Optimization of Preparative Conditions for Polylactide (PLA) Microspheres Containing Ovalbumin

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Polylactide (PLA) microspheres containing ovalbumin (OVA) as a model protein were prepared by a water-in-oil-in-water (w/o/w) emulsion solvent evaporation method. The optimization of preprative parameters for the PLA microspheres containing OVA were performed, and the *in vitro* characteristics of the obtained microspheres were examined. Firstly, a smaller internal aqueous phase volume was found to be advantageous in obtaining high loading efficiency. Secondly, the addition of 2—10% (w/v) NaCl into the external aqueous phase (0.5% (w/v) polyvinyl alcohol solution) also improved OVA loading efficiency. Prepared products showed a sharp release of OVA at the initial phase, but the following phase was characterized by a slow release rate of OVA that continued at least 28 d. The release rate of OVA from microspheres made of PLA with a molecular weight of 15400 was faster than that from microspheres made of PLA with a molecular weigh of 58300. However, the LA/GA (lactide/glycolide) ratio was not likely to have much effect on the release profile of OVA. Finally, the effect of PLA microsphere particle size on the release profiles of OVA was examined. The extent of burst release at the initial phase increased as the mean diameter of prepared PLA microspheres decreased. For example, the PLA microspheres with a small mean diameter (5.0 μm) showed a 40% burst release, but almost 30% of OVA remained in the PLA microspheres (confirmed by HPLC method) after the 28 d release test, suggesting the possibility of using this carrier as a long-acting protein delivery system.

Key words polylactide; poly(lactide-co-glycolide); microsphere; ovalbumin protein delivery; burst release; loading efficiency

Microspheres developed for use in medicine consist of a drug dispersed or encapsulated in a polymer. Microspheres usually have a particle size ranging between 1 and 2000 μm. Microspheres have been employed for various purposes, including the sustained release of drugs,[1,2] taste masking,[3] environmental protection[4,5] and liquid to solid conversion.[6] Recently, a once-a-month injectable poly(lactide-co-glycolide) (PLGA) microsphere of leuprolide acetate was developed.[7,8] This success in developing a commercial microsphere product containing a peptide, with advantages such as biocompatibility and biodegradability of the PLGA and the reported sustained release characteristics[8] of obtained microspheres encouraged many researchers to further study the delivery of peptides or proteins using PLGA microspheres. Simultaneous studies on PLGA microspheres containing proteins or active reagents have been performed.[9] Nevertheless, few studies have focused on proteins with a comparatively large molecular weight.

We recently reported a preparative method for PLGA microspheres (< 10 μm in diameter) containing ovalbumin (OVA) using a water-in-oil-in-water (w/o/w) emulsion solvent evaporation method. The microspheres thus obtained showed an excellent vaccine efficacy by oral and subcutaneous inoculations.[10,11] Nevertheless, the poor loading efficiencies of OVA in microspheres were demonstrated in the published studies. In relation to water-soluble compounds like peptides or proteins, there have been very few reports demonstrating high loading efficiency in the process of microsphere manufacturing. Attempts to improve the loading efficiency in the preprative process of PLGA microspheres for water soluble compounds such as OVA seem to be important goals.

In the present study, therefore, we describe the modification of the above w/o/w emulsion solvent evaporation method and an improvement of the loading efficiency of OVA (molecular weight (Mw), 45000) as a water-soluble model protein in PLGA 100/0 (PLA) microspheres. The purpose of the present study was to perform the optimization of preparative parameters for the PLA microspheres containing OVA, and the *in vitro* characteristics of obtained microspheres such as size, loading efficiency, morphology, and dissolution profiles, and finally to evaluate the possibility of their use as a protein (antigen) delivery system.

Experimental

Chemicals PLA (PLGA100/0; Mw = 58000, 15400), PLGA85/15 (Mw = 101000), PLGA75/25 (Mw = 10500), and PLGA50/50 (Mw = 53000) were purchased from Medisorb Technologies (Cincinnati, OH, U.S.A.). Polyvinyl alcohol (PVA; 87—89% hydrolized, Mw = 85000—146000) was supplied by Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Ovalbumin (grade V) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NaCl and D-glucitol were from Wako Chemicals (Osaka, Japan). Other reagents are all of special reagents grade.

