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Research paper

Preparation and evaluation of tacrolimus-loaded nanoparticles for lymphatic delivery

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ABSTRACT

In an effort to improve lymphatic targeting efficiency and reduce the toxicity of tacrolimus, the emulsification–diffusion method was used to load the drug into nanoparticles (NP). Poly(lactide-co-glycolide) (PLGA) and PLGA surface-modified with poly(ethylene glycol) (PEG) were used as polymers. Mean particle size and drug encapsulation efficiency of PLGA were 218 ± 51 nm and 60.0 ± 1.2% and for PEG–PLGA NP were 220 ± 33 nm and 60.3 ± 2.0%. NP were characterized by thermal analyzer and X-ray diffractometry (XRD), and their shapes were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *In vitro* release profiles were affected by the pH of dissolution media. The prepared NP and commercial product of tacrolimus (Prograf® inj.) were intravenously administered to rats to compare their pharmacokinetic characteristics and lymphatic targeting efficiency. The area under the whole blood concentration–time curve (AUC), mean residence time (MRT), and total clearance (CL_t) of PEG–PLGA NP were significantly different ($P < 0.05$) compared with those of Prograf® inj., and lymphatic targeting efficiencies of both NP formulations at the mesenteric lymph node significantly increased ($P < 0.05$). These results showed that the prepared tacrolimus-loaded NP are good possible candidates as a lymphatic delivery system of tacrolimus.

1. Introduction

Tacrolimus, a macrolide lactone immunosuppressant from the fungus *Streptomyces tsukubaensis*, is used for the prophylaxis of liver and kidney transplant rejection. It is also used for heart, pancreas, bone marrow, small bowel, and lung transplant and for the treatment of T cell-mediated autoimmune diseases [1]. It acts as a calcineurin inhibitor that blocks interleukin-2 production, leading to a decrease in T cell proliferation [2]. Today, calcineurin inhibitors are the basis of most immunosuppressive protocols after organ transplantation [3]. Compared with cyclosporine A, tacrolimus provides a better side effect profile and increased long-term survival in patients [4,5].

However, tacrolimus shows high between- and within-subject pharmacokinetic variability. Due to its narrow therapeutic index, close drug monitoring programs are required to optimize its efficacy and limit its toxicity [6,7]. Because tacrolimus is a substrate for *CYP3A5* and *MDR1* genes, polymorphisms of the genes are major reasons for the large interindividual variations in the pharmacokinetic characteristics of tacrolimus and are important factors in tacrolimus-induced toxicity [6,8,9]. Among the known adverse events, such as nephrotoxicity, neurotoxicity, hypertension, and diabetogenic effects, neurotoxicity (tremor, seizure and encephalopathy) is frequently observed and causes discontinuation of the treatment [8,10].

Nanoparticles (NP) are stable, solid colloidal particles consisting of biodegradable polymer or lipid materials and range in size from 10 to 1000 nm. Drugs can be absorbed onto the particle surface and are entrapped inside the polymer/lipid or dissolved within the particle matrix [11,12]. Over the past few decades, there have been considerable interests in developing biodegradable polymeric NP as potential and effective drug delivery devices due to their ability to control drug release and delivery. They can effectively deliver the drug to target sites and thus increase the therapeutic benefit while minimizing side effects [13–16].

One of the main methods for the preparation of long-circulating NP is to modify their surface with a hydrophilic, flexible, and non-ionic polymer, poly(ethylene glycol) (PEG) [17,18]. Surface modification using PEG enables NP to escape the arrest of the mononuclear phagocytic system (MPS) so as to prolong their half-life in plasma [17,19]. Biodegradable PEG-coated NP have

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been found to be important in potential therapeutic applications as injectable colloidal systems for controlled drug release [19–21].

The lymphatic system consists of lymph, lymphatic pathways and some lymphatic organs including lymph nodes, thymus and spleen [22]. T cells, target cells of tacrolimus, are also highly concentrated in lymphatic fluid. NP are one of the most promising approaches for lymphatic targeting [23–25]. The walls of lymphatic capillaries consist of a single layer of epithelial cells that enables small particles to freely pass through [22,26].

In this study, the purpose is to improve the drug targeting to the lymphatic system using NP to reduce the systemic toxicity or adverse effects of tacrolimus. Poly(lactide-co-glycolide) (PLGA) and PEG–PLGA NP were prepared to reduce the adverse effects of systemic immunosuppression and simultaneously enhance immunosuppressive efficacy by selectively transferring tacrolimus into the lymphatic system. We selected the emulsification–diffusion method to prepare NP because of its simplicity, reproducibility, and stability [27,28]. This method presents clearer advantages compared to the other existing methods such as the use of pharmaceutically acceptable organic solvents, high yield of encapsulation, high reproducibility, better control of particle size, and easy scaling-up [28–30]. We characterized the prepared NP according to their physicochemical properties and also evaluated pharmacokinetic characteristics and lymphatic targeting efficiencies of the formulations in rats for intravenous (i.v.) administration. The results were compared to those of the tacrolimus commercial product, Prograf® inj.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Tacrolimus and cyclosporine A were kindly supplied by Chong Kun Dang Pharm. (Seoul, Korea). Prograf® inj. was kindly supplied by Astellas Pharma Korea, Inc. (Seoul, Korea). PLGA (lactide:glycolide ratio 50:50, molecular weight = 50,000–75,000) and Pluronic® F-68 (poloxamer 188) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Poly(lactide-co-glycolide)-methoxy poly (ethylene glycol) (PLGA-mPEG) was purchased from Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany). Trehalose dihydrate was purchased from Calbiochem (Darmstadt, Germany). Ethyl acetate was purchased from Dae-Jung (Incheon, Korea). Normal saline, heparin sodium (25,000 IU/5 ml) (Choongwae Pharma. Co., Seoul, Korea), zinc sulfate (Sigma Chemical Co., St. Louis, MO, USA), 70% ethanol, and sodium hydroxide (Dae-Jung, Incheon, Korea) were used for animal experiments and sample extractions.

