Testosterone-encapsulated Surfactant-free Nanoparticles of Poly(DL-lactide-*co*-glycolide): Preparation and Release Behavior

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Received September 24, 2001

Since surfactant or emulsifiers remained on the nanoparticle surface significantly affect the physicochemical properties, the biodegradation rate, the biodistribution, and the biocompatibility of nanoparticles, surfactant-free nanoparticles should be good candidate. surfactant-free PLGA nanoparticles were successfully prepared by both the dialysis method and the solvent diffusion method. The PLGA nanoparticles prepared using the solvent diffusion method has a smaller particle size than the dialysis method. The solvent diffusion method was better for a higher loading efficiency than the dialysis method but the nanoparticle yield was lower. Testosterone (TST) release from the PLGA nanoparticles was dependent on the particle size rather than the drug contents. Testosterone release from the PLGA nanoparticles prepared by the solvent diffusion method using acetone was faster than those prepared by the dialysis method. TST release from the PLGA nanoparticles prepared by the solvent diffusion method using acetone and the dialysis method using dimethylformamide (DMF) was completed for 4 days while the PLGA nanoparticles prepared by the dialysis method using acetone showed approximately 80% TST release after 4 days. Since the PLGA nanoparticle degradation ratio was below 20% within 5 days at all samples while TST release completed within 4 days, TST release was dependent on the diffusion mechanism rather than degradation.

Key Words : Surfactant-free, Poly(DL-lactide-*co*-glycolide), Nanoparticles, Dialysis, Solvent diffusion

Introduction

Since the size of nanoparticles range 10 to 1000 nm, they have been extensively investigated for use as targeted drug delivery systems. They are useful for treating several disease such as cancer chemotherapy, gene therapy, viral disease, and microbial infection.[1-3] The polymers used to make the nanoparticles for administration into the human body are significantly limited to a few types of polymers due to their biocompatibility and biodegradation although various polymers can be employed to make nanoparticles. PLGA and its homo- or copolymers are the most widely used biodegradable polymers for making nanoparticles. Emulsion solvent evaporation techniques are most frequently employed to fabricate nanoparticles using PLGA and a significant amount of poly(vinyl alcohol) (PVA), the most abundant stabilizing agent, is required. PVA has some problems associated with its use because it remains at the particle surface making its removal difficult. PVA frequently modifies the surface characteristics of the particles, resulting change in the biodegradation rate, the body distribution, the drug release characteristics, and biocompatibility.[4-6]

From these viewpoints, surfactant-free nanoparticles or microspheres are of significant interest and investigated by several groups in the last decade.[7-10] Surfactant-free nanoparticles of poly(DL-lactide) prepared by the interfacial polymer deposition technique were first reported by Fessi *et al.*.[7] Several investigators have extensively employed this method for a decade. A surfactant-free nanoparticulate system has many advantages such as easy preparation, prevention of side-effect from the nanoparticle surface-located surfactant on the human body, and avoiding complexation of the physicochemical properties of the polymer nanoparticulate system from the surfactant on the drug release mechanism and polymer degradation.

In this study, surfactant-free nanoparticles was prepared by a dialysis and solvent diffusion method. The changes in the physicochemical properties, drug release behavior, and biodegradation rate of the surfactant-free nanoparticles were investigated and compared.

Experimental Section

PLGA 50 : 50 and testosterone (TST) were purchased from Sigma Co. USA. Acetone, methanol, dimethylformamide (DMF), and tetrahydrofuran (THF) were used as reagent grade.

The surfactant-free PLGA nanoparticles by the dialysis method were prepared as follows: 50 mg of PLGA and 10 mg of testosterone were dissolved in either acetone or DMF. The resulting solution was introduced into a dialysis tube

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(molecular weight cut-off (MWCO) size: 12,000 g/mol, Sigma Co. USA) and dialyzed against 1 L of deionized water for 12 h. Water was exchanged at 2 h intervals to remove the solvent. The dialyzed aqueous solution was adjusted to 50 ml for either drug release test and analysis, or it was freeze-dried. The surfactant-free PLGA nanoparticles by the solvent diffusion method were prepared as follows: 50 mg of PLGA and 10 mg of testosterone were dissolved in acetone and dropped into 50 mL of deionized water. The resulting solution was stirred for 30 min under reduced pressure and the residual solvent was removed using a rotary evaporator. The nanoparticle solution was used for either the drug release test and analysis, or it was freeze-drying for further use.

