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In vitro and in vivo studies of cyclosporin A-loaded microspheres based on copolymers of lactide and ε-caprolactone: Comparison with conventional PLGA microspheres

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Abstract

A hydrophobic peptide, cyclosporin A (CyA), was incorporated in microspheres based on poly(lactide-*b*-ε-caprolactone) (P(LA-*b*-CL), LA/CL (in molar ratio): 78.7/21.3 and 48.1/51.9) and poly(lactide-co-glycolide) (PLGA, LA/GA: 80/20) using oil-in-water (O/W) emulsion solvent evaporation method. The microspheres were characterized by SEM, DSC and X-ray diffraction, and CyA release rate was determined by HPLC. It was revealed that CyA can be efficiently loaded into all the microspheres (exceed 96%). Compared to PLGA microspheres, P(LA-*b*-CL) microspheres liberated CyA more rapidly. Within the first day, about 75, 50 and 12% of CyA released from P(LA-*b*-CL) (48.1/51.9), P(LA-*b*-CL) (78.7/21.3) and PLGA microspheres, respectively, which can be attributed to the partial crystallization occurring in P(LA-*b*-CL) microspheres. CyA levels in whole blood were also tested. In comparison with PLGA microspheres, P(LA-*b*-CL) microspheres provided a higher blood level of CyA. The maximum CyA concentration in whole blood (∼520, 450 and 400 ng ml^{-1} for P(LA-*b*-CL) (48.1/51.9) P(LA-*b*-CL) (78.7/21.3) and PLGA microspheres, respectively) was reached at the second day post administration. And then P(LA-*b*-CL) microspheres showed a constant CyA level (about 100–200 ng ml^{-1}) for extended periods of time (several weeks). Such CyA-loaded P(LA-*b*-CL) microspheres displaying higher CyA concentration during the first few days and similar constant blood CyA level thereafter showed more advantages than those prepared with PLGA and could meet clinical needs more efficiently.

Keywords: Cyclosporin A; P(LA-*b*-CL); PLGA; Microspheres

1. Introduction

Cyclosporin A (CyA) is a lipophilic cyclic undecapeptide of fungal origin, which has the selective

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property of suppressing various T-lymphocyte functions, particularly the production of interleukin-2 (IL-2) (Christopher et al., 2001). During the last two decades, CyA has become the most frequently used drug in the prophylaxis and therapy of graft rejection in all types of solid organ and bone marrow transplantation. Its introduction considerably improved the management of graft versus host disease, a major cause of treatment failure in bone marrow transplantation. CyA is also a useful tool in the treatment of a number of autoimmune diseases (Kahan, 1989; Roy, 1994). The conventional formulations of CyA (Sandimmun®) caused marked intra- and inter-individual variation in drug pharmacokimetics. Neoral®, another formulation, is a microemulsion of pre-concentrated CyA designed to provide better consistent absorption of the drug. Although this orally administered CyA has more stable drug metabolism, its gastrointestinal absorption is still incomplete and variable due to CyA's extremely hydrophobic character (Gennery et al., 1999; Tom et al., 2000). Some researchers (Loosli et al., 1985; Altschuh et al., 1992; Ko and Dalvit, 1992) have indicated that the intramolecular H-bonds (produced by NH groups) result in a very rigid configuration, which play a dominating role in the incomplete and erratic bioavailability of CyA from all dosage forms. Furthermore, CyA is costly and time consuming (Manuel et al., 2002), and in clinical, the prolonged repeated treatment with CyA is not advisable because it has severe side effects like nephrotoxicity, gingivitial hyperplasia and neurological disorders. The development of a means of enhancing absorption and bioavailability of systemically effective but poorly absorbed CyA is thus urgently needed.