Methanol (Fischer Scientific., Fair Lawn, NJ, USA), formic acid (Sigma Chemical Co., St. Louis, MO, USA), and ammonium acetate (Yakuri Pure Chemicals Co. Ltd., Osaka, Japan) were used for high performance liquid chromatograph (HPLC) assay. HPLC grade water was obtained from a Milli-Q water purification system (Millipore Co., Milford, MA, USA) and was used throughout the study. Dialysis tubing cellulose membrane with molecular weight cut-off (MWCO) at 12,000 (Sigma–Aldrich Co., St. Louis, MO, USA) was used for *in vitro* release tests. All other chemicals and solvents were of analytical grade or the highest quality available.

2.1.2. Instruments

Homogenizer (IKA-WERKE, T10 basic, GmbH & Co., Staufen, Germany), freeze dryer (FDU-506, EYELA, Tokyo Rikakikai Co. Ltd., Tokyo, Japan), and ultra-speed centrifuge (CENTRIKON T-2070, KONTRAN, Switzerland) were used for the preparation of NP.

A particle size analyzer (Autosizer Lo-C, Malvern Instruments, Worcesterahire, UK), zeta potential analyzer (ELS-8000, OTSUKA Electronics, Osaka, Japan), thermal analyzer (differential scanning calorimeter (DSC)-2920, TA Instruments, Inc., New Castle, DE, USA), X-ray diffractometer (XRD, D/MAX-1200, Rigaku, Japan), field emission-scanning electron microscopy (FE-SEM, S-4700, Hitachi Ltd., Tokyo, Japan), transmission electron microscopy (TEM, JEM-200 FXII, JEOL Ltd., Tokyo, Japan), and shaking incubator (BS-11, JEIO TECH, Kimpo, Kyunggi-do, Korea) were used for the evaluation of prepared NP.

A HPLC–mass spectroscopy (HPLC–MS) system consisting of a pump (LC-20AD, Shimadzu Corp., Kyoto, Japan), degasser (DGU-$20A_3$, Shimadzu Corp., Kyoto, Japan), MS detector (LCMS-2010EV, Shimadzu Corp., Kyoto, Japan), system controller (CBM-20A, Shimadzu Corp., Kyoto, Japan), and 4.6 mm × 250 mm analytical column (Luna 5u C18(2) 100A, Phenomenex, Torrance, CA, USA) was used for the determination of tacrolimus. The mobile phases were (A) 2 mmol/l ammonium acetate and 1 ml/l formic acid in water and (B) 2 mmol/l ammonium acetate and 1 ml/l formic acid in methanol. The gradient condition was initially 50% B and then set to the linear gradient of B from 50% to 100% for 2 to 4 min, 100% B for 4 to 19 min, the linear gradient of B from 100% to 50% for 19 to 20 min, and 50% B for 20 to 30 min. The flow rate was 0.2 ml/min, and the temperature for the column was set to 50 °C. The injection volume was 5 μl. Nitrogen was used as nebulizing gas at a pressure of 0.1 MPa and as drying gas at 1.5 l/min and 200 °C. The capillary voltage was set at 1500 V and the fragmentor voltage at 150 V. MS detection of the ions $[M+Na]^+$ of tacrolimus (m/z 826.15) and cyclosporine A (m/z 1224.70) was carried out in the selected ion monitoring mode.

2.1.3. Experimental animals

Male Sprague–Dawley rats weighing 190–210 g were obtained from Dae Han Biolink Co., Ltd. (Eumseong, Korea). The rats were fed with tap water and food (Cheil Food and Chemical, Icheon, Korea). Animals were housed separately in a cage in a ventilated animal room with controlled temperature (19 ± 1 °C) and relative humidity (50 ± 5%) and kept in a 12 h light/dark cycle. Rats weighing 240–280 g were used.

2.2. Methods

2.2.1. Preparation of tacrolimus-loaded NP

The tacrolimus-loaded NP were prepared by the emulsification–diffusion method. More specifically, an organic phase consisting of polymer (PLGA and PLGA-mPEG, typical concentration, 10 mg/ml) and drug (tacrolimus, typical concentration, 1 mg/ml) dissolved in ethyl acetate (typical volume, 10 ml) was added to an aqueous phase containing a surfactant (poloxamer 188, typical concentration, 2%, 20 ml) and emulsified by homogenization using homogenizer (20,000 rpm, 10 min). The addition of a large volume of water (three times the volume of the emulsion) to the emulsion under gentle stirring with a magnetic bar allowed the ethyl acetate to leave the droplets. The organic solvent and a part of the water were thereafter removed by evaporation under reduced pressure for 3 h to produce a purified and concentrated suspension. Afterwards, the final product was obtained by centrifugation (12,000 rpm, 20 min), re-dispersion and freeze-drying. Poloxamer 188 and trehalose were added to the suspension as cryoprotectants (the weight ratio of NP:each cryoprotectant was 1:2) before freeze-drying [31].

2.2.1.1. Determination of optimal concentration of poloxamer 188. Ten milligrams of tacrolimus and 100 mg of PLGA or PEG–PLGA were dissolved in 10 ml of ethyl acetate. Poloxamer 188 aqueous solution was added to this mixture at concentrations of 1%, 2%, 3%, and 5% (w/v). NP were prepared as previously described. The optimal concentration of poloxamer 188 was determined from size and encapsulation efficiency (EE) of the prepared NP [30].

2.2.2. Characterization of tacrolimus-loaded NP

2.2.2.1. Determination of particle size of NP. The mean particle size and size distribution analysis of NP were performed by the dynamic light scattering (DLS) method at 25 °C with a 90° scattering angle for optimum detection. Ten milligrams of tacrolimus-loaded NP was dispersed in 10 ml of deionized water. The dispersion was sonicated for 1 min and sized immediately. The results shown (mean ± standard deviation (SD)) are representatives of three independent experiments.

