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Investigation on structural integrity of PLGA during ammonolysis-based microencapsulation process

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ABSTRACT

The objective of this study was to gain insights into the structural integrity of PLGA during an ammonolysis-based microencapsulation process. PLGA (lactide:glycolide ratio = 75:25; M_w = 25,925 g/mol) was dissolved in ethyl acetate or isopropyl formate (3–6 ml), which was emulsified in an aqueous phase. Ammonia, being added to the emulsions, reacted with the dispersed solvents to yield water-miscible solvents. Consequently, emulsion droplets were solidified into microspheres. To evaluate the impact of ammonia upon PLGA, the molar ratio of ammonia to a dispersed solvent varied from 1 to 2 and 3. After preparation of microspheres by the ammonolysis-based procedure, the lactide:glycolide composition and Mw of PLGA were analyzed by ^{1}H NMR and GPC. Our results demonstrated that ammonia did indeed catalyze the cleavage of PLGA ester bonds during microencapsulation. Strikingly, PLGA degradation was affected by solvent type and volume, as well as ammonia concentration. For instance, when 6 ml of ethyl acetate was used and the molar ratio of ammonia to the solvent was 3, the glycolide content and M_w of the microspheres considerably decreased to 17.56% and 10,814 g/mol, respectively. There were little changes in these terms, however, when microspheres were prepared using 3 ml of isopropyl formate and an equimolar amount of ammonia. Depending upon microencapsulation conditions, progesterone encapsulation efficiency ranged from 71.6 to 98.8%. Also, its release behavior was significantly influenced by ammonolysis-related process parameters. Our study demonstrated that all these contrasting results arose from differences in solvent reactivity toward ammonolysis, the rate of microsphere solidification, and the availability of ammonia to PLGA ester linkages.

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1. Introduction

Poly-D,L-lactide-co-glycolide (PLGA) microspheres are being increasingly utilized as injectable depot systems for extended release of various bioactive materials. Oil-in-water (o/w) emulsion-based solvent extraction/evaporation processes have been frequently used in the practice of microencapsulation processes (Gouin, 2004; Jain, 2000; Katou et al., 2008). Such preparation techniques include the preparation of a polymeric dispersed phase, its emulsification in an aqueous continuous phase, and solvent removal. A solvent evaporation process typically uses a water-immiscible halogenated solvent such as methylene chloride having strong volatility. A solvent extraction process opts to use ethyl acetate with relatively high water miscibility. There have been demands on the development of an alternative microencapsulation process that does not use a halogenated organic solvent and improvement of the processing of solvent extraction (Freitas et al., 2005; Ng et al., 2010; Yeo and Park, 2004).

Recently, a new concept has been proposed for solvent removal: Instead of conventional physical methods to proceed with solvent extraction, a chemical approach such as ammonolysis is utilized as a means to complete solvent removal (Im and Sah, 2009). For instance, a dispersed phase consisting of PLGA and isopropyl formate is first emulsified in an aqueous phase. Ammonia, when added into the emulsion, reacts with the water-immiscible solvent in emulsion droplets into water-miscible isopropanol and formamide. Their leaching to the aqueous continuous phase leads to the solidification of emulsion droplets into microspheres. This new ammonolysis-based microencapsulation procedure does not require a quenching liquid, nor does it need a sophisticated manufacturing device. However, the use of ammonia for the ammonolysis-based microencapsulation process raises a concern because the nucleophilic compound may bring about destabilization of PLGA and/or drug (Wischke and Schwendeman, 2008). It was reported elsewhere that nucleophilic compounds (e.g., risperidone, olanzapine, thioridazine and ketotifen) catalyzed the cleavage of ester bonds in PLGA during microencapsulation (Maulding et al.,

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1986; Thanoo et al., 2005; Wright et al., 2001). Therefore, there is a question such as 'how does PLGA react to ammonia during the ammonolysis-based microencapsulation process?' On recognition of this issue, this study aimed at gaining insights into the structural integrity of PLGA microspheres during the ammonolysis-based microencapsulation process. It attempted to scrutinize critical parameters influencing PLGA degradation during the microencapsulation process. At present, ethyl acetate is a solvent of choice in the practice of a typical solvent extraction-based microencapsulation process (Freitas et al., 2005). As mentioned before, isopropyl formate has been found to be a useful solvent for an ammonolysis-based microencapsulation process. Therefore, the two non-halogenated organic solvents were chosen as dispersed solvents for PLGA, and their reactivity toward ammonolysis was compared to each other. A design of experiment was applied for randomizing the order of microsphere preparation under different microencapsulation conditions. The characteristics of these PLGA microspheres were assessed, especially in terms of their lactide:glycolide composition, molecular weight and polydispersity. Finally, it should be mentioned that long-acting progesterone dosage forms have been used for controlling human fertility and maintaining veterinary pregnancy (Beck et al., 1979; Gangrade and Price, 1991; Latha et al., 2000; Vanderwall et al., 2007). The drug has also been frequently chosen as a hydrophobic model drug in order to study its release behavior from polymeric dosage forms and to investigate the physical status of polymer and drug (Benoit et al., 1986; Izumikawa et al., 1991; Kawaguchi, 2000). Based on these earlier studies, progesterone was selected as a model drug in order to evaluate the influence of ammonolysis-based microencapsulation process parameters upon drug release behavior.

2. Materials and methods

2.1. Materials

Poly-D,L-lactide-co-glycolide with a lactide:glycolide ratio of 75:25 (inherent viscosity = 0.25 dl/g in $CHCl_3$ at 30 °C; Lakeshore Biomaterials™) was obtained from SurModics Pharmaceuticals (AL, USA). This polymer was abbreviated as PLGA in text. Polyvinyl alcohol (PVA; 88% hydrolyzed, MW = 25,000) was supplied from Polysciences, Inc. (PA, USA). Isopropyl formate and ethyl acetate were purchased from Alfa Aesar (MA, USA) and Fisher Scientific (PA, USA), respectively. Progesterone was obtained from Sigma–Aldrich (MO, USA). A 28% ammonia solution was from Junsei Chemical Co., Ltd. (Tokyo, Japan). Butanol was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). Tetrahydrofuran was obtained from Burdick & Jackson (MI, USA). The supplier of $CDCl_3$ was Cambridge Isotope Laboratories, Inc. (MA, USA).

2.2. Measurement of solvent reactivity toward ammonolysis

Two organic solvents (isopropyl formate and ethyl acetate) were used as dispersed solvents for the ammonolysis-based microencapsulation process. Upon reaction with ammonia, isopropyl formate and ethyl acetate give rise to isopropyl alcohol and ethanol, respectively (Im and Sah, 2009). Therefore, measuring dynamic changes in their aqueous concentrations as a function of time would allow comparing the reactivity of ethyl acetate and isopropyl formate toward ammonolysis. To do so, 3 ml of a 28% ammonia solution were added into 40 ml of a 0.5% PVA aqueous solution. Four milliliters of ethyl acetate or isopropyl formate were carefully layered on the top of the solution. To keep the two layers from being emulsified, the mixture was gently stirred at 180 rpm using a digital hotplate magnetic stirrer (model 400 series; VWR Scientific, PA, USA). Aliquots (300 μl) of the aqueous phase were taken out at time intervals of 3, 8, 15, 45, and 60 min. The sample solutions were immediately diluted to 50 times with butanol, which were treated with $MgSO_4$ to remove residual water. After filtration through a 0.45 μm pore-size PTFE syringe filter, a portion (500 μl) of the filtrate was spiked with 100 μl of methanol used as an internal standard for GC analysis.

