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Preparation of Cyclosporin A-loaded Nanoparticles Containing Ethyl Myristate or Chitosan and Pharmacokinetics in Rats

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ABSTRACT – An oil-in-water solvent evaporation method was used to prepare the cyclosporin A (CyA)-loaded nanoparticles varying in poly(D,L-lactide-co-glycolide) (PLGA) polymer (RG 502H, RG 503H) and the amount of additive ethyl myristate (EM) or chitosan (CS). The particles were characterized for drug loading and entrapment efficiency by HPLC, surface morphology by scanning electron microscopy, particle size by dynamic light scattering and surface charge by Zeta-potential. The results showed drug loadings ranging from 10.9% to 15.8% with high encapsulation efficiency (82.0-97.8%). SEM and DLS studies showed discrete and spherical particles with smooth surfaces and mean size ranging 257.6-721.7 nm. The additive EM or CS did not change the mean sizes of the nanoparticles, whereas by the coating effect of CS, the Zeta-potential values of the CS-added nanoparticles were moved to the more positive direction as the amount of CS was increased. From the pharmacokinetic analysis, the nanoparticles formulations showed the higher bioavailability and MRT than Neoral®. While little adding effect of EM or CS was detected in pharmacokinetic profile when RG 503H was used as polymer carrier, more noticeable different pharmacokinetic behaviors could be observed in case of RG 502H. EM incorporation was found to elevate the K_{el}, whereas CS coating resulted in the decrease of F and K_{el}, which seems to be due to the function of CS as a barrier and a mucoadhesive coating.

Key words – Nanoparticles, Cyclosporin A, Oral, Neoral

Cyclosporin A (CyA), a highly hydrophobic cyclic peptide, has been extensively used in the prevention of xenograft rejection following organ transplantation[1] and is also applied in the treatment of patients with selected autoimmune diseases such as rheumatoid arthritis[2] and Behcet's disease.[3] However, there are some problems with the treatment with the available dosage forms of CyA in spite of the great therapeutic interest in the drug. When administered orally, the bioavailability of CyA in its conventional oral preparation displayed considerable inter- and intra-patient variability presumably due to its poor and highly bile-dependent absorption as well as the involvement of intestinal metabolism,[4-6] which is the reason why CyA is one of the drugs that require careful monitoring of blood levels. The blood levels of CyA above the therapeutic range were related to adverse effects, such as nephrotoxicity and neurotoxicity.[7,8] Below the therapeutic levels of CyA, high episodes of organ rejection have been reported.[9] The severity of symptoms observed outside the therapeutic window of CyA requires the development of formulations that can control the blood levels of CyA in the therapeutic ranges during the whole dosing interval.

Recently a new oral CyA formulation was developed (Sandimmun Neoral®), which incorporates the peptide in a microemulsion. This microemulsion formulation displays significantly less inter-individual variation,[10] supposed to improve the absorption characteristics, thus leading to a reduction in the doses administered. However, this improved efficiency of the microemulsion formulation is open to further discussion because toxic and sub-therapeutic levels were also reported,[11] and therefore one cannot be assured of a uniform CyA exposure after a given dose of Sandimmun Neoral®. In this respect, a recent study has demonstrated that low bioavailability of CyA is due to extensive CyA metabolism in the gut wall.[12] Thus protection against degradation in the GI tract would be a first key factor in improving CyA oral formulations.

On the other hand, the immunosuppressive activity of CyA is related to a selective action against T lymphocytes, which mainly circulate in the lymphatic system. Consequently, targeting lymphatics has been suggested as second key factor in improving commercial CyA formulations.[13]

Based on these considerations, one approach is that using polymers in the form of biodegradable microparticles and nanoparticles. Biodegradable polymeric nanoparticles have proven their ability to increase the oral bioavailability of CyA in rats.[14,15] Indeed, *in vivo* studies with CyA-loaded NP demonstrated that polycaprolactone nanoparticles could be used for drug administration to experimental animals with significant