2.2.2.2. Determination of drug EE. Drug concentrations were determined by HPLC–MS. Ten milligrams of NP containing tacrolimus were dissolved in 10 ml of methanol. The solution was stirred for 1 h and sonicated for 5 min. Afterwards, the solution was diluted with a specific volume of the mobile phase and injected into HPLC. EE was calculated with the following equation:

$$\text{EE}\,(\%, \text{W/W}) = \frac{\text{weight of drug in nanoparticles}}{\text{weight of drug used in preparation of nanoparticles}} \times 100 \quad (1)$$

2.2.2.3. Measurement of zeta potential of NP. The zeta potential of prepared NP was measured by the ELS-8000 zeta potential analyzer of triplicate among different batches to assess the surface charge and stability of NP. Zeta potential of NP was measured in aqueous dispersion. The measurements were carried out using the medium of water. The results (mean ± SD) are representatives of three independent experiments.

2.2.2.4. Morphological characterization.

2.2.2.4.1. SEM observation. The morphology of tacrolimus-loaded NP was observed using FE-SEM. The NP samples were coated with platinum (~20 nm thick) using an Ion Sputter (JFC-1100, JEOL Ltd., Tokyo, Japan) for 5 min at 20 mA. Observation was performed at an accelerating voltage of 5 kV and a working distance of 10 mm. The magnification for the SEM images was 20,000×.

2.2.2.4.2. TEM observation. A drop of tacrolimus-loaded NP dispersion containing 1% of phosphotungstic acid (pH 7.0 adjusted with 1 M KOH solution) was placed on carbon film coated on a copper grid for TEM. Observation was performed at 120 kV in a JEM-2000 FXII. The magnification for the TEM images was 50,000×.

2.2.2.5. Thermal analysis and XRD analysis. The endothermic melting temperature for tacrolimus, empty NP, physical mixture of tacrolimus/empty NP, and tacrolimus-loaded NP were determined with a DSC-2920. Fifty milligrams of samples were scanned from 20 °C to 160 °C at a rate of 10 °C/min.

XRD patterns of tacrolimus, empty NP, physical mixture of tacrolimus/empty NP, and tacrolimus-loaded NP were obtained using a D/MAX-1200. The scanning range of 2θ was from 2° to 60°. The X-ray source was Cu Kα radiation (40 kV, 20 mA).

2.2.2.6. In vitro release studies. The *in vitro* release tests of tacrolimus from tacrolimus-loaded NP in different pH media (1.2, 4.0, 6.8 and 7.4) were employed by a dialysis method. Each tacrolimus-loaded NP solution was diluted with each medium, and the final concentration of tacrolimus was adjusted to 100 μg/ml. Thereafter, each sample was put into a dialysis tube (MWCO: 12,000), which was placed into 50 ml of a screw-capped falcon tube with 10 ml of each dissolution medium. The falcon tubes were incubated in a shaking water bath at 37 °C and shaken at a rate of 100 opm. The whole-media change method was used for the prevention of drug saturation. At predetermined time intervals, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 240, 288, 336, and 384 h after starting incubation, whole medium (10 ml) was taken and replaced with the same volume of fresh medium (10 ml). The amount of tacrolimus released was determined by HPLC–MS.

2.2.2.7. In vivo studies. The femoral vein and artery of rats were cannulated with a polyethylene tube under light ether anesthesia. Cannulated rats were kept in restraining cages under normal housing conditions for 1–2 h until they recovered from the anesthesia prior to the experiments. The rats were divided into three groups: (1) Prograf® inj., commercial product of tacrolimus, (2) tacrolimus-loaded PLGA NP, and (3) tacrolimus-loaded PEG–PLGA NP. A single dose (1 mg/kg as tacrolimus) [32] of each formulation was given to the rats intravenously at the same time. At predetermined time intervals (5, 10, 15, 30, 45, 60, 90, 120, 240, and 360 min), whole blood (about 150 μl) was withdrawn from the femoral artery into a Vacutainer® tube with EDTA, thoroughly mixed and stored at −80 °C until assay [33]. A whole blood (approximately 1 ml) collected from untreated rats was infused via the femoral artery at 45 and 240 min, respectively, to replace blood-loss due to blood sampling.

Also, in order to evaluate the lymphatic delivery of tacrolimus, the rats were divided into three groups and administered each formulation as mentioned earlier. At 1 h after administration, whole blood was taken from the abdominal aorta, and the mesenteric and axillary lymph nodes were isolated and weighed (25 mg for each lymph node). These lymph node samples were suspended by homogenization for 1 min in a phosphate-buffered saline (PBS, pH 7.4) so as to achieve a final concentration of 25 mg/ml in the suspension and stored at −80 °C until assay. All *in vivo* tests were started at 10 am to avoid circadian-dependent changes in the pharmacokinetics of tacrolimus [34]. The Ethical Review Committee on Animals Experiments at Chonnam National University approved the study.

2.2.2.8. Determination of tacrolimus in rat whole blood and lymph node suspension. The whole blood and lymph node suspension concentration of tacrolimus was determined by HPLC–MS using a method from previous reports [33,35] with some modification. The stock solutions of tacrolimus were prepared at 1 mg/ml in methanol and stored at 4 °C. The stock solutions of internal standard (I.S.) were prepared by dissolving cyclosporine A in methanol at a concentration of 1 mg/ml. Calibration standards of the rat whole blood and lymph node suspension were prepared at concentrations of 10, 20, 50, 100, 200, 500, and 1000 ng/ml in a drug-free pooled rat whole blood and lymph node suspension. The calibration curves were obtained by plotting the peak-area ratios of the drug to I.S. versus the concentration of tacrolimus.

In short, 100 μl of rat whole blood or lymph node suspension samples were put into an Eppendorf tube, then 10 μl of I.S. (cyclosporine A in methanol at a concentration of 100 μg/ml) was added and vortex-mixed for 5 min with 100 μl of 0.1 M NaOH and 200 μl of a mixture of methanol and 0.3 M $ZnSO_4$ (70:30, by volume). After centrifugation for 10 min at 12,000 rpm, the supernatants were decanted, followed by vortexing for 30 s, and 5 μl of the sample was injected into the HPLC–MS system.