2.3. Gas chromatography

The aqueous concentrations of ethanol and isopropanol were measured by gas chromatography (model GC-2010AF; Shimadzu Corp., Kyoto, Japan). The Zebron ZB-624 column (30 m × 0.32 mm) with a 0.18 μm film of 6%-cyanopropylphenyl–94%-methyl polysiloxane was used as a stationary phase. Nitrogen was used as a mobile phase at the flow rate of 1.3 ml/min. The injector and detector temperature were set at 200 and 220 °C, respectively. The oven temperature was maintained at 80 °C for 5.1 min, and its temperature was increased to 180 °C at the rate of 200 °C/min. The sample injection volume was 1 μl.

2.4. Application of a design of experiment in the preparation of microspheres

Microspheres were prepared according to an ammonolysis-based microencapsulation process as follows. PLGA (0.25 g) was dissolved in a dispersed solvent (solvent type = ethyl acetate or isopropyl formate; solvent volume = 3, 4.5 or 6 ml). Each dispersed phase was emulsified at 550 rpm in 40 ml of the 0.5% PVA aqueous solution. Added this emulsion was 2.2–13.5 ml of the ammonia solution (the molar ratio of ammonia to a dispersed solvent = 1, 2 or 3), and the mixture was subject to stirring for 2 h. One hundred fifty milliliters of water were poured into the microsphere suspension, which was subject to another 1-h stirring. After filtration, the microspheres were redispersed in 200 ml of a 0.1% PVA aqueous solution for 2 h. They were collected by filtration and dried inside a vacuum oven. Three replicates of a microsphere batch were prepared for a given manufacturing condition.

In the above experiments the level of treatment for solvent volume (independent variable) was 3 (3, 4.5, and 6 ml; In all cases, the amount of PLGA used to prepare microspheres was fixed at 0.25 g). This selection was based on the fact that typical microencapsulation procedures used dispersed phases having 4–10% PLGA concentration (a dispersed phase having PLGA concentrations below 4% usually leads to the formation of nonspherical and flake-type microspheres. The opposite case of using too concentrated dispersed phase tends to yield compact microspheres that are prone to aggregation and contain high amounts of residual solvents). Three levels of treatment was also given to another independent variable (ammonia:solvent molar ratio), based on the reaction stoichiometry of ammonolysis—one molecule of ammonia reacted with one molecule of each organic solvent (obviously, a microencapsulation condition using a 3:1 molar ratio indicates that an excessive amount of ammonia is used for ammonolysis). After preparation of microspheres, the impact of variations in the independent variables upon the structural integrity of PLGA was evaluated by measuring two major dependent variables, i.e., the lactide:glycolide composition of PLGA and its molecular weight.

The Design-Expert® (Version 7.1, State-Ease) software was used to generate a randomized run order for microsphere preparation. Solvent volume and ammonia concentration would be of great importance with regard to PLGA degradation that might occur during the ammonolysis-based microencapsulation process. Therefore, three levels were set up for each factor, as described in the above section of microsphere preparation. To increase statistical power, three replicates of a microsphere formulation were prepared under the same manufacturing condition. Table 1 displays

Table 1
Design layout of microsphere preparation in randomized run order with ethyl acetate used as a dispersed solvent (3 levels of treatment for each independent variable).

Run order	Independent variables	
	Solvent volume (ml)	Molar ratio of ammonia to solvent
1	4.5	2
2	6	2
3	6	3
4	6	1
5	6	1
6	3	2
7	3	3
8	4.5	3
9	6	1
10	3	3
11	3	3
12	4.5	3
13	4.5	1
14	6	3
15	4.5	2
16	6	2
17	3	1
18	4.5	2
19	3	1
20	3	2
21	3	2
22	6	3
23	6	2
24	4.5	1
25	3	1
26	4.5	1
27	4.5	3

27 runs in random order from the 3 × 3 factorial design with three replicates. In this case, ethyl acetate was used as a dispersed solvent for PLGA. After ethyl acetate was replaced with isopropyl formate, another 27 microsphere batches were also prepared following the same experimental conditions.

2.5. Observation of physical status of emulsion droplets by light microscopy

PLGA (0.25 g) was dissolved in 6 ml of isopropyl formate or ethyl acetate. The dispersed phase was emulsified under the experimental condition described above. Added into this o/w emulsion was 4.5 ml of the ammonia solution (the ammonia:organic solvent molar ratio = 1:1). Aliquots of the o/w emulsion were sampled in 5, 10 and 15 min, and their physical status was observed under a light microscope (Eyecam 2.0, MICro Scopes Inc., MO, USA).

2.6. Scanning electron microscopy (SEM)

Microsphere morphology was evaluated by use of a scanning electron microscope (model JSM-5200; Jeol Inc., Tokyo, Japan). The microspheres prepared using an organic solvent and ammonia (the ammonia:solvent molar ratio was 1:1) were mounted onto metal stubs with a double-sided adhesive tape and sputter-coated under vacuum in an argon atmosphere (model SC7620 sputter coater, VG Microtech, West Sussex, UK).

2.7. Nuclear magnetic resonance (NMR) spectroscopy

A 400-MHz spectrometer was used to measure ^{1}H spectra of PLGA raw materials and PLGA microspheres prepared under different manufacturing conditions. These PLGA samples were dissolved in a deuterated chloroform ($CDCl_3$) containing tetramethylsilan internal reference. PLGA has three different sets of chemically equivalent protons in lactide and glycolide. Therefore, the ^{1}H NMR spectrum of PLGA gives rise to three major signal strengths from the protons of the CH group (peak A), the CH_2 group (peak B) and the CH_3 group (peak C) in the copolymer backbone. The area under each peak was electronically integrated, and the contents of lactide and glycolide in PLGA were calculated by the following formula.

$$\text{Lactide}\,\% = \frac{(\text{peak area of A} + \text{peak area of C})/4}{\{(\text{peak area of A} + \text{peak area of C})/4 + (\text{peak area of B})/2\}} \times 100 \quad (1)$$

$$\text{Glycolide}\,\% = \frac{(\text{peak area of B})/2}{\{(\text{peak area of A} + \text{peak area of C})/4 + (\text{peak area of B})/2\}} \times 100 \quad (2)$$

2.8. Gel permeation chromatography (GPC)

A Hewlett Packard GPC system with a refractive index detector was used to measure the molecular weight of PLGA before and after microencapsulation. A sample (ca. 16 mg) was dissolved in 4 ml of tetrahydrofuran. Three analytical columns of PLgel™ 100 Å, Phenogel™ 10^3 Å, and PLgel™ 10^4 Å were connected in series, and their column temperature was kept at 25 °C. Tetrahydrofuran was used as a mobile solution at a flow rate of 1 ml/min. Fifty microliters of the sample solution were injected into the GPC system. A standard calibration curve was constructed by use of polystyrene standards with 5 known molecular weights (Varian Inc., Santa Clara, CA, USA). An Agilent ChemStation software program was used to calculate the molecular weights of various samples.

2.9. Drug release study

Progesterone-loaded microspheres were prepared under different ammonolysis-based microencapsulation conditions. The initial amount of progesterone dissolved in a dispersed phase varied from 50 to 150 mg. After progesterone encapsulation efficiency was determined, release behavior of progesterone from these microspheres was investigated by a modified water-bath method (Im and Sah, 2009). Briefly, microsphere samples (ca. 40 mg) were suspended in 20 ml of a 5% sodium dodecyl sulfate (SDS) solution. The microsphere suspensions were gently shaken at room temperature using a reciprocal shaking water bath (because microspheres were severely aggregated at 37 °C due to their low Tg, the release study was performed at room temperature). At predetermined time intervals, 0.5 ml of the aqueous phase was sampled for analyzing progesterone concentration.

