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Articles

The Evaluation of Fabrication Parameters Process Effect on the Formation of Poly(lactic-*co*-glycolic acid) (PLGA) Microspheres

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In this study, a poly(lactic-*co*-glycolic acid) (PLGA) microspheres was fabricated using emulsion solvent evaporation technique. During the procedure fabrication, some parameters process have effected on the formation of micro-carriers. The structure and morphology of micro-carriers were evaluated by SEM observation. Beside, heparin incorporated into microspheres was determined using toluidine blue method. Specifically, the effects of some parameters process such as ultrasonic levels, PLGA concentrations and freeze-dry times on the size, structure, porous formation and heparin entrapment of micro-carriers were studied carefully. We found that, the morphology and structure of carriers were influenced by the all above parameters. The diameter of the carriers varied from 20 to 400 μm depending on experimental conditions. At suitable freeze-dry time, the pores were automatically formation on surface of microspheres with a significantly in the numbers of pore. After heparin incorporated porous PLGA microspheres, it was suggested that the highly heparin incorporated into porous PLGA microspheres could enhance of angiogenesis for tissue regeneration easily.

Key Words : Porous PLGA microspheres, Drug delivery system, Heparin.

Introduction

Due to the excellent biocompatibility, biodegradability, thermoplastic aliphatic poly(esters), such as poly(lactide) (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), and PLGA, are most commonly used as a drug carrier. PLGA has recently been used more widely in drug delivery systems because of its efficacy in pharmaceutical and biomedical applications [1]. In addition, PLGA can be degraded into non-toxic substances and removed from the human body [2]. This process can be accelerated by altering the diffusion and release rate of the drug [3].

For drug delivery study, the use of microspheres polymeric carriers in the therapeutic applications is a promising approach to inject or implant into the injure organs of the body [4]. Beside, functionalize the surface of microspheres have become necessary in the biomedical and pharmaceutical applications [5]. Moreover microspheres polymeric as drug delivery systems have been widely investigated for inducing angiogenesis. In particular, PLGA microspheres loaded with various growth factors have received much attention, because they can be directly injected into a tissue defect site to induce angiogenesis by local sustained release actions [6]. In satisfactory medical therapy, an understanding of physicochemical characteristics of microsphere, such as size, structure, composition of organization, loading efficiency and release property was often of critical concerns [7]. On the other hand, it has some methods to fabricate microspheres but the most widely used method for preparing microspheres encapsulating drugs or proteins was a water-in-oil-in water ($W_1/O/W_2$) double emulsion solvent evaporation technique [6]. In this process, some parameters such as stirring speed, inner water volume, outer water volume, polymer concentration and drug loading have effected on the physicochemical characteristics of microsphere [8]. However, in our research not only above mentioned parameters but also ultrasonic conditions and freeze-dry times have investigated to know deeply about the formation of porous PLGA microspheres aiming apply as drug delivery system for tissue regeneration.

Heparin is a polysaccharide macromolecule [9], an efficacious substrate for the functionalized surfaces of biomaterials [10]. Furthermore, it plays a pivotal role in process such as cell adhesive, cell growth [9] because of its association with the cell surface and extra cellular matrix [5] and it has been recently reported to have ability to accelerate angiogenesis [11].

In this study, we investigated the influence of parameters process on the formation of carriers to enhance well ability of drug delivery system for tissue regeneration. PLGA microspheres were fabricated by $W_1/O/W_2$ emulsion solvent evaporation method. After that the microspheres' morphology, microstructure were evaluated and quantifying the amount of heparin incorporated into PLGA microspheres were determined.

Materials and Methods

Materials. Poly(vinyl alcohol) was purchased from Sigma-Aldrich (St, Louis, MO U.S.A). Poly (lactic-*co*-glycolic acid, 50:50, inherent viscosity 0.15-0.25) was obtained from Aldrich (St, Louis, MO U.S.A). Heparin ammonium salt (porcine intestinal mucosa) was purchased from Sigma-Aldrich Chemie GmbH. Dichloromethane 99.5% was obtained from Samchun Pure Chemical Co., ltd (Pyeongteak city, Gyeonggi-do, Korea). All other chemicals and solvents were of analytical reagent grade.