The nanoparticle morphology was observed using Field Emission-Scanning Electron Microscope (FE-SEM, P-4700, Hitachi Co., Ltd., Japan). One drop of the nanoparticle solution was placed onto a cover glass and freeze-dried. The cover glass was placed onto a copper grid using double-sided tape. The sample was then coated with gold/palladium using an ionsputter (JFC-1100). The observations were performed at 25 kV for SEM and 5.0 kV for FE-SEM. The particle size distribution was measured by photon correlation spectroscopy (Zetasizer 3000, Malvern Instruments, England) equipped with a He-Ne laser beam at a wavelength of 633 nm at 25 °C (scattering angle of 90°). The X-ray powder diffraction (XRD) were obtained with a Rigaku D/Max-1200 (Rigaku) using Ni-filtered CuKα radiation (35 kV, 15 mA).

The drug content in the nanoparticles were determined as follows: 10 mg of the freeze-dried nanoparticles was dissolved in acetone and measured at 238 nm with a UV spectrometer (UV-1200, Shimadzu Co., Ltd., Japan). The drug release test was performed using a dialysis tube as follows: 10 mL of the nanoparticle solution that was prepared using the above method was introduced into a dialysis tube and the dialysis tube was then placed into a bottle with 100 mL of phosphate buffered saline (PBS, 0.1 M, pH 7.4). The release test was performed at 37 °C with stirring rate of 100 rpm. At specific time intervals, the whole media was discarded and replaced with fresh PBS to prevent drug saturation. The amount of TST released was measured by a UV spectrophotometer at 238 nm.

The molecular weight (Mw) of the PLGA 50/50 was measured by gel permeation chromatography (GPC). The GPC system used was a Waters LC system coupled with a Waters 410 Differential Refractometer using Waters Styragel™ HR1, HR2 and HR4 column at a flow rate of 1 mL/min. THF was used as the eluent. The average M.W. was evaluated by standard polystyrene. Weight-average molecular weight (Mw), number-average molecular weight (Mn), and Mw/Mn of PLGA 50 : 50 were aproximately 48,000, 40,000, and 1.2.

For the degradation study of the surfactant-free PLGA nanoparticles, the nanoparticle solution (20 mg as a PLGA weight) was introduced into the dialysis tube (12,000 g/mol) and incubated at 37 °C with 80 mL of PBS at 100 rpm. At specific time intervals, dialysis tube samples were taken and dialyzed against distilled water for 6 hrs. The resulting solution was freeze-dried for analysis of the molecular weight change by GPC as described above. The degradation ratio was calculated as follows;

$$\text{Degradation ratio} = \frac{(\text{Initial M.W. of PLGA} - \text{M.W. of PLGA at time. t})}{(\text{Initial M.W. of PLGA})} \times 100$$

Results and Discussion

PLGA nanoparticles were prepared by dialysis technique or solvent diffusion and evaporation method. When dichloromethane, which is a water-insoluble organic solvent, was used to make the PLGA nanoparticles, the nanoparticles could not be prepared by solvent evaporation method without use of a surfactant such as PVA. A water-miscible solvent such as acetone or DMF is essential for making PLGA nanoparticles without a surfactant. In the solvent diffusion method, the solvent is immediately diffused into an aqueous phase and the nanoparticles contaning the drug are precipitated.[7] Since Lasic[11] reported the dialysis procedure to make liposomes using amphiphilic materials, several groups have used this method to fabricate nanoparticles or polymeric micelles.[12,13] In our case, significant amount of precipitants was formed using the solvent diffusion method and the nanoparticle yield was 76.3 wt.-% whereas the nanoparticle yield using the dialysis method was 90.6 wt.-% by DMF and 97.5 wt.-% by acetone. However, the drug content in the nanoparticles produced by the solvent diffusion method was higher than dialysis method as shown