Up to date, most work has been focused on the study of CyA-loaded microspheres (Rojas Silva et al., 1999; Aberturas et al., 2002; Kim et al., 2002a; Vallelado et al., 2002), nanoparticels (Guzman et al., 1993; Chacon et al., 1996; Chacon et al., 1999; Molpeceres et al., 2000; Gref et al., 2001; Chen et al., 2002; Ugazio et al., 2002) and emulsion (Kim et al., 2002b). The majority of materials used are biodegradable aliphatic polyesters, such as poly(ε-caprolactone) (PCL), poly(lactide) (PLA) and its glycolide copolymers (PLGA). Most of these CyA controlled delivery systems showed little practical feasibility when optimal drug concentration time profile at the sites of action, as well as sufficiently long effective period, is concerned (Campos et al., 2001; Lee et al., 2001). In order to gain more satisfactory release rate, a wider range of loading materials need to be screened. However, there were still no reports on using other materials, such as the copolymers based on lactide and ε-caprolactone, for controlled CyA release.

In this paper, we pursued the development of a new microsphere formulation of the extremely hydrophobic peptide CyA. The objectives of the present study were: (i) to incorporate CyA into biodegradable microspheres based on poly(lactide-*b*-ε-caprolactone) (P(LA-*b*-CL)) with two different ratios, as well as PLGA; (ii) to characterize these microspheres and in vitro release study; (iii) to take preliminary in vivo study, thus to compare these new copolymers with PLGA for controlled CyA release.

2. Materials and methods

2.1. Materials

Cyclosporin A was obtained from East-China Pharmaceutical Factory (Hangzhou, China) and used as supplied. Cyclosporin D was purchased from Fujian Kerui Pharmaceutical Co. Ltd. (Fuqing, China). P(LA-*b*-CL) was prepared in Professor Yasuda's lab. (Hiroshima Univ., Japan), PLGA was synthesized in our lab. All samples were purified by extraction with hydrochloric acid (0.1 M) to ensure removal of trace catalyst residuals, followed by washing with distilled water and dried. The characteristics of all samples are presented in Table 1. Poly(vinyl alcohol) (PVA, MW 88,000, degree of hydrolysis 88%) was purchased from Aldrich Chemical Company (St. Louis, MO, USA). All the other solvents and chemicals were of analytical grade and used as received.

2.2. Preparation of microspheres

CyA-loaded microspheres were prepared by the oil-in-water (O/W) solvent evaporation method described in previous documents (Sánchez and Alonso, 1995a). Briefly, about 160 mg of polymers and 40 mg of CyA were dissolved in 4 ml of dichloromethane (DCM) at room temperature, then the organic phase was emulsified into 50 ml of PVA aqueous solution (0.4%, w/w) at 1000 rpm with mechanic stirring. After the complete evaporation of DCM under magnetic agitation (about

Table 1
Characteristics of block copolymers of LA/ε-CL and PLGA used in this experiment

Sample	Monomer composition (wt.%)[a]		M_n[a]	M_w/M_n[a]	T_g[b] (°C)	T_m[b] (°C)
PLGA (LA/GA)	80.0	20.0	27,300	1.58	46.8	n.d.[c]
P(LA-*b*-CL) (LA/CL)	78.7	21.3	31,000	1.78	n.d.[c]	61.6
P(LA-*b*-CL) (LA/CL)	48.1	51.9	22,400	1.34	n.d.[c]	67.7

[a] Monomer composition, M_n and M_w/M_n were calculated from ^{1}H NMR.
[b] T_g and T_m were determined by DSC.
[c] Not detectable.

3 h, at room temperature), the microspheres were collected by centrifugation at 4000 rpm, washed three times with double-distilled water and dried in vacuum at 4 °C.

2.3. Determination of drug content in the microspheres

CyA entrapment in the microspheres was calculated by the difference between the theoretical feed and that found in the external aqueous phase after centrtfugation of the suspension at 4000 rpm for 15 min at room temperature. CyA concentration in the external aqueous phase was assayed by a high performance liquid chromatography system (HPLC, Waters, MA, USA) with an Agilent C_{18} column (5 μm, 4.6 mm × 250 mm). The HPLC system consists of Waters 510 pump and Waters 486 UV detector set at 210 nm, the mobile phase was methanol/water (80/20, v/v) and the flow rate was 1.0 ml/min.

2.4. Particle size analysis

The particle size and size dispersion of the microspheres were measured by the laser light scattering technique using a Coulter LS-230 Particle Size Analyzer (Miami, USA). The microspheres were first dispersed in 100 ml of double-distilled water containing 0.05% Tween 80 and sonicated for 15 s to re-disperse the microspheres. The particle size at 60% of the total volume fraction was taken as the average diameter ($n = 3$).