Preparation of PLA (PLGA) Microspheres A w/o/w emulsion solvent evaporation method adopted in the present study was performed as follows. First of all, 2.2, 11.1, and 22.2 mg of OVA (corresponding to 1, 5, and 10% (w/w) of theoretical loading, respectively) was dissolved in 50—1000 μl water. The solution was emulsified with 5 ml of methylene chloride containing 200 mg of PLA (PLGA) for 1 min using an ultrasonic disruptor (UD-200; Tomy Seiko Co., Ltd., Tokyo, Japan). This w/o emulsion was poured into 200 ml of 0.5% (w/w) PVA solution. Emulsification was continued using a homogenizer (NS-60; Nichionirikakikai Co., Ltd. (Tokyo, Japan) at 3000 rpm (in the case of the particle size study, stirring rates were further increased) for 1 min. 0—10% (w/v) of NaCl was added into the 0.5% (w/w) PVA solution as an external aqueous phase if necessary. This dispersion was gently agitated in a 500 ml beaker on a stirring plate containing a 3.75 cm stirring bar for 4 h at room temperature. The microspheres were collected by centrifugation at 3000 rpm for 10 min. The obtained microspheres were washed with water and freeze dried (FD-1, Tokyo Rikakikai Co., Ltd.,

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Tokyo) for at least 12 h.

Determination of Loading Amount of OVA About 10 mg of microspheres were precisely weighed and dissolved in 2 ml of actetone to further dissolve them in PLA (PLGA) polymer in a glass of the vial. The polymer solution containing suspended OVA was centrifuged at 3000 rpm for 10 min. The acetone was decanted and replaced with fresh acetone. The procedure was repeated three times. Thereafter, residual acetone was dried up by a nitrogen stream, and the remaining precipitate was dissolved in 4 ml of purified water. The OVA concentration in the solution was determined using HPLC. Twenty microlitres were injected onto a chromatograph (Shimadzu LC-10A, Kyoto, Japan) equipped with a UV detector (Shimadzu SPD-10AV), an integrator (Shimadzu C-R6A) and reversed phase C8 column (SG 300, 4.6 × 150 mm, Shiseido, Tokyo, Japan). The mobile phases employed were A (0.10% (w/v) trifluoroacetic acid in water) and B (0.10% (w/v) trifluoroacetic acid in acetonitrile); buffer B was linearly varied from 45 to 60% (v/v) over 10 min. The flow rate was 2.0 ml/min. The wavelength was set at 214 nm and the column was operated at 40 °C. The loading was calculated from the weight of the initial microspheres and the amount of drug incorporated.

Morphology and Microsphere Size The microspheres were coated with gold using an Fine Coat Ion Spatter (JFC-100, Tokyo, Japan) under a vacuum of 0.1 Torr and at a voltage of 1.2 kV) and 10 mA. Samples were coated for 5 min to achieve continuous coverage. The PLA microspheres obtained by this procedure were examined with a scanning electron microscope (Akashi WS 250, Tokyo, Japan) at 15 kV. The average diameters were measured by a Microtrac Particle Size Analyzer (model 7995-30, Leeds and Northrup, North Wales, PA, U.S.A.).

***In Vitro* OVA Release Test** The *in vitro* OVA release profiles of OVA from PLA (PLGA) microspheres were determined as follows. Microspheres corresponding to 100 μg of OVA were suspended in 5 ml of 10 mM Tris buffer (pH 7.4) containing 0.02% (w/v) Tween 80 and shaken horizontally at 75 stroke/min at 37 °C. At predetermined intervals, 200 μl of the suspension was taken as a sample, centrifuged (12000 rpm, 5 min) and the concentration of the supernatant was analysed by HPLC as described above. The suspension picked up as a sample was not compensated.

Results and Discussion

Effect of the Internal Aqueous Phase Volume on OVA Loading Efficiency Figure 1 shows the effect of the internal aqueous phase volume on OVA loading efficiency in PLA microspheres. The theoretical OVA content was 10.0% (w/w). When a comparatively large volume of the internal aqueous phase (500, 1000 μl) was used, a low loading efficiency was observed. On the other hand, the usage of a smaller volume as an internal aqueous phase produced a comparatively high loading efficiency (39% was obtained when 50 μl of the internal aqueous phase volume was employed). This result seems reasonable. Loading efficiency is likely to depend on the internal aqueous phase volume employed. The bigger the internal aqueous phase volume, the thinner was the oily PLA layer (methylene chloride phase). Because this oily PLA layer acts as a barrier through which internal OVA is diffused to the external aqueous phase, a thinner oily PLA layer is expected to give rise to a larger diffusion rate. Thereby, the probability of OVA leakage from the internal aqueous phase to the external aqueous phase will increase. The smaller internal aqueous phase volume was found to be advantageous for obtaining high loading efficiency. The mean diameter of obtained microspheres was 19.2 ± 2.2 μm.