Table 1. Characteristics of surfactant-free nanoparticles of PLGA

Method	Solvent	Drug loading contents (wt.-%)[a]	Loading efficiency (wt.-%)[b]	Particle size (nm)		
				Intentisy ave.	Volume ave.	Number ave.
Dialysis	DMF	9.1	50.1	164.1 − 32.5 (58.3%)	163.4 − 54.0 (35.9%)	161.1 − 55.6 (87.8%)
				363.5 − 75.6 (41.7)	381.6 − 122.5 (64.1%)	376.6 − 126.1 (12.2%)
Dialysis	Acetone	8.5	46.4	732.8 − 190.7	761.4 − 352.6	758.2 − 354.4
Solvent	Acetone	11.2	63.1	81.3 + 10.4 (23.9%)	81.2 + 13.6 (72.1%)	80.9 − 13.3 (97.1%)
diffusion				192.3 − 29.3 (76.1%)	191.7 − 36.2 (27.9%)	190.2 − 36.0 (2.9%)

[a]Drug contents = [weight of remained drug in the nanoparticle (weight of remained drug in the nanoparticle + polymer weight)] × 100. [b]Loading efficiency = (weight of remained drug in the nanoparticle feeding weight of drug) × 100.

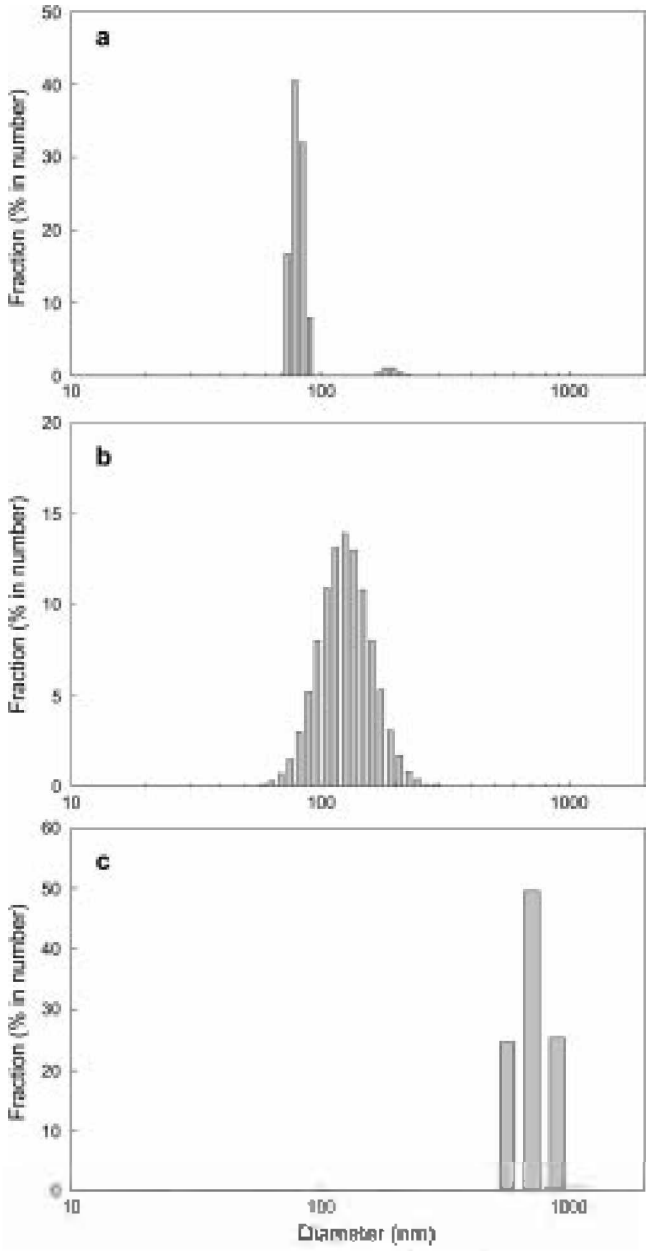

Figure 1. Particle size distribution of surfactant-free nanoparticles of PLGA prepared by solvent diffusion method using acetone (a), dialysis method using DMF(b) and acetone (c).