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increases of the oral bioavailability after therapeutic dosing. Besides polycaprolactone, several CyA delivery methods using poly (D,L-lactide-co-glycolic acid) (PLGA) or poly (D,L-lactic acid) (PLA) microparticles and nanoparticles have been reported.[16,17] Sanchez et al. prepared PLGA nanoparticles with different sizes and showed that a single subcutaneous dose of microencapsulated CyA provided constant levels of the drug in blood and plasma for extended periods of time (several weeks). In another study, the release rate of CyA from PLA microspheres was controlled by adding fatty acid esters (ethyl myristate, EM), and the PLA microspheres with fatty acid ester improved the symptoms such as the decrease in body weight and the increase in paw swelling occurring with adjuvant-induced arthritis in rats.[18] To compare with conventional PLA particulate carriers, the stability of tetanus toxoid-loaded PLA-poly (ethylene glycol) nanoparticles in simulated digestive fluids was evaluated in terms of their aggregation, PLA degradation, release of the encapsulated macromolecule and *in vivo* absorption.[19] PLA-PEG nanoparticles showed the reduced interaction between the nanoparticles and enzymes of the digestive fluid and the higher level of encapsulated radioactive antigen in the blood stream and lymphatics. *In vitro* release experiments revealed that PLA-PEG particles provided a more adequate control of CyA release than conventional PLA micro- and nanoparticles.[20] From these studies, although the particulate oral delivery system for CyA seems to have promising characteristics, in our knowledge, the successful oral delivery of CyA using PLGA or PLA has not been reported. Our previous study investigated the factors influencing the release rates of CyA-loaded micro- and nanoparticles prepared by high-pressure homogenizer.[21]

However, oral administration of these particulate formulations to rat did not show the better pharmacokinetic result compared with conventional CyA microemulsion of Neoral® (data not shown). To enhance the bioavailability and prolong the therapeutic blood concentration of CyA, the particulate formulations were modified using chitosan (CS) and EM. The cationic polymer CS is one of mucoadhesive polymers and has attracted a great deal of attention because of its unique properties, such as acceptable biocompatibility and biodegradability[22,23] and ability to enhance the paracellular transport of drugs.[24]

Mucoadhesive chitosan coating of D,L-lactide/glycolide copolymer nanospheres improved oral delivery of elcatonin.[25] When the chitosan-coated nanospheres with elcatonin were administered intragastrically to fasted Wistar rats, the chitosan-coated nanospheres reduced significantly the blood calcium level compared with elcatonin solution and uncoated nanospheres, and the reduced calcium level was sustained for a period of 48 hr. EM was used as an additive to increase the release rate and bioavailability of nanoparticles.[18,21]

In present report, several formulations of nanoparticles were prepared by high-pressure homogenizer and investigated for their physico-chemical properties and *in vivo* efficiency as oral delivery formulation. Moreover, this study shows how the factors such as polymer molecular weight, chitosan coating and additive influence the pharmacokinetic profile.

Materials and Methods

Materials

Cyclosporin A (CyA) was kindly donated by Hanmi Pharmaceutical Co. (Korea). Poly (D,L-lactide-co-glycolic acid) (PLGA) with molar ratio of lactide: glycolide (1:1; Resomer RG 502H, Resomer RG 503H) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Inherent viscosities (i.v.) of Resomer RG 502H (502H) and Resomer RG 503H (503H) in chloroform are 0.19 and 0.38 dl/g, respectively. Tween 80, and poly-vinylalcohol (PVA, MW 30000-70000) were obtained from Sigma (Mo, USA). Ethyl myristate (EM) was purchased from Aldrich (WI, USA) and chitosan (CS) was purchased from Fluka (Japan). All other reagents were of analytical grade and used without further purification

