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Ammonolysis-based microencapsulation technique using isopropyl formate as dispersed solvent

Hye-Yeum Im, Hongkee Sah*

College of Pharmacy, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, South Korea

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ABSTRACT

The objectives of this study were to develop an ammonolysis-based microencapsulation technique using a nonhalogenated isopropyl formate and to evaluate its feasibility in preparing poly-D,L-lactide-*co*-glycolide microspheres. The choice of isopropyl formate was based on its great reactivity toward ammonolysis and acceptance as a flavoring agent for human food by regulatory agencies. Progesterone was used as a model drug for microencapsulation. In the practice of this microencapsulation process, a dispersed phase consisting of isopropyl formate, the polymer and progesterone was emulsified in an aqueous phase. Solvent removal from emulsion droplets was rapidly achieved by ammonolysis at ambient conditions, not by typical solvent evaporation and/or extraction. Depending upon microsphere formulations, its encapsulation efficiency ranged from 88.0 ± 3.6 to 97.0 ± 3.6%. Analysis of FTIR spectra suggested that there were no significant chemical interactions between prednisolone and the polymer. Both DSC and XRD data substantiated that the magnitude of an actual progesterone loading influenced its physical status in the microspheres. Interestingly, the microspheres prepared in this study contained noticeably lower levels of solvent residues: a gas chromatographic analysis demonstrated that the levels of residual isopropyl formate found in different microspheres were not more than 0.34 ± 0.07%. It was seen to be feasible from these results that the ammonolysis-based approach using isopropyl formate might have a potential as an alternative microencapsulation technique.

1. Introduction

Microencapsulation of pharmaceuticals into microspheres is an invaluable technique for achieving controlled release of bioactive materials to animals and humans. One advantage of a microsphere dosage form is that pharmaceuticals of interest can be administered less frequently at lower doses, thereby helping improve drug efficacy and patient compliance. Microspheres can be made using a variety of polymeric materials, but an exceptionally attractive biodegradable polymer is poly-D,L-lactide-*co*-glycolide (PLGA) with proven records of safety and regulatory approval. There are several PLGA products in the market such as Arestin, Lupron Depot, Risperdal Consta, Sandostatin LAR Depot, and Vivitrol. Even though versatile microencapsulation methods are found in literature, common techniques are based on solvent removal such as evaporation, extraction, spray drying and supercritical fluid technology (Jain, 2000; Yeo and Park, 2004; Li et al., 2008). Among them, an emulsion-based solvent evaporation/extraction process is frequently used to prepare PLGA microspheres. This method includes emulsification of a polymeric dispersed phase in an aqueous phase. Emulsion droplets are then converted to microspheres by removing the organic solvent either by causing evaporation while stirring or by extracting the organic solvent out of the droplets with a large volume of water. The hardened microspheres are collected by either filtration or centrifugation.Methylene chloride is typically used as an organic solvent, when microspheres are prepared following solvent evaporation. However, high volatility makes it an inhalation hazard to humans. Overexposure to methylene chloride can cause respiratory or central nervous system failure, posing an increased risk of cancer. Because of such serious adverse effects, the US Department of Labor's Occupational Safety and Health Administration (OSHA) designates methylene chloride as carcinogen groups. In the practice of solvent extraction, ethyl acetate is preferably used as a substitute for methylene chloride (Soppimath and Aminabhavi, 2002; Lagarce et al., 2005).

However, conventional emulsion-based processes bear some critical issues in relation to difficulty in the removal of an organic solvent, limitations in manufacturing facility, instability and coalescence of emulsion droplets during hardening, and so on. Recently, we have proposed an ammonolysis-based technique for microencapsulating drugs into PLGA microspheres. In this process, solvent removal was achieved by ammonolysis – the chemical reaction converted a water-immiscible solvent in emulsion droplets into water-miscible solvents diffusing to an aqueous continuous phase.

* Corresponding author. Tel.: +82 2 3277 4367; fax: +82 2 3277 2851.
E-mail address: hsah@ewha.ac.kr (H. Sah).

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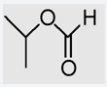
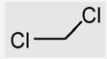
Table 1
Major physicochemical properties of isopropyl formate, ethyl acetate and methylene chloride[a].

Property	Isopropyl formate	Ethyl acetate	Methylene chloride
Structure			
Density (g/cm^3)	0.87	0.92	1.33
Boiling point (°C)	68.2	77.1	40
Log *P* (octanol–water)	0.73	0.73	1.25
Water solubility (g/100 ml)	2.1	6.4–8.0	1.3
Vapor pressure (mm Hg)	138	93.2	435

[a] *Source*: ChemIDplus Advanced Database (http://chem.sis.nlm.nih.gov/chemidplus) of United States National Library of Medicine.

This event led to the transformation of emulsion droplets to hardened microspheres in an efficient way. In the practice of this technique, halogenated ester organic solvents such as methyl chloroacetate and ethyl chloroacetate were chosen as dispersed solvents (Kim et al., 2007; Chung et al., 2009). Because the polarization status of carbonyl groups in ester backbones influences their ester reactivity, the earlier studies have used the halogenated ester organic solvents for the purpose of facilitating ammonolysis. The data relevant to gas chromatographic residual solvent analysis was not provided at that time. Also, there have been concerns in the use of halogenated solvents in the pharmaceutical industry, due to their toxicity to humans and environment (Matsumoto et al., 2008; Wischke and Schwendeman, 2008).

To address these issues, for the first time, we have chosen isopropyl formate as a dispersed solvent for the ammonolysis-based microencapsulation process. Table 1 compares the major physicochemical properties of isopropyl formate with those of ethyl acetate and methylene chloride which are commonly used as dispersed solvents. Isopropyl formate, which is formic acid isopropyl ester, does not belong to chlorinated solvents. According to International Programme on Chemical Safety, there is no safety concern for isopropyl formate being used as a flavoring agent. The European Food Safety Authority categories isopropyl formate into flavoring substances to be used in human foodstuffs (European Food Safety Authority, 2008). The US FDA also lists isopropyl formate among food additives permitted for human consumption. Recognizing the acceptance of isopropyl formate by worldwide authorities, its feasibility as a dispersed solvent for the ammonolysis-based microencapsulation process was investigated in this study. Progesterone was used as a model drug throughout this study. After microsphere preparation, their characteristics were evaluated in terms of solidification of emulsion droplets, microsphere morphology, encapsulation efficiency, structural integrity of progesterone, and thermal behavior. Also, a gas chromatographic analytical procedure was developed to measure the level of residual isopropyl formate in the microspheres.