2.2.2.9. Pharmacokinetic analysis and evaluation of lymphatic targeting efficiency. The pharmacokinetic parameters associated with i.v. administration were estimated by noncompartmental methods using a WinNonlin™ program [36]. The pharmacokinetic parameters, such as mean residence time (MRT), total clearance (CL_t), and half-life ($t_{1/2}$), were calculated from standard equations using WinNonlin™ [37]. The area under the whole blood

Table 1
The effect of poloxamer 188 concentration (%, w/v) on particle size and EE of tacrolimus-loaded NP.

Polymer	Poloxamer 188 concentration (%, w/v)	Particle size (nm)	EE (%)
PLGA	1	439 ± 85	59.3 ± 1.9
	2	218 ± 51	60.0 ± 1.2
	3	287 ± 48	52.9 ± 1.0
	5	369 ± 36	50.1 ± 1.6
PEG–PLGA	1	489 ± 96	57.7 ± 1.8
	2	220 ± 33	60.3 ± 2.0
	3	260 ± 29	52.4 ± 2.4
	5	327 ± 38	51.2 ± 2.0

Each value represents the mean ± SD (n = 3).

concentration–time curve (AUC) was calculated by trapezoidal rule with time extrapolated to infinity.

Furthermore, targeting efficiency of tacrolimus to the lymphatic system was calculated as the ratio of the tacrolimus concentration in lymph node (mesenteric and axillary lymph nodes) to the concentration in whole blood at 1 h after i.v. administration of each formulation.

2.2.2.10. Statistical evaluation. All data were analyzed for statistical significance by the Student's t-test ($\alpha = 0.05$). All calculated values were expressed as their mean ± SD.

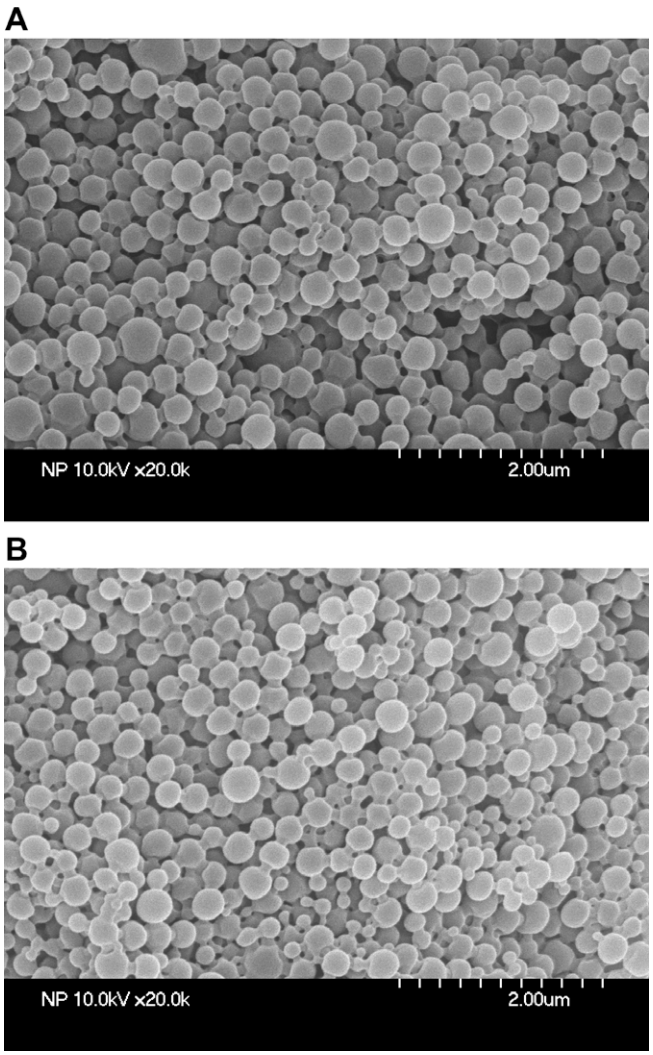

Fig. 1. SEM images of tacrolimus-loaded (A) PLGA NP and (B) PEG–PLGA NP. Bar = 2 μm (20,000×).

3. Results and discussion

3.1. Preparation of tacrolimus-loaded NP

3.1.1. Determination of optimal concentration of poloxamer 188

The surfactant concentrations from 1% to 5% (w/v) of poloxamer 188 in the external phase were used to evaluate the effect of surfactant concentration on particle size and drug EE (Table 1). As shown in Table 1, the mean sizes of NP were maintained at 100–500 nm. Our results showed that the smallest particle size and the highest drug EE were found at 2% (w/v) of poloxamer 188. At this poloxamer 188 concentration, the mean sizes of PLGA and PEG–PLGA NP were 218 ± 51 nm and 220 ± 33 nm, respectively, and the drug EE for PLGA and PEG–PLGA NP were 60.0 ± 1.2% and

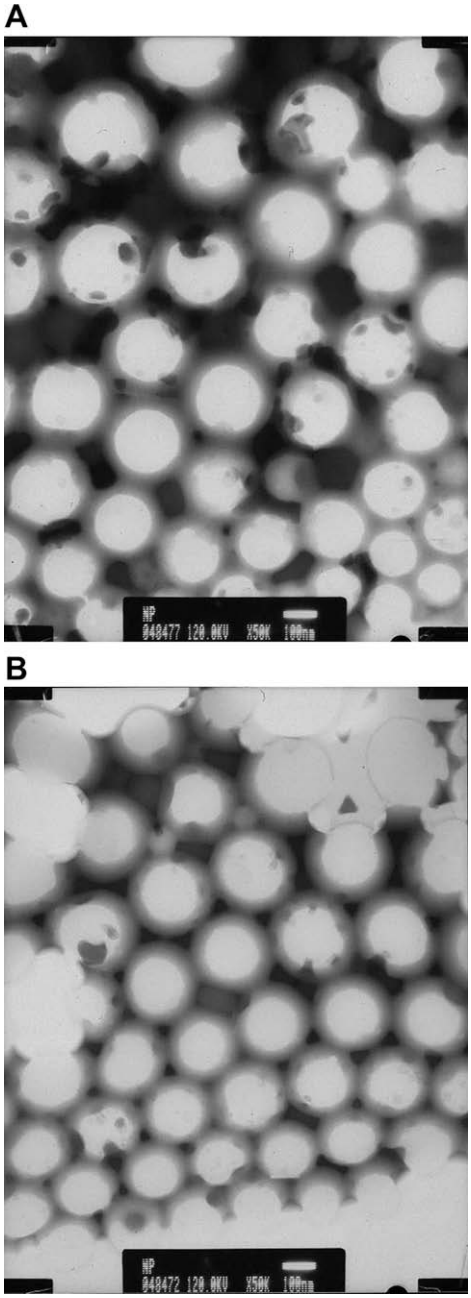

Fig. 2. TEM images of tacrolimus-loaded (A) PLGA NP and (B) PEG–PLGA NP. Bar = 100 nm (50,000×).