Preparation of PLGA nanoparticles

PLGA nanoparticles entrapping CyA were prepared by o/w emulsion solvent evaporation method. Briefly, 200 mg of PLGA, 20 mg of CyA, and fatty acid ester (EM; none, 10, 20 or 40%) were dissolved in 4 ml of methylene chloride. This solution was emulsified in 1% (w/v) poly-vinylalcohol (PVA) aqueous solution containing chitosan (CS; none, 0.025, 0.5, 0.1%) using a high-pressure homogenizer (Inc EmulsiFlex-Cs, Avestin, Canada) to form an oil-in-water emulsion, followed by repetition of passage of emulsion at 5 times. In this emulsifying step, the pressure of the regulator in the high-pressure homogenizer was set at 60 psi for nanoparticles. The resulting o/w emulsion was stirred for 5 hr at room temperature under reduced pressure to evaporate methylene chloride. After removing the volatile organic solvent, the resulting aqueous suspension of nanoparticles was centrifuged and washed twice with distilled water to remove residual polyvinyl alcohol and unencapsulated CyA, followed by resuspending the particles in distilled water prior to the freeze-drying.

Morphological characterization

The morphological examination of PLGA nanoparticles entrapping CyA was performed by scanning electron micro-

scope (SEM, JSM 840-A, Jeol Ltd., Tokyo, Japan). PLGA nanoparticles were placed on an aluminum stub to get a uniform layer of particles, coated with gold to a thickness of about 100 Å using gold sputter, then observed using SEM. The particles were examined for shape, size and surface characteristics such as pores, the extent of aggregation and the presence of non-particulate materials.

Particle size analysis

The lyophilized particles were redispersed in water and the mean particle size was measured using a BI-200SM Goniometer (Brookhaven instruments corporation, Holtsvile, NY, USA) which works on the principle of dynamic light scattering. Surface charge was investigated by measuring the Zeta-potential using the same instrument.

Evaluation of drug contents and encapsulation efficiency

The amount of CyA encapsulated was determined by a reversed-phase high-performance liquid chromatography (RP-HPLC). A specific amount of CyA-loaded micro- and nanoparticles was dissolved in acetonitrile and applied to an HPLC system (Waters, Mass, USA), which consisted of Waters™ 600 controller, 600 pump, and 486 tunable absorbance detector (210 nm). Separation was achieved by using a reversed-phase column (Symmetry C_{18}, Waters, Mass, USA) thermostated at 75°C and with a flow rate of the mobile phase of 1 ml/min. The encapsulation efficiency was expressed as the percentage of CyA incorporated in the carrier relative to the total amount of CyA in the preparation medium.

***In vivo* studies**

Male Sprague-Dawley rats weighing 250-290 g were obtained from the Laboratory Animal Center, Seoul National University (Seoul, Korea). The rats were fasted overnight before oral administration of CyA and had access to water ad libitum. After appropriate dilution (Neoral®) or dispersion (nanoparticles formulations), 1ml of a single oral dose (15 mg/kg) was given to each rat by gastric gavage through a metallic tube. Whole blood samples ware withdrawn from the tail vein of lightly anaesthetized rats at 0.5, 1, 2, 4, 6, 9, 14, 24, 34 and 48 h post-treatment, and frozen until analysis. The concentrations of CsA in whole blood were measured using CYCLO-Trac SP-Whole Blood Radioimmunoassay for Cyclosporin kit (Dia-Sorin Inc., Stillwater, MN, USA).[27]

Analysis of pharmacokinetic parameters

The area under the whole blood concentration curve (AUC) and the area under the first moment curve (AUMC) were calculated using trapezoidal rule. The mean residence time (MRT) was calculated as the ratio of AUMC/AUC. The elimination rate constant (K_{el}) was calculated with log-linear regression, from three to five observations of each curve at the terminal phase, and the elimination half-life ($t_{1/2}$) was derived from $0.693/K_{el}$. Maximum whole blood concentration (C_{max}) and time at the maximum whole blood concentration (T_{max}) were obtained directly from the experimental data. The absolute bioavailability (F%) for each formulation was calculated as the ratio of AUC of each formulation to AUC of Neoral®.

