of solidification on the physicochemical properties of the microspheres was presented in Table 3 and Fig. 8. The results showed that the solidification time had negligible effect on the particle size of the microspheres. Besides, solidification time had nearly no effect on the encapsulation efficiency of drug-loaded microspheres ($P > 0.05$), which might be due to the fast surface solidification of emulsified droplets hindering the drug leakage from the droplets as the solvent evaporated. This result further implied that the drug was mainly lost in the process of forming O/W emulsified droplets. However the duration of the evaporation process had a slight effect on the release kinetics. The microspheres produced with the solidification time of 20 min had the largest "initial burst". It could be ascribed to the incomplete solidification of the microspheres and further migration of the drug occurred during subsequent drying. The similar in vitro release profiles of the microspheres were obtained when the solidification time exceeded 30 min.

Table 3
The effect of solidification time on the properties of AZI-loaded microspheres prepared under reduced pressure (460 mmHg) ($n = 3$, $\bar{x} \pm$ S.D.).

Formulation code	Solidification time (min)	Mean particle size (μm)	Actual loading (%)	E.E. (%)	Burst release (%)
F4a	20	12.38 ± 0.82	8.30 ± 0.14	49.83 ± 0.86	12.58 ± 1.93
F4	30	14.45 ± 0.57	8.33 ± 0.33	49.97 ± 1.98	6.62 ± 0.71
F4b	40	11.80 ± 0.74	8.47 ± 0.22	50.82 ± 1.32	5.16 ± 0.28
F4c	60	12.57 ± 0.91	7.63 ± 0.08	45.79 ± 0.46	4.33 ± 0.10
F4d	120	11.62 ± 0.64	8.11 ± 0.37	48.49 ± 2.24	5.05 ± 0.32

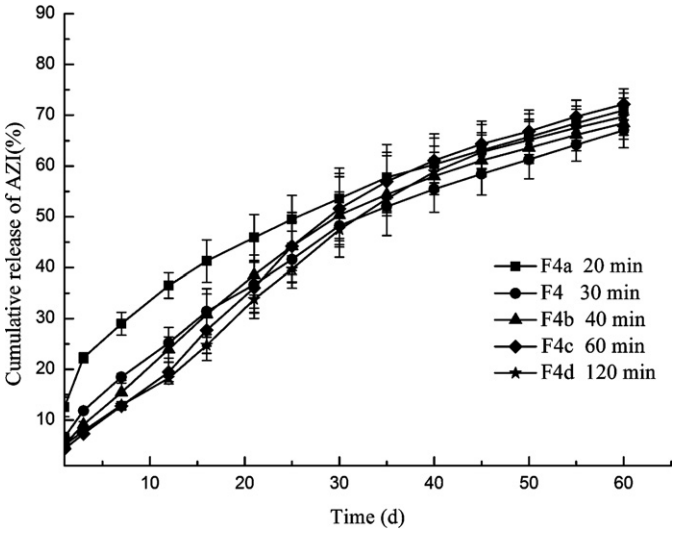

Fig. 8. Release profiles of AZI from microspheres prepared with different solidification time under reduced pressure (460 mmHg) ($n = 3$) (F4a: 20 min; F4: 30 min; F4b: 40 min; F4c: 60 min; F4d: 120 min).

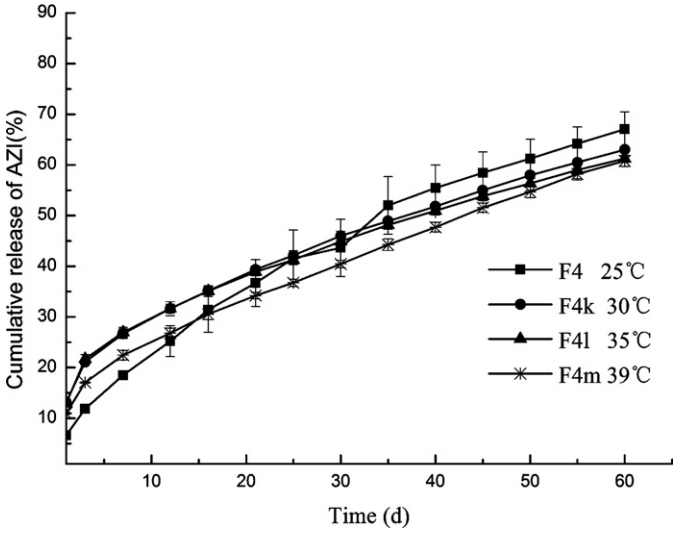

Fig. 9. Release profiles of AZI from microspheres prepared at different temperatures under reduced pressure (460 mmHg) ($n = 3$) (F4: 25 °C; F4k: 30 °C; F4l: 35 °C; F4m: 39 °C).