Effect of NaCl Added in the External Aqueous Phase in the Preparative Process on PLA Microsphere Characteristics We previously reported[12] that the addition of NaCl or D-glucitol to the external aqueous phase in the preparation process significantly improved the loading

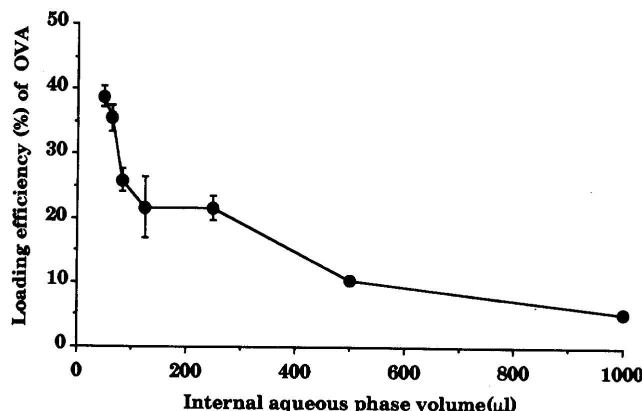

Fig. 1. Relationship between the Internal Aqueous Phase Volume (μl) and Loading Efficiency (%) of OVA for Prepared PLA Microspheres

Each point and vertical line represents the mean and S.D. of three experiments.

efficiency of brilliant blue as a water-soluble model dye into the PLA microspheres. The formation of a stable w/o/w emulsion seems to be essential to efficiently entrapping OVA into PLA microspheres. The presence of additives such as electrolytes, sugars or alcohols was reported to be advantageous for preparing a stable w/o/w emulsion.[13] Therefore, NaCl or D-glucitol were selected as representative additives, and added to the external aqueous phase in the manufacure of OVA loaded PLA microspheres. In this experiment, the volume of the internal aqueous phase was fixed at 50 μl. Theoretical OVA loading was 10% (w/w). The results are shown in Fig. 2a. The addition of NaCl into the external aqueous phase improved OVA loading efficiency compared to the case of no additives (75% efficiency at 2—10% (w/v) of NaCl). Although D-glucitol improved OVA loading efficiency to some extent, its effect was inferior to NaCl. Even in the microsphere formulation with other theoretical OVA contents (1 and 5% (w/w)), NaCl was also more effective for improving OVA loading efficiency, as shown in Fig. 2b. The mean diameter of obtained OVA-loaded PLA microspheres was 18.5 ± 2.4 μm.

In our previous paper,[12] we demonstrated that the addition of NaCl into the external aqueous phase in the preparative process significantly improved loading efficiency (80—90% of theoretical value; theoretical loading was 10% (w/w)) of brilliant blue compared to the case of no additives (<10%), and to the addition of D-glucitol (40—50%) to the external aqueous phase. In that article, in the presence of NaCl in the external aqueous phase, microscopic examination suggested that internal aqueous droplets were stable and were not expelled during the second emulsification. On the other hand, in the absence of NaCl, almost internal aqueous droplets containing a high concentration of dye solution were expelled immediately after the second emulsification. Even in the case of the addition of D-glucitol (corresponding to the same molar concentration as NaCl) to the external aqueous phase, many internal aqueous droplets containing a high concentration of dye were expelled gradually during the second emulsification. These results suggested that differences in the stability of w/o/w emulsion could not explained only by differences in osmotic pressure, even