in Table 1. In the dialysis method, a significant amount of drug must have leaked during the dialysis procedure, resulting in the low loading efficiency. When the dialysis method was used to make nanoparticles, the particle size was higher than that of the solvent diffusion method. In the solvent diffusion method, the loading efficiency was less than that expected because of a significant amount of drug had aggregated with the polymer and precipitated (the precipitants were readily discarded from the nanoparticle solution by gentle centrifuging) whereas residue were not observed in the dialysis method. Figure 1 shows the particle size distribution of the surfactant-free nanoparticles. As shown in Figure 1(a), the nanoparticles produced by the solvent diffusion method using acetone showed narrower

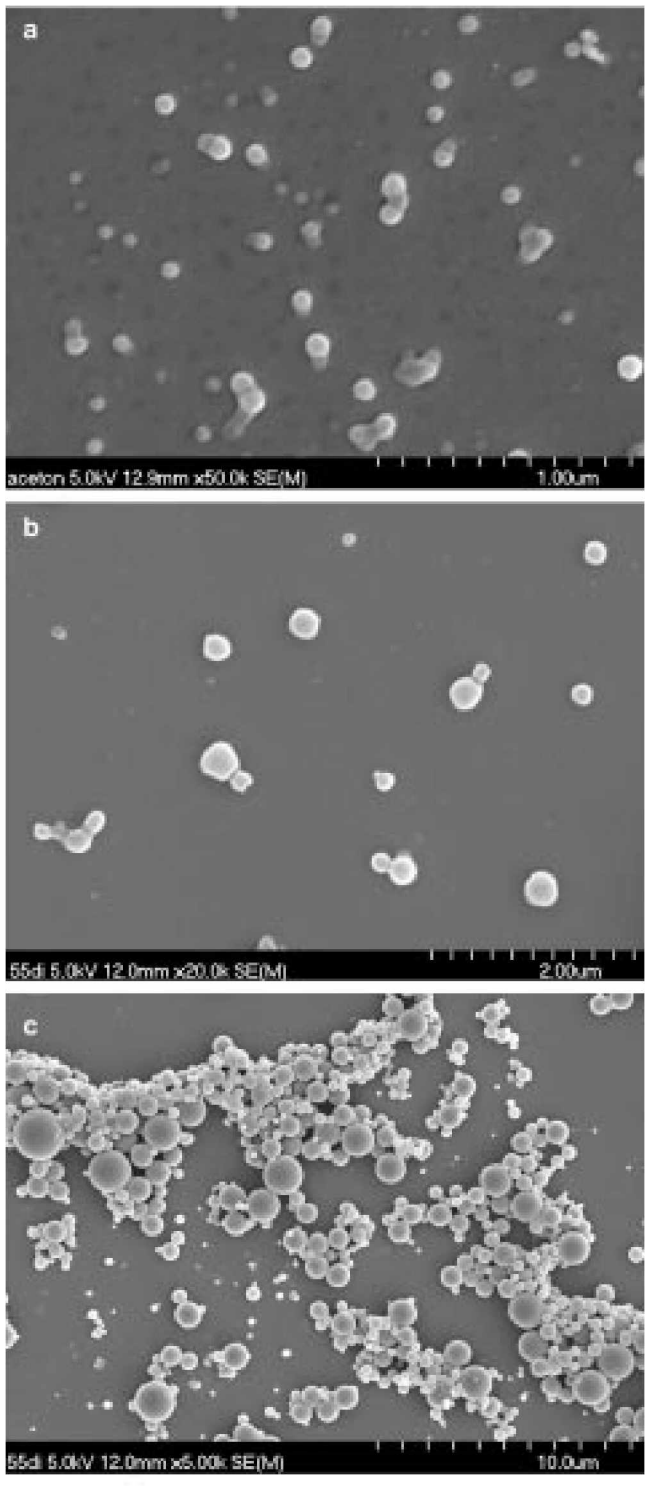

Figure 2. SEM photographs of surfactant-free nanoparticles of PLGA prepared by solvent diffusion method using acetone (a), dialysis method using DMF(b) and acetone (c).