2.5. DSC analysis

To investigate the physical state of CyA and polymers in the microspheres, the thermal analysis was performed by differential scanning calorimetry (DSC, Pyris 1 Perkin-Elmer Corp., USA). All the samples were heated in crimped aluminum pans, and the first scan was measured at a heating rate of 10 °C/min from room temperature to 200 °C, subsequent scan was from −60 °C to 100 °C followed by cooling the sample to −60 °C.

2.6. X-ray diffraction analysis

In order to investigate the crystallization behavior of the copolymers after forming microspheres, the dry powders was examined with the Rigaku X-ray diffractometer (D/MAX-2000/PC Series, Japan) equipped with a graphite monochromator-filtered Cu-Kα radiation.

2.7. Characterization of the microspheres by SEM

Dried microspheres were firstly mounted onto stubs using double-sided adhesive tape without being gold-coated, then directly observed using a scanning electron microscope (JSM-5501LV, Tokyo, Japan) at an accelerating voltage of 20 kV.

2.8. In vitro release study

About 10 mg of microspheres were incubated in 4.5 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.2% sodium dodecyl sulphate (SDS) at 37 °C. The samples (3 ml) were withdrawn at predetermined time intervals and centrifuged for 10 min at 4000 × *g*, the supernatant was collected and replaced by the same amount of fresh buffer solution. The amount of CyA released into the medium was determined by HPLC.

2.9. Animal studies

Sixteen Wistar rats (weighted from 200 to 250 g) were obtained from the Central Stabulary of Zhe-

jiang College of Traditional Chinese Medicine, and randomly divided into four groups of four animals each. Three groups of CyA-loaded microspheres and one group of CyA suspension in 1 ml of 0.1% (w/v) sodium carboxymethyl cellulose (CMC-Na) for comparison were administered subcutaneously at a dose of 15 mg kg^{-1} to nuchal regions of rats. The venous blood samples were collected from the postorbital vein sinus at predetermined intervals up to 1 month after the drug administration.

2.10. Determination of CyA in whole blood

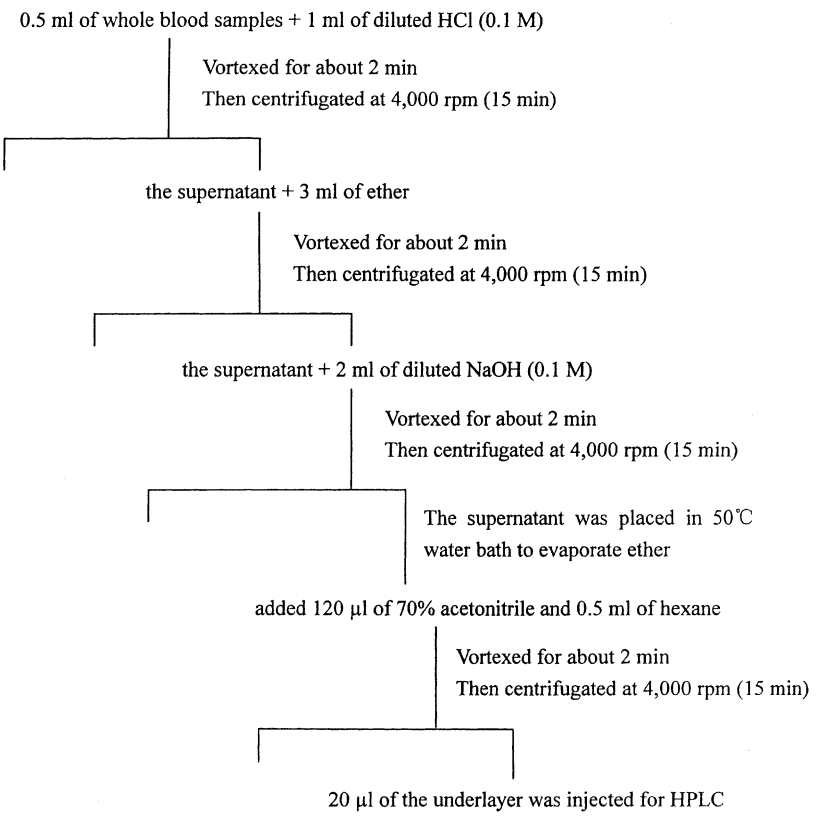
Since 90–98% of CyA is bound to plasma proteins in the blood (Christopher et al., 2001), it is necessary to detect its content in the whole blood. In this experiment, CyA concentration in whole blood was determined by HPLC and the assay procedures are as Scheme 1.