Preparation of Microspheres. The PLGA microspheres were prepared by the emulsion solvent evaporation technique. The method was based on the use of a homogenizer in the two-step emulsification process. Briefly, 100 μL of a heparin solution (or distilled water) was first emulsified in dichloromethane (DCM) containing the PLGA by sonication using a probe sonicator (Sonifier-Brason 450). The resulting water-in-oil (W_1/O) emulsion was then poured into 4 mL of PVA (1%) and stirred for 2 minutes to form a multiple water-in-oil-in-water ($W_1/O/W_2$) emulsion. The resulting $W_1/O/W_2$ emulsion was added to 200 mL of a PVA aqueous solution (0.1%) and stirred for 45 minutes. The organic solvent was allowed to evaporate while being stirred at atmospheric pressure. After a few minutes, the microspheres were isolated by centrifugation. Finally, they were washed three times with distill water to remove unincorporated substances before freeze-dry. All parameters process effect to the fabrication of PLGA microspheres were summarized in Table 1.

Morphology of Microspheres. The morphology of the microspheres and microspheres' surface were analyzed by SEM (SEM, JSM-5410LV, Jeol). After washing by distill water, the samples were placed in a glass vial and dried using a freeze-dry machine. The samples were then coated with a fine gold layer and the morphology was observed using a JSM-5410LV scanning microscope.

Determination of Heparin Incorporated in PLGA Microspheres. Colorimetric determination of the heparin incorporated PLGA microspheres was performed using the toluidine blue method. All operations were conducted at room temperature. First, 2 g NaCl was dissolved in 1000 mL H_2O to create a 0.2% NaCl solution and then 0.25 g toluidine was dissolved in 500 mL of HCl 0.01 N containing 0.2% NaCl. The heparin solution was prepared by adding 0.05 g of heparin diluted to 50 mL in 0.2% NaCl, resulting in a 1 mg/mL heparin solution. Hexane liquid was then prepared. The method used to prepare a set of test samples was as follows: 17 μg heparin loaded microspheres (test sample) was dissolved in 10 mL 0.2% NaCl solution by slowly increasing the temperature. When the sample had completely changed to a suspension, it was stirred for 2 hours at room temperature. After the above reagents were ready, the experiment was conducted. Two and a half milliliters of the toluidine blue solution was pipetted into 15 different test vials. Varying amounts of the standard heparin solution were added to vials 1-11 and these vials were used to establish a standard curve in the concentration range of approximately 0.25-10 μg/mL. Vials 12-14 contained 2 mL of the test sample. Vial 15 contained 2 mL of only the PLGA sample. All 15 vials were agitated using a Vortex mixer for 30 seconds. Hexane (3 mL) was then added to each vial and the vials were shaken vigorously for another 30 seconds. Two μL from each of the 15 vials were then placed into a 96-well plate and the absorbance at 630 nm was measured using an ELISA plate reader.

Results and Discussion

Figure 1 shows the SEM micrographs of PLGA microspheres, which were synthesized at different ultrasonic conditions. In sample S1, which was synthesized using 40 W of output for 2 minutes, the microspheres stuck together and created a network structure, as shown in Figure 1(a). It was found that the surface of the particles was uneven. In sample S2, which was synthesized using 50 W of output for 2 minutes, the particles were granular shape with a diameter larger than 100 μm (Fig. 1(b)). Beside the diameter of the microspheres in sample S3 was around 90 μm and the microparticles did not aggregate. The formation of microspheres with small in diameters and homogeneous structures was achieved when the ultrasonic condition was set at 60 W of output for 2 minutes (Fig. 1(c)). From these results showed that ultrasonic conditions can be used to control the size and shape of PLGA microspheres in the fabrication of PLGA microspheres.