3. Results

Measurement of solvent reactivity toward ammonolysis demonstrated that there was a pronounced difference in ammonolysis rates between ethyl acetate and isopropyl formate. Fig. 1 illustrates GC chromatograms showing dynamic changes in aqueous concentrations of ethyl acetate and isopropyl formate, as well as their ammonolysis products (isopropanol and ethanol). As time elapsed, the aqueous concentration of ethyl acetate increased progressively (Fig. 1A). This suggests that ethyl acetate diffuses slowly into the aqueous phase under our experimental condition. In the meantime, ethanol was hardly noted in the aqueous solution. Interestingly, an experiment with isopropyl formate exhibited unlikeness on comparison with that of ethyl acetate (Fig. 1B). The peak area of isopropyl formate reached a plateau at a faster rate, compared to that of ethyl acetate. Besides, there was a continual rise in the aqueous concentration of isopropanol as a function of time. These results indicate that isopropyl formate has much better reactivity toward ammonolysis than does ethyl acetate. Fig. 2 illustrates the time-dependent concentrations of ethyl acetate and isopropyl formate in the aqueous phase, whereas Fig. 3 compares quantitatively their ammonolysis rates. In fact, the reactivity of ethyl acetate was so poor that less than 10% of the organic solvent was subject to ammonolysis for 60 min. By sharp contrast, isopropyl formate

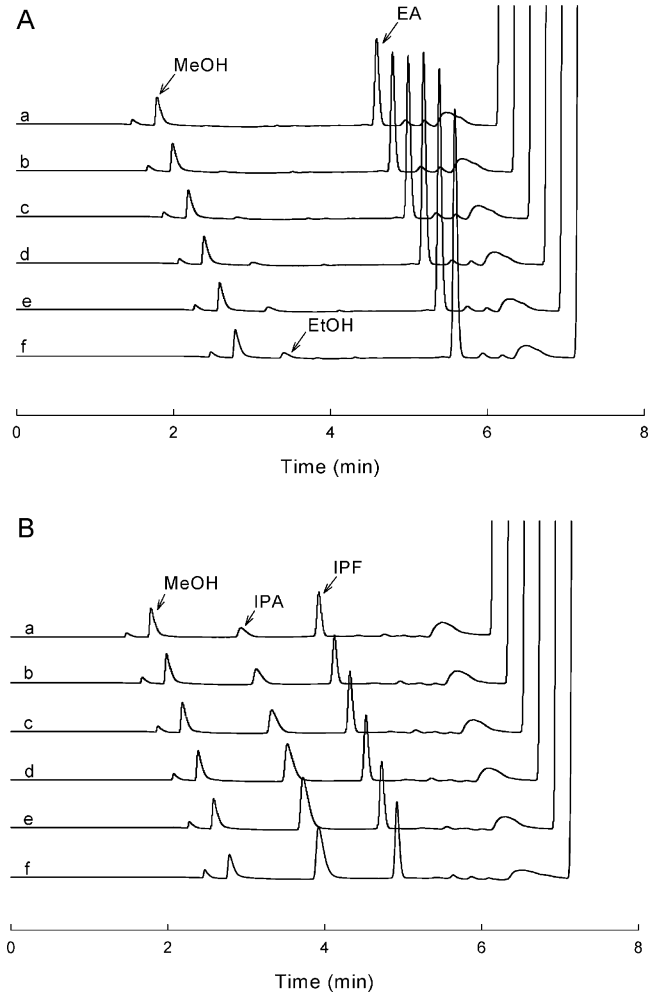

Fig. 1. GC chromatograms illustrating time-dependent changes in the concentrations of organic solvents and their ammonolysis products (ethanol and isopropanol) in the aqueous phase. GC samples were taken at (a) 3-, (b) 8-, (c) 15-, (d) 30-, (e) 45-, and (f) 60-min stirring. There are big differences in the reactivity of ethyl acetate (EA) and isopropyl formate (IPF) toward ammonolysis. EtOH and IPA represent ethanol and isopropanol, respectively. MeOH stands for methanol used as an internal standard.

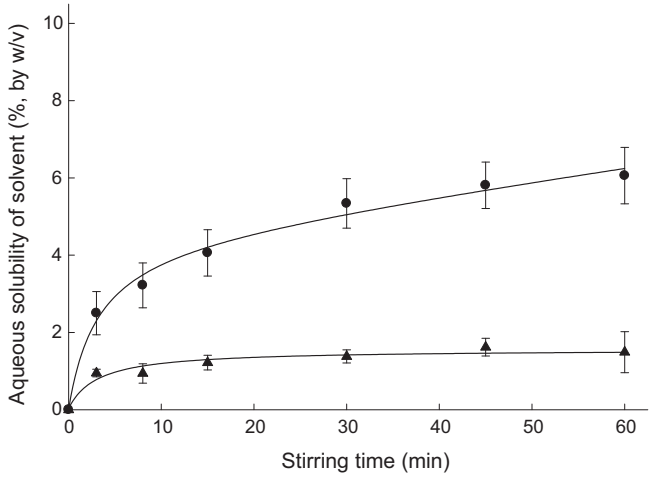

Fig. 2. Dynamic changes in aqueous concentrations of (●) ethyl acetate and (▲) isopropyl formate as a function of time. Ethyl acetate has better water solubility than does isopropyl formate.

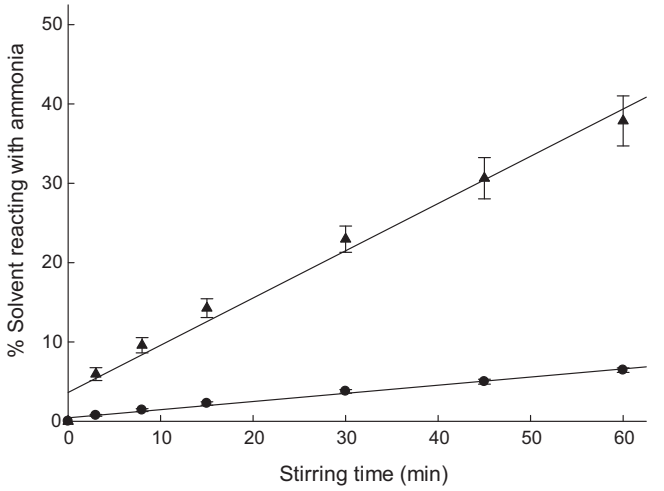

Fig. 3. Ammonolysis rates of (●) ethyl acetate and (▲) isopropyl formate. On comparison to ethyl acetate, isopropyl formate exhibits greater reactivity toward ammonolysis.

reacted fast with ammonia, and its average ammonolysis rate was 6 times greater than that of ethyl acetate.

It was speculated that, after a dispersed phase being emulsified in an aqueous phase, an organic solvent having poor ammonolysis rate would remain in emulsion droplets. On the contrary, solvent removal would have occurred effectively, if a dispersed solvent displayed a fast ammonolysis rate. Based on this rationale, the physical status of emulsion droplets during the microencapsulation process was evaluated. When ethyl acetate was used as a dispersed solvent, emulsion droplets sampled in 5–15 min were still liquids. Being mounted on a glass for observation under a light microscope, they coalesced together to form polymeric films (Fig. 4A). This demonstrates that microsphere hardening does not take place over this time frame. In opposition to what was observed above, the isopropyl formate-based microencapsulation process allowed emulsion droplets to become hardened microspheres over a short period of time (Fig. 4B). The ease at which isopropyl formate reacts with ammonia is an essential feature required for the practice of an ammonolysis-based microencapsulation process.

A difference in solvent reactivity toward ammonolysis also affected the morphology of PLGA microspheres prepared in this study. Microspheres prepared using ethyl acetate were seriously aggregated (Fig. 5A). It is likely that its poor reactivity toward ammonolysis results in incomplete solvent extraction, thereby causing microsphere aggregation. In contrast, the ammonolysis-based process using isopropyl formate led to the formation of discrete microspheres with an excellent flow property (Fig. 5B).