60.3 ± 2.0%, respectively. From these results, the optimal concentration was determined to be 2% (w/v) of poloxamer 188.

3.1.2. Zeta potential of tacrolimus-loaded NP

Zeta potential can greatly influence the stability of NP in suspension through electrostatic repulsion between the particles. Zeta potential of PLGA and PEG–PLGA NP were −28.2 ± 4.3 mV and −24.5 ± 5.7 mV, respectively. This decrease in the average surface charge for PEG–PLGA NP is considered enough to be due to masking the negative charges [17].

Based on the zeta potential data from the previous studies for NP, these absolute values of zeta potential indicate a high electric charge on the surface of the NP which can cause strong repellent forces among particles to prevent aggregation of the NP [38].

3.2. Morphological characterization

In the SEM observation (Fig. 1), the prepared tacrolimus-loaded NP were about 200 nm in size and had a spherical shape with a smooth surface, which were almost same results as the TEM observation (Fig. 2) as well as DLS measurements. These results showed that tacrolimus-loaded NP about 200 nm in size and ideal morphology were successfully prepared by the emulsification–diffusion method.

3.3. Thermal analysis and XRD analysis

DSC studies were performed in order to characterize drug status inside the NP as well as the melting points of the compounds of each formulation. Fig. 3 shows the DSC thermograms. The melting point of tacrolimus is 127–129 °C [39]. As shown in Fig. 3, the endothermic peak of tacrolimus could be observed at around 128 °C and was also visible in the pattern obtained from the physical mixture of tacrolimus and empty NP. However, such peaks were found neither in the empty NP nor in the tacrolimus-loaded NP.

The XRD spectra of tacrolimus, empty NP, physical mixture of tacrolimus/empty NP, and tacrolimus-loaded NP are shown in Fig. 4. Tacrolimus exhibited several intense peaks, and the two polymers showed different patterns due to different crystallinities [40]. However, these peaks were not observed in the XRD patterns from samples of the tacrolimus-loaded NP.

From these results, it can be concluded that the drug entrapped in NP was in an amorphous or disordered crystalline phase of molecular dispersion [41].

3.4. In vitro release studies

Fig. 5 compares the release profiles of tacrolimus from (A) PLGA and (B) PEG–PLGA NP in media with various pH values (pH 1.2, 4.0,

