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Preparation, Characterization, and Mucoadhesive Properties of Chitosan-Coated Microspheres Encapsulated with Cyclosporine A

Bizhan Malaekeh-Nikouei
Department of Pharmaceutics, School of Pharmacy and Pharmaceutical Research Centre, Mashhad University of Medical Sciences, Mashhad, Iran

Sayyed A. Sajadi Tabassi
Department of Pharmaceutics, School of Pharmacy and Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran

Mahmoud R. Jaafari
Department of Pharmaceutics, School of Pharmacy and Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

The aim of this study was to prepare and characterize chitosan-coated microspheres containing cyclosporine A (CyA). Microspheres encapsulated with CyA were prepared by solvent evaporation-emulsification methods. Microspheres were immersed in chitosan solution (0.5% w/w) to be coated. Morphology, mean size, and encapsulation efficiency of chitosan-coated microspheres were evaluated. To assess the mucoadhesive properties of this drug delivery system, the percent of mucin adsorption to the surface of coated microspheres was determined. Microspheres were spherical in shape. Encapsulation efficiency of different microsphere formulations varied from 78% to 92%. According to the mucin adsorption results, this particulate system showed suitable mucoadhesive properties. It can be concluded that surface modification of microspheres by chitosan coating would increase the prospects of their usefulness as oral drug delivery systems for CyA.

Keywords cyclosporine A; microsphere; chitosan coating; oral delivery

INTRODUCTION

Cyclosporine A (CyA) is a neutral, lipophilic cyclic undecapeptide, purified from two strains of fungi imperfecti, *Tolypocladium inflatum Gams* and *Cylindrocapon lucidum Booth* (Allison, 2000; Matsuda & Koyasu, 2000). This drug is a unique immunosuppressant used to prevent rejection of transplanted organs like the kidneys, liver, skin, pancreas, and bone marrow, and also for the treatment of selected autoimmune disorders such as uveitis, rheumatoid arthritis, and early treatment of type I diabetes (Brody, Lanser, & Minneman, 1998; Lake, Akporiaye, & Hersh, 2001). CyA is poorly absorbed after oral administration due to its relatively high molecular weight, very high lipophilicity, and poor solubility in aqueous media (Guo, Chen, & Ping, 2001; Lee, Choi, Lee, & Kim, 2001). With conventional delivery systems, bioavailability after oral intake varies from 20% to 50% (Jaifwal, Gupta, & Kreuter, 2004).

To solve these problems, the use of carrier systems like liposomes and microspheres has been suggested, but the retention time of the drug carrier system in the gastrointestinal (GI) tract is a determining factor that controls bioavailability of the drug (Takeuchi, Matsui, Yamamoto, & Kawashima, 2003). Microspheres as a novel drug delivery system can protect the loaded drug from the hostile environment of the GI tract and provide a controlled time release of the drug at the site of action (Galović Rengel et al., 2002). To increase the retention time of dosage forms at absorption sites, mucoadhesive carrier systems have been examined in recent years (Chen, Win, & Fang, 2001; Takeuchi et al., 2003; Takeuchi, Yamamoto, Niwa, Hino, & Kawashima, 1996). These mucoadhesive dosage forms are among the attractive means of improving the bioavailability of drugs (Takeuchi et al., 1996). Mucoadhesive carrier systems can be tailored to adhere to any mucosal tissue, including those found in the eye, nasal cavity, and urinary and GI tracts. Application of these systems to the mucosal tissues of the ocular cavity, gastric, and colonic epithelium can be used for administration of drugs for localized action. Also, they undergo selective uptake by the M cells of Peyer's patches in

Address correspondence to Dr. Bizhan Malaekeh-Nikouei, Pharmaceutical Research Center, Buali Square, P.O. Box 9196773117, Mashhad, Iran. E-mail: malaekehb@mums.ac.ir

GI mucosa (Chowdary & Rao, 2004). These carriers are expected to remain in the GI tract, protecting the entrapped drugs from aggressive conditions in the GI so that they may be absorbed as a released or intact particulate form (Takeuchi, Matsui, Sugihara, Yamamoto, & Kawashima, 2005). These dosage forms facilitate intimate contact of the formulation with the underlying absorption surface. This allows modification of tissue permeability for absorption of macromolecules (Chowdary & Rao, 2004).