As shown in Figure 2, the morphology of the PLGA microspheres was dependent upon the concentration of PLGA. In sample S4, which was synthesized using 0.2 g/1 mL DCM, the size of the microspheres was less than 400 μm in diameter and the shape of the particles was a uniform in

Table 1. Difference parameters process of the fabrication PLGA microspheres

Samples	Ultrasonic	Concentration of PLGA	Concentration of Heparin	Freeze-dry time
S1	40 W of output for 2 minutes	0.1 g PLGA/2 mL DCM	0	0
S2	50 W of output for 2 minutes	0.1 g PLGA/2 mL DCM	0	0
S3	60 W of output for 2 minutes	0.1 g PLGA/2 mL DCM	0	0
S4	60 W of output for 2 minutes	0.2 g PLGA/1 mL DCM	0	8 h
S5	60 W of output for 2 minutes	0.1 g PLGA/1 mL DCM	0	8 h
S6	60 W of output for 2 minutes	0.1 g PLGA/2 mL DCM	0	8 h
S7	60 W of output for 2 minutes	0.1 g PLGA/1 mL DCM	0.04 g heparin/1 mL D.I water	8 h
S8	60 W of output for 2 minutes	0.1 g PLGA/1 mL DCM	0.04 g heparin/1 mL D.I water	20 h

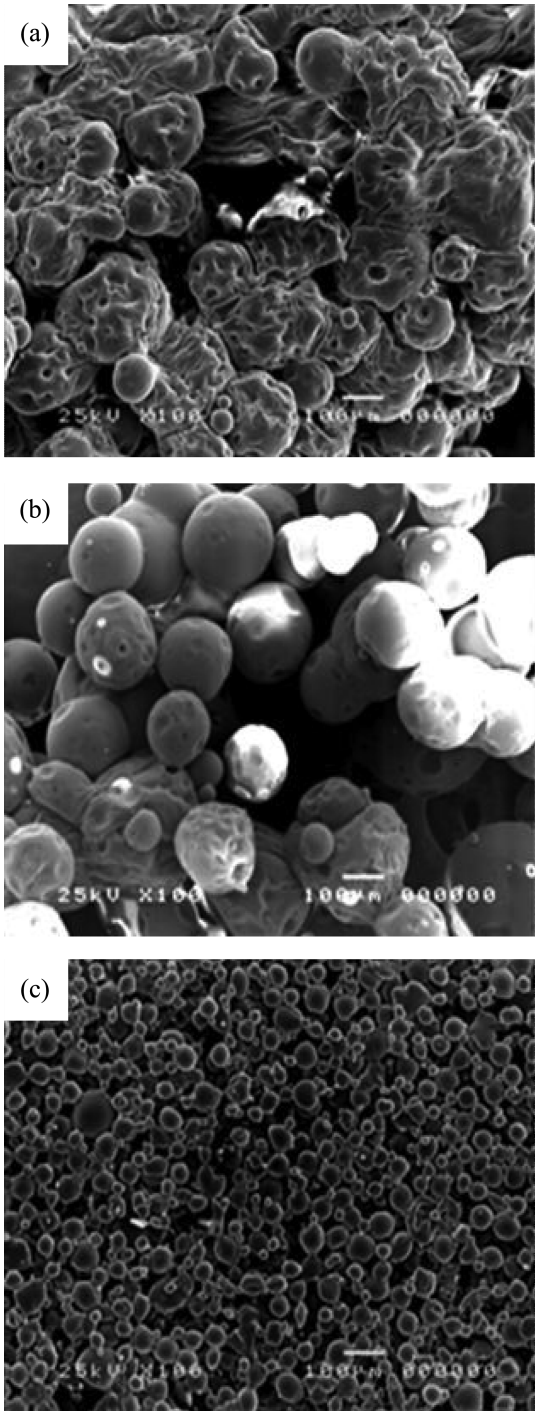

Figure 1. SEM micrographs of PLGA microspheres fabricated at different ultrasonic levels: (a) S1 (40 W of output for 2 minutes) (b) S2 (50 W of output for 2 minutes) (c) S3 (60 W of output for 2 minutes).