Result and Discussion

CyA-loaded nanoparticles with different inherent viscosity (i.v.) and varying contents of EM and CS were prepared using an o/w emulsion solvent evaporation method to examine PLGA nanoparticles as an oral delivery system for CyA. As mentioned in the previous report,[21] the nanoparticles could be made by setting the regulator pressure of high-pressure homogenizer at 60 psi, irrespective of the contents of EM or CS.

The drug contents and encapsulation efficiency of the nanoparticles are summarized in Table I. When the drug contents in

Table I–*Drug Content and Encapsulation Efficiency of PLGA Nanoparticles.* *The amount of CyA encapsulated was determined by a reversed-phase high-performance liquid chromatography (RP-HPLC). The encapsulation efficiency was expressed as the percentage of CyA incorporated in the carrier relative to the total amount of CyA in the preparation medium. ; results are expressed as mean± S.D. (n = 5)*

	Drug contents (%)	Encapsulation efficiency (%)
RG 502H (NP1)	15.8±0.3	94.5±1.7
502H+EM 10%	14.0±0.4	90.8±2.6
502H+EM 20% (NP2)	12.9±0.2	90.3±1.1
502H+EM 40%	10.9±0.4	87.5±3.4
502H+CS 0.025%	14.1±0.4	84.6±2.4
502H+CS 0.05% (NP3)	14.9±0.3	89.1±1.5
502H+CS 0.1%	13.7±0.5	82.0±2.8
RG 503H (NP4)	15.8±0.3	94.8±1.7
503H+EM 10%	15.0±0.2	97.3±1.0
503H+EM 20% (NP5)	13.4±0.4	93.5±3.1
503H+EM 40%	11.5±0.5	92.2±3.8
503H+CS 0.025%	15.5±0.5	93.0±2.9
503H+CS 0.05% (NP6)	14.6±0.4	87.3±2.6
503H+CS 0.1%	14.0±0.2	83.8±1.4

PLGA nanoparticles and drug encapsulation efficiency measured by the methods of HPLC, high drug encapsulation efficiency was obtained in the range of 82.0-97.3% using a high-pressure homogenizer as reported by another group[26] and in our previous report.[21] This high encapsulation efficiency was not dependent on polymers, and contents of EM or CS.

The size distribution and the mean sizes of PLGA nanoparticles were measured by the dynamic light scattering method. As shown in Figure 1, a narrow size distribution was observed independent of the formulation change. A high-pressure homogenizer appears to be an effective instrument to produce nanoparticles with a narrow size distribution and high