Chitosan (poly[b-(1-4)-2-amino-2-deoxy-D-glucopyranose]) is a natural, biocompatible and biodegradable polymer with low toxicity (Galović Rengel et al., 2002; He, Davis, & Illum, 1998; Ribeiro, Neufeld, & Arnaud, 1999; Ribeiro, Silva, Ferreira, & Veiga, 2005). This water-soluble polymer swells indefinitely upon contact with water and eventually undergoes complete dissolution (Chowdary & Rao, 2004). It is commercially available in a range of molecular weights, degrees of deacetylation, and types of salts, such as glutamate, hydrochloride, and lactate (He et al., 1998). Because of its bioadhesive and permeation enhancing properties, chitosan has received substantial attention in the development of novel bioadhesive drug delivery systems (Galović Rengel et al., 2002; Ribeiro et al., 1999).

Different studies have been done in this field. To modify glycolic acid release, chitosan microspheres, chitosan-loaded liposomes, and chitosan-coated liposomes were prepared. Results showed that liposomes can modulate the release rate of glycolic acid (Perugini et al., 2000). Chitosan-coated PLGA nanospheres were prepared to improve pulmonary delivery of calcitonin. The findings demonstrated that these nanospheres are useful for improving peptide delivery via a pulmonary route due to prolonged mucoadhesion for sustain drug release at the absorption site and the absorption-enhancing action of the surface modifier, that is, chitosan (Yamamoto, Kuno, Sugimoto, Takeuchi, & Kawashima, 2005). In another study, chitosan-coated microspheres containing ketoprofen were prepared, and in vitro and in vivo evaluation of these microspheres were done (Yamada, Onishi, & Machida, 2001). Also, chitosan-coated alginate microspheres containing a lipophilic marker were prepared by emulsification/internal gelation method and coated with chitosan. The results showed a decrease in the release rate of the marker (Ribeiro et al., 1999).

The aim of the present study was to prepare and characterize chitosan-coated PLGA microspheres. The mucoadhesive properties of these microspheres were also evaluated. Finally, a preliminary in vitro immunosuppressive test was carried out to evaluate the effect of microspheres on T cell proliferation.

MATERIALS AND METHODS

Materials

CyA was purchased from LC Laboratories. PLGA 50:50 (Mw 40000–75000) and PLGA 85:15 (Mw 50000–126000) were obtained from Sigma (St. Louis, MO, USA). Dichloromethane, dimethyl sulfoxide (DMSO), and polyvinyl alcohol (PVA; Mw 27000) were supplied by Merck (Munchen, Germany). Ficoll, RPMI-1640 growth medium, and tissue culture grade MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) were from Sigma (St. Louis, MO, USA). Chitosan (low molecular weight) was ordered from Fluka (Steinheim, Germany). Phytohemagglutinin (PHA) was from Gibco (Langley, OK, USA), and fetal calf serum was from Biochrome (Berlin, Germany). All materials were of analytical grade unless otherwise stated.

Preparation of Chitosan-Coated Microspheres Encapsulated with CyA

The solvent evaporation-emulsification method was used to prepare microspheres encapsulated with CyA. Briefly, CyA and PLGA (with 1:5 and 1:10 ratios) were dissolved in dichloromethane. This solution was emulsified in 25 ml of an aqueous solution containing 0.3% w/w PVA using a homogenizer (Ultraturax, IKA, Staufen, Germany) at 21,000 rpm for 5 minutes and left to be stirred for 24 hours. To prepare chitosan-coated microspheres, microspheres were immersed into chitosan solution (0.5 % w/v). After 10 minutes, microspheres were collected by centrifugation at 15,000 g for 15 minutes and washed twice with distillated water. Finally, the microspheres were vacuum dried in a freeze drier (Heto, DW3, Roskilde, Denmark; Ribeiro et al., 1999).