3.5.3. Temperature of continuous phase

As shown in Table 4, a higher preparation temperature seemed to lead to smaller size of the microspheres. The possible reason was that the decrease in the viscosity of the dispersed phase caused by the increasing temperature facilitated high dispersion of the O/W emulsion. The encapsulation efficiency had a tendency of increasing with an increase in the temperature, which coincided with the result of Miyazaki et al. (2006).

The probable reason was that faster solvent evaporation rate at the high temperature facilitated the formation of the microspheres and the rapid solidification of the polymer impeded the migration of the drug that dispersed in or on the surface of the microparticles.

The microspheres prepared at the higher temperature showed higher "initial burst" in the in vitro release study, and this might be due to the increased porosity and decreased particle size of microspheres produced at higher temperature (Fig. 5D). The microspheres prepared at 39 °C also showed a first-order release behavior ($R^2 = 0.9972$). The results were presented in Fig. 9.

3.5.4. Drug to polymer ratio

The effect of the ratio of drug to polymer on the physicochemical properties of the microspheres was illustrated in Table 5 and Fig. 10. In this study, the drug to polymer ratio was modulated by varying the amount of the drug. Therefore, particle size did not show any noticeable change as the concentration of the polymer retained constant (Table 5). However, the encapsulation efficiency was improved with an increase in drug–polymer ratio. The possible reason was that the more drug was added into the oil phase, the more drug would be encapsulated as the concentration of the drug in the continuous phase reached the saturation solubility during the emulsification process. As a result, the amount of drug in per unit polymer with a higher drug–polymer ratio formulation was greater than that in other formulations. Microspheres with largest drug

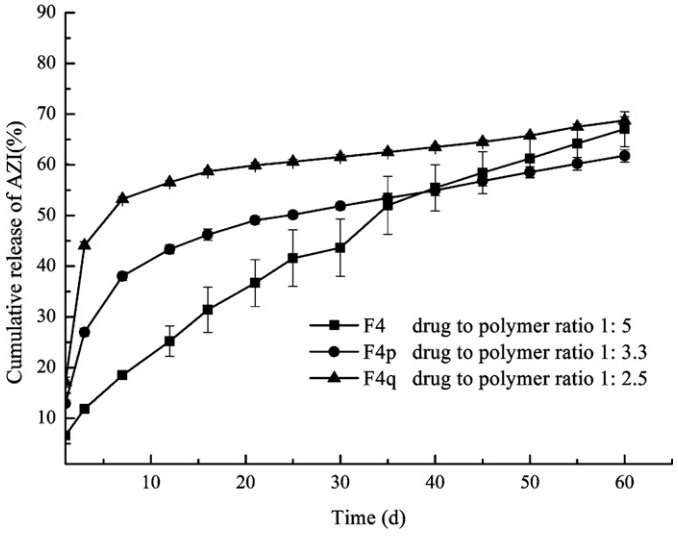

Fig. 10. Release profiles of AZI from microspheres prepared with different drug to polymer ratios under reduced pressure (460 mmHg) ($n = 3$) (F4: Drug to polymer ratio 1:5 (w/w); F4p: 1:3.3 (w/w); F4q: 1:2.5 (w/w)).

content presented a biphasic release style, which had a quick drug release in the first 15 days and then followed by a slowly zero-order release profile ($R^2 = 0.9901$) in the in vitro release experiments.

3.5.5. pH value of continuous phase

It has been reported that azithromycin is a weak base with a high solubility of 440 mg/ml at pH 2.9 and 5 mg/ml at pH 7.4; but when the pH value increases to 10.3, the solubility is only 0.005 mg/ml (Hagen et al., 2005; William, 2011). In this study, the PVA solution (1%, w/v) was found to have a pH value of 6.7 (25 °C). In view of

Table 4
The effect of temperature of the continuous phase on the properties of AZI-loaded microspheres prepared under reduced pressure (460 mmHg) ($n = 3$, $\bar{x} \pm$ S.D.).