size distribution than that of the dialysis method using DMF (Figure 1(b)). The nanoparticles produced by the dialysis method using DMF showed a broad size distribution and larger particle size than the solvent diffusion method. Furthermore, the nanoparticles produced by the dialysis method using acetone (Figure 1(c)) showed the largest particle size. Although the differences in the particle size and

size distribution according to the preparation method and solvent used are unclear, the nanoparticle size may be variably altered by several factors: such as the solubility of the polymer into the solvent, the solubility of the solvent into water, the solvent-water exchange rate, the solvent-diffusion rate into water, and the entropy between the polymer, drug, solvent, and water.[1,14,15] The size of the nanoparticles is important for establish drug delivery strategies to specific sites of the body, since particles of several micrometers in diameter are filtered by the lung capillaries[16,17] and submicron particles are rapidly cleared by the reticuloendothelial system (RES).[18-20] In particular, nanoparticles below 200 nm have advantages for site-specific drug delivery or long blood circulation by avoiding the RES.[21] Figure 2 shows the nanoparticle morphology observed by FE-SEM. Figure 2(a) shows the PLGA nanoparticles obtained using the solvent diffusion method with a small particle size of less than 100 nm, which is similar to the results obtained using particle size analysis. The PLGA nanoparticles prepared by the dialysis method using acetone (Figure 2(c)) has largest particle size. Nanoparticles smaller than 100 nm, which is a similar size to viruses, are much more acceptable for targeting the drug to specific sites in the human body and the long blood circulation.[12,18]

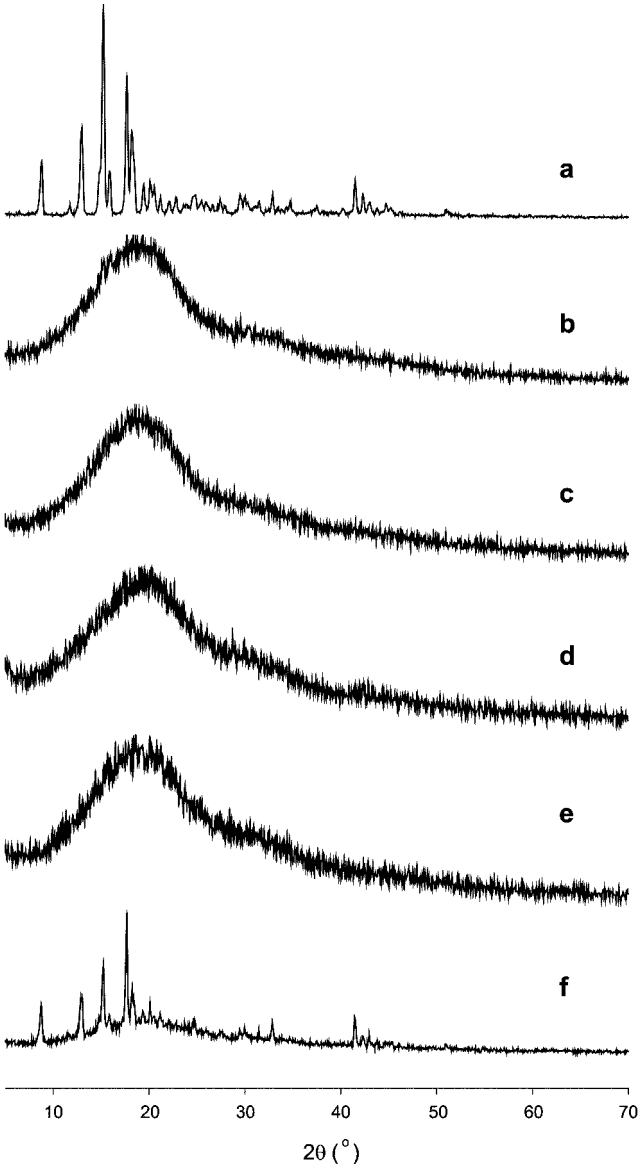

Figure 3. X-ray powder diffraction spectra of surfactant-free PLGA nanoparticles. (a) TST, (b) empty nanoparticles, (c) TST-entrapped nanoparticles prepared from DMF by dialysis, (d) TST-entrapped nanoparticles prepared from acetone by dialysis, (e) TST-entrapped nanoparticles prepared from acetone by solvent diffusion method, (f) physical mixture of TST and empty PLGA nanoparticles (prepared by solvent diffusion using acetone, weight ratio of TST : empty nanoparticle = 1.5 : 10).