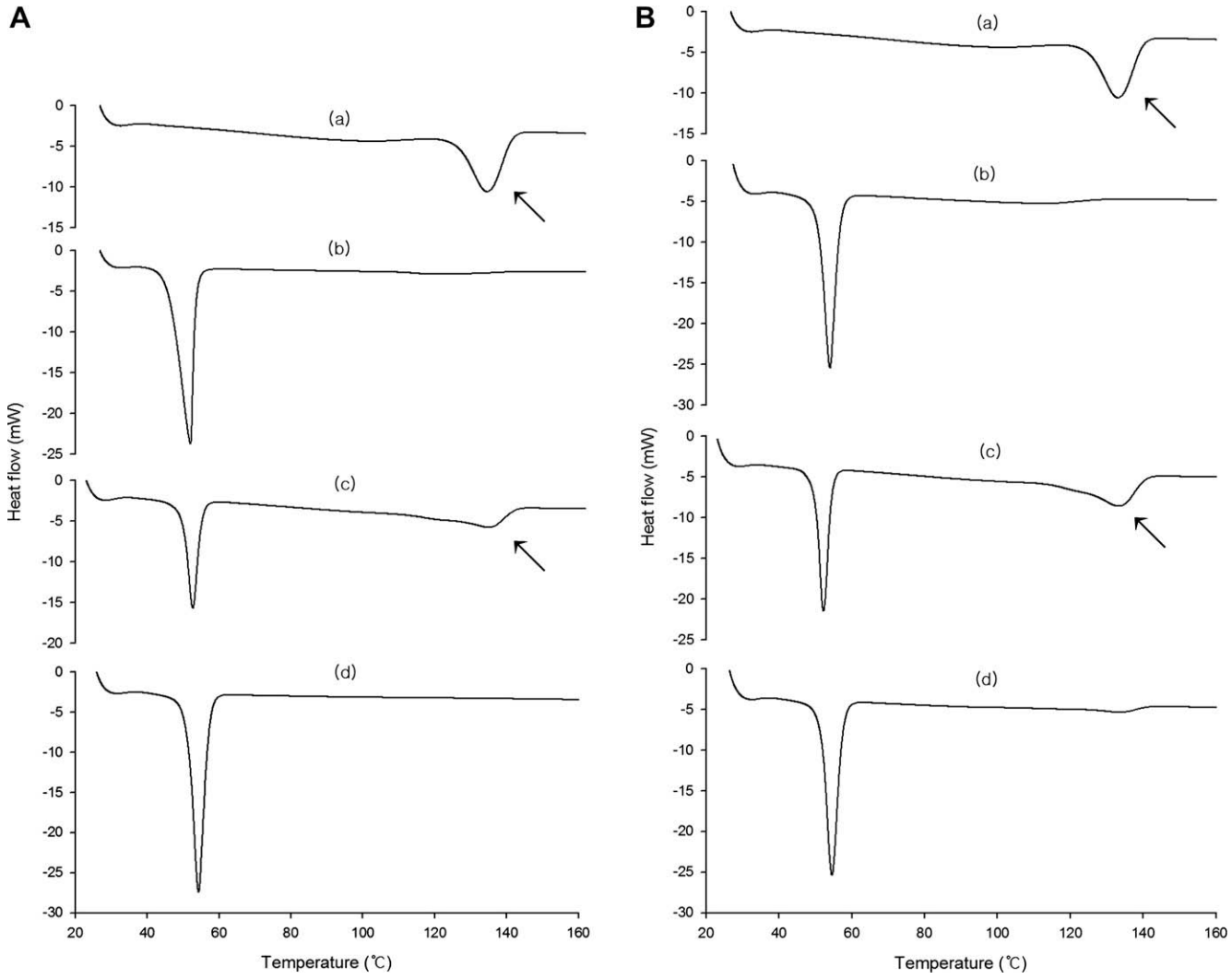

Fig. 3. (A) DSC thermograms of (a) tacrolimus, (b) empty PLGA NP, (c) physical mixture of tacrolimus and empty PLGA NP (weight ratio of tacrolimus/empty NP = 1/10), and (d) tacrolimus-loaded PLGA NP. (B) DSC thermograms of (a) tacrolimus, (b) empty PEG–PLGA NP, (c) physical mixture of tacrolimus and empty PEG–PLGA NP (weight ratio of tacrolimus/empty NP = 1/10), and (d) tacrolimus-loaded PEG–PLGA NP (↖: endothermic peak of tacrolimus).

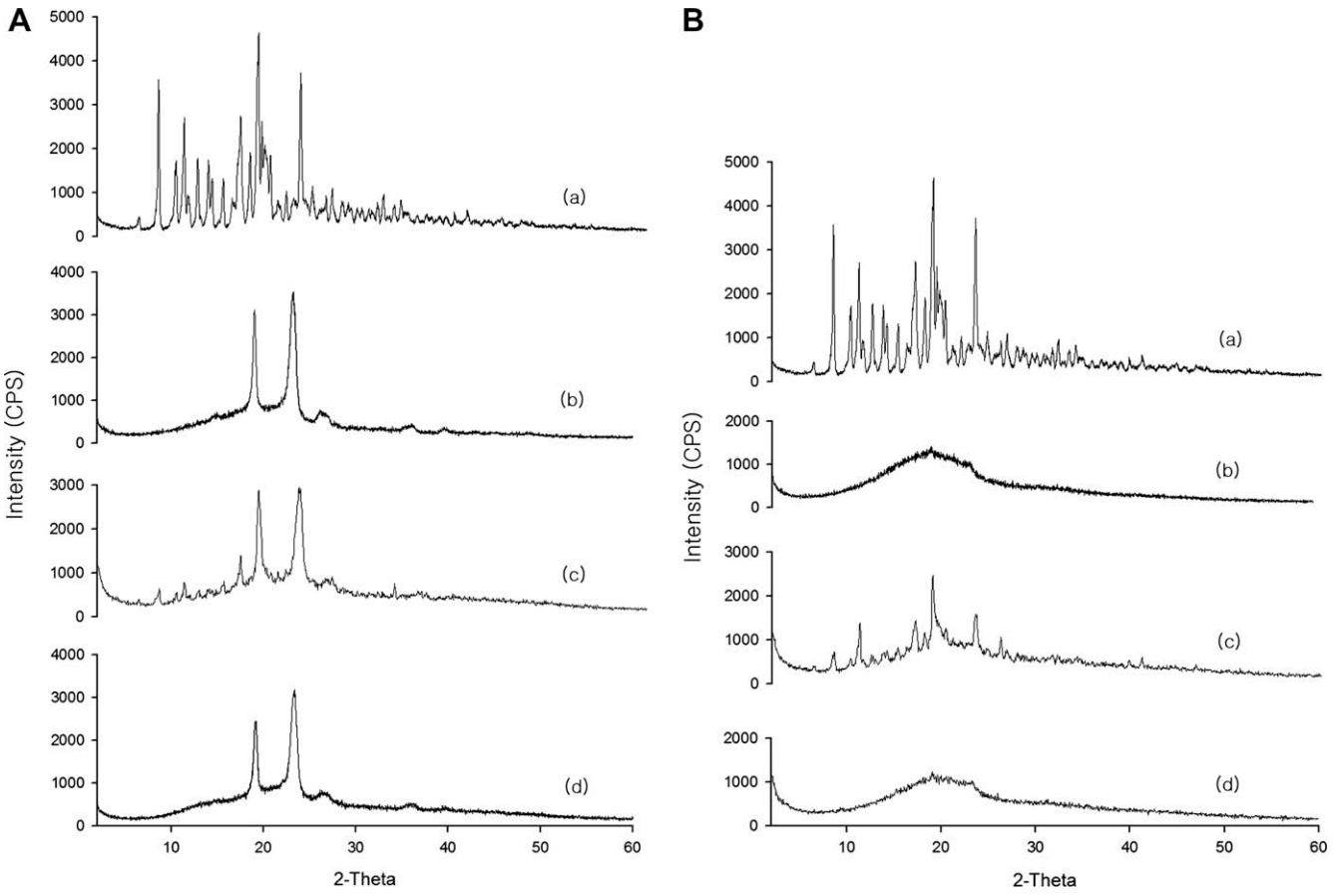

Fig. 4. (A) XRD patterns of (a) tacrolimus, (b) empty PLGA NP, (c) physical mixture of tacrolimus and empty PLGA NP (weight ratio of tacrolimus/empty NP = 1/10), and (d) tacrolimus-loaded PLGA NP. (B) XRD patterns of (a) tacrolimus, (b) empty PEG–PLGA NP, (c) physical mixture of tacrolimus and empty PEG–PLGA NP (weight ratio of tacrolimus/empty NP = 1/10), and (d) tacrolimus-loaded PEG–PLGA NP.