Formulation code	$C{\cdot}P{\cdot}T^a$ (°C)	Mean particle size (μm)	Actual loading (%)	E.E. (%)	Burst release (%)
F4	25	14.45 ± 0.57	8.33 ± 0.33	49.97 ± 1.98	6.62 ± 0.71
F4k	30	12.11 ± 0.86	8.42 ± 0.22	50.52 ± 1.32	13.15 ± 0.23
F4l	35	12.22 ± 0.92	8.63 ± 0.09	51.77 ± 0.56	12.89 ± 0.60
F4m	39	11.65 ± 0.78	9.08 ± 0.19	54.46 ± 1.15	10.97 ± 0.18

[a]The temperature of the continuous phase.

Table 5
The effect of drug to polymer ratio on the properties of AZI-loaded. microspheres prepared under reduced pressure (460 mmHg) ($n = 3$, $\bar{x} \pm$ S.D.).

Formulation code	Drug to polymer ratio	Mean particle size (μm)	Actual loading (%)	E.E. (%)	Burst release (%)
F4	1:5	14.45 ± 0.57	8.33 ± 0.33	49.97 ± 1.98	6.62 ± 0.71
F4p	1:3.3	13.83 ± 0.80	13.21 ± 0.27	57.26 ± 1.15	12.93 ± 0.56
F4q	1:2.5	13.44 ± 0.79	18.80 ± 0.14	65.78 ± 0.48	17.15 ± 0.99

Table 6
The effect of the pH value of the continuous phase on the properties of AZI-loaded microspheres prepared under reduced pressure (460 mmHg) ($n = 3$, $\bar{x} \pm$ S.D.).

Formulation code	pH value	Mean particle size (μm)	Actual loading (%)	E.E. (%)	Burst release (%)
F4	6.7	14.45 ± 0.57	8.33 ± 0.33	49.97 ± 1.98	6.62 ± 0.71
F6	10.3	13.62 ± 0.79	14.06 ± 0.35	84.04 ± 2.09	14.73 ± 1.06

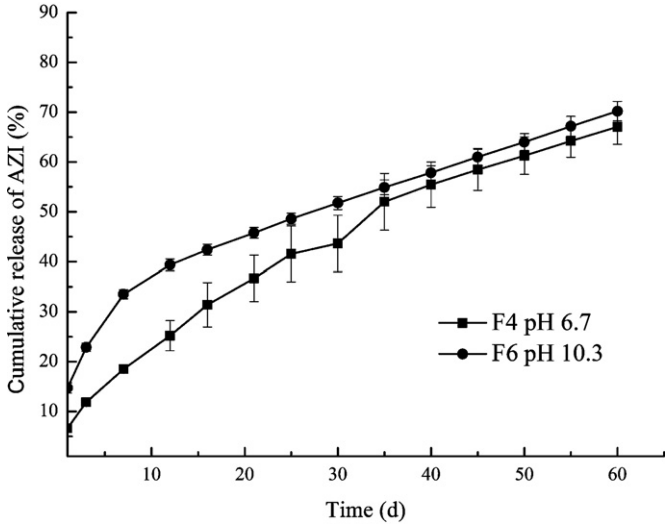

Fig. 11. Release profiles of AZI from microspheres fabricated in the different pH circumstances under reduced pressure (460 mmHg) ($n = 3$) (F4: pH 6.7; F6: pH 10.3).

the pH-dependent solvability of azithromycin, the pH value of the continuous phase was adjusted to 10.3 to decrease the drug solubility. As was shown in Table 6, the encapsulation efficiency of the microspheres could be high up to 84.04 ± 2.09%. The less drug loss might happen in the process of microsphere washing. Besides, the pH value showed no obvious effect on the particle size ($P > 0.05$). The results of the in vitro release studies were given in Fig. 11. Microspheres with higher drug loading had larger initial burst and cumulative release. This could be ascribed to the larger osmotic pressure difference existed in the higher drug loading formulations when kept in the in vitro environment.