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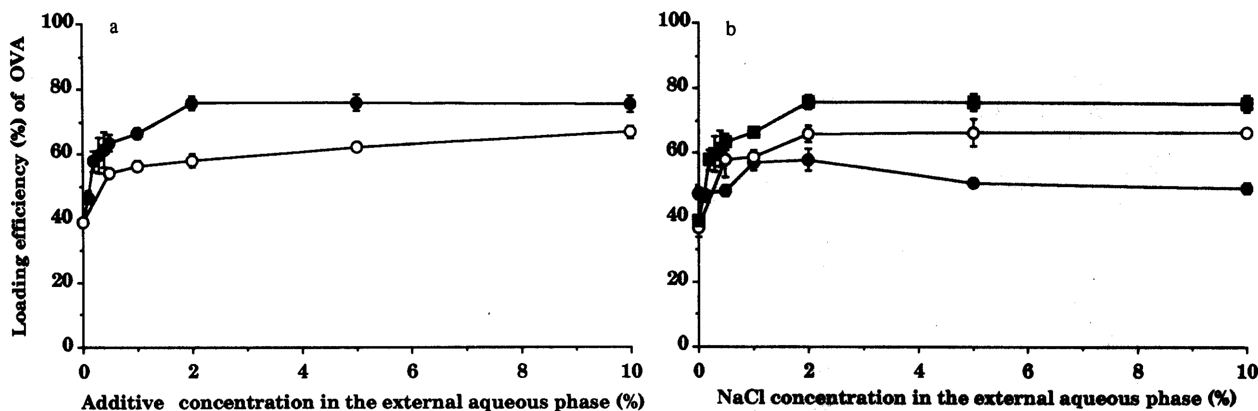

Fig. 2a. Relationship between Additive Concentrations (%, w/v) and Loading Efficiency (%) of OVA for Prepared PLA Microspheres

Key: ●, NaCl; ○, D-glucitol. Each point and vertical line represents the mean and S.D. of three experiments.

Fig. 2b. Relationship between NaCl Concentration % (w/v) and Loading Efficiency (%) of OVA for Prepared PLA Microspheres When the Theoretical OVA Loading Was (●), 1%; (○), 5%; (■), 10%.

Each point and vertical line represents the mean and S.D. of three experiments.

though osmotic pressure might act as a barrier for the expulsion of internal aqueous droplets into the external aqueous phase to some extent. Brodin *et al.* reported the release of naltrexone hydrochloride from the internal aqueous phase of a w/o/w emulsion.[14] In their article, a 73% decrease in the diffusion coefficient of the drug was obtained with 9% (w/v) NaCl dissolved in the internal aqueous phase. Sorbitol also caused a decrease in the diffusion coefficient, but its effect reached a maximum level at about 6% (w/v) as a NaCl equivalent, and the extent of the effect was almost same as with 9% (w/v) NaCl. These results also indicate that factors other than osmotic gradients affect the passage of the drug, even though our case was different from Brodin's case since NaCl was added into the external aqueous phase in our case.

In the present study, nearly 40% of OVA loading efficiency was obtained in the absence of NaCl, and the value was almost four times higher than the value (< 10%) that was obtained in the previous entrapment study of brilliant blue in PLGA microspheres. This fact seems to be due to the high viscosity of OVA solution as the internal aqueous phase (viscosities were not measured), as Ogawa *et al.* reported previously.[7]

Figure 3 shows the release profiles of OVA from PLA microspheres prepared using various NaCl concentrations in the preparative process. The volume of the internal aqueous phase was fixed at 50 μl. Actual OVA loading % (w/w) in PLA microspheres used in the release study, prepared in the presence of 0, 0.5, 1, 2, 5, and 10% (w/v) NaCl in the preparation process, were 3.9, 6.3, 6.7, 7.6, 7.6, and 7.5% (w/w), respectively. Theoretical OVA loading was 10% (w/w). In the absence of NaCl in the preparation process, obtained PLA microspheres showed fast OVA release, and all OVA was dissolved into Tris buffer medium (pH 7.4) within several hours. The PLA microspheres prepared using various NaCl concentrations in the preparative process produced sustained-release profiles of OVA, even though burst release was observed to some extent. A morphology study suggested that PLA microspheres prepared in the presence of NaCl (5% (w/v))