Morphology, Zeta Potential and Size Analysis of Microspheres

An optical microscope (OLYMPUS, Hamburg, Germany) was used for studying the morphological features of microspheres. Surface characteristics of microspheres were examined by SEM (LEO, 1450 VP, Cambridge, UK). In this case, samples were prepared on stubs and coated with platinum. The mean diameter of microspheres was determined by a particle size analyzer (Klotz, Birmingham, UK). The zeta potential was determined using Zetasizer (3000HSA, Malvern, UK) in the basis of laser Doppler spectroscopy.

Determination of Encapsulation Efficiency

A weighed amount of CyA-loaded microspheres (10 mg) was dissolved in 1 ml dichloromethane, and after filtration through a 0.45-μm filter, 20 μl of this solution was injected into High Pressure Liquid Chromatography (HPLC). The HPLC system consisted of a pump and ultraviolet detector. Separation was achieved by using a reverse phase column. The mobile phase was methanol:water (90:10) with a flow rate of 1.5 ml/minute, and the concentration of CyA was determined at 214 nm using a standard curve. Results were reported as mean ± standard deviation (*SD*) of three measurements.

Determination of Mucin Adsorption on Chitosan-Coated Microsphere Surface

To study the adsorption of mucin on the surface of chitosan-coated microspheres, the procedure described by Galović Rengel and colleagues (2002) was applied with some modification. Briefly, microspheres (10 mg) were dispersed in the above of mucin aqueous solution (0.5 mg/ml), vortexed, and shaken at room temperature for 60 minutes. The dispersion was then centrifuged at 12,000 rpm for 20 minutes and the supernatant was used to determine the free mucin content. For determination of mucin concentration in the supernatant, Bradford protein assay was used. The mucin adsorbed on the surface of the chitosan-coated microspheres was calculated from the total and free mucin. For every formulation, the study was done in triplicate, and results were expressed by mean ± *SD*.

In Vitro Immunosuppressive Activity

Five ml of heparinized whole blood from volunteers was mixed with an equal volume of normal saline. This mixture was slowly poured over 5 ml of Ficoll solution, and tubes were centrifuged at 2,500 rpm (750 × g; Pars Azma, Iran) for 20 minutes at room temperature. Then the middle layer containing the mononuclear cells was removed and placed in a clean tube, washed by normal saline, and centrifuged at 1,500 rpm (250 g) for 10 minutes. The washing step was repeated twice.

The remaining pellet of cells after centrifuge was resuspended in 2 ml of growth medium (RPMI 1640, fetal calf serum 10% v/v, L-glutamine 5 Mm, penicillin 50 IU/ml, and streptomycin 50µg/ml). Viability of cells was tested by trypan blue. Cells were counted with a hemocytometer. An aliquot of cell suspension was placed in a 96-well microtiter plate with each well containing 50,000 cells, and 20 µl of PHA solution (5 µg/ml) was added to each well for induction of T cell proliferation. Fifty µl of microsphere formulations containing different amounts of CyA were added to each well. The final CyA concentration in the wells was adjusted between 10^{-5} and 10^{-2} mM. Some wells containing only cell medium or cell suspension in the presence of PHA or cell suspension without PHA were used as a control. The plates were incubated at 37°C in 5% CO_2/95% O_2 for 4 days. After the incubation period was over, 20 µl of MTT dye (5 mg/ml) was added, and they were incubated in the dark for 4 hours. The plates were centrifuged at 2,500 rpm for 15 minutes, the growth medium was removed, and 200 µl of DMSO and 20 µl of glycine buffer were added and it was shaken for 5 minutes. Absorbance of each well was measured by an ELISA reader (Statfax–2100, Awareness Technology, Los Angeles, USA) at 570 nm, and the percent inhibition of T cell proliferation was calculated for each formulation. In all cases, percentage of T cell proliferation inhibition was expressed in comparison with the cells treated with PHA that was taken as 100%. IC50 was determined according to the Litchfield and Wilcoxon method using PCS software.