2. Materials and methods

2.1. Materials

A poly-D,L-lactide-*co*-glycolide with a lactide:glycolide ratio of 75:25 (inherent viscosity, 0.70 dl/g in $CHCl_3$ at 30 °C) was purchased from Lactel Absorbable Polymers (AL, USA). This polymer was abbreviated as PLGA in text. An ammonia solution (28%) was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). Isopropyl formate, progesterone and sodium dodecyl sulfate (SDS) were obtained from Sigma–Aldrich (MO, USA). Butanol, methanol, tetrahydrofuran, and methylene chloride of HPLC grade were obtained from Burdick & Jackson (MI, USA). Polysciences, Inc. (PA, USA) was the supplier of polyvinyl alcohol (88% hydrolyzed, Mw = 25,000).

2.2. Preparation PLGA microspheres

Microspheres were prepared by an ammonolysis-based microencapsulation process as follows: PLGA (0.25 g) and progesterone (60, 160, and 250 mg) were dissolved in 4 ml of isopropyl formate. The dispersed phase was emulsified in 40 ml of a 0.5% polyvinyl alcohol solution by agitation at 550 rpm, in order to make an oil-in-water (o/w) emulsion. After 3 min, 6 ml of the ammonia solution was added to the emulsion, which was subjected to stirring for another 30 min. After addition of 40 ml, stirring was continued for 5 more minutes. The resultant suspension was first passed through a 425 μm sieve and filtered. The microspheres collected were redispersed and stirred in 80 ml of a 0.1% polyvinyl alcohol solution for 2 h. They were then collected by filtration and dried for 3 days in a vacuum oven. At least 3 batches were prepared for a given microsphere formulation. Progesterone-free microspheres were also prepared following the same procedure. Provided in Fig. 1 is an illustration of the ammonolysis-based microsphere formation.

2.3. Monitoring the status of an emulsion by light microscopy

After the ammonia solution was added to the primary o/w emulsion as described above, its aliquot was taken out by a pasteur pipet, mounted on a glass, and was observed under a light microscope (model Axiovert-200, Carl Zeiss MicroImaging Inc.; New York, USA). As control, an emulsion without being treated with the ammonia solution was also stirred for 15 min and then sampled for observation under the light microscope.

2.4. Elemental analysis

The total amounts of carbon, hydrogen and nitrogen present in raw PLGA powders and progesterone-free PLGA microspheres were quantified by the Fisons-EA1108 elemental analyzer. After both samples were subject to flash combustion, their individual element components were determined. Acetanilide was used as a standard, and the elemental analyzer was operated by the Eager 200 software.

2.5. HPLC systems

Chromatographic measurements were performed on a Shimadzu model LC-20A series HPLC equipped with a binary pump (LC-20AD), a UV/VIS detector (SPD-20A), and an autosampler (SIL-20A). The system was controlled by the Shimadzu LC Solution 1.12 software. A Luna C_{18} 5 μm column (150 mm × 4.6 mm) was used as an analytical column. A mixture of methanol and water (80:20, by v/v) was used as a mobile phase at the flow rate of 0.8 ml/min. The elution of progesterone was detected at a wavelength of 254 nm. The injection volume of a sample solution was fixed at 20 μl. In the meantime, progesterone standard solutions (16.6, 76.1, 133.1,

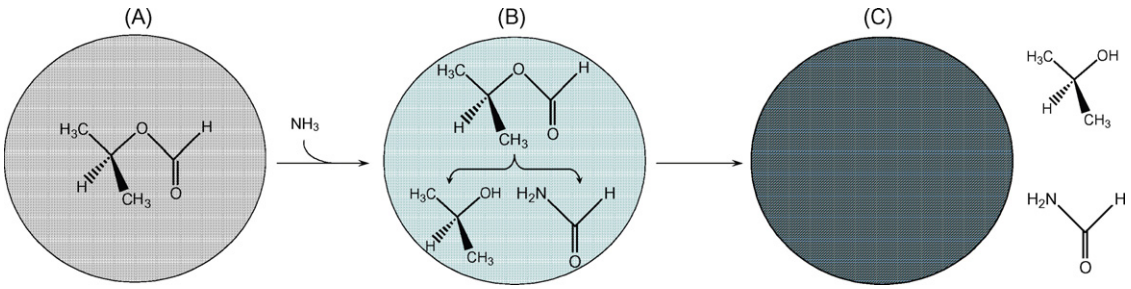


Fig. 1. An illustration of the process of the ammonolysis-based microsphere formation. (A) An emulsion droplet consisting of PLGA, progesterone and isopropyl formate is dispersed in the aqueous phase. (B) Ammonia reacts with the water-immiscible isopropyl formate to yield water-soluble products. (C) Their leaching to the aqueous continuous phase leads to microsphere hardening.

199.7, and 266 μg/ml) were prepared to construct a standard calibration curve.

2.6. Determination of progesterone microencapsulation efficiency

Microsphere samples, accurately weighed, were dissolved in 4 ml of tetrahydrofuran. One part of the solution was further diluted with 5 parts of methanol, in order to precipitate the PLGA polymer. The suspension was filtered with a nylon syringe filter (0.45 μm pore size) to remove PLGA precipitates. Aliquots of the filtrate (20 μl) were injected onto the Luna C_{18} 5 μm column. The samples were eluted isocratically with the mobile phase, following the HPLC conditions described earlier. Progesterone peaks in the chromatograms were quantitated by peak area measurement using the Shimadzu data processing system. The percentage microencapsulation efficiency of progesterone was calculated as follows: 100 × (actual loading ÷ theoretical loading) = 100 × (wt. of progesterone in microspheres ÷ wt. of microsphere samples taken for analysis) ÷ (wt. of progesterone used ÷ total wt. of progesterone and PLGA used to prepare microspheres).