4. Conclusions

Different physical characteristics and drug release profiles were observed in the azithromycin-loaded microspheres prepared with the atmosphere-solvent evaporation (ASE) method and the reduced pressure-solvent evaporation (RSE) method. Both X-ray diffractograms and DSC graphs showed that poly(L-lactide) in the microspheres produced under reduced pressure was in an amorphous state while crystalline forms of poly(L-lactide) were present in the ASE formulations, suggesting that the crystallinity of polymers was significantly affected by the rate of solvent removal in solidification process of emulsified droplets. Smoother surface, higher encapsulation efficiency as well as lower "initial burst" and slower cumulative release rate were obtained in the RSE microspheres compared with the ASE microspheres. An increase in the temperature of the continuous phase could result in an increased encapsulation efficiency, but a higher "initial release". Due to the pH-dependent solubility of azithromycin, incorporation efficiency was improved greatly in the higher pH environment. Other process parameters, i.e. solidification time and drug–polymer ratio were also found to have obvious effects on the physicochemical characteristics and release behaviors of the microspheres. The overall improvement of microspheres in appearance, encapsulation efficiency and controlled drug release could be easily fulfilled using the reduced pressure-solvent evaporation method. The features including short preparation time, effective solvent recovery as well as scaled-up potential enable the RSE method a promising technique to design biodegradable polymeric microspheres for bioactives.

Acknowledgements

This work was partially supported by the National Natural Science Foundation of China (nos. 20836005 and 21176173). And the authors also wished to thank Japanese Synthetic Chemical Industry Co., Ltd. for their kindness in supplying the PVA.

References

Angadi, S.C., Manjeshwar, L.S., Aminabhavi, T.M., 2011. Stearic acid-coated chitosan-based polymer network microspheres: controlled release characteristics. Ind. Eng. Chem. Res. 50, 4504–4514.
Ballow, C.H., Amsden, G.W., 1992. Azithromycin: the first azalide antibiotic. Ann. Pharmacother. 26, 1253–1261.
Berkland, C., King, M., Cox, A., Kim, K.K., Pack, D.W., 2002. Precise control of PLG microsphere size provides enhanced control of drug release rate. J. Control. Release 82, 137–147.
Chaturvedi, K., Kulkarni, A.R., Aminabhavi, T.M., 2011. Blend microspheres of poly(3-hydroxybutyrate) and cellulose acetate phthalate for colon delivery of 5-fluorouracil. Ind. Eng. Chem. Res. 50, 10414–10423.