Statistical Analysis

One-way analysis of variance was used with Instat software (GraphPad Software, Inc., San Diego, CA, USA) to assess the significance of differences among various groups. In the case of significant *F* value multiple comparison a Tukey test was used to compare the means of different treatment groups. Results with $p < .05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Evaluation of microspheres by optical and electron microscopy showed that microspheres were spherical in shape. Noncoated and chitosan-coated microspheres had similar morphologic features. SEM studies revealed that islands of CyA formed on the surface of noncoated microspheres, but for coated microspheres, surface characteristics were different compared with the noncoated ones. In this case, microsphere surface was covered by chitosan except those areas that were occupied by CyA islands, and CyA islands on the surface of microsphreres remained intact (Figure 1).

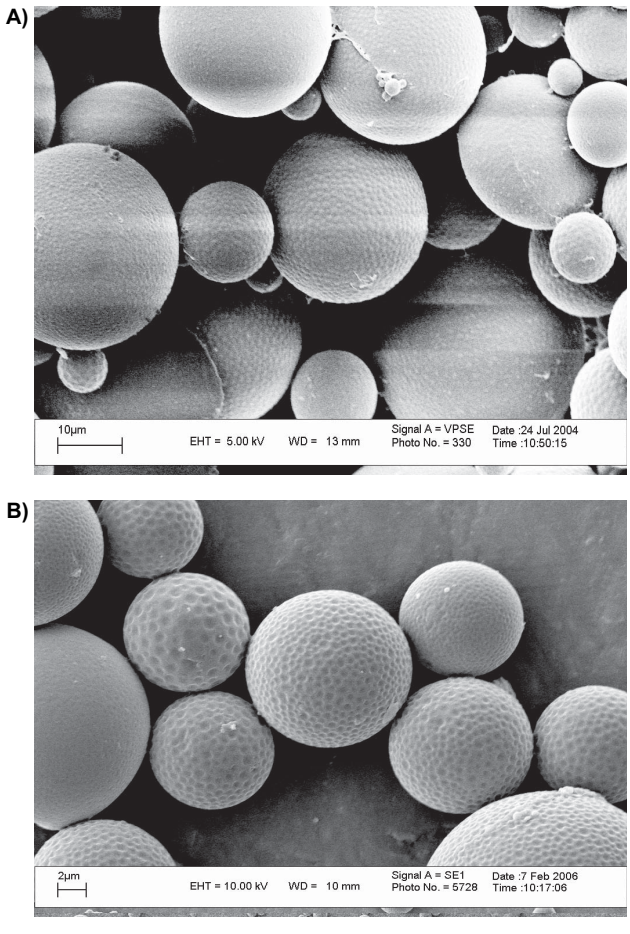

FIGURE 1. SEMs of (A) noncoated and (B) chitosan-coated microspheres. Magnification of images from top to bottom is × 3,000 and × 5,000, respectively.

TABLE 1
Mean Size, Zeta Potential, and Encapsulation Efficiency of Chitosan-Coated Microspheres Encapsulated with CyA

Polymer Grade	Drug to Polymer Ratio	Mean Size (μm)	Zeta Potential (mV)
PLGA (50:50)	1:5	3.30 ± 1.25	3.1
PLGA (50:50)	1:10	5.28 ± 1.79	5.5
PLGA (85:15)	1:5	6.53 ± 2.54	0.4
PLGA (85:15)	1:10	8.10 ± 2.11	2.2

Mean size and zeta potential data of different microsphere formulations are summarized in Table 1. Coated PLGA (50:50) microspheres were smaller than those of PLGA (85:15), and chitosan-coated microspheres in 1:10 ratio were larger than those of 1:5 ratio. As expected, all chitosan-coated microsphere formulations carried a positive charge.