2.7. Particle size analysis

The mean diameter and size distribution of microsphere samples were determined by a particle size analyzer (model Mastersizer 2000, Malvern Instruments; Malvern, UK). This instrument measures the size of particles dispersed in a medium by the scattering pattern of a laser light passing through the medium. For measurement, microsphere samples were added to the deionized water in a sample cell for counting. At least 3 batches were prepared for a given microsphere formulation, and the data were presented as mean ± SD.

2.8. Scanning electron microscopy (SEM)

The external and internal morphology of microspheres were evaluated by using a scanning electron microscope (model JSM-5200, Jeol Inc.; MA, USA). In case of observing their internal morphology, they were first embedded in epoxy resin and cross-sectioned. The microsphere samples were mounted onto metal stubs with a double-side adhesive tape and were sputter-coated under vacuum in an argon atmosphere (model SC7620 sputter coater, VG Microtech; West Sussex, England). The microsphere samples were then observed under the scanning electron microscope.

2.9. FTIR spectrometry

The infrared absorption spectra of progesterone, blank microsphere and those laden with 48.5% progesterone were separately recorded by an infrared spectrophotometer (model FTS135, Bio-Rad Laboratories; PA, USA). The instrument was operated by the IR Mentor Pro™ 2.0 software. Samples were prepared following the potassium bromide disk method, and their absorption spectra were determined between 4000 and 400 cm^{-1}.

2.10. Differential scanning calorimetry (DSC)

Thermal behavior of progesterone, raw PLGA polymer, and PLGA microspheres loaded with different amounts of progesterone were analyzed by differential scanning calorimetry. A DSC-Q1000 (TA Instrument; DE, USA) was used under nitrogen atmosphere. Samples were loaded in stainless steel pans, and a temperature ramp from 25 to 180 °C was then applied at a heating rate of 10 °C/min.

2.11. X-ray powder diffractometry (XRD)

Raw PLGA polymer, progesterone powder, blank microspheres, and those containing different progesterone payloads were placed into aluminum sample holders and exposed to Cu Kα radiation (40 Kv × 30 mA) in a wide-angle X-ray powder diffractometer (model D5005, Bruker AXS; Karlsruhe, Germany).

2.12. Drug release study

A water-bath method was used to investigate the release of progesterone from microspheres. Briefly, microsphere samples (40–45 mg) with different progesterone payloads were suspended in 20 ml of a 5% SDS solution. The microsphere suspensions were gently shaken at 37 °C for 5 days (Reciprocal Shaking Bath, Precision Scientific, Inc.; IL, USA). At predetermined time intervals, 0.5 ml of its aqueous portion were taken out for the determination of progesterone concentration by the HPLC method described earlier.

2.13. Gas chromatography (GC)

Microsphere samples (25.30–29.18 mg) were dissolved in 2 ml of methylene chloride and diluted to make a final volume of 10 ml with butanol. The appearing PLGA precipitates were removed by a 0.45 μm nylon syringe filter. An aliquot of the filtrates (1 μl) was injected to a GC-2010 gas chromatograph (Shimadzu Corp.; Kyoto, Japan) equipped with a split-injector (split ratio 15) and flame ionization detector. Nitrogen was used as a carrier gas at a column flow rate of 1.3 ml/min, whereas the crosslinked methylpolysiloxane ZB-624 column (30 m × 0.32 mm) was used as a stationary phase. The temperature of its injector and detector was maintained at 200 and 220 °C, respectively. During sample running, the oven temperature was set at 80 °C for 5 min and increased to 180 °C at a rate of 200 °C/min. For GC analysis of unknown samples, isopropyl formate standard solutions were prepared by dissolving it in butanol and diluting successively with butanol. The concentrations of its

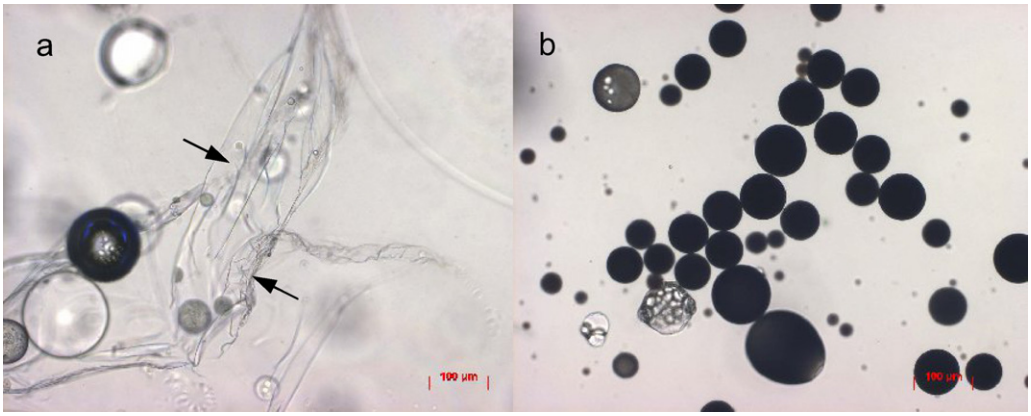


Fig. 2. LM photographs of emulsions sampled at 15-min stirring. In the absence of ammonia, emulsion droplets coalesce together to form films as indicated by arrows (a). In contrast, treatment with ammonia leads to quick microsphere solidification (b).

standard solutions ranged from 1.21 to 32.63 μg/ml. A standard calibration curve was constructed by integrating peak areas obtained with the isopropyl formate standard solutions.

2.14. Thermogravimetric analysis (TGA)

Microsphere samples were housed inside a platinum pan which was subject to heating at a rate of 10 °C/min. Weight changes of blank microspheres and those with different progesterone contents were automatically monitored as a function of temperature, using a TGA 2050 (TA Instruments; DE, USA). Dry nitrogen gas was used as a purge gas at a rate of 50 ml/min.

3. Results and discussion

Progesterone was well dissolved in isopropyl formate: its solubility at room temperature was determined to be 139.5 mg/ml. PLGA polymers with different lactide:glycolide ratios and molecular weights, as well as PLGA used in this study, also showed good solubility in the organic solvent. For instance, it was possible to prepare ≥35 wt.% PLGA solutions.