Fig. 6 depicts ^{1}H NMR spectra of a raw PLGA polymer, PLGA microspheres prepared using ethyl acetate, and those prepared using isopropyl formate. Based on Eqs. (1) and (2), their lactide:glycolide compositions were determined. Our ^{1}H NMR experiment demonstrated that the raw PLGA polymer consisted of $75.03 \pm 0.25\%$ of lactide and $24.97 \pm 0.29\%$ of glycolide (the number of determinations = 6). The ethyl acetate-based microencapsulation procedure caused significant changes in its lactide:glycolide composition (Fig. 7A). When 3 ml of ethyl acetate and an equimolar amount of ammonia were used for microencapsulation, the lactide:glycolide composition of the resultant PLGA microspheres was 76.64:23.36%. A change in the lactide:glycolide composition represented that some degree of degradation occurred to PLGA. Increasing solvent volume and ammonia concentration exacerbated PLGA degradability, thereby producing more severe changes in its lactide:glycolide composition. For instance, when the solvent

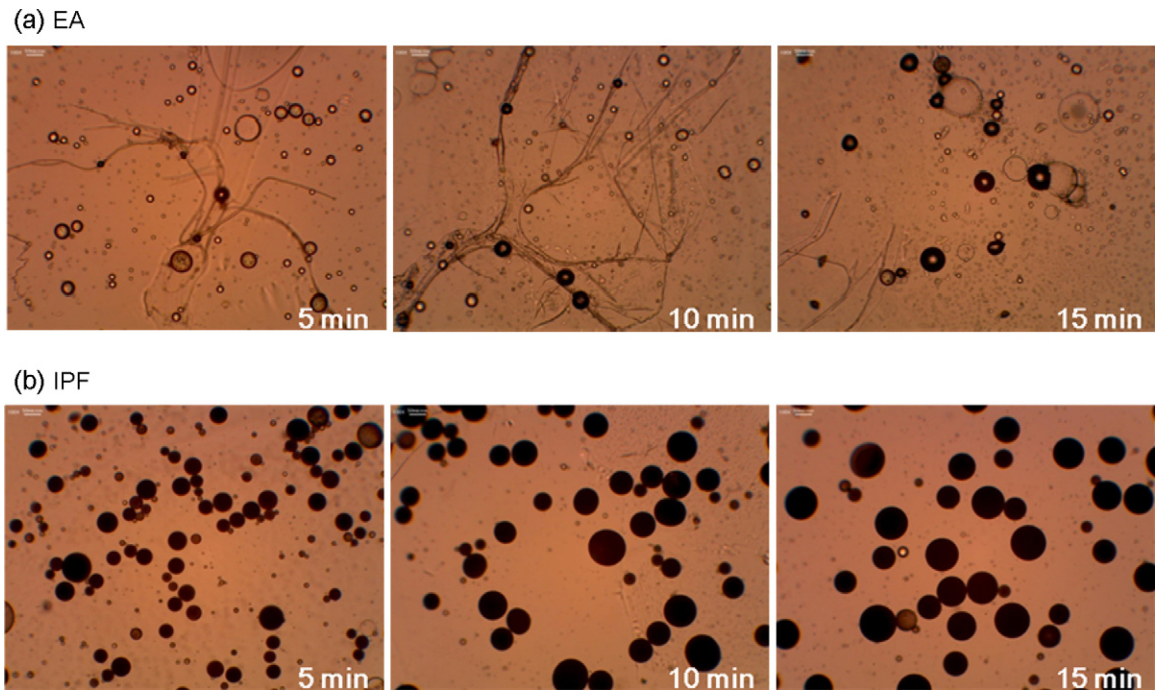

Fig. 4. LM photographs of oil-in-water emulsions sampled at 5-, 10-, and 15-min stirring. (a) When the dispersed phase is made of ethyl acetate and PLGA, emulsion droplets remain liquid. (b) By contrast, emulsion droplets consisting of isopropyl formate and PLGA become solidified into microspheres. Solvent reactivity toward ammonolysis determines the rate of microsphere solidification.

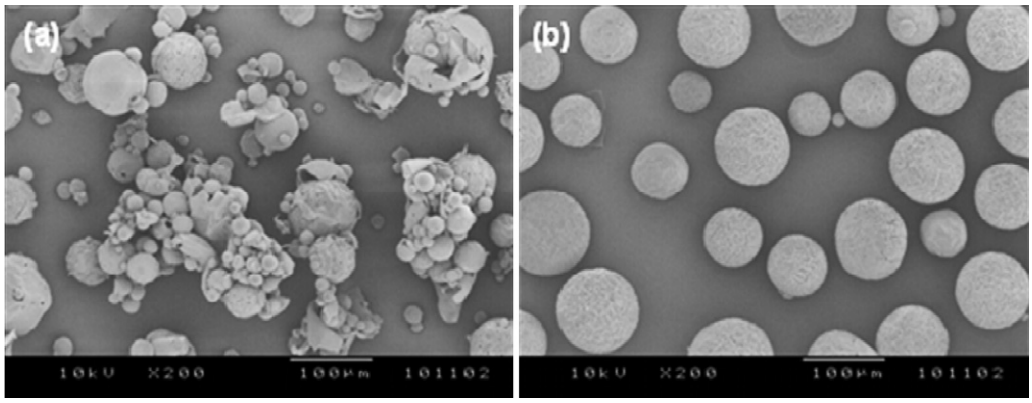

Fig. 5. SEM micrographs of PLGA microspheres prepared following (a) ethyl acetate- and (b) isopropyl formate-based ammonolysis processes. The degree of microsphere aggregation and/or dispersibility is influenced by solvent type. The bar size is 100 μm.

Table 2
ANOVA results for the 3 × 3 factorial models with 3 replicates.

Variation source	Sum of squares	d.f.	Mean square	*F* value	Prob > *F*
Model	103.66	8	12.96	178.01	<0.0001
EA[a]	8.28	2	4.14	56.88	<0.0001
NH_3	85.73	2	42.87	588.9	<0.0001
EA × NH_3	9.65	4	2.41	35.14	<0.0001
Pure error	1.31	18	0.073		
Cor. total	104.97	26			
Model	15.53	8	1.94	43.25	<0.0001
IPF[b]	6.58	2	3.29	73.34	<0.0001
NH_3	7.79	2	3.9	86.88	<0.0001
IPF × NH_3	1.16	4	0.29	6.44	0.0021
Pure error	0.81	18	0.045		
Cor. total	16.33	26			

[a] EA represents ethyl acetate.
[b] IPF represents isopropyl formate.