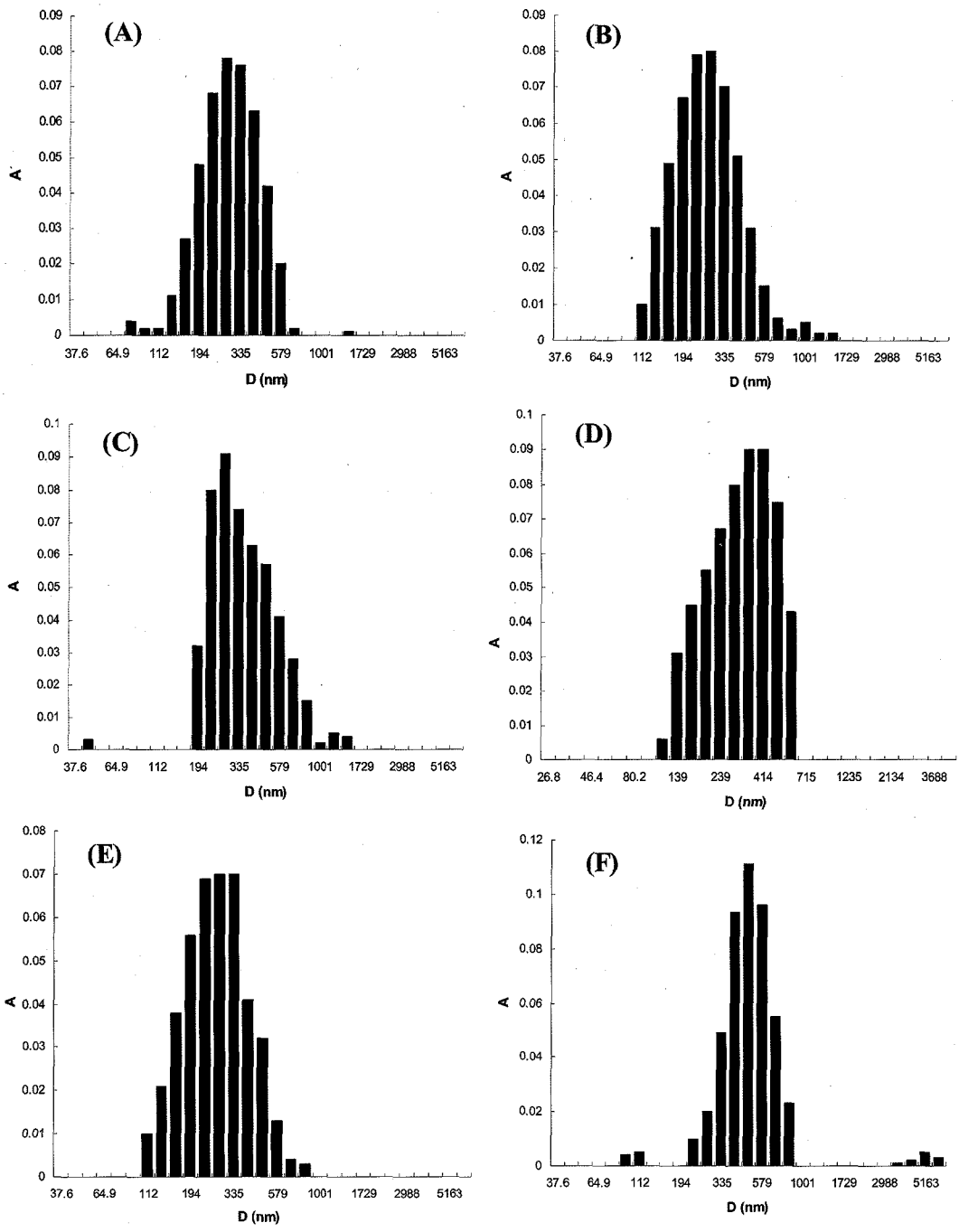

Figure 1—Dynamic light scattering analysis of nanoparticles. The size distribution of nanoparticles was measured using the instrument of BI-200SM Goniometer which works on the principle of dynamic light scattering. (A) RG 502H (NP1), (B) RG 502H+EM 20% (NP2), (C) RG 502H+CS 0.05% (NP3), (D) RG 503H (NP4), (E) RG 503H+EM 20% (NP5), (F) RG 503H+CS 0.05% (NP6).

Table II–*Physico-chemical Characteristics of PLGA Nanoparticles.* *The mean particle size and zeta-potential were measured using the instrument of BI-200SM Goniometer which works on the principle of dynamic light scattering. ; results are expressed as mean± S.D. (n = 5)*

	Mean size (nm)	Zeta potential (mV)
RG 502H (NP1)	315.3±25.7	-15.0±1.3
502H+EM 10%	289.9±17.8	-15.2±1.0
502H+EM 20% (NP2)	628.7±9.3	-17.4±1.9
502H+EM 40%	349.6±24.6	-17.7±0.7
502H+CS 0.025%	342.5±25.0	-8.9±1.6
502H+CS 0.05% (NP3)	382.7±24.9	-7.7±1.3
502H+CS 0.1%	411.3±17.1	-4.5±1.2
RG 503H (NP4)	337.3±9.7	-12.9±1.1
503H+EM 10%	257.6±19.2	-14.2±1.0
503H+EM 20% (NP5)	423.9±24.7	-12.7±0.6
503H+EM 40%	721.7±16.8	-13.4±0.8
503H+CS 0.025%	363.5±24.8	-8.9±1.9
503H+CS 0.05% (NP6)	535.9±27.3	-7.5±2.3
503H+CS 0.1%	590.8±25.9	-0.60±0.08