When the initial o/w emulsion was stirred without exposure to ammonia, hardening did not occur to emulsion droplets under our experimental condition. When an aliquot of the emulsion was sampled in 15-min and mounted on a glass for observation under a light microscope, irregular polymeric films appeared (Fig. 2a). This indicated that the emulsion droplets, if exposed to air, coalesced to form polymeric precipitates in the absence of mechanical stirring. By sharp contrast, treating the emulsion with the ammonia solution only for 15 min led to transformation of emulsion droplets into hardened microspheres (Fig. 2b). This phenomenon results from the fast completion of ammonolysis reaction of isopropyl formate. The conversion of water-immiscible isopropyl formate into water-soluble solvents of formamide and isopropyl alcohol serves as an effective tool to achieve fast microsphere solidification. It should be pinpointed that the volume of water used for conventional solvent extraction is on the order of 3–10 times the aqueous saturation solubility of an organic solvent. Large amounts of water are also used in order to minimize the solvent concentration in water, which contributes to preventing microsphere aggregation during collection and drying (Katou et al., 2008). If such attributes of typical solvent extraction processes are taken into account, it is definitely worthy mentioning that the use of a smaller amount of water suffices to this ammonolysis-based procedure.

It was speculated that ammonia might be entrapped physically and/or chemically inside the microspheres prepared by the ammonolysis-based method. To address this issue, elemental components (carbon, hydrogen and nitrogen) of blank PLGA75:25 microspheres were quantified by the elemental analyzer. The same experiment was performed on raw PLGA75:25 powders. The amounts of carbon and hydrogen present in both samples were the same, compared to each other. It was also found that both samples contained negligible amounts of nitrogen (less than 0.3 wt.%). These results indicate that the microspheres do not contain any significant amounts of nitrogenous residues.

Microspheres prepared in this study displayed excellent flowability. When progesterone was encapsulated into PLGA microspheres by the ammonolysis-based procedure, its encapsulation efficiency was high. When microspheres were made of 60, 160, or 250 mg of progesterone and 250 mg of PLGA, the corresponding encapsulation efficiency (mean ± SD) was 88.0 ± 3.6, 96.2 ± 1.2, and 97.0 ± 3.6%. Based on these encapsulation efficiency data, the actual contents of these microspheres were determined to be 17.0, 37.5, and 48.5%, respectively.

The extent of progesterone payload in microspheres affected their external and internal morphology (Fig. 3). The microspheres with 17.0% progesterone showed smooth external morphology. An increase of progesterone payload to 37.5% led to appearance of some drug particles on the external region of microspheres. A further increase to 48.5% progesterone led to distortions in the external structure of the resultant microspheres. In addition, increases in progesterone payloads induced the appearance of bigger cavities inside their microspheres. Phase separation occurring at higher progesterone loadings, as suggested elsewhere (Kim et al., 2007), might be partly responsible for the formation of drug crystals and bigger cavities. Such phenomena are also likely to be due to the spontaneous removal of isopropyl formate and/or insufficient amounts of PLGA available for microencapsulating progesterone. In fact, increasing the amount of PLGA used to prepare microspheres helps not only reduce the amount of drug crystals but also decrease the size of inside cavities.

Fig. 4 shows the size distribution patterns of microspheres containing various amounts of progesterone. Significant differences were not observed among them. Their mean volume-weighted diameters ranged from 123 to 141 μm which could be considered appropriate sizes for subcutaneous injection. Even though microspheres with sizes of 3–30 μm were present, their proportion was not more than 2.21% of the total microspheres.

Sometimes, a microencapsulation procedure can influence polymer property and induce drug–polymer interactions (Thanoo et al., 2005). A nucleophilic ammonia, being used in our microencapsulation process, might have reactivity toward prednisolone and/or ester linkages of PLGA. To answer for progesterone's structural integrity, the FTIR spectra of prednisolone, blank microspheres, and 48.5% progesterone-containing microspheres were determined (Fig. 5). As far as the IR spectrum of progesterone-free PLGA