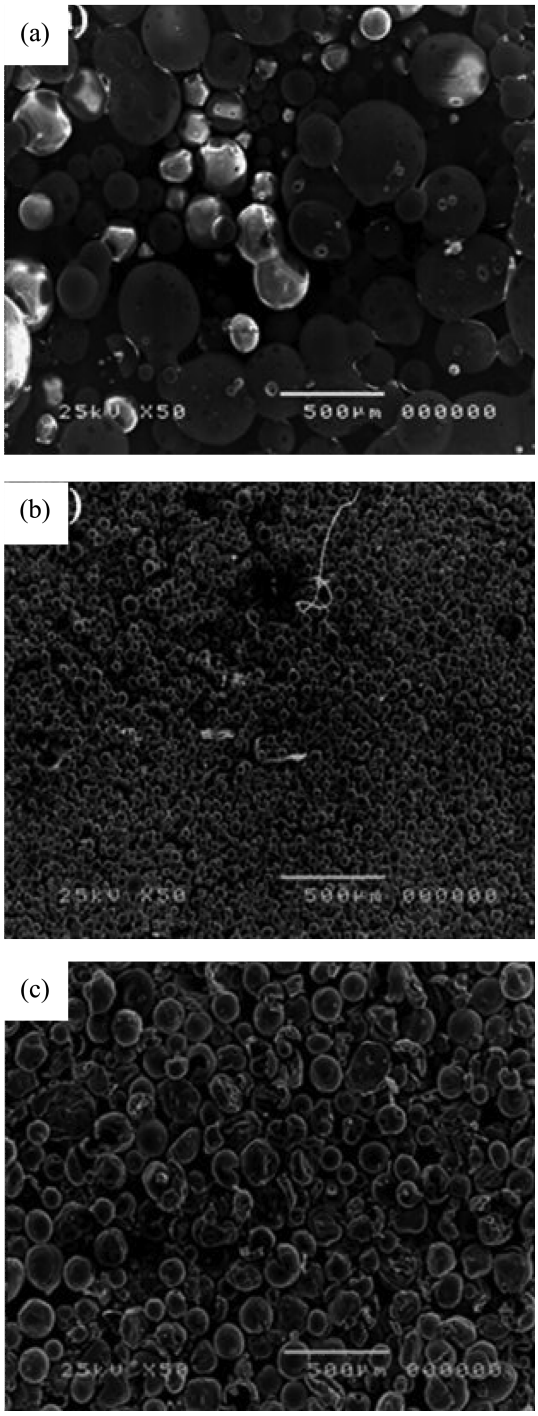

Figure 2. SEM micrographs of PLGA microspheres fabricated at different PLGA concentrations: (a) S4 (0.2 g PLGA/1 mL DCM) (b) S5 (0.1 g PLGA/1 mL DCM) (c) S6 (0.1 g PLGA/2 mL DCM).

circle form, as shown in Figure 2(a). The diameter of microspheres in sample S5 that was synthesized at a PGLA concentration of 0.1 g/1 mL DCM. It was significantly smaller than the diameter of the microspheres in sample S4, as shown in Figure 2(b). Thus, it is probable that lower concentration of polymers resulted the formation of smaller of droplet during the first emulsion state; therefore, the diameter of the microspheres was smaller [12]. As previous report, the stirring rate was one of conditions that affected on the formation of microspheres [13]; however, it was not extensively examined in this study. So that in sample S6, although it was synthesized at a concentration of 0.1 g/2 mL DCM of PLGA but the diameter of the microspheres was still larger than the diameter of sample S5 because of the effected of stirring rate, as shown in Figure 2(c).