Mucoadhesiveness can be related to the amount of mucin adhered to the microspheres. In the present study, chitosan-coated microspheres were the most mucoadhesive of all types. Figures 2 and 3 display the percent of mucin adsorbed on the surface of noncoated and chitosan-coated microspheres, respectively. Noncoated microspheres with 1:5 drug to polymer molar ratio showed better mucoadhesive properties than 1:10 microspheres, but in chitosan-coated microspheres, the differences of mucin adsorption between different formulations was not significant ($p > .05$). Mucin adsorption on coated microspheres was at least two times more than that of noncoated microspheres. The amounts of chitosan adsorbed on the surface of PLGA (50:50) and PLGA (85:15) microspheres in 1:5 and 1:10 ratio, respectively, were 70.56% ± 5.42, 66.73% ± 6.55, 69.79% ± 4.10, and 65.24% ± 7.50.

Figure 4 shows the result of T cell proliferation inhibition assay of chitosan-coated microspheres with different concentrations. The IC50 value for PLGA (50:50) and PLGA (85:15) microspheres in 1:5 ratio was 11.41 and 21.12 mM, respectively. Statistical analysis of MTT test data showed that the effect of polymer grade on the inhibitory effect of microspheres was not significant in both 1:5 and 1:10 ratio ($p > .05$) but microspheres in 1:5 ratio inhibit T cell proliferation significantly ($p < .05$) in comparison with microspheres in 1:10 ratio.

In order to solve the problems associated with poor absorption of drugs after oral administration, the use of carrier systems like liposomes and microspheres are preferable. Considering poor absorption of CyA owing to its high molecular weight and lipophilicity, the retention time of the drug carrier systems in the GI tract is an important factor controlling the drug's bioavailability (Takeuchi et al., 2003, 2005). To increase the retention time of dosage forms at the absorption site, mucoadhesive carrier systems have been used in recent years (Chen et al., 2001; Galović Rengel, 2002; Ribeiro et al, 1999; Takeuchi et al., 2003). It has been shown that delayed GI transit induced by bioadhesive polymers could lead to increased oral bioavailability of drugs (Longer, Chong, & Robinson, 1985). Indeed, in this situation, improved drug absorption is expected with a combination of mucoadhesiveness and controlled drug release from devices (Takeuchi et al., 2005). Also, several authors have used chitosan-related polymers as liposome- and microsphere-coating material to improve the bioavailability of drugs by prolonging their retention time at the site of absorption, hence undergoing a slower clearance from the GI tract (Barratt, 2000; Galović Rengel, 2002; Takeuchi et al., 2003).

Since localization and retention of the drug on the absorption site is known to influence the absorption and chitosan coating, which was reported to enhance the accessibility and localization to the absorptive membrane via bioadhesion, in the present study, chitosan-coated microspheres containing CyA were prepared and characterized. Various mucoadhesive polymers such as HPMC (Kawashima, Takeuchi, & Yamamoto,