After above treatment, CyA concentration in the underlayer was determined by HPLC using cyclosporin D (CyD) as internal standard. The injection volume was 20 μl. The HPLC system consists of a Spectra-physics model SP 8800/8810 LC pump (San Jose, CA), a model SP 100 UV detector and a SP 4400 integrator; the mobile phase consisted of 62% acetonitrile and 38% H_2O. Separation was performed with a reverse phase-type column (μBondapak-C18 250 mm × 4.6 mm, 10 μm) thermostated at 70 ± 0.1 °C, the flow rate was 1.4 ml min^{-1}. The retention times were 6.5 and 7.9 min for CyA and CyD, respectively.

3. Results and discussion

3.1. Microspheres preparation and characterization

CyA-loaded microspheres based on P(LA-*b*-CL) (78.7/21.3), (LA-*b*-CL) (48.1/51.9) and PLGA (80/20) were successfully prepared by the oil-in-water emul-



Scheme 1. The treating process of whole blood before HPLC determination.

Table 2
Entrapment efficiency and particle size of three kinds of microspheres[a] ($n = 3$, mean ± S.D.)

Sample	Entrapment efficiency[b] (%)	Mean diameter (μm)
PLGA (80/20)	97.87 ± 0.36	36.16 ± 1.80
P(LA-*b*-CL) (78.7/21.3)	96.64 ± 1.48	34.43 ± 1.38
P(LA-*b*-CL) (48.1/51.9)	97.98 ± 0.67	31.14 ± 2.64

[a] All microspheres were prepared under the same experimental condition as follows: polymer concentration: 4% (w/v); theoretical CyA-loading: 20% (w/w); PVA concentration: 0.2% (w/v); stirring speed: 1000 rpm.
[b] Entrapment efficiency was calculated by the ratio of the actual drug loading to theoretical drug loading.

sion solvent evaporation method. In this study, like many other reports (Sánchez et al., 1995b; Allemann et al., 1998), high CyA entrapment efficiency can be achieved for all types of microspheres, which were prepared under the same conditions and show similar diameter (Table 2). Such high efficient entrapment can be resulted from two reasons. On one hand, owing to its high hydrophobic nature, CyA solubility in water is only about 4 μg ml^{-1} (Ismailos et al., 1991; Ford et al., 1999); for another reason, since all these polymers used in our experiments are hydrophobic, lipophilic CyA is more prone to distribute in the loading matrices than in external aqueous.

The surface morphologies of the three types of microspheres were observed by SEM, which are displayed in Fig. 1. PLGA microspheres showed a compact and smooth surface; a smooth surface with a few concaves can be observed for P(LA-*b*-CL) (78.7/21.3) and the surface of the microspheres composed of P(LA-*b*-CL) (48.1/51.9) became rough and irregular. The distinct differences can be resulted from the different physicochemical character of these three copolymers (Table 1). For PLGA microspheres, due to its amorphous character, solvent evaporation occurred at an even rate during the solidification of the emulsion drops, thus produced microspheres with smooth surface. As for the copolymers of lactide and ε-caprolactone, the introduction of ε-CL segments changed the character of PLA to a great degree. Owing to the strong crystallization of ε-CL segments, as well as the amorphous character of PLA, phase separation could happen during the process of solvent evaporation, thus leaded to the different surface morphology. DSC traces of the microspheres