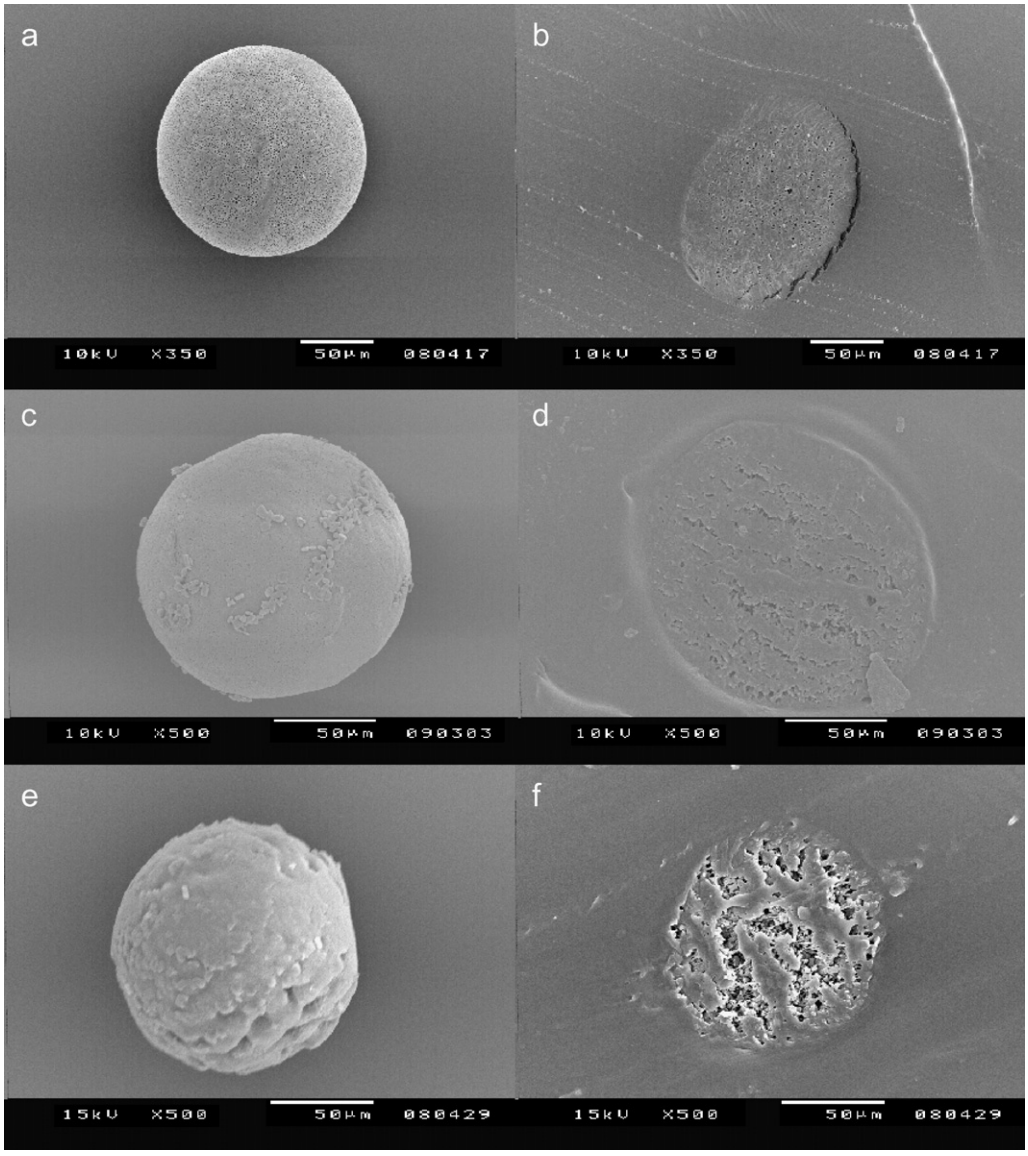


Fig. 3. SEM micrographs of the external/internal morphology of microspheres laden with (a/b) 17.0%, (c/d) 37.5%, and (e/f) 48.5% progesterone. The size of bar is 50 μm.

microspheres is concerned, the utmost characteristic band of the PLGA polymer is related to the band of ester groups occurring in 1772–1790 cm^{-1} (Silva-Júnior et al., 2008). In case of progesterone, it contains one vinyl keto group in C3 and another aliphatic methyl keto group in C20. The two carbonyl-stretching bands of C3 and C20 are manifested by the peaks at 1664 and 1699 cm^{-1}, respectively. The OH group of progesterone showed characteristic absorption bands around 3400 cm^{-1}. It was emphasized before that absorption bands representing the C=O spectral regions were important with regard to elucidating the molecular state of progesterone in a delivery vehicle (Zoppeti et al., 2007). For instance, inclusion of prednisolone by a cyclodextrin derivative resulted in the modification of the relevant C=O absorption bands. When the IR spectrum of PLGA microspheres laden with 48.5% progesterone was scrutinized, all the major bands of both prednisolone and PLGA were identified. This suggested that the environment of the two C=O spectra of progesterone remained unchanged, after it was microencapsulated into PLGA microspheres. These results support that there are no significant chemical interactions between prednisolone and PLGA during the ammonolysis-based microencapsulation process.

Of course, the ammonolysis-based technique does not lead to microencapsulating all drug substances into microspheres. Ammonia can react with a number of organic compounds in the absence of catalysts. For instance, chemical reactions between ammonia and lactones yield difunctional groups of alcohol and amide. Carboxylic esters and anhydrides react with ammonia to form amides. Drug substances without those backbones might be eligible for the ammonolysis-based microencapsulation procedure. If summarized, the stability requirement for eligible drug substances should be carefully evaluated on a case-by-case basis.

Fig. 6 shows the DSC thermograms of progesterone, blank microspheres, and those with different payloads of progesterone. In accordance with literature, progesterone exists in two major polymorphic forms of α (bp = 129–131 °C) and β (bp = 121–123 °C) (Lancaster et al., 2007). In our experiment progesterone showed a sharp endotherm at 129 °C, representing the melting point of α form. Raw PLGA powders showed Tg transition, due to their amorphous nature. Interestingly, the Tg of blank PLGA microspheres occurred at a slightly higher temperature, compared to that of raw PLGA powders. The microspheres containing 17.0% progesterone were subjected to glass transition at a lower temperature at which

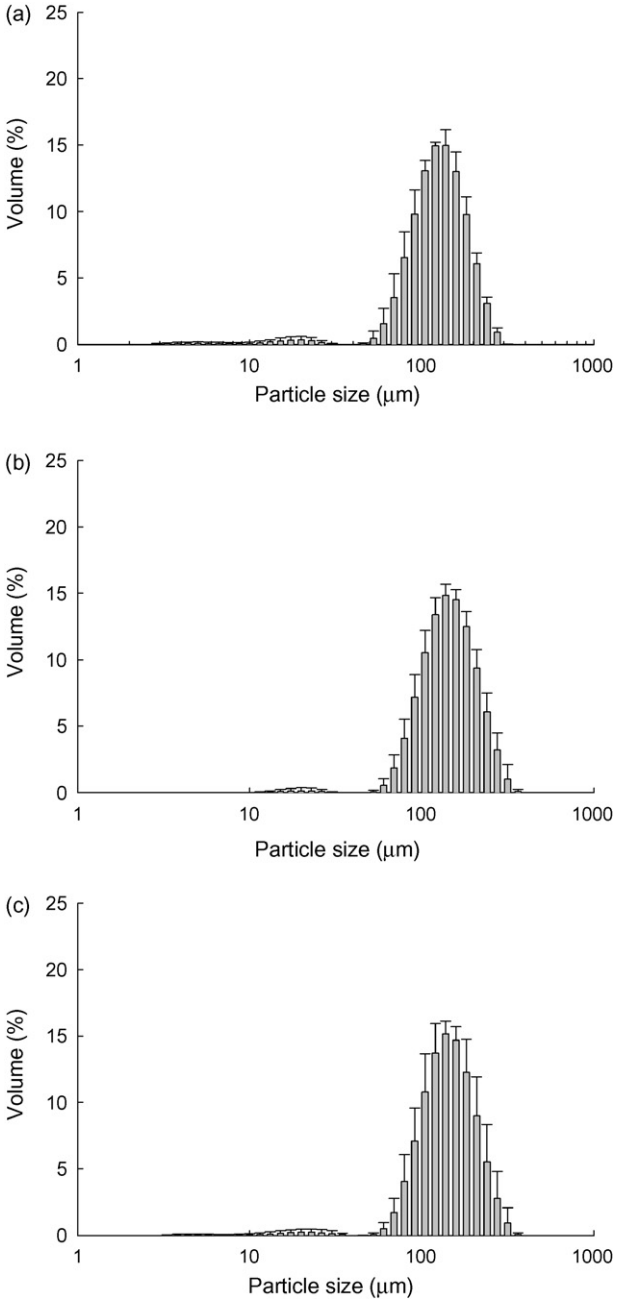


Fig. 4. Particle size distribution patterns of (a) 17.0%, (b) 37.5%, and (c) 48.5% progesterone-loaded microspheres. Their size distribution data are expressed as mean ± SD.