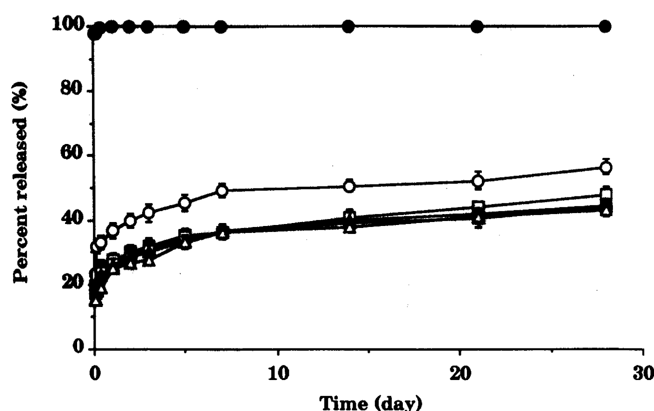

Fig. 3. Release Profiles of OVA from PLA Microspheres Prepared under Different NaCl Concentrations

Key: ●, 0%; ○, 0.5%; ■, 1%; □, 2%; ▲, 5%; △, 10%. Each point and vertical line represents the mean and S.D. of three experiments. See the text in relation to loading and diameter.

had a smooth surface, as shown in Fig. 4b. On the other hand, the PLA microspheres prepared in the absence of NaCl had pores in the surface of the microspheres, and microspheres with irregular shapes were observed, as shown in Fig. 4a. These differences in morphology seems to be the primary reason for the differences in release profiles from microspheres prepared in the absence or presence of NaCl.

Effect of PLGA Type on Release Profiles of OVA from Microspheres Several articles reported the effect of PLGA type on the release profiles of various drugs from PLGA microspheres. Heya *et al.* reported that the initial burst of thyrotropin releasing hormone differed with changes in the molecular weight of PLGA.[15] Simultaneously, the effect of PLGA type on the release rate of OVA from microspheres was examined in the present study. The PLGA microsphere preparations were performed in the presence of 5% (w/w) NaCl. The theoretical OVA loading was 10% (w/w). Prepared microspheres were different in OVA loading, but were almost the same in mean diameters ($18.7 \pm 2.5\,\mu$m). Actual OVA loading % (w/w) in the microspheres made of PLGA75/25 (Mw=10500), PLA

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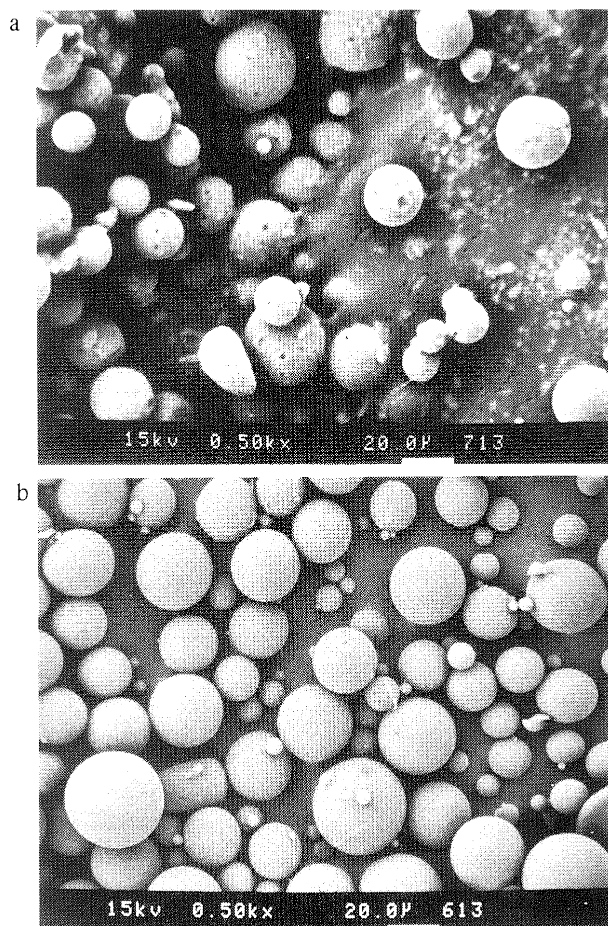

Fig. 4. Scanning Electron Micrographs of PLA Microspheres Prepared in the Absence (a) or Presence (b) of 5% (w/v) NaCl

Each product contained 3.9%, 7.6 (w/w) of OVA, respectively.