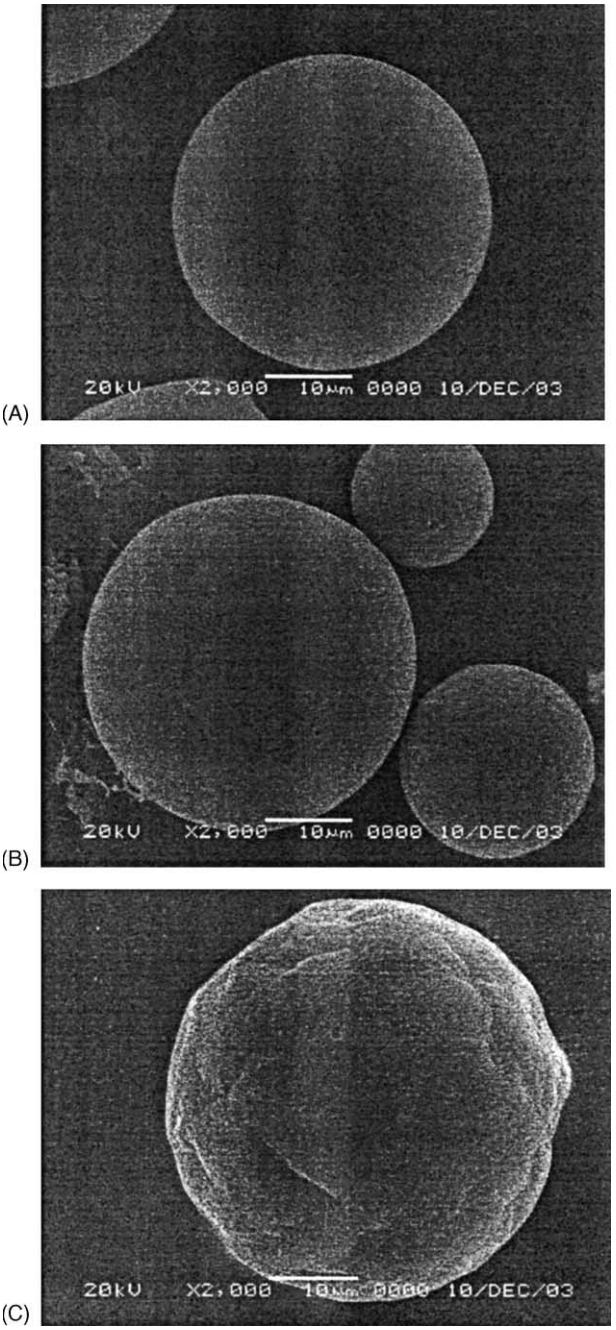


Fig. 1. Scanning electron micrographs of cyclosporin A-loaded microspheres made from (A) PLGA (80/20); (B) P(LA-*b*-CL) (78.7/21.3) and (C) P(LA-*b*-CL) (48.1/51.9).

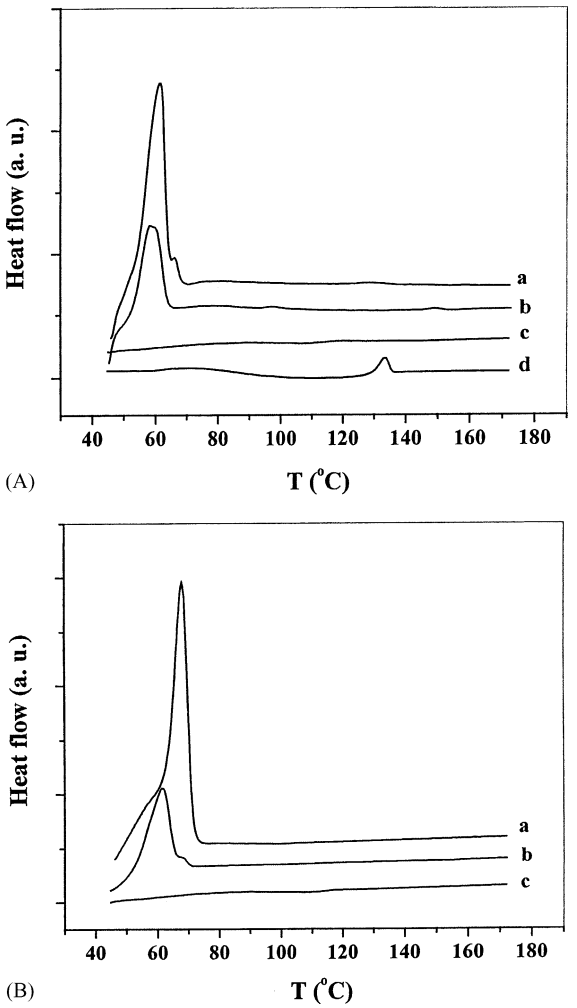


Fig. 2. DSC thermograms of (A) cyclosporin A-loaded microspheres and (B) blank polymers: a, P(LA-*b*-CL) (48.1/51.9); b, P(LA-*b*-CL) (78.7/21.3); c, PLGA (80/20) and d, plain CyA crystals.