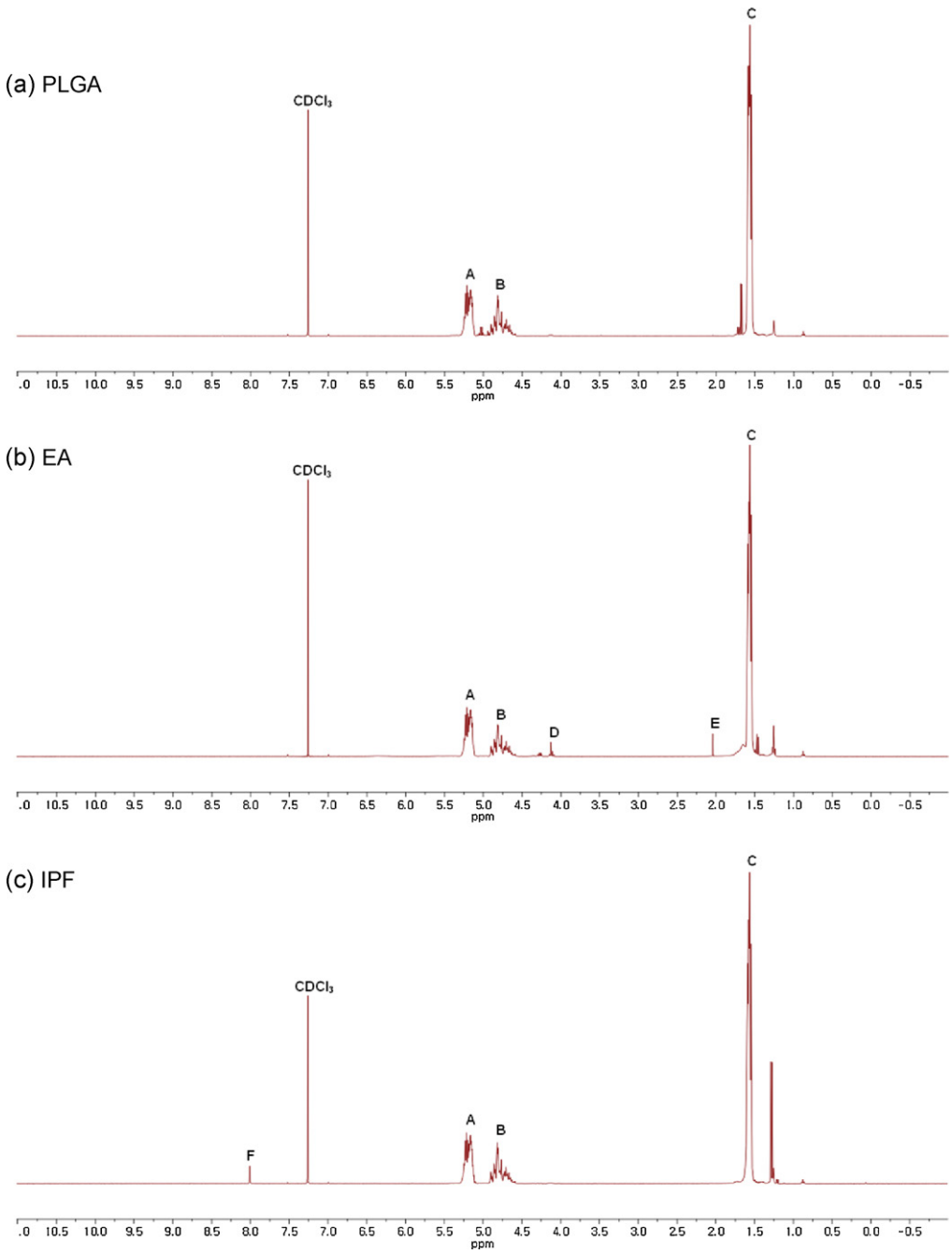

Fig. 6. ^{1}H NMR spectra of (a) a raw PLGA material and (b and c) PLGA microspheres prepared using ethyl acetate or isopropyl formate. Three major signal strengths arise from the CH group (A), the CH_2 group (B), and the CH_3 group (C) in the backbone of PLGA. Residual solvents in PLGA microspheres give rise to solvent-specific peaks (D–F).

volume and molar ratio of ammonia to the solvent were fixed at 6 ml and 3, the lactide:glycolide content of PLGA was further changed to 82.44:17.56%.

Interestingly, supplanting ethyl acetate with isopropyl formate contributed to preserving the structural integrity of PLGA (Fig. 7B). There was little change in the lactide:glycolide composition of PLGA microspheres prepared by treating 3 ml of isopropyl formate with an equimolar amount of ammonia. PLGA degradation was facilitated by use of larger amounts of isopropyl formate and higher molar ratios of ammonia to the solvent. However, it is noteworthy that PLGA degradability becomes much more alleviated, when compared to the cases attained with ethyl acetate.

Statistical analyses were performed on the data shown in Fig. 7, in order to see whether or not our lactide:glycolide data were compounded by variability in the determination by ^{1}H NMR. The Design-Expert® software that generated a randomized run order for microsphere preparation was used for data analysis. Based on the complete analysis of variance shown in Table 2, it stands to reason that solvent volume, the molar ratio of ammonia to solvent and their interaction are significant model terms. In other words, the lactide:glycolide composition of PLGA microspheres is considerably influenced by these terms, regardless of solvent type. Normal plots of residuals shown in Fig. 8 substantiate that there is no indication of any abnormality in the lactide:glycolide data collected from our design of experiments. It was also confirmed that all data points fell within the limits calculated at 95% confidence interval.

The molecular weights of PLGA before and after microencapsulation were determined by GPC. Fig. 9 illustrates GPC

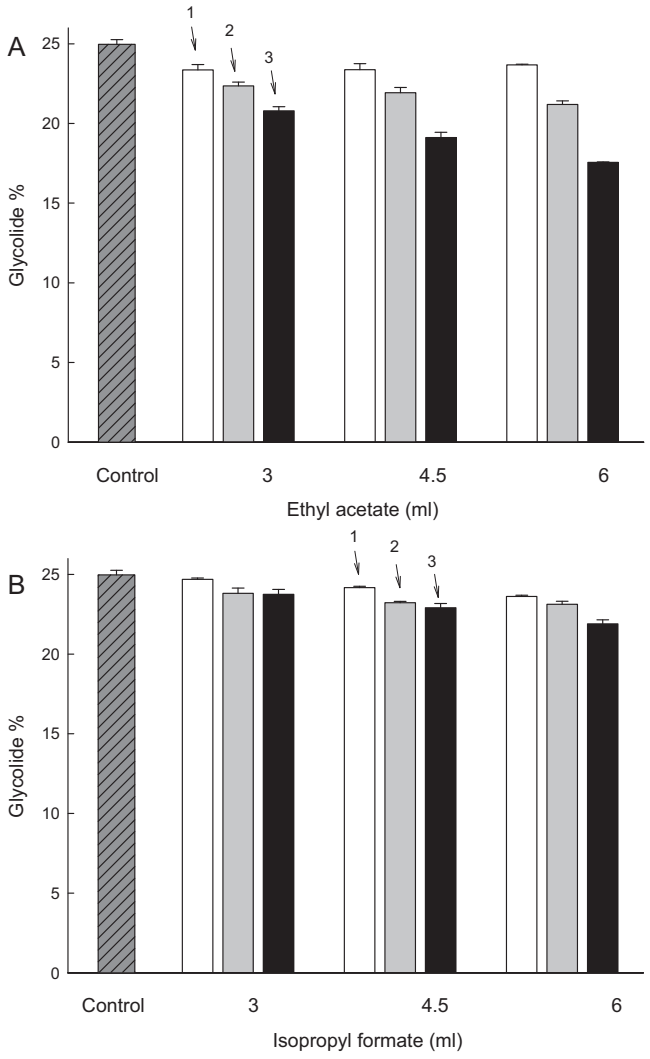

Fig. 7. Effect of microencapsulation conditions upon the glycolide content in PLGA microspheres. Two different solvents were used as dispersed solvents (3–6 ml), and the molar ratio of ammonia to solvent varied from 1 to 2 and 3.