blank microspheres did. At the same time, the melting peak of progesterone disappeared, indicating that the drug was in molecular dispersion or a solid solution state in the microspheres (Corrigan, 1995). Such a physical status leads to a plasticization of PLGA microspheres, and its consequence is a lower Tg. This result is quite comparable with that observed with the microspheres prepared by solvent evaporation process (Rosilio et al., 1998). In contrast, different thermal behavior was manifested at higher progesterone payloads. For example, increases in progesterone payloads to 37.5 and 48.5% accompanied Tg shifts back to 54 °C, the same temperature at which blank microspheres underwent glass transition. These results indicate that, when microspheres are loaded with higher amounts of progesterone exceeding the solubility in PLGA, drug molecules start to crystallize. Because drug crystals do not help improve the chain flexibility of PLGA, the Tg of these microspheres seems to be shifted back to its original characteristic value.

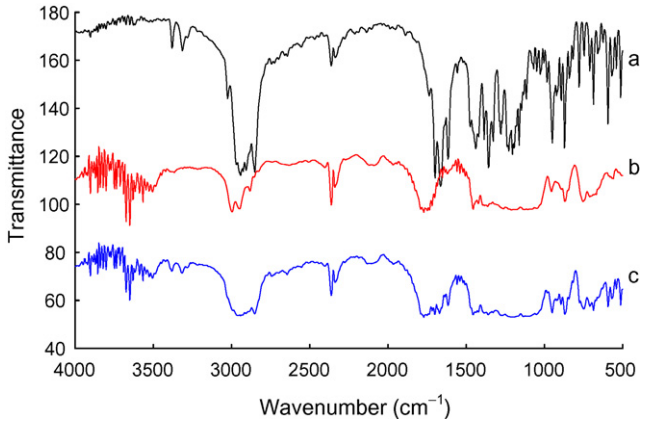


Fig. 5. Infrared spectra of (a) progesterone, (b) progesterone-free microspheres, and (c) 48.5% progesterone-loaded microspheres.

Interestingly, the microspheres laden with 48.5% progesterone showed another melting endotherm at 122 °C, demonstrating that a portion of α-form was prone to polymorphic transition into β-form. Polymorphic transformation of progesterone is known to take place on several occasions. As far as the microencapsulation process is concerned, the contact of progesterone with poly-D,L-lactide is attributed to causing the polymorphic transition of progesterone. For instance, when progesterone was spray-dried in combination of poly-D,L-lactide, β form prevailed in the resultant microspheres. In contrast, progesterone crystallized predominantly in α form, when spray-dried alone (Bodmeier and Chen, 1988). Another interesting study also reports the physical status of progesterone in the microspheres prepared by a solvent evaporation process (Rosilio et al., 1998). When excessive amounts of progesterone were loaded into the microspheres, progesterone underwent polymorphic transformation. The proportions of α and β forms in the microspheres were affected not only by the type of PLGA but also by the degree of progesterone loading. In particular, the latter was speculated to decide the proportions of α and β forms by affecting a drug crystal volume to drug crystals/polymer interface ratio. The appearance of the β form in our 48.5% progesterone-loaded microspheres resembles those previous trends. Our results also substantiate that polymorphic transition of a drug substance is dependent upon its concen-

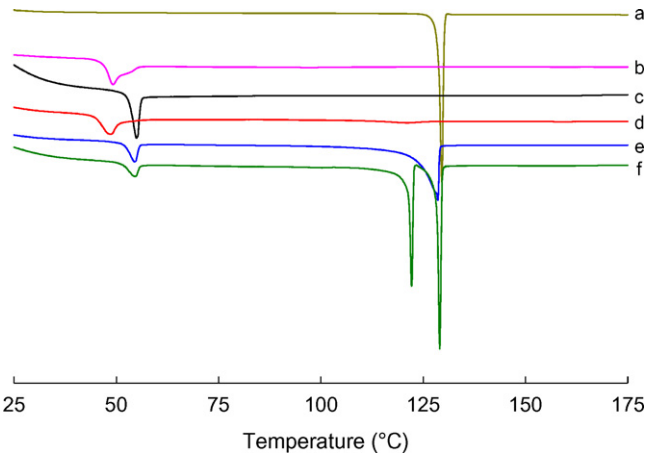


Fig. 6. DSC thermograms of (a) progesterone, (b) PLGA, (c) blank microspheres, and microspheres laden with (d) 17.0%, (e) 37.5%, and (f) 48.5%. The degree of progesterone payload affects its physical status and thermal behavior of PLGA.

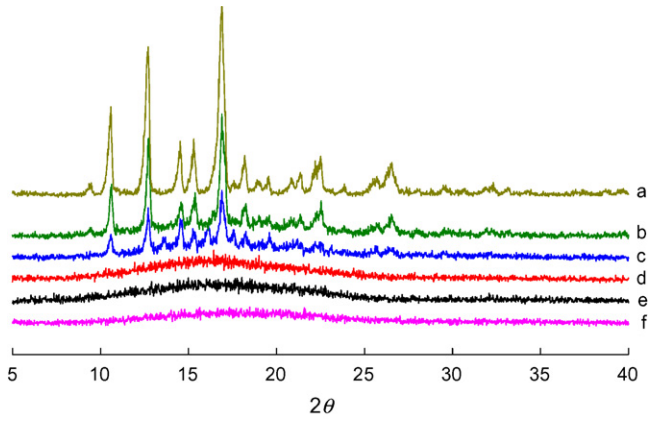


Fig. 7. XRD patterns obtained with (a) progesterone, (b) 48.5%, (c) 37.5%, and (d) 17.0% progesterone-loaded microspheres, (e) blank microspheres, and (f) raw PLGA polymer.