encapsulation efficiency by controlling the pressure on a regulator. As mentioned in other previous report,[21] the nanoparticles with narrow size distribution could be made by controlling the pressure regulator of high-pressure homogenizer irrespective of the contents of EM or CS. Table II showed the mean sizes and the Zeta-potential values of the nanoparticles formulations. Although the PLGA nanoparticles of various formulations had the submicron mean size, the value showed increasing tendency as the amount of the additive such as EM or CS was increased, which was more remarkable in case of RG 503H than RG 502H. This tendency was also observed when other PLGA polymers were used and EM was incorporated to nanoparticles.[21] The reason of this increasing tendency was not clear. It seems to be related to the incorporation of EM in the matrix of nanoparticles or the coating effect of CS. This hypothesis could be confirmed by Zeta-potential analysis as shown next column in Table II. PLGA nanoparticles displayed negative surface charge because of the terminal -COOH groups of PLGA, which are more generated by the hydrolysis of PLGA for the incubation of water. However, EM and CS had different effect on the nanoparticles. While the EM addition did not influence the surface charge of the nanoparticles, the increased amount of CS led the surface characteristic to more positive direction in both cases of RG 502H and RG 503H. Thus, EM was likely to be incorporated into matrix of PLGA nanoparticles rather than adsorbed to the nanoparticles surface and did not modify the property of surface charge. On the other hand, CS has positive amine groups and appeared to coat at the nanoparticles surface, which rendered the absolute negative value of Zeta-potential to be decreased. However, the nanoparticles were thought to be partially coated with CS, since the CS coated nanoparticles displayed negative values rather than positive values even if the decreased values.

The morphological analysis of PLGA nanoparticles was performed by scanning electron microscope (SEM). SEM allowed the visualization of nanoparticles with homogeneous size distributions, and these observations were in good agreement with the results of the dynamic light scattering analysis experiment (Figure 2). The particles produced were discrete spheres with a smooth surface. Although, in case of large microparticles, the incorporation of EM significantly influenced the surface morphology resulting in the production of particles with gross surface defects as mentioned in the report,[21] the morphological change of the nanoparticles with the incorporation of EM could not be detected, which was likely due to the resolution limit of SEM. Thus, in SEM analysis, EM and CS seems to make a morphological change, because a noticeable difference in surface property could not be observed with the formulation changes.

In vivo experiments were carried out by the oral administration of 6 samples out of the particulate formulations to Sprague-Dawley rats, and by the collection of blood samples from the tail vein at predetermined interval. The pharmacokinetic results were obtained from the analysis of the concentration of the blood samples by the method of radioimmunoassay and shown in Figure 3(A) and (B) for the RG 502H and RG 503H, respectively. The pharmacokinetic parameters obtained from the calculations as non-compartment model were summarized in Table III. Neoral® and NP5 (RG 503H+EM 20%) showed maximum CyA concentration at 1 hour after oral injection, whereas other particulate formulations at 2 hr, which suggested the slower initial release rate of the nanoparticles formulations than microemulsion formulation.

While little adding effect of EM or CS was detected in pharmacokinetic profile when RG 503H was used as polymer carrier as shown in Figure 3(B), more noticeable different pharmacokinetic behaviors could be observed in case of RG 502H if EM or CS was added to formulation (Figure 3(A)). When EM was added to the NP1, C_{max}, F and K_{el} were elevated, and $t_{1/2}$ was decreased, which indicated that the EM incorporation increased the initial release rate, resulting in higher C_{max} and F. However, the additive EM also accelerated the elimination of CyA, which led to a decreased $t_{1/2}$. On the