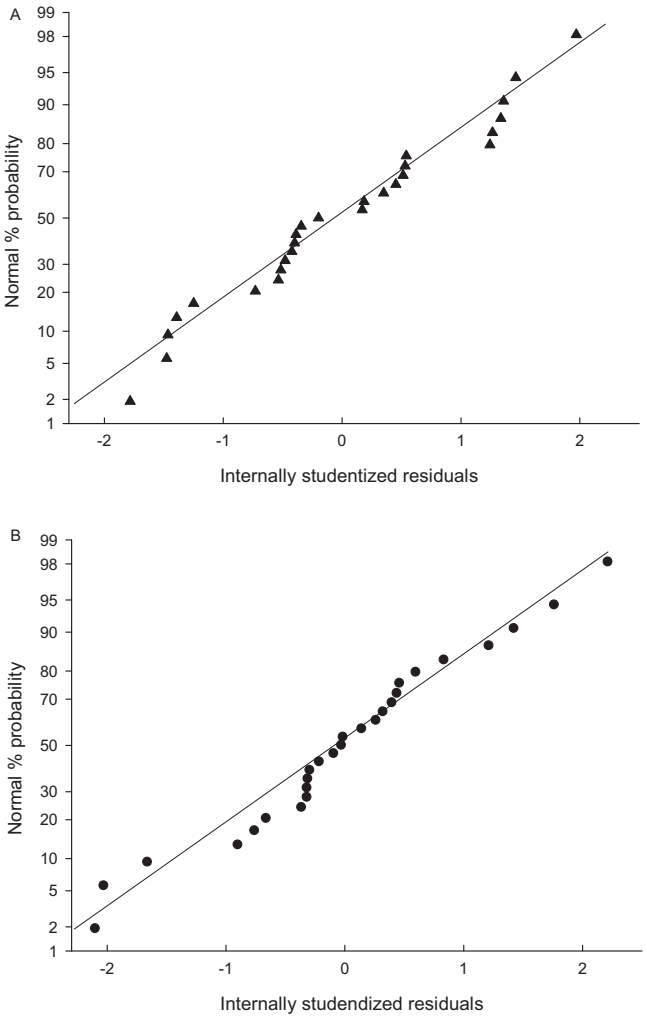

Fig. 8. Normal (%) probability plots of studentized residuals. Linearity of the data points indicates that there are no signs of abnormality. All data are collected from PLGA microsphere prepared using (A) ethyl acetate and (B) isopropyl formate.

chromatograms of various PLGA samples. When microencapsulation proceeded with 3 ml of ethyl acetate and an equimolar amount of ammonia, the resultant microspheres had a molecular weight of 20,660 ± 295 g/mol (Fig. 10A). In fact, the molecular weight of the raw PLGA material before microencapsulation was determined to be 25,925 ± 671 g/mol. Therefore, it can be safely concluded that the ethyl acetate-based microencapsulation process leads to a molecular weight reduction of PLGA. When the molar ratio of ammonia to the solvent increased to 2 and 3, the molecular weight of the PLGA microspheres further decreased to 19,894 ± 186 and 14,171 ± 379 g/mol, respectively. Increases in solvent volume to 4.5 and 6 ml also caused more pronounced reductions in the molecular weight of PLGA.

The magnitude of a molecular weight reduction of PLGA considerably lessened, when the microencapsulation process utilized isopropyl formate as a dispersed solvent (Fig. 10B). When 3 ml of the solvent was treated with an equimolar amount of ammonia, the molecular weight of the resultant microspheres was 24,461 ± 569 g/mol. Even though increases in the solvent volume and the amount of ammonia accompanied reductions in the molecular weight of PLGA microspheres, such changes were much smaller compared to the cases observed with ethyl acetate. All these results were consistently relatable to those shown in term of the changes in

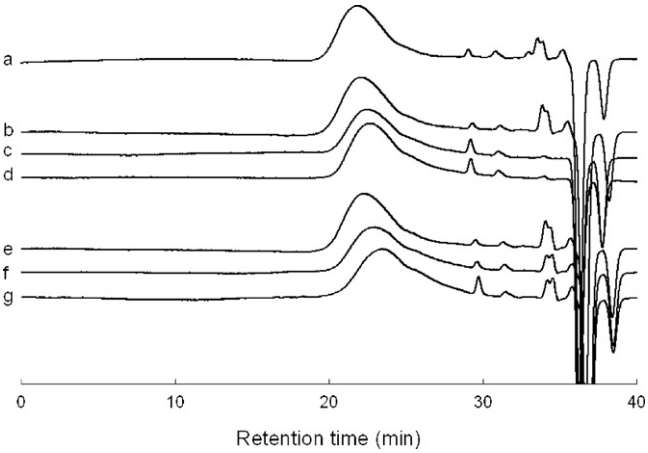

Fig. 9. GPC chromatograms of (a) PLGA before microencapsulation and (b–g) PLGA microspheres. The solvent volume used for microencapsulation was fixed at 3 ml, but the molar ratio of ammonia to isopropyl formate was changed from (b) 1 to (c) 2 and (d) 3. Similarly, the molar ratio of ammonia to ethyl acetate varied from (e) 1 to (f) 2 and (g) 3.

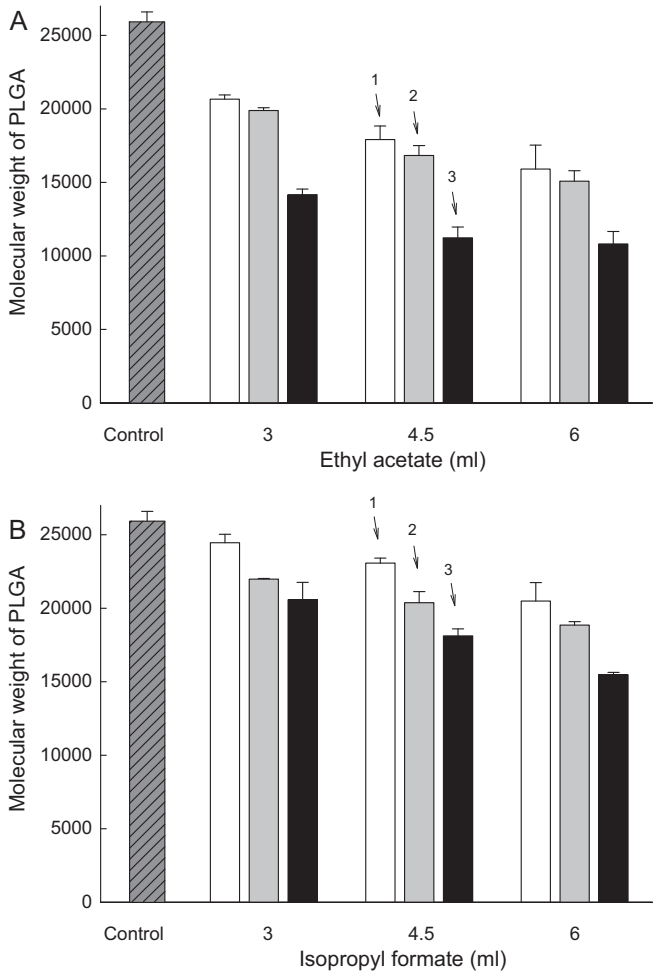

Fig. 10. Effect of microencapsulation conditions upon the degree of reduction in the molecular weights of PLGA microspheres. They were prepared using (A) ethyl acetate or (B) isopropyl formate. For the preparation of microspheres, solvent volume and the ammonia:solvent molar ratio were changed as indicated.

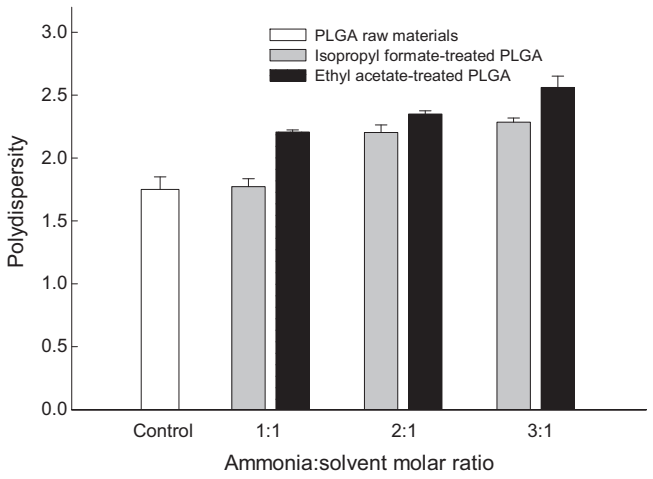

Fig. 11. Effect of solvent type and ammonia:solvent molar ratio upon polydispersity index of PLGA. Solvent volume used for microencapsulation was maintained at 3 ml. A change in the lactide:glycolide content of PLGA accompanies an increase in its polydispersity index.

the lactide:glycolide compositions of PLGA (Fig. 7). A polydispersity index of PLGA also was affected by a change in its lactide:glycolide composition. Whenever there was a noticeable change in the lactide:glycolide composition of PLGA, its polydispersity index also varied accordingly (Fig. 11).