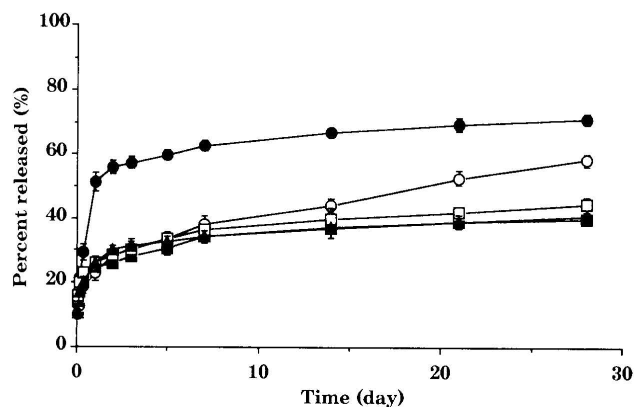

Fig. 5. Release Profiles of OVA from PLGA Microspheres Prepared with Different Types of PLGA

Key: ●, PLGA 75/25 (Mw 10500); ○, PLA (Mw 15400); ■, PLGA 50/50 (Mw 53100); □, PLA (Mw 58300); ▲, PLGA 85/15 (Mw 101000). Each point and vertical line represents the mean and S.D. of three experiments. See the text in relation to loading and diameter.

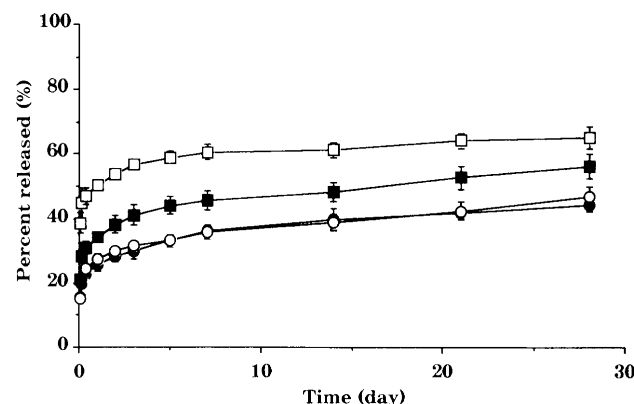

Fig. 6. Release Profiles of OVA from PLA Microspheres with Different Size

Key: ●, 18.7 μm; ○, 13.4 μm; ■, 8.8 μm; □, 5.0 μm. Each point and vertical line represents the mean and S.D. of three experiments. See the text in relation to loading.

(Mw = 15400), PLGA50/50 (Mw = 53100), PLA (Mw = 58300), and PLGA85/15 (Mw = 101000) were 3.4, 5.3, 7.3, 7.6, and 8.5% (w/w), respectively. The theoretical OVA loading was 10% (w/w). The loading efficiency of OVA in PLGA microspheres increased as the molecular weight of PLGA increased. This seemed to be attributed to the stronger barrier characteristics of PLGA with a larger molecular weight as an oily methylene chloride phase. The release rate of OVA from PLA microspheres seems to decrease with an increase in molecular weight of PLGA as shown in Fig. 5. For example, the release rate of OVA from microspheres made of PLA with a molecular weight of 15400 was faster than that from microspheres made of PLA with a molecular weight of 58300. However, the LA/GA (lactide/glycolide) ratio was not likely to affect the *in vitro* release profile of OVA so much, even though LA/GA is another factor affecting the release of the drug from PLGA microspheres. One explanation for this phenomenon is the flocculation of PLGA microspheres, observed during the release test in spite of the addition of 0.02% (w/w) of Tween 80 to 10 mM Tris buffer, and a subsequent increase in the apparent diameter of the microspheres, and finally a decrease in the total surface area of the microspheres (in fact, slight flocculations were sometimes observed during the release test). Another possibility may be the comparatively low concentration of the buffer (10 mM Tris buffer) employed in the present study, since the previous report[15] suggested that a higher buffer concentration or ionic strength accelerated the *in vitro* drug release rate from PLGA microspheres. Even in the PLGA 75/25 (Mw = 10500), the microspheres showing the fastest OVA release rate, about 30% (w/w) of OVA remained unreleased at 28 d, suggested sustained release characteristics.

Effect of Particle Size on Release Profiles of OVA from PLA Microspheres Some articles have reported that the particle size of microspheres played a critical role in the release rate, or the effectiveness of the product. In particular, the particle size seemed to be an important factor in the antigen delivery system, as described in previous papers.[10,16] Therefore, the effect of microsphere particle size on the release profiles of OVA was examined. The result is demonstrated in Fig. 6.