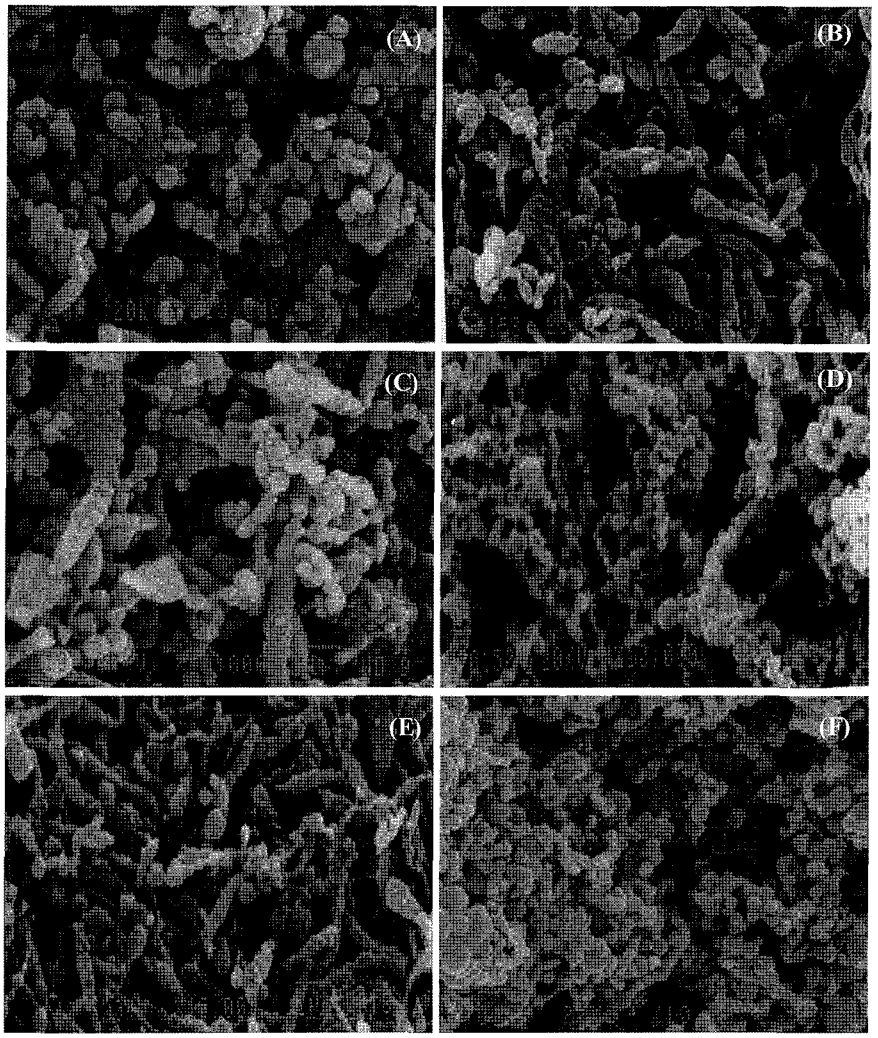

Figure 2–Scanning electron microscopy images of PLGA nanoparticles. The morphological investigation for nanoparticles was carried out using scanning electron microscopy. (A) RG 502H (NP1), (B) RG 502H+EM 20% (NP2), (C) RG 502H+CS 0.05% (NP3), (D) RG 503H (NP4), (E) RG 503H+EM 20% (NP5), (F) RG 503H+CS 0.05% (NP6).