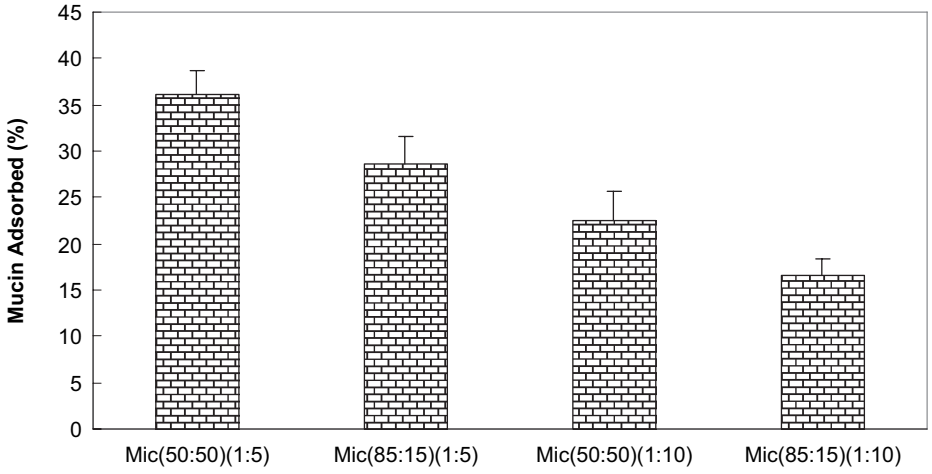

FIGURE 2. Percent of mucin adsorption on noncoated microspheres. The results are expressed as mean ± *SD* ($n = 3$).

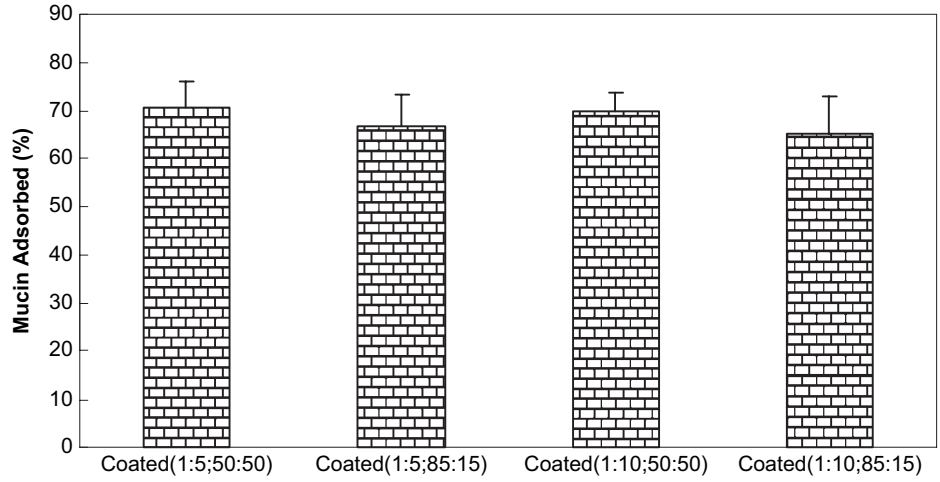

FIGURE 3. Percent of mucin adsorption on chitosan-coated microspheres. The results are expressed as mean ± *SD* (*n* = 3).

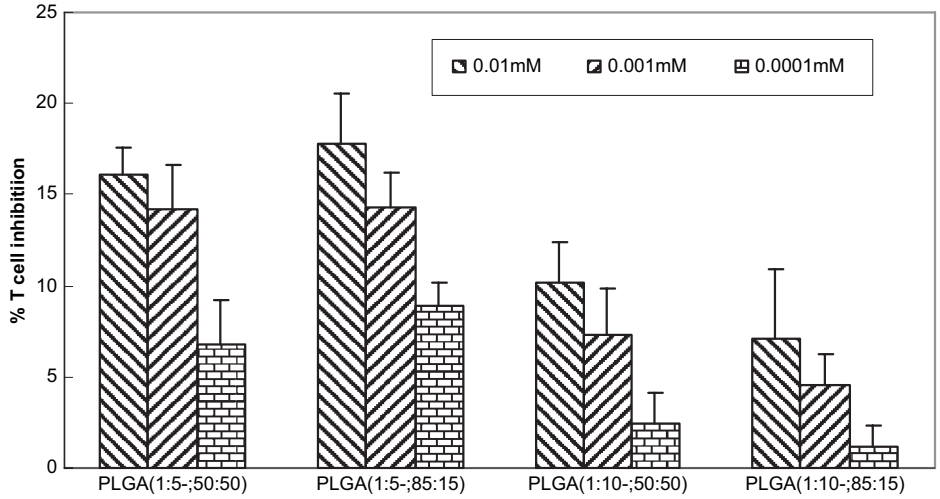

FIGURE 4. T cell proliferation inhibition induced by various concentration of chitosan-coated microspheres encapsulated with CyA (*M* ± *SD*, *n* = 8). T cells treated with PHA as a control were considered 100%.