6.8 and 7.4). The tacrolimus-loaded NP formulations showed slow release for 16 days. The release rate of tacrolimus in pH 1.2 dissolution medium was higher and faster than those of all other media. The release rate of tacrolimus was dependent on the pH of dissolution media (order of pH 1.2 > 6.8 > 7.4 > 4.0). These differences related to pH of the media suggested that degradation or swelling of polymers or diffusion process through the matrix of NP is affected by pH as exemplified in cases of doxorubicin and ganciclovir [42]. Therefore, it could be concluded that the prepared tacrolimus-loaded NP are pH sensitive and have potential applications in controlled drug release in blood or intracellular fluid such as with clozapine [43].

3.5. Pharmacokinetic analysis and evaluation of lymphatic targeting efficiency

The peaks of tacrolimus and I.S. (cyclosporine A) were well resolved and free of interference from endogenous compounds in the rat whole blood and lymph node suspension. The retention time for tacrolimus and I.S. were 12.3 and 13.7 min, respectively. The regression equations from three replicate calibration curves for rat whole blood and lymph node suspension: $y = 0.001054x + 0.02567$ for rat whole blood and $y = 0.0009319x + 0.05045$ for lymph node suspension (where, y = peak-area ratio, x = concentration of tacrolimus), showed significant linearities ($r = 0.9904$ and 0.9993, respectively).

Fig. 6 shows the mean rat whole blood concentration–time profiles of tacrolimus after i.v. administration of Prograf® inj. and tacrolimus-loaded NP to rats (1 mg/kg as tacrolimus). Table 2 lists the pharmacokinetic parameters obtained by noncompartmental methods using WinNonlin™. After i.v. administration, the AUC, MRT, and CL_t of tacrolimus-loaded PEG–PLGA NP (39,526.18 ± 3411.35 ng min/ml, 227.26 ± 50.44 min and 7.90 ± 0.62 ml/min, respectively) were significantly different ($P < 0.05$) compared with those of Prograf® inj. (28,058.66 ± 4531.07 ng min/ml, 97.78 ± 34.89 min and 10.84 ± 0.32 ml/min, respectively). $T_{1/2}$ was highest for PEG–PLGA NP (269.32 ± 136.16 min), followed by PLGA NP and Prograf® inj. (189.43 ± 76.43 and 129.15 ± 70.88 min, respectively), but there were no significant differences. These results were consistent with other pharmacokinetic studies in relation to parameter values [32]: increased AUC, MRT and $t_{1/2}$, a decreased CL_t of NP [44], and extended circulation of PEGylated NP [17].

The concentrations of tacrolimus in mesenteric and axillary lymph nodes after i.v. administration are shown in Fig. 7A. The concentrations after administration of tacrolimus-loaded NP were higher than those of Prograf® inj. in both lymph nodes. In the case of PEG–PLGA NP, there was a significant difference ($P < 0.05$) in the concentration of mesenteric lymph node compared with Prograf® inj.

The lymphatic targeting efficiencies of tacrolimus calculated as the ratio of the lymph node concentration to the whole blood concentration are shown in Fig. 7B. Both tacrolimus-loaded NP showed higher lymphatic targeting efficiencies than those of Prograf® inj. In the case of the mesenteric lymph node, there were significant differences ($P < 0.05$) between tacrolimus-loaded NP and Prograf® inj, and PEG–PLGA NP showed higher average targeting efficiencies than those of PLGA NP. These increased lymphatic targeting efficiencies can lower the blood drug concentration needed to show

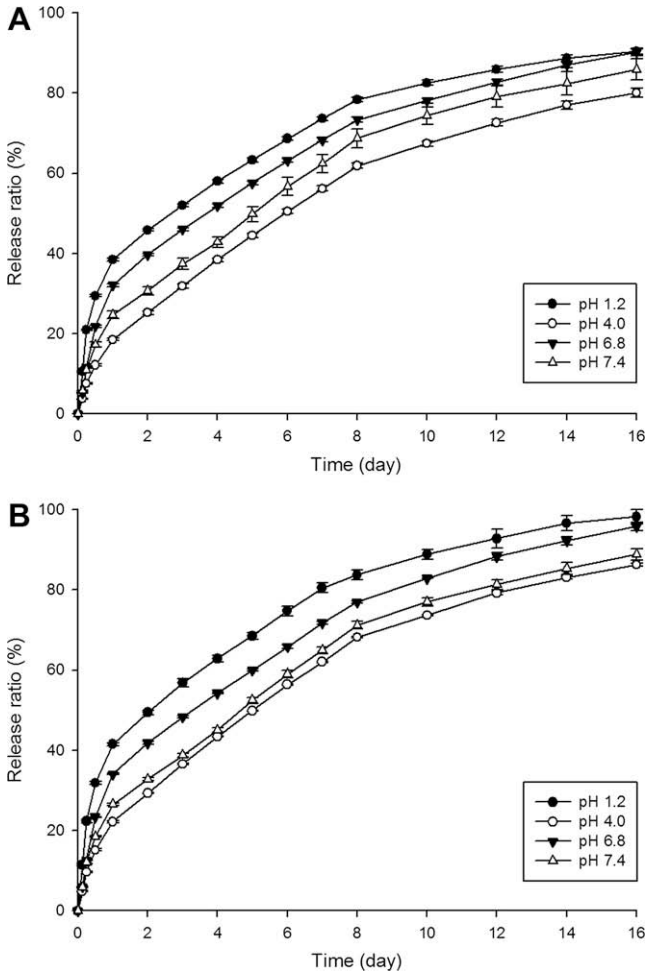

Fig. 5. *In vitro* release profiles of tacrolimus-loaded (A) PLGA and (B) PEG–PLGA NP in various dissolution media (pH 1.2, 4.0, 6.8, and 7.4). Each value represents the mean ± SD (n = 3).

Table 2
Pharmacokinetic parameters of tacrolimus in rat whole blood after i.v. administration of the prepared tacrolimus-loaded NP and Prograf® inj. to rats[a].