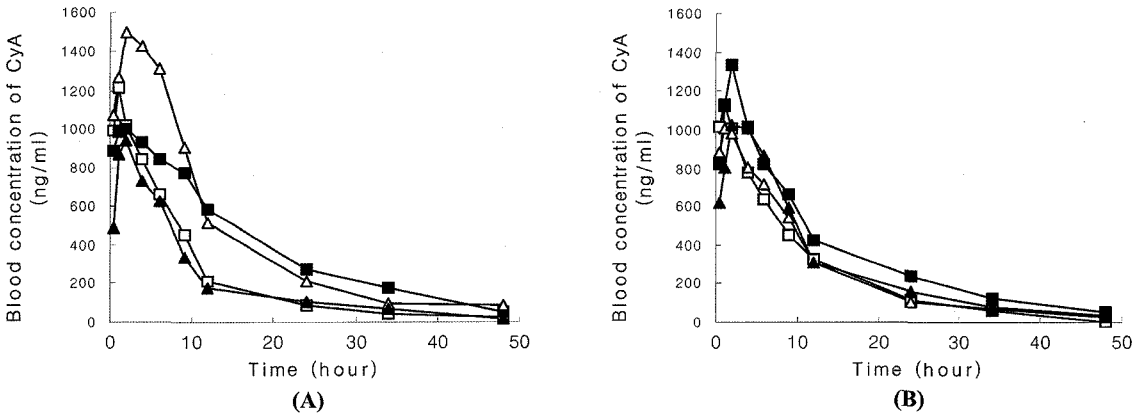

Figure 3–Pharmacokinetic profiles. Pharmacokinetic profiles were obtained by measuring the blood concentration of CyA in rats after oral administration of Neoral® and nanoparticles.
(A) (□) Neoral, (■) RG 502H (NP1), (△) RG 502H+EM 20% (NP2), (▲) RG 502H+CS 0.05% (NP3)
(B) (□) Neoral, (■) RG 503H (NP4), (△) RG 503H+EM 20% (NP5), (▲) RG 503H+CS 0.05% (NP6)

Table III–***Pharmacokinetic Parameters.*** *Pharmacokinetic parameters were calculated by measuring the blood concentration of CyA in rats after oral administration of Neoral and six formulations of nanoparticles (**NP1** : RG 502H, **NP2** : RG 502H + EM 20%, **NP3** : RG 502H + CS 0.05%, **NP4** : RG 503H, **NP5** : RG 503H + EM 20%, **NP6** : RG 503H + CS 0.05%)*

	Neoral	NP1	NP2	NP3	NP4	NP5	NP6
T_{max} (hr)	1	2	2	2	2	1	2
C_{max} (ng/ml)	1213	998	1498	942	1335	1005	1023
AUC (hr·ng/ml)	11194	19766	20598	10246	17589	12556	14183
AUMC (hr·ng/ml)	119303	318581	248657	140141	252681	147590	192295
MRT (hr)	10.66	16.12	12.07	13.68	14.37	11.75	13.56
F (%)	100	177	184	92	157	112	127
K_{el} (hr^{-1})	0.0699	0.0653	0.0731	0.0511	0.0685	0.0762	0.0598
$t_{1/2}$ (hr)	9.92	10.62	9.48	13.57	10.11	9.1	11.6

other hand, NP3 showed lower blood level of CyA and lower F than that of NP1. This phenomenon was likely due to the coating effect of CS. Although the CS coating functioned as a barrier for the initial release resulting in lower F, its mucoadhesive property also effected the pharmacokinetic behavior as extended half-life.

The reason why EM and CS influenced the pharmacokinetic behavior in a different degree to RG 502H and RG 503H was not clear. This seems to be related to the pharmacokinetic property of NP1 and NP4. While NP1 showed the lower C_{max} and the slower decrease of CyA concentration, the pharmacokinetic property of NP4 was the fast release and clearance. Thus, in case of NP4, the partial amount of CyA appears to be distributed in the area close to the surface of nanoparticles, which led to the initial release and little change by EM or CS. However, further investigation needs to be performed to elucidate this hypothesis.

Conclusion

In this study, we prepared CyA-loaded PLGA nanoparticles, which had a high encapsulation efficiency and a monodispersed property, using a high-pressure homogenizer. The additive EM or CS did not change the mean sizes of the nanoparticles, whereas by the coating effect of CS, the Zeta-potential values of the CS-added nanoparticles were moved to the more positive direction as the amount of CS was increased. From the pharmacokinetic analysis, the nanoparticles formulations showed the higher bioavailability and MRT than Neoral®. When the effect of EM or CS on pharmacokinetic behavior was investigated, the EM incorporation was found to elevate the K_{el}, whereas CS coating led to the decreased F and K_{el} valued, which seems to be due to the function of CS as a barrier and a mucoadhesive coating. However, the modification by the additive EM or CS was more noticeable in case of RG 503H. This difference needs further investigation.

Acknowledgments

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