PLA microspheres with different diameters were prepared by increasing stirring rates (3000 (control), 5000, 10000, and 20000 rpm) at the second emulsification step in the presence of 5% (w/v) NaCl. The theoretical OVA loading was 10% (w/w). The corresponding mean diameters of the obtained PLA microspheres were 18.7 (con-

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trol), 13.4, 8.8, and 5.0 μm, respectively. The observed OVA loading % (w/w) in the microspheres decreased with an increase in stirring rates (7.6% (3000 rpm), 7.3% (5000 rpm), 6.1% (10000 rpm), and 4.4% (20000 rpm), respectively). In the preparation of the PLA microspheres by the w/o/w emulsion solvent evaporation process, the expulsion of the internal aqueous phase (internal droplet containing OVA) to the external aqueous phase was observed during the second emulsification, and its extent was proportional to stirring rates by microscopy observation (data not shown). The extent of burst release at the initial phase increased with a decrease in the mean diameter of prepared PLA microspheres. For example, PLA microsphere with a small mean diameter (5.0 μm) showed about a 40% burst release. Lai *et al.* reported the simultaneous burst release of isoproterenol (about 70%) from PLGA microspheres with a mean diameter of 4.5 μm.[9] In the present study, in spite of the initial burst release, almost 30% of OVA remained in PLGA microspheres (confirmed by the HPLC method described in the experimental section) after the 28 d dissolution test, suggesting the possibility of this carrier system as a long-acting protein delivery system.

These phenomenon coincided well with our previous paper[11] in which about 20% (w/w) ovalbumin remained inside poly(lactide-co-glycolide) microspheres after a three week release test. This phenomena in the present study may be caused by the electrical interaction between basic amino acids in the OVA molecule and an acidic carboxy group in PLGA as described in the previous paper.[7]

In conclusion, we were successful in developing a modified method to efficiently entrap ovalbumin into PLGA microspheres using a w/o/w emulsion solvent evaporation method by adding NaCl into the external aqueous phase. This method will be applied to other water soluble proteins in the near future.

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References

1) Nixon J. R., Maleka M. R., *J. Microencapsulation*, **1**, 53—64 (1984).
2) Jalsenjak I. I., Kondo T., *J. Pharm. Sci.*, **70**, 456—457 (1981).
3) Bakan J. A., *Food Technology*, **27**, 34—44 (1973).
4) Bakan J. A., Anderson J. L., "The Theory and Practice of Industrial Pharmacy," 2nd ed., ed. by Lachman L., Lieberman H. A., Kanig J. L., Lea & Febiger, Philadelphia, 1976, pp. 420—438.
5) Klaui H. M., Housheer W., Huschke G., "Fat-Soluble Vitamins," ed. by Morton R. A., Pergamon, London, 1970, pp. 113—159.
6) Nang L. S., Calier P. F., Delort P., Gazzola J., Lafont D., *J. Pharm. Sci.*, **62**, 452—455 (1973).
7) Ogawa Y., Yamamoto M., Takada S., Okada H., Shimamoto T., *Chem. Pharm. Bull.*, **36**, 1095—1103 (1988).
8) Okada H., Heya T., Ogawa Y., Toguchi H., Shimamoto T., *Pharm. Res.*, **8**, 584—587 (1991).
9) Lai Y.-L., Mehata R. C., Thacker A. A., Yoo S.-D., McNamara P. J., DeLuca P. P., *Pharm. Res.*, **10**, 119—125 (1993).
10) Uchida T., Goto S., *Chem. Pharm. Bull.*, **17**, 1272—1276 (1994).
11) Uchida T., Martin S., Foster T. P., Wardley R. C., Grimm S., *Pharm. Res.*, **11**, 1009—1015 (1994).
12) Uchida T., Yoshida K., Goto S., *J. Microencapsulation*, **12**, in press.
13) Florence A. T., Whitehill D., *Int. J . Pharm.*, **11**, 277—308 (1982).
14) Brodin A. K., Kavaliunas D. R., Frank S. D., *Acta Pharmaceutical Suec.*, **15**, 1—12 (1978).
15) Heya T., Okada H., Ogawa Y., Toguchi H., *J. Pharm. Sci.*, **83**, 636—640 (1994).
16) Eldridge J. H., Hammond C. J., Meulbroek J. A., Staas J. K., Gilley R. M., Tice T. R., *J. Contr. Rel.*, **11**, 205—214 (1990).

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