XRD was used to confirm the characteristics of the TST-loaded PLGA nanoparticles. Figure 3 shows the XRD patterns of the TST-loaded PLGA nanoparticles and the corresponding physical blend. The characteristic XRD peaks of TST, which were also visible in the pattern obtained for the physical blend (Figure 3(f)), disappeared in the scans corresponding to the TST-entrapped nanoparticles in all nanoparticle formulations. These results suggest that the TST existed as a molecular dispersion in the polymeric nanoparticles and became entrapped into the nanoparticles without free drug on their surfaces. Although the drug cannot be completely entrapped and free drug may exist on the nanoparticle surface, the amount of free drug on the nanoparticle surfaces was below the XRD detectation level.

Figure 4 shows the drug release from the PLGA nanoparticles. Drug release was mainly dependent on the particle size rather than the drug content. Drug release from the PLGA nanoparticles prepared by the dialysis method using DMF and the solvent diffusion method using acetone was almost complete within 3 days but drug release by the dialysis method using acetone exhibited pseudo-zero order release kinetics. There are several reports[22] showing that a hydrophobic drug is generally released slowly in higher drug contents than in lower drug contents because of a hydrophobic drug crystallizes inside the nanoparticles and phase separation occurs at a higher drug load, reducing the drug release rate. Although the PLGA nanoparticles prepared by

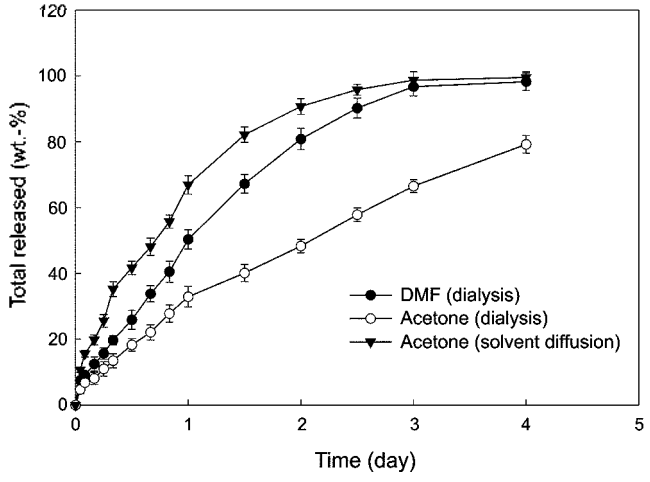

Figure 4. Drug release from surfactant-free nanoparticles of poly(DL-lactide-co-glycolide).