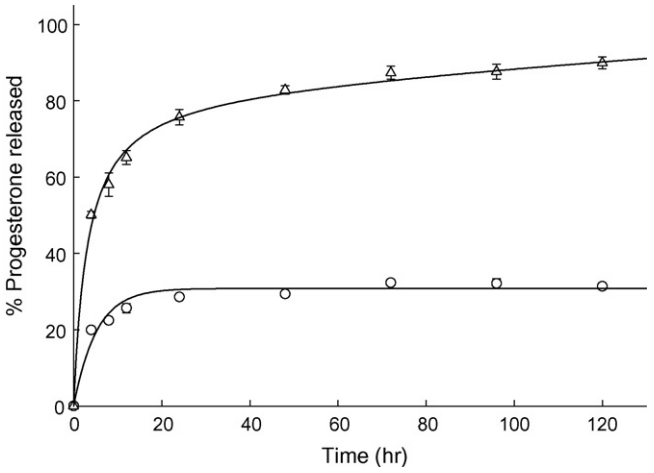


Fig. 8. *In vitro* progesterone release profiles observed with (◯) 17.0% and (△) 48.5% progesterone-containing microspheres.

tration in a vehicle – polymorphic transition does not occur readily at a low drug concentration, or vice versa (Silva-Júnior et al., 2009).

Shown in Fig. 7 are XRD patterns of progesterone, the raw PLGA polymer and microspheres containing different progesterone contents. The diffraction pattern of progesterone substantiates its crystalline nature. On the contrary, the XRD patterns of both PLGA itself and blank microspheres suggest that they are amorphous. The microspheres laden with 17.0% progesterone presented a diffuse diffraction pattern, indicating progesterone is molecularly dispersed in the polymeric matrix to exist as an amorphous status. However, increases in progesterone payloads triggered the presence of crystalline progesterone. All these data are consistent with the thermal analysis data shown in Fig. 6. Both XRD and DSC data substantiate that the extent of a progesterone payload plays a role in deciding its physical status in PLGA microspheres.

Fig. 8 shows the release profiles observed with 17.0 and 48.5% progesterone-containing microspheres. The drug release study was carried out with use of a 5% SDS solution. SDS has been frequently used in dissolution studies of many poorly water-soluble drugs listed in official monographs. Since progesterone was practically insoluble in water, SDS was dissolved in water to make a 5% solution. Progesterone solubility in the SDS solution was determined to be 9.7 mg/ml. Under our experimental conditions the release medium provided a sink condition. The microspheres loaded with 17.0% progesterone provided only an initial burst ($28.6 \pm 0.5\%$ of the dose in 1 day). A gradual release of progesterone did not follow, and a major portion of progesterone still remained inside the microspheres. Drug release is generally driven by a combined process of hydrolysis and erosion of PLGA microspheres. Such incomplete release of progesterone suggests that the microspheres do not undergo significant degradation over a 5-day period. On the contrary, the microspheres containing 48.5% progesterone acted in a different way: $89.9 \pm 1.6\%$ of progesterone was released in 5 days. Our results demonstrate that at 48.5% loading, progesterone crystals are ubiquitously present in the microspheres. SDS-mediated micellar solubilization of progesterone crystals and their leaching might lead to the generation of interconnected pores and/or channels, thereby facilitating progesterone release. This conclusion is supported by the SEM micrographs of the microspheres taken at the end of the release study (Fig. 9).

In the previous practice of the ammonolysis-based microencapsulation, we used methyl chloroacetate and ethyl chloroacetate with boiling points of 129.5 and 142.9 °C, respectively (Kim et al., 2007; Chung et al., 2009). All blank microspheres prepared by the halogenated organic solvents had residual solvents of not less than 2.67%. At that time, when microspheres were prepared with 0.25 g of PLGA and 250 mg of progesterone using the two halogenated ester solvents, solvent residues were in the range of 1.62–1.67%. To provide quantitative information on the residual isopropyl formate (bp = 68.2 °C), a gas chromatographic analytical procedure was developed in this study. Fig. 10 shows a typical GC chromatogram obtained under our experimental conditions. When the test was repeated with 5 times with 1 μl of an isopropyl formate

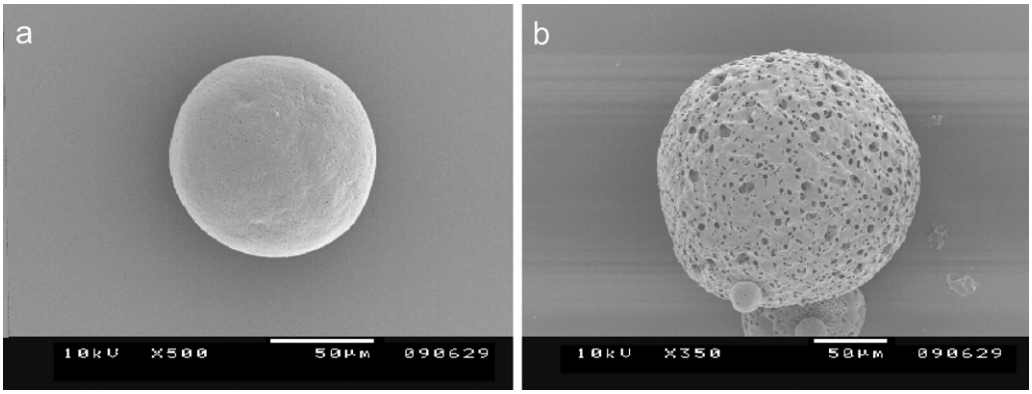


Fig. 9. SEM micrographs of (a) 17.5% and (b) 48.5% progesterone-loaded microspheres taken at the end of the drug release study. Numerous big cavities and/or pores are observed with 48.5% progesterone-loaded microspheres. The size of bar is 50 μm.