In sample S7, the PLGA microspheres were incorporated

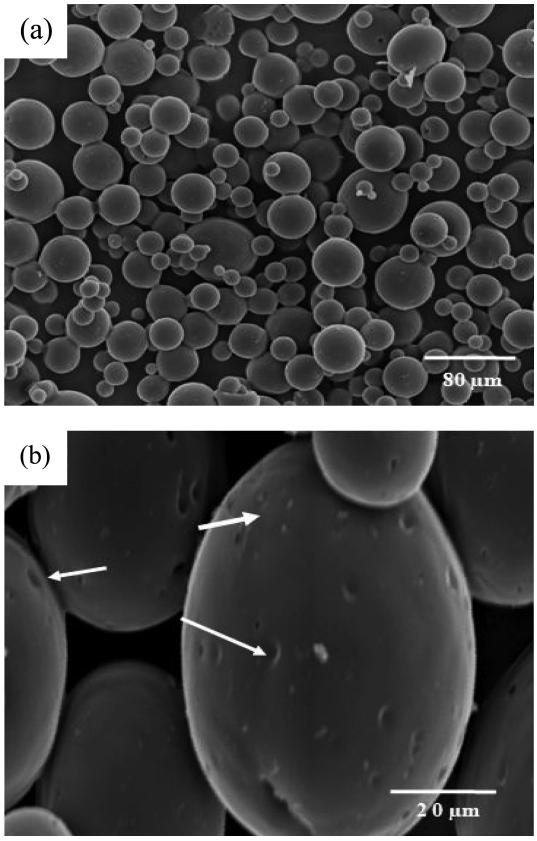

Figure 3. SEM micrographs of PLGA microspheres (S7) containing heparin: (a) at low magnification and (b) at high magnification.

with heparin in order to examine the size and shape of the microspheres. It clearly exhibited the effect of heparin on the surface morphology of PLGA microspheres. Figure 3(a) shows the morphology of the microspheres in sample S7, which were the most homogenous in the spherical shape in all previous exhibition of micro-carriers. In addition, the surface of the microspheres had several indents, as shown in Figure 3(b). It was reasoned by the solvent evaporation when the sample was made freeze-dry under −80 ºC and 5 mTorr for 8 hours. Simultaneously, many dots appeared on the surface of the microspheres, as shown in Figure 3(b). The appearance of these dotted points was hypothesised to be due to the incorporation of heparin. These results indicate that heparin affected on the morphology of microspheres. Although, sample S7 was synthesized using the same conditions as sample S5; however, the morphology and diameter of the microspheres in samples S5 and S7 were different clearly. It was reasoned by the incorporation of heparin into PLGA microspheres in the case of sample S7.

Figure 4 shows a typical standard curve for various knew concentrations of native heparin solutions. This curve was the standard curve of native heparin concentration range of 0.25-10 μg/mL that was used to extrapolate concentration of heparin. On the other hand, the heparin incorporated PLGA microspheres (test sample) were dissolved slowly in NaCl solution by slowly increasing the temperature to 100 ºC. The sample was then placed into a 96-well plate to measure the

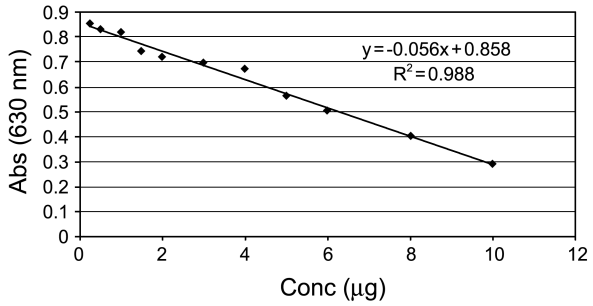

Figure 4. Standard curve shows the decrease in absorbance in the toluidine blue solution at 630 nm with increasing concentrations of native heparin.