All the above experiments were carried out using blank microspheres. In the following experiments progesterone was actually loaded into microspheres using two extreme microencapsulation conditions specified in Table 3. When F1 and F2 microspheres were prepared at a manufacturing condition that preserved the structural integrity of PLGA, they tended to have smooth surface (Fig. 12A). On the contrary, when F3 and F4 microspheres were prepared under a manufacturing condition that caused harm to PLGA, their surface was irregular and covered with some drug crystals. It was also quite interesting to note that solvent type, molar ratio of ammonia:solvent, and progesterone influenced release patterns of progesterone to greater extents (Fig. 13). For example, even though both F1 and F3 microspheres had similar progesterone payloads, their release patterns of progesterone were very different from each other—F1 microspheres provided sustained release of progesterone, whereas F3 microspheres released progesterone in a burst mode. When 150 mg of progesterone was used for microencapsulation, drug release profiles were always characterized with burst release. A higher progesterone payload seemed to cause ubiquitous existence of drug crystals across microsphere matrices. Their solubilization and leaching would generate numerous pores and/or microchannels, thereby facilitating burst drug release. This speculation was supported by the SEM micrographs of the microspheres taken at the end of our release study (Fig. 12B).

4. Discussion

A number of strategies have been proposed to complete solvent extraction in the practice of o/w and w/o/w emulsion-based microencapsulation processes. Relevant examples include using an organic solvent having high water miscibility, extracting a dispersed solvent with large amounts of a quenching liquid, or manipulating the composition of a continuous phase (Freitas et al., 2005; Sawalha et al., 2008; Yeo and Park, 2004). In order to increase the efficiency of solvent extraction, an organic solvent dissolved in an aqueous phase is sometimes eliminated by use of devices such as hollow fiber filter and ultrafiltration. In contrast to these techniques, the microencapsulation approach reported in this study makes use of a chemical approach to transform a dispersed solvent into water soluble products. It might be worth providing pro and con arguments about the ammonolysis-based process. There are several advantages of using the new process for the preparation of polymeric microspheres. A nonhalogenated organic solvent (e.g., isopropyl formate) can be used as a dispersed solvent for microencapsulation. The fast rate of ammonolysis contributes to completing the process of microsphere hardening over a short period of time. Furthermore, the ammonolysis-based

Table 3
Effect of microsphere batch formulation upon progesterone encapsulation efficiency (EE).

Code	Solvent	Volume (ml)	Progesterone (mg)	Molar ratio (ammonia:solvent)	EE% (mean ± SD)
F1	IPF	3	50	1:1	71.6 ± 7.7
F2	IPF	3	150	1:1	85.8 ± 3.6
F3	EA	6	50	3:1	96.1 ± 3.8
F4	EA	6	150	3:1	98.8 ± 2.8

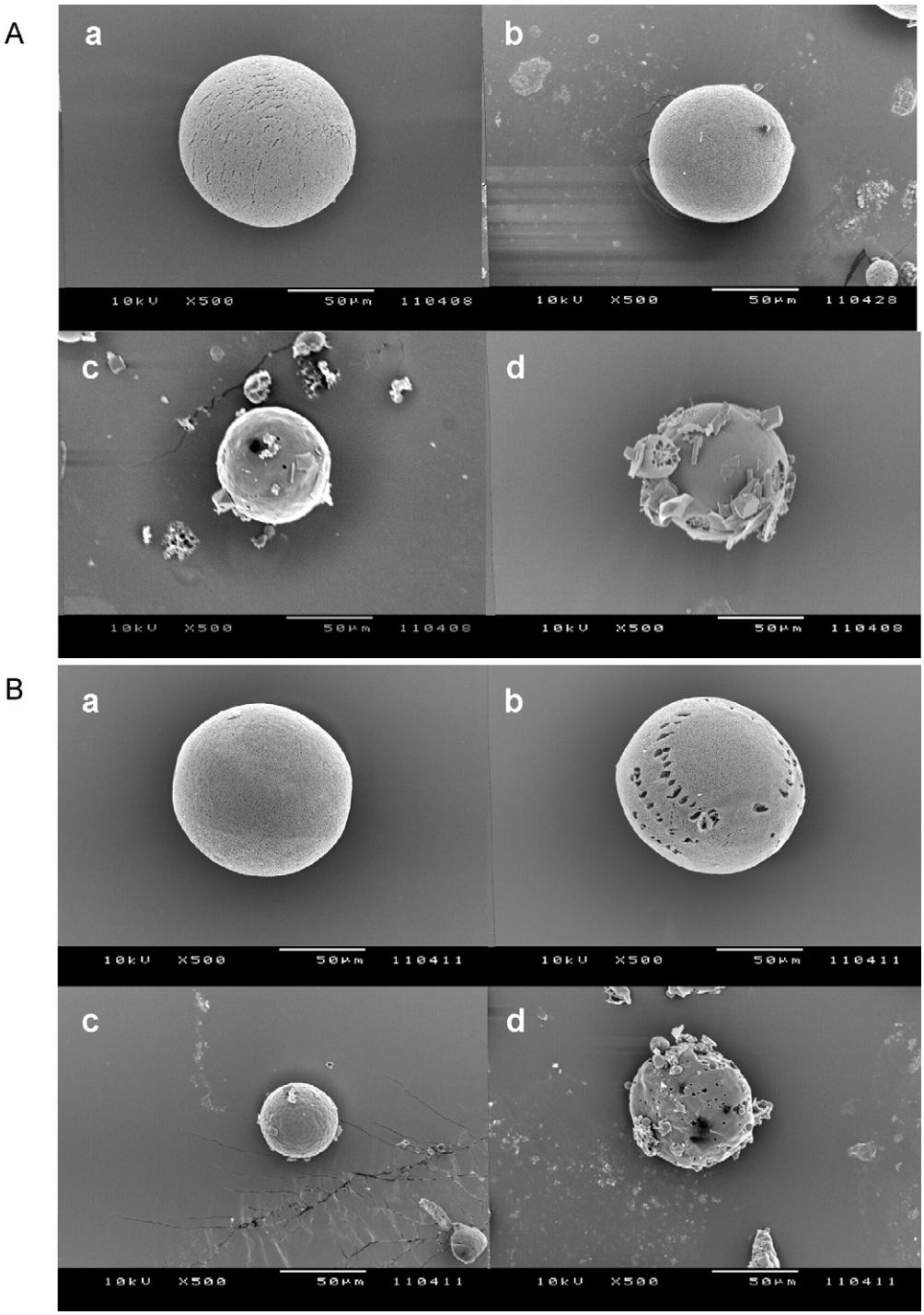

Fig. 12. SEM micrographs of (a) F1, (b) F2, (c) F3, and (d) F4 microspheres taken (A) before and (B) after progesterone release study.

process does not require a vast amount of a quench liquid and a series of complicated equipments which are usually required for typical solvent extraction. However, ammonia is a strong nucleophile that can react with various electrophilic groups. Subsequently, the ammonolysis-based process might raise some stability issues toward drugs with backbones of acid halide, lactone, carboxylic ester, and anhydride. As demonstrated in this study, ammonia can also attack the ester backbone of PLGA under stressed conditions, thereby bringing changes in the lactide:glycolide composition and Mw of PLGA. Therefore, the stability issues of both drug and PLGA should be carefully evaluated in the practice of the ammonolysis-based microencapsulation process.