Parameters	i.v. Administration		
	Prograf® inj.	Tacrolimus-loaded PLGA NP	Tacrolimus-loaded PEG– PLGA NP
AUC (ng min/ml)	28058.66 ± 4531.07	33971.19 ± 14100.49	39526.18 ± 3411.35*
MRT (min)	97.78 ± 34.89	136.01 ± 42.97	227.26 ± 50.44*
$t_{1/2}$ (min)	129.15 ± 70.88	189.43 ± 76.43	269.32 ± 136.16
CL_t (ml/min)	10.84 ± 0.32	10.29 ± 4.81	7.90 ± 0.62*

[a] Mean ± SD (n = 5–6).
* $P < 0.05$ between Prograf® inj. and PEG–PLGA NP.

effect. Considering this along with a long circulation of NP, improvement in efficacy at the target sites and reduction in systemic side effects seem to be within reach. From these results, it was suggested that the prepared tacrolimus-loaded NP can be effective formulations for lymphatic delivery of tacrolimus.

4. Conclusions

Tacrolimus-loaded NP with diameters of about 200 nm could be prepared easily and are reproducible by the emulsification–

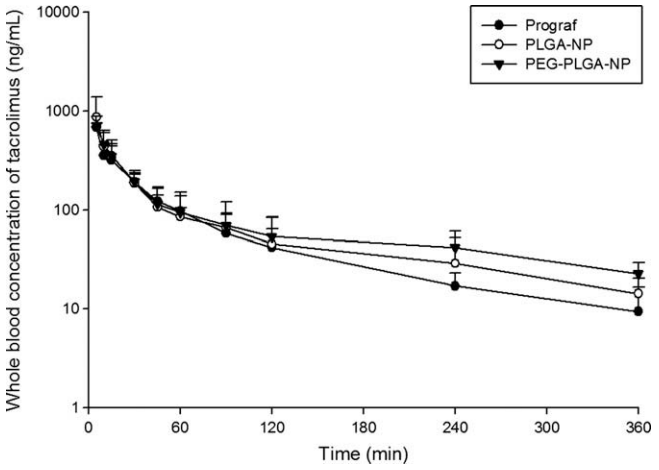

Fig. 6. Rat whole blood concentration–time profiles of tacrolimus after i.v. administration of Prograf® inj. (●), tacrolimus-loaded PLGA NP (○) and PEG–PLGA NP (▼) to rats. Each value represents the mean ± SD (n = 5–6).

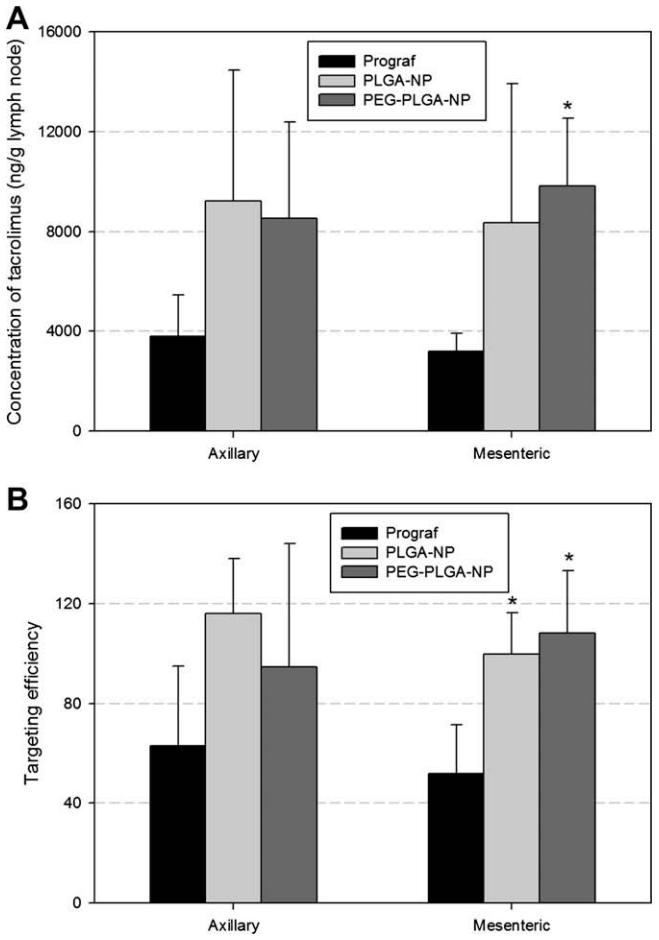

Fig. 7. (A) Concentrations of tacrolimus in mesenteric and axillary lymph nodes at 1 h after i.v. administration (1 mg/kg) of Prograf® inj. (■), tacrolimus-loaded PLGA NP (■), and PEG–PLGA NP (■) to rats. (B) Lymphatic targeting efficiencies of tacrolimus to mesenteric and axillary lymph nodes at 1 h after i.v. administration (1 mg/kg) of Prograf® inj. (■), tacrolimus-loaded PLGA NP (■), and PEG–PLGA NP (■) to rats.Vertical bars represent the mean ± SD (n = 5–6). * $P < 0.05$.

diffusion method. Their drug EE were about 60% at the optimal condition (2% w/v of poloxamer 188 concentration). And there is a possibility that the tacrolimus release from PLGA is pH

dependent. From *in vivo* studies, the AUC, MRT, and CL_t of tacrolimus-loaded PEG–PLGA NP were significantly different from those of Prograf® inj. The prepared NP showed significantly higher lymphatic targeting efficiencies than that of Prograf® inj. at the mesenteric lymph node. In addition, PEGylated PLGA NP showed higher distribution of tacrolimus to the mesenteric lymph node than PLGA NP, and the increased PK parameters (AUC, MRT, and $t_{1/2}$) were observed for PEGylated PLGA NP compared with PLGA NP.

Generally, most of NP accumulate to the target lymph site during continuous systemic circulation due to the physicochemical characteristics such as particle size, surface coated with biodegradable polymers, and so on. As mentioned earlier, polymeric NP can easily pass through the walls of lymphatic capillaries during systemic circulation, and this 'passive targeting' enables the drug to be targeted to the lymph site. Based on the results of our study, we could conclude that this mechanism was related to drug delivery system of tacrolimus-loaded NP. Therefore, it was suggested that PEGylation of NP has an effect on targeting tacrolimus to the lymph node, and the prepared tacrolimus-loaded NP can be good candidates as a lymphatic delivery system of tacrolimus.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2009.08.006.

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