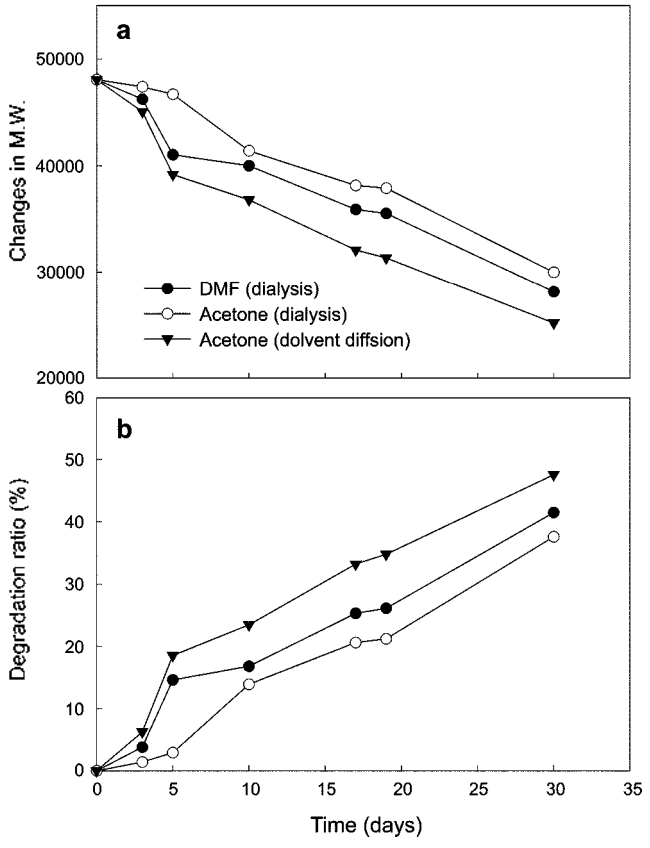

Figure 5. *In vitro* degradation profiles of surfactant-free nanoparticles of PLGA. Changes in M_w (a) and degradation ratio (b). Equation of degradation ratio was described in Experimental part.

solvent diffusion method using acetone had the highest drug content, drug release was faster than in those produced by the dialysis method. It was reported that the drug release rate from the larger nanoparticles was slower than the small size nanoparticles.[23] These results suggest that differences in the particle size are a significant factor affecting the drug release rate in the nanoparticle system.

The degradation behavior of polymers is frequently associated with chain cleavage and a drop in the molecular weight. Lactide/glycolide polymers undergo erosion in an aqueous environment. The biodegradation of lactide/glycolide polymer microspheres or nanoparticles has been characterized in terms of the change in particle morphology and changes in the polymer molecular weight. To observe the biodegradation behavior of the PLGA 50/50 nanoparticles, the PLGA nanoparticles prepared by the dialysis method and the solvent diffusion method using either DMF or acetone were incubated in PBS (0.1 M, pH 7.4) and the residual molecular weight was analyzed by GPC. The degradation rate of PLGA nanoparticles is expected to be faster than other drug delivery carriers such as microspheres, a matrix system, and hydrogels because of their large surface area.

However, the biodegradation rate was slower than expected as shown in Figure 5, *i.e.* only 16.8% with DMF, 13.9% with acetone prepared by the dialysis method and 23.4% with acetone by the solvent diffusion method for 10 days. Although the differences in the degradation ratio against the initial solvent was not significantly altered, the biodegradation rate of the PLGA nanoparticles prepared from DMF was faster than that from acetone, indicating that the PLGA nanoparticle degradation was size-dependent, *i.e.* the larger the particle size, the slower the degradation rate. Generally, PLGA nanoparticles degradation can be affected by the molecular weight of the polymers, the lactide/glycolide ratio, the particle external and internal morphology, the nanoparticle or microsphere preparation method, and the surfactant used. Of course, in our system, the surfactant effect on the PLGA nanoparticle biodegradation rate was not considered due to the PLGA nanoparticles were prepared without surfactant.

Generally, a surfactant must be used to make small-sized nanoparticles in conventional emulsion solvent evaporation systems[23] and the surfactant used can absorb onto the nanoparticle surface.[4] The absorbed surfactant is known to affect the particle size, the biodegradation rate, the biodistribution, and the physicochemical properties of the nanoparticles.[4] These results suggest that the mechanism of TST release from the PLGA nanoparticles is dependent on the diffusion mechanism rather than biodegradation mechanism because the molecular weight of the PLGA 50/50 nanoparticles was not significantly lowered until 3 days but almost all the drug was released after 1 day.

In conclusion, surfactant-free PLGA nanoparticles were successfully prepared by both the dialysis method and the solvent diffusion method. The PLGA nanoparticles prepared using the solvent diffusion method has a smaller particle size than the dialysis method. The solvent diffusion method was better for a higher loading efficiency than the dialysis method but the nanoparticle yield was lower. Drug release from the PLGA nanoparticles was dependent on the particle size rather than the drug contents. PLGA nanoparticle degradation was significantly slow, *i.e.* 13.9-23.4% after 10 days, indicating that drug release was dependent on the diffusion mechanism rather than degradation.

Acknowledgment. This work was supported by the Postdoctoral Fellowship Program of Korea Science & Engineering Foundation (KOSEF).

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