Previously, several reports suggested that the composition of a dispersed phase used for an o/w-based microencapsulation process influenced a molecular weight of PLGA. For example, when a dispersed phase consisting of risperidone, benzyl alcohol, PLGA and ethyl acetate was kept at 25 °C, its initial molecular weight loss was about 17% and an additional loss took place at a rate of about 5.7% per incubation time (h) (Wright et al., 2001). When drugs such as thioridazine and ketoifen were loaded in poly-lactide microcapsules, the polymer molecular weight decreased during microencapsulation. At that time, the temperature at which the microencapsulation process was carried out affected the degree of poly-lactide degradation (Maulding et al., 1986). Sometimes, there

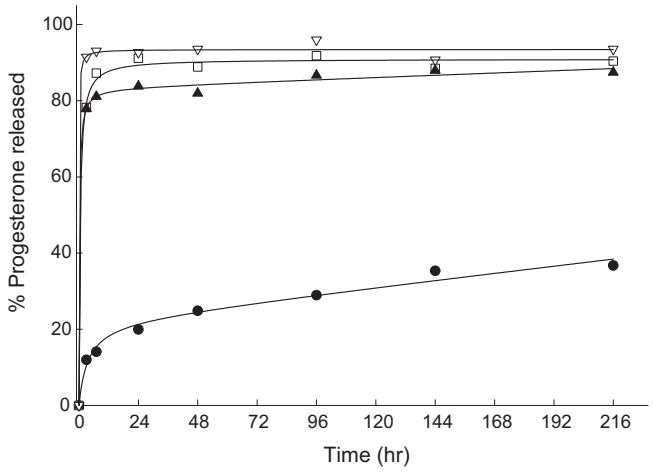

Fig. 13. Progesterone release profiles observed with (●) F1, (□) F2, (▲) F3, and (▽) F4 microspheres.

is a contradictory opinion that the presence of a drug in the matrix of PLGA microspheres can decrease the hydrolytic cleavage of its ester bonds (Chen et al., 2006). All these studies shed light on the influence of a dispersed phase formulation upon PLGA degradability. Our ammonolysis-based microencapsulation process uses a nucleophilic ammonia that reacts with ester organic solvents. Besides its reactivity toward the organic solvents, ammonia might also be capable of facilitating the hydrolysis of ester bonds in PLGA by nucleophilic catalysis. To address this issue, the concentration of ammonia used to trigger ammonolysis varied on the ground of solvent type and volume. First of all, when the reactivity of ethyl acetate and isopropyl formate toward ammonolysis was assessed, it was found that the latter solvent had by far better reactivity than did ethyl acetate (Figs. 1 and 3). In general, the polarization status of the carbonyl carbon in a solvent ester backbone and its steric environment influence ammonolysis rate. Since isopropyl formate does not have a bulky methyl group adjacent its carbonyl carbon, its ammonolysis rate is faster than that of ethyl acetate having the bulky group near its carbonyl carbon.

Our ^{1}H NMR study was able to detect even a subtle change in the lactide:glycolide composition of PLGA. When a higher solvent volume constituted a dispersed phase, changes in its lactide:glycolide composition became more and more prominent (Fig. 7). Such changes were well correlated to reductions in its molecular weight (Fig. 10). Our ^{1}H NMR and GPC data are in line with the supposition that degradation of PLGA accompanies an increase in its lactide/glycolide ratio. For instance, when PLGA50:50 with intrinsic viscosity of 1.05 dl/g was incubated for 7 days, its lactide/glycolide ratio increased from 1.08 ± 0.02 to 1.30 ± 0.03 (Tang and Singh, 2008).

It was interesting to note that the structural integrity of PLGA was considerably affected by solvent type (ethyl acetate, isopropyl formate) and its volume (3–6 ml). The effect of solvent type and volume upon PLGA degradability would be understandable, if the following is taken into consideration. The solubility of ethyl acetate in a 0.5% PVA solution was 61 mg/ml (Fig. 2). When 3 ml of ethyl acetate is used to dissolve PLGA and emulsified in 40 ml of the aqueous phase, a dispersed phase would lose a major proportion of ethyl acetate. As a consequence, the dispersed phase has barely enough ethyl acetate to fully dissolve PLGA, which makes the polymer start to precipitate to form microspheres. When the solvent volume increases to 4.5 and 6 ml, the resultant dispersed phases emulsified in 40 ml of the aqueous phase remain to be liquid—they can hold sufficient amounts of ethyl acetate in which PLGA is completely dissolved. Besides, ammonia is hardly consumed by ethyl acetate having poor reactivity toward ammonolysis. Under these situations, the likelihood of the ammonia attack on ester linkages in PLGA would increase to greater extents. As a consequence, an increase in solvent volume accelerates the degradation of PLGA. Of course, the rate of ammonolysis would be proportional to the concentration of ammonia.

Supplanting ethyl acetate with isopropyl formate provides a quite different situation. Due to poor water solubility of isopropyl formate, most of the solvent used to dissolve PLGA is confined to the dispersed phase after emulsification. However, its notable reactivity toward ammonolysis leads to the depletion of ammonia. As a result, the availability of ammonia to ester linkages in PLGA is limited. Furthermore, once microspheres are formed, PLGA degradation would be suppressed. Therefore, the structural integrity of PLGA is preserved better in the practice of the isopropyl formate-based microencapsulation process, compared to the ethyl acetate-based one. In particular, using 3 ml of isopropyl formate and an equimolar amount of ammonia caused little distress on the lactide:glycolide composition and molecular weight of PLGA. It should be emphasized that changes in chemical properties of PLGA have many ramifications for the characteristics of microspheres including their morphology, physical status of drug, and behavior of drug release (Figs. 12 and 13). Therefore, relevant parameters (solvent type, ammonia concentration, solvent volume, and ammonia:solvent molar ratio) should be taken into consideration, in order to tailor microsphere attributes for specific needs.

It is generally known that degradation of PLGA occurs in three stages (de Jong et al., 2001; Jung et al., 2006). An initial stage is characterized by random chain scission that cleavages ester bonds in random order. At this time the molecular weight of PLGA decreases significantly with little weight loss, but no considerable changes are observed with regard to the ratio of lactide to glycolide. During a second stage, chain end scission takes place so that glycolide ester bonds at the end of PLGA become unzipped faster than do lactide ones. These events bring pronounced changes in polydispersity and increases in the ratio of lactide to glycolide (Hakkarainen et al., 1996; Lee et al., 2001; Shih, 1995). In the following final stage, weight loss is experienced and soluble oligomers and monomers are produced. Our ^{1}H NMR and GPC data indicate that chain end scission might predominate over random chain scission when PLGA is subject to the ammonolysis-based microencapsulation process. This might be true especially when microencapsulation proceeds with use of a lower solvent volume and an equimolar amount of ammonia. If random chain scission took place, not only PLGA molecular weight would have undergone significant decrease but also its lactide:glycolide content would have remained relatively unchanged. When the microencapsulation process was carried out under other manufacturing conditions, there always were decreases in the proportion of glycolide in the resultant PLGA microspheres. Such increases in the ratio of lactide to glycolide suggest that glycolide ester linkages have greater hydrolytic reactivity than lactide ones and chain end scission continues to participate in the process of PLGA degradation.

5. Conclusions

The results shown in this study demonstrate that the structural integrity of ester bonds in PLGA is greatly affected by the type and volume of a dispersed solvent, the molar ratio of ammonia to solvent, and the interaction between solvent volume and ammonia concentration. Adjustments in these parameters are prerequisites to not only preserving the structural integrity of PLGA but also controlling the magnitude of its molecular reduction. The manipulation of these parameters can also be utilized as a strategic approach to prepare microsphere products of varying molecular weights and/or

lactide:glycolide contents by use of the same starting PLGA material.

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