and blank polymers are represented in Fig. 2. The results suggest the distinct crystallization of ε-CL segments in all the samples although a decrease of T_m for P(LA-*b*-CL) (78.7/21.3) (from 67.7 to 61.27 °C) and P(LA-*b*-CL) (48.1/51.9) (from 61.6 to 58.76 °C) after forming microspheres can be observed. It seemed that the effect of preparing conditions on the crystallinity of ε-CL segments was insignificant. These results could also be proven by X-ray diffraction pattern, which are shown in Fig. 3.

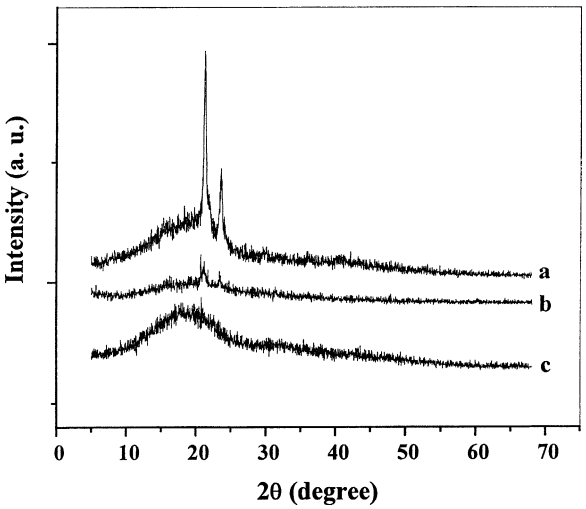


Fig. 3. Powder X-ray diffraction diagrams of CyA-loaded microspheres made from: a, P(LA-*b*-CL) (48.1/51.9); b, P(LA-*b*-CL) (78.7/21.3) and c, PLGA (80/20).

3.2. In vitro release behavior

Fig. 4, compares CyA release profiles from the three types of microspheres. On the whole, in vitro release behaviors of CyA from P(LA-*b*-CL) microspheres are quite different from that of PLGA microspheres. The drug release rate decreased in the order of

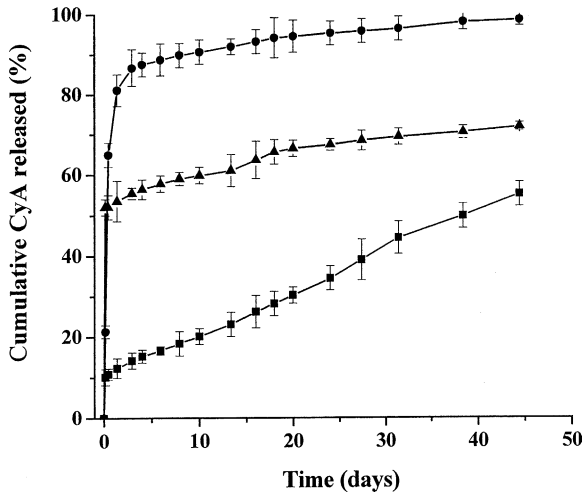


Fig. 4. In vitro release profiles of cyclosporin A-loaded microspheres in 0.1 M phosphate buffer solution (pH 7.4, containing 0.2% SDS) at 37 °C. (●) CyA-P(LA-*b*-CL) (48.1/51.9); (▲) CyA-P(LA-*b*-CL) (78.7/21.3) and (■) CyA-PLGA (80/20).