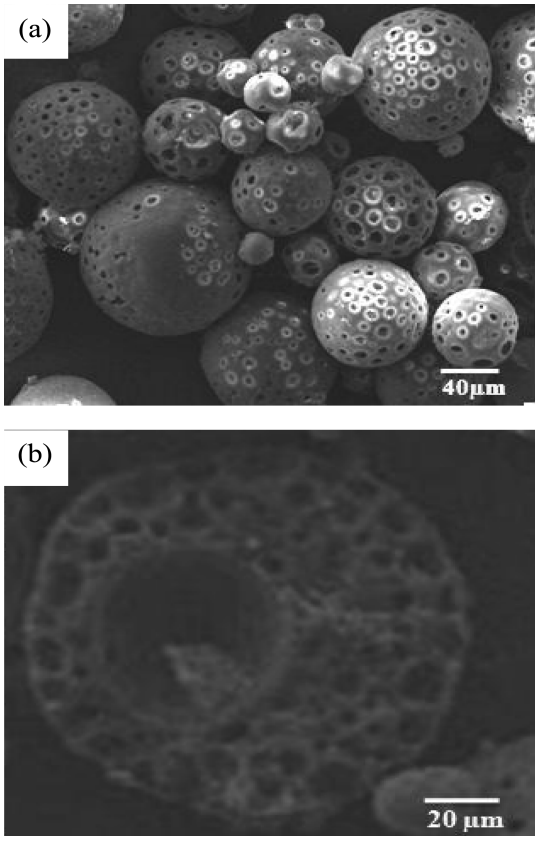

Figure 5. SEM micrographs of porous PLGA microspheres (S8) after freeze-dry 20 h (a) and its cross section (b).

absorbance using an ELISA plate reader at 630 nm. By directly extrapolating these absorbance readings on the standard heparin curve, the heparin incorporated in PLGA microspheres was determined to be 14.9%.

Figure 5 shows the SEM micrographs of porous PLGA microspheres that had a highly open external and internal porous structure. The open-pores were measured to be about 10 μm in diameter. These open-pores were scattered throughout the surface and occupied almost the entire volume of the microspheres. The pore structures can act as a reservoir to potentially hold drugs or any other additives. From this result showed that samples S8 was highly different structure compared to samples from S1 to S7. For example, sample S8

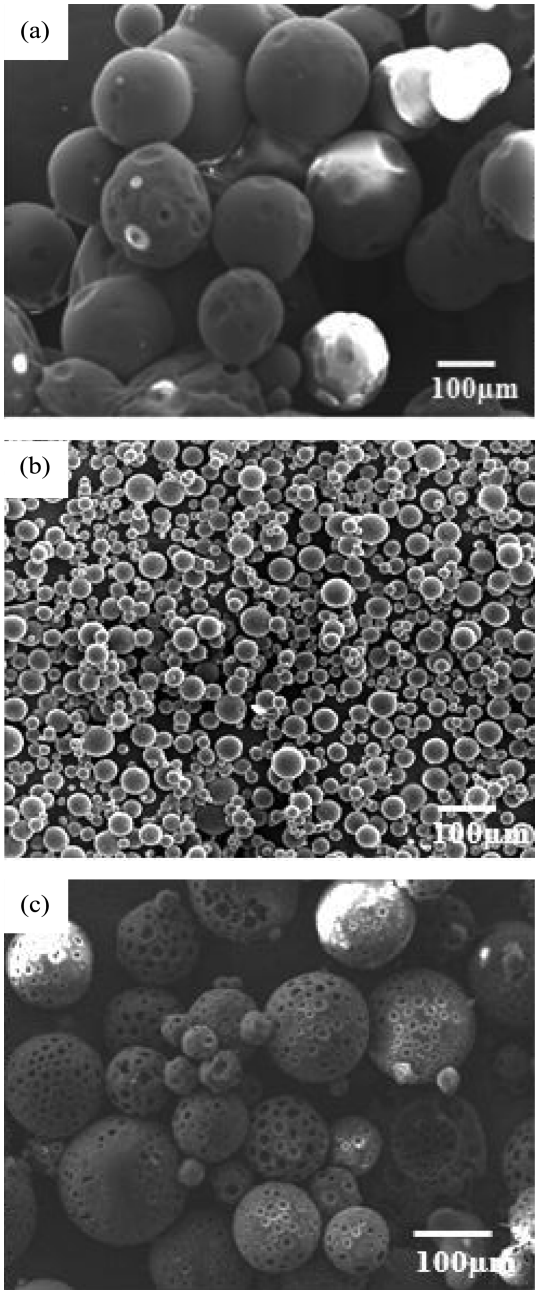

Figure 6. SEM micrographs of PLGA microspheres were synthesized at: non using freeze-dry (a), after 8 h freeze-dry (b) and after 20 h freeze-dry (c).

contained many pores on its surface, as shown in Figure 5(a), which were not observed from S1 to S7. In addition, the inner structure contained many pores that were dispersion throughout of inner spherical carriers. We can conclude that this was due to the use of the freeze-dry technique. It induced the remaining water or solvent inside of the microspheres, which was evaporated after freeze-dry in several hours. The freeze-dry technique is interested discovery phenomenon in the formation porous structure of PLGA microspheres that have not been mentioned in the synthesis of porous PLGA microspheres in the previously studies.