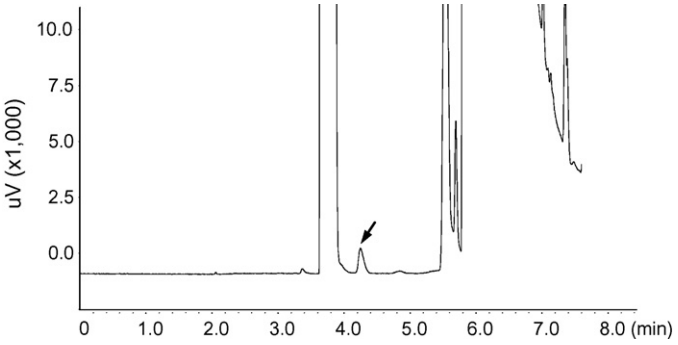


Fig. 10. A typical gas chromatogram of microsphere samples prepared by the analytical procedure described in text. An arrow indicates the peak of isopropyl formate.

standard solution (3.63 μg/ml), the relative standard deviation of the peak area of isopropyl formate was 1.14%. It can be inferred from the system repeatability data that the GC method was reliable and could be used for determining isopropyl formate content in microsphere samples. Fig. 11 shows that the levels of isopropyl formate remaining in the microspheres are very low: not more than 0.34 ± 0.07% of residual isopropyl formate was found in all the microsphere formulations. The amount of a residual solvent is generally known to be inversely proportional to drug content in microspheres. In our case, progesterone-free microspheres and those with 48.5% progesterone had similar levels of residual isopropyl formate. These data were strikingly different from and much better than the earlier data obtained with methyl chloroacetate and ethyl chloroacetate. The lower boiling point of isopropyl formate, as well as better water solubility of its ammonolysis products, might have contributed to minimizing the amounts of solvent residues. Another great advantage of isopropyl formate arises from the fact that it is a nonhalogenated, safer organic solvent being currently used as a flavoring agent in human food.

To complement the GC data shown in Fig. 11, TGA was also performed on blank microspheres and those laden with different amounts of progesterone. When progesterone-free microspheres were heated to 125 °C (a temperature higher than boiling points of water and isopropyl formate), 99.62% of their original mass remained (Fig. 12). This suggested that the microspheres contained equal to or less than 0.38% of residual isopropyl formate. Upon heating to 125 °C, masses of the microspheres with various amounts of progesterone also remained almost unchanged: the residual amount of isopropyl formate was not more than 0.31%. These values are quite in line with the GC data shown in Fig. 11. In fact, the residual level of an organic solvent is of paramount issue in developing a microsphere dosage form. Let alone environmental and health risks caused by halogenated organic solvents, their presence in microspheres often causes aggregation, affects syringeability, and/or changes drug release profiles. Before experiment, it was supposed that relatively higher amounts of isopropyl formate would be found in microspheres, because its boiling point was much higher than that of methylene chloride (40 °C) commonly used in microencapsulation. However, it turned out that the ammonolysis-based microencapsulation process utilizing isopropyl formate allowed the preparation of microspheres with very low levels of the residual solvent. For instance, 1.36–3.8% of methylene chloride was shown to be present in PLGA microspheres, when the solvent was used for typical emulsion-based solvent evaporation/extraction processes (Spenlehauer et al., 1986; Wantier et al., 1997).

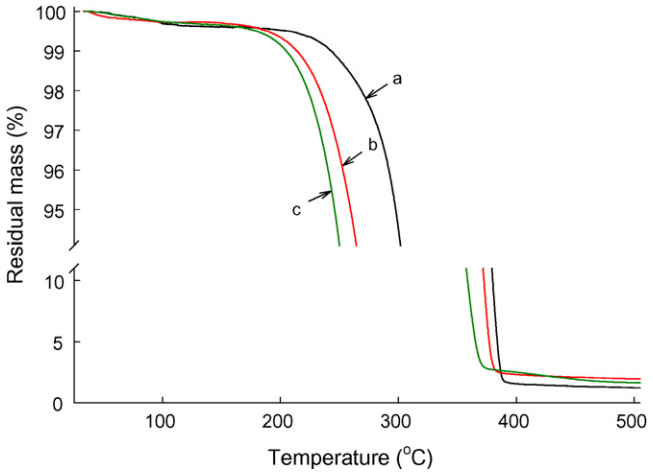


Fig. 12. TGA curves obtained with (a) progesterone-free microspheres and the microspheres laden with (b) 17.0% and (c) 48.5% progesterone.

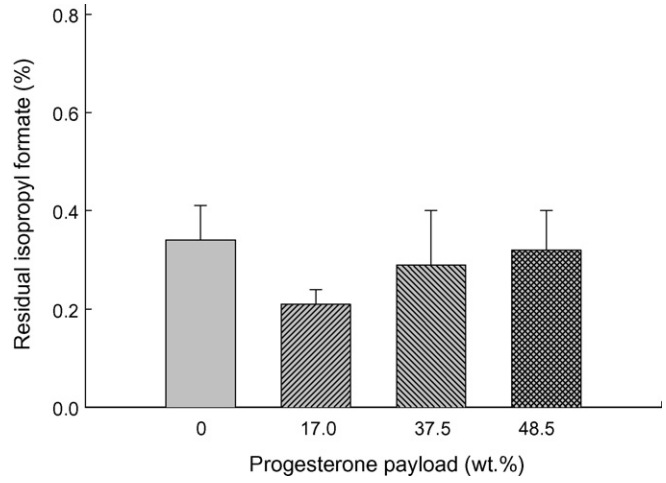


Fig. 11. The levels of residual isopropyl formate found in (a) blank microspheres and those laden with (b) 17.0%, (c) 37.5%, and (d) 48.5% progesterone. The ammonolysis-based microencapsulation helps keep its residual amount low.

4. Conclusions

An important quality concern in the development of PLGA microsphere products includes toxicity of a dispersed solvent and its residue. Since solvent residues accelerate instabilization of pharmaceuticals and change microsphere characteristics, it is highly desirable to minimize solvent residues to an acceptable level by regulatory agencies. Therefore, there have always been increasing demands to develop a microencapsulation process that removes solvent residues from microspheres in an efficient way. As far as a manufacturing process is concerned, sophisticated equipment is often needed to proceed with solvent removal. Solvent extraction also tends to require a large volume of a quench liquid and subsequently generates a vast amount of waste stream. The ammonolysis-based microencapsulation process reported in this study uses a safer, nonhalogenated isopropyl formate and makes use of a chemical method to achieve solvent removal at ambient conditions. It is found that the technique contributes to keeping solvent residues at low levels. In addition, this simple microencapsulation technique does not require a large volume of a quench liquid, thereby minimizing the generation of waste stream in which an organic solvent is dissolved. It is expected that the microencapsulation technique might find some applications in developing microsphere dosage forms.

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