P(LA-*b*-CL) (48.1/51.9), P(LA-*b*-CL) (78.7/21.3) and PLGA. In all the cases, CyA release can be divided by two different phases: burst release within the first few days and the subsequent sustained release. In addition, it can be seen that P(LA-*b*-CL) (48.1/51.9) microspheres showed more serious burst release within the first day (almost 75%) than P(LA-*b*-CL) (78.7/21.3) (about 50%) and PLGA microspheres (12%). Thereafter, CyA continued release at a reduced rate in the case of P(LA-*b*-CL) microspheres; but for the PLGA microspheres, CyA release rate was almost constant. 60% of CyA can be liberated from the microspheres in 50 days. With respect to PLGA microspheres, our results are in good accord with other reports (Urata et al., 1999; Lee et al., 2002).

It is generally accepted that drug release rate from microspheres is strongly dependent on polymer crystalline behavior and drug dispersion state. In this study, the absence of the endothermic peak at 133.29 °C in the DSC traces (curve a–c in Fig. 2A) which corresponds to T_m of CyA for all the three types of microspheres suggests that no crystallization of CyA occurred during microsphere preparation. The crystallinity of the microsphere was also studied by DSC and X-ray diffraction. High crystallization of P(LA-*b*-CL) can be apparently observed. It was reported that the crystallization of the polymers during micirosphere formation may produce micro voids in the microspheres, which can function as channels for water penetration (Urata et al., 1999). In addition, owing to the contemporaneous presence of the amorphous and crystalline regions in P(LA-*b*-CL), it is conceivable that the tendency of phase separation would become more evident when the content of ε-CL segments increased from 21.3 to 51.9% (as shown in Fig. 1).

Above explanation mainly accounted for the burst release. The subsequent slower CyA release from P(LA-*b*-CL) microspheres could be attributed to the more slower degradation of such polymers compared with PLGA. As Lee et al. (2002) indicated, the release of CyA from PLA/PLGA particles is dependent on the dissolution diffusion of drug from the matrices as well asl the matrices erosion.

3.3. In vivo studies

In order to evaluate and compare these three CyA microsphere formulations, we tested CyA concentration in whole blood after single dosage administration to Wistar rats. Figs. 5 and 6, show CyA concentrations after subcutaneous injection of CyA-loaded microspheres based on P(LA-*b*-CL), PLGA and CyA suspension using 0.1% CMC-Na as a suspending agent. For CyA suspension, CyA blood levels rapidly reached 700 ng ml^{-1} within 2 days, followed by quick decline to 100 ng ml^{-1} after 10 days. For microsphere formulations, a maximum blood level occurred at the second day post administration of P(LA-*b*-GL) microspheres (∼520 and 450 ng ml^{-1} for P(LA-*b*-CL) with high and low ε-CL content, respectively). Thereafter, CyA levels gradually decreased to 200 ng ml^{-1} after 12 days and maintained between 100 and 200 ng ml^{-1} during the 24-day period. In comparison, PLGA microspheres showed the lowest drug concentration during the first 2 days. The maximum CyA concentration in whole blood (400 ng ml^{-1}) was reached 2 days after injection, during the following 22 days, CyA level could maintain at about 200 ng ml^{-1}. On the whole, the blood level profiles showed essentially similar pattern as those found in vitro for all formulations studied.

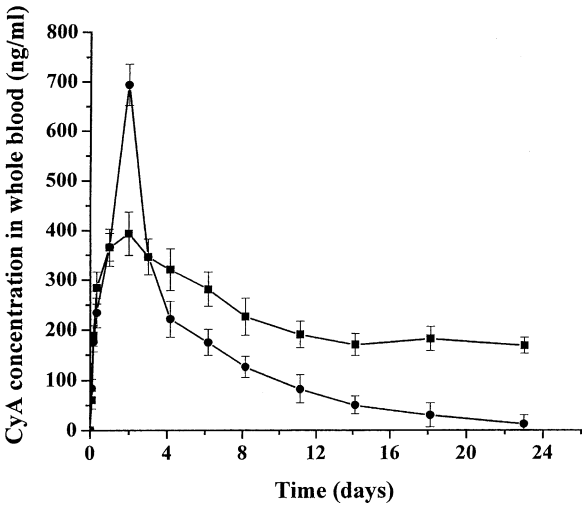


Fig. 5. In vivo release profiles of cyclosporlin A-loaded microspheres (suspended in 1 ml of 0.1% CMC-Na) after subcutaneously injected in Wistar rats. (●) CyA suspension and (■) CyA-PLGA (80/20). Each point represents the mean ± S.D. of four animals.