Figure 6 shows the significant influence of the freeze-dry

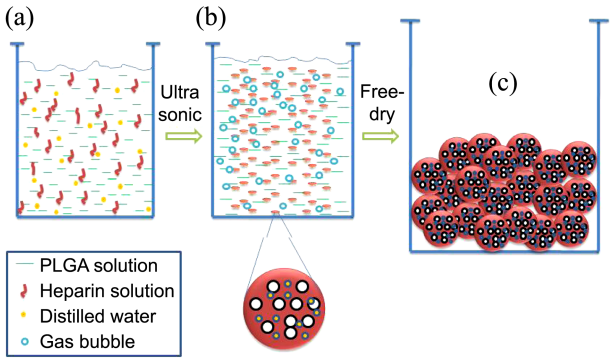

Figure 7. Schematic diagram showed the formation of porous PLGA microspheres.

time on the pores formation of microspheres. Using this technique, it was easy to create the porous structure of PLGA microspheres. The freeze-dry method is generally considered to be a new technique since it has not been discovered in the fabrication of porous PLGA microspheres. Without the use of freeze-dry, it was difficult to remove water or solvent inside the microspheres in the first emulsion step; thus, the microspheres adhered to one another, as shown in Figure 6(a). Prior to this, microspheres were separate and homogeneous. When the sample was freeze-dried for 8 hours, the outer surface of the polymer was weakened; thereby it was easy to separate from each other ones, as shown in Figure 6(b). Beside, Figure 6(c) shows sample S8 that was made freeze-dry for 20 hours. The microspheres in this sample appeared with many pores on its body because of increased the freeze-dry time. Thus, the increasing in freeze-dry time caused water or solvents easy to evaporate and create porous structure of the PLGA microspheres.

Figure 7 shows the schematic diagram of the sequential formation of porous PLGA microspheres. After the mixture of distilled water containing heparin (or only distilled water) was added to the PLGA solution (Fig. 7(a)), the solution was subjected by ultrasonic. The mixture became an emulsion solution; then PLGA micro-droplets was formed and slowly separated from the other ones to create microspheres. At this time, many gas bubbles and water droplets appeared and were incorporated into the microspheres. In the first emulsion step, gas bubbles and water droplets were blended with PLGA micro-droplets through using ultrasonic technique, as shown in Figure 7(b). In the second emulsion step, solvent was evaporated at a slow rate; simultaneously, gas bubbles collapsed and consequently created pores in the body of the microspheres. Finally, when the sample was made freeze-dry, all solvent and water that remained in the microspheres evaporated easily, gas bubbles collapsed quickly and formed many pores, as shown in Figure 7(c).

Conclusions

In conclusion, heparin-incorporated PLGA microspheres were successfully fabricated using the water-in-oil-in-water

(W1/O/W2) emulsion solvent evaporation method. In this process, ultrasonic levels, PLGA concentrations, heparin incorporation and freeze-dry times were investigated in regards to the characteristics of the PLGA microsphere and it was found that the size of microspheres were uniform and decreased in diameter when the ultrasonic level increased. In addition, the concentration of PLGA effected to the morphology of the microspheres; when the concentration of PLGA decreased, the diameter of the microspheres decreased as well expected the effect of stirring rate on the formation of microspheres. After heparin was incorporated into the microsphere, the outer surface showed the significantly with the contained many dots on microspheres' surface. The calculation based on the standard heparin curve was shown to have a performance of 14.9% heparin incorporation inside microspheres. Simultaneously, the results of this study demonstrated that freeze-dry techniques contribute to the creation of many pores, which have a diameter of approximately 10 μm.

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