Chung, T.W., Huang, Y.Y., Liu, Y.Z., 2001. Effects of the rate of solvent evaporation on the characteristics of drug loaded PLLA and PDLLA microspheres. Int. J. Pharm. 212, 161–169.
Freiberg, S., Zhu, X.X., 2004. Polymer microspheres for controlled drug release. Int. J. Pharm. 282, 1–18.
Freytag, T., Dashevsky, A., Tillman, L., Hardee, G.E., Bodmeier, R., 2000. Improvement of the encapsulation efficiency of oligonucleotide-containing biodegradable microspheres. J. Control. Release 69, 197–207.
Gao, Y., Cui, F.D., Guan, Y., Yang, L., Wang, Y.S., Zhang, L.N., 2006. Preparation of roxithromycin-polymeric microspheres by the emulsion solvent diffusion method for taste masking. Int. J. Pharm. 318, 62–69.
Girard, A.E., Girard, D., English, A.R., Gootz, T.D., Cimochowski, C.R., Faiella, J.A., Haskell, S.L., Retsema, J.A., 1987. Pharmacokinetic and in vivo studies with azithromycin (CP-62,993) a new macrolide with an extend half-life and excellent tissue distribution. Antimicrob. Agents Chemother. 31, 1948–1954.
Hagen, T., Lo, J.B., Thombre, A., Herbig, S., Appel, L., Crew, M., 2005. Azithromycin dosage forms with reduced side effects. Patent No.: EP1, 537, 859B1.
He, J.T., Su, H.B., Li, G.P., Tao, X.M., Mo, W., Song, H.Y., 2006. Stabilization and encapsulation of a staphylokinase variant (K35R) into poly(lactic-*co*-glycolide acid) microspheres. Int. J. Pharm. 309, 101–108.
Igartua, M., Hernandez, R.M., Rosas, J.E., Patarroyo, M.E., Pedraz, J.L., 2008. Gamma-Irradiation effects on biopharmaceutical properties of PLGA microspheres loaded with SPf66 synthetic vaccine. Eur. J. Pharm. Biopharm. 69 (2), 519–526.
Izumikawa, S., Yoshioka, S., Aso, Y., Takeda, Y., 1991. Preparation of poly(L-lactide) microspheres of different crystalline morphology and effect of crystalline morphology on drug release rate. J. Control. Release 15, 133–140.
Jeyanthi, R., Mehta, R.C., Thanoo, B.C., Deluca, P.P., 1996. Effects of solvent removal technique on the matrix characteristics of polylactide: glycolide microspheres for peptide delivery. J. Control. Release 38, 235–244.
Li, M., Rouaud, O., Poncelet, D., 2008. Microencapsulation by solvent evaporation: state of the art for process engineering approaches. Int. J. Pharm. 363, 26–39.
Lin, W.J., Yu, C.C., 2002. The effect of solvent removal conditions on performance and release property of protein-loaded microparticles. J. Microencapsulation 19, 767–773.
Liu, R., Huang, S.S., Wan, Y.H., Ma, G.H., Su, Z.G., 2006. Preparation of insulin-loaded PLA/PLGA microcapsules by a novel membrane emulsification method and its release in vitro. Colloids Surf. B: Biointerface 51, 30–38.
Matsumoto, A., Matsukawa, Y., Suzuki, T., Yoshino, H., Kobayashi, M., 1997. The polymer-alloys method as a new preparation method of biodegradable microspheres: principle and application to cisplatin loaded microspheres. J. Control. Release 48, 19–27.
Meng, F.T., Ma, G.H., Liu, Y.D., Qiu, W., Su, Z.G., 2004. Microencapsulation of bovine hemoglobin with high bio-activity and high entrapment efficiency using a W/O/W double emulsion technique. Colloids. Surf. B: Biointerface 33, 177–183.
Miyazaki, Y., Onuki, Y., Yakou, S., Takayama, K., 2006. Effect of temperature-increase rate on drug release characteristics of dextran microspheres prepared by emulsion solvent evaporation process. Int. J. Pharm. 324, 144–151.
Mohammadi, G., Valizadeh, H., Mohammad, B.J., Lotfipour, F., Adibkia, K., Milani, M., Azhdarzadeh, M., Kiafar, F., Nokhodchi, A., 2010. Development of azithromycin-PLGA nanoparticles: physicochemical characterization and antibacterial effect against *Salmonella typhi*. Colloids Surf. B: Biointerface 80, 34–39.
Moore, J.W., Flanner, H.H., 1996. Mathematical comparison of curves with an emphasis on in vitro dissolution profiles. Pharm. Technol. 20, 64–74.
Mundargi, R.C., Babu, V.R., Rangaswamy, V., Pradip, P., Aminabhavi, T.M., 2008. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. J. Control. Release 125, 193–209.
National Institute of Standards and Technology (NIST), 2011. http://webbook.nist.gov/cgi/cbook.cgi?ID=C75092&Units=SI&Mask=4#Thermo-phase (accessed 08.10.11).
Oh, Y.J., Lee, J., Seo, J.Y., Rhim, T., Kim, S.H., Yoon, H.J., Lee, K.Y., 2011. Preparation of budesonide-loaded porous PLGA microparticles and their therapeutic efficacy in a murine asthma model. J. Control. Release 150, 56–62.
Pekarek, K.J., Dyrud, M.J., Ferrer, K., Jong, Y.S., Mathiowitz, E., 1996. In vitro and in vivo degradation of double-walled polymer microspheres. J. Control. Release 40, 169–178.
Pollauf, E.J., Pack, D.W., 2006. Use of thermodynamic parameters for design of double-walled microsphere fabrication methods. Biomaterials 27, 2898–2906.
Shah, V.P., Tsong, Y., Sathe, P., Liu, J.P., 1998. In vitro dissolution profile comparison-statistics and analysis of the similarity factor, f_2. Pharm. Res. 15, 889–896.
Sultana, N., Arayne, M.S., Hussain, F., Fatima, A.F., 2006. Degradation studies of Azithromycin and its spectrophotometric determination in pharmaceutical dosage forms. Pak. J. Pharm. Sci. 19, 98–103.
Tan, E.C., Lin, R., Wang, C.H., 2005. Fabrication of double-walled microspheres for the sustained release of doxorubicin. J. Colloid Interface Sci. 291, 135–143.
Tan, H.X., Ye, J.D., 2008. Surface morphology and in vitro release performance of double-walled PLLA/PLGA microspheres entrapping a highly water-soluble drug. Appl. Surf. Sci. 255, 353–356.
William, C., 2011. Interdisciplinary science and the design of a single-dose antibiotic therapy. Pharm. Res. 28 (9), 2059–2071.
Yang, Y.Y., Chung, T.S., Bai, X.L., Chan, W.K., 2000. Effect of preparation conditions on morphology and release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion method. Chem. Eng. Sci. 55, 2223–2236.
Ye, M.L., Kim, S.W., Park, K., 2010. Issues in long-term protein delivery using biodegradable microparticles. J. Control. Release 146, 241–260.