In clinical, immediately after transplantation the immune response against the graft is especially strong. In

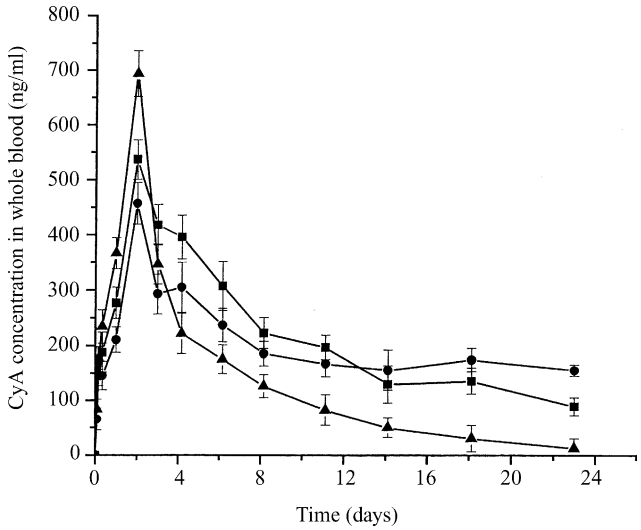


Fig. 6. In vivo release profiles of cyclosporin A-loaded microspheres (suspended in 1 ml of 0.1% CMC-Na) after subciutaneously injected in Wistar rats. (▲) CyA suspension; (■) CyA-P(LA-*b*-CL) (48.1/51.9) and (●) CyA-P(LA-*b*-CL) (78.7/21.3). Each point represents the mean ± S.D. of four animals.

order to avoid early destruction of the graft, a repeated high dose of CyA should be administered by oral or injection route everyday during the initial stage. Many literatures regarding solid organ transplantation reflects a general relationship between higher concentrations of CyA and the prevention of acute rejection (Strong et al., 1992; Strome et al., 1993; Haug et al., 2003). After the immunologically stormy early stage, the immune system gradually adapts to the new organ, therefore, the continued use of CyA at reduced blood concentration was needed. In addition, since most patients even will require life-long immunosuppression to maintain stable graft acceptance (Reyes et al., 1993; Burke et al., 1994), such low CyA concentration is more favorable for patients to mitigate side effects. From above results, it can be seen that compared with conventional PLGA microspheres, the microsphere formulation based on P(LA-*b*-CL) microspheres enable CyA to reach blood levels which is higher to have a pharmacological response to inhibit the incidence of severe acute rejection episodes. In addition, this formulation can maintain the levels in whole blood for extended period of time compared with CyA suspension. Therefore, such formulation could overcome the limitations of those on the market and better meet the requirement in clinical.

4. Conclusions

In this paper, CyA-loaded microspheres based on P(LA-*b*-CL) with different ε-CL content (21.3 and 51.9%) and PLGA were prepared using the oil-in-water (O/W) solvent evaporation method. Under the same preparing conditions, all types of microspheres with the mean size of about 35 μm showed high drug encapsulation efficiency (exceed 96%). DSC and X-ray diffraction indicate that CyA was amorphously dispersed in all polymer matrices. In vitro release rate decreased in the order of P(LA-*b*-CL) (48.1/51.9), P(LA-*b*-CL) (78.7/21.3) and PLGA microspheres. P(LA-*b*-CL) showed more evident initial burst release behavior compared with PLGA microspheres. This difference was attributed to the different crystalline character of loading materials after forming microspheres. In vivo evaluation reveals that, compared with PLGA microspheres, a single shot of P(LA-*b*-CL) microspheres could provide a higher blood level of CyA during the initial 2 days, as well as constant levels for the followed extended periods of time (several weeks). Taking into account that almost all conventional CyA formulatibns has a narrow therapeutic window and dominant dose-dependent immunomodulatory effects,

these findings may lead to an improvement in the efficiency of the drug and the compliance of the patients.

Acknowledgments

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