2000), polyacrylic acid (Takeuchi et al., 1996), carbopol (Takeuchi et al., 2003), and PVA (Takeuchi et al., 1998) have been used in different studies, but in the present study, chitosan was used as polymer for coating microspheres.

Although evaluation of microspheres by optical microscopy showed the same morphologic features for coated and noncoated microspheres, but SEM studies confirmed that the interaction of chitosan to the surface changed the surface characteristics of the chitosan-coated microspheres. The electrostatic attraction between positively charged chitosan and the opposite charge of PLGA causes formation of a chitosan coat on the surface of the microspheres. All amino groups of chitosan were not always bound to the carboxyl group of PLGA, as suggested by positive zeta potential (Yamamoto et al., 2005). Surface charge variation of chitosan-coated microsphere before and after coating confirmed the coating of this drug delivery system by chitosan.

Mucoadhesiveness was calculated as the amount of mucin adhered on the surface of microspheres. The result of mucin adsorption on the surface of microspheres showed that adsorption of mucin for all chitosan-coated microspheres was identical. This confirms that coating for all microsphere formulations was the same and that they have mucoahdesive properties. Mucin adsorption for noncoated microspheres was different in such a way that 1:5 drug to polymer ratio microspheres adsorb more mucin. This result is related to the surface properties of microspheres. According to our previous research (Malaekeh-Nikouei, Sajadi Tabassi, & Jaafari, 2006), the dimension of CyA islands on microsphere surfaces in 1:5 drug to polymer microspheres were larger. Consequently, in 1:10 microspheres, more polymer

is exposed to the mucin and the same charge of mucin and polymer can decrease mucin adsorption for these microspheres.

Taking into account the fact that most microspheres were smaller in size than 10 μm, these particles can be suitable for oral delivery due to the possibility of uptake by the lymphoid system M cells in Peyer's patches (Hoshi, Uchino, Kusanagi, Ihara, & Ueda, 1999; Hussain, Jaitley, & Florence, 2001). Based on the mechanism of drug effect (T cell inhibition), direct uptake by the lymphoid system will improve the therapeutic effect of CyA.

Furthermore, the polycationic nature of chitosan could provide a strong electrostatic interaction with mucus or the negatively charged mucosal surface. The negative charge of mucin is due to the ionization of sialic acid (He et al., 1998). The flexible structure of chitosan on the surface might also participate in the physical entanglement between chitosan molecular chains and the mucus component on the surfaces, leading to prolonged residence time of delivery systems in different membranes as well (Yamamoto et al., 2005).

It is expected that drug molecules may be released upon interaction with mucin or degradation of the microsphere structure in the mucous layer (Takeuchi et al., 2003, 1996). For microspheres, since localization and retention of the drug on the absorption site are known to influence the absorption, chitosan coating enhances the accessibility and localization to the absorptive membrane via bioadhesion (Yamada et al., 2001). It has been reported that chitosan has preferable properties for improving drug absorption, such as protection of the drug against enzymatic degradation and absorption-enhancing effects in the GI tract (Guo, Ping, Jiang, Huang, & Tong, 2003; Yamamoto et al., 2005). It is suggested that the absorption-enhancing mechanism of chitosan might be due to opening of the intercellular tight junctions of the epithelia of intestinal, nasal, and pulmonary membranes (Yamamoto et al., 2005).

In this study, the effect of chitosan-coated microspheres containing CyA on T cell proliferation in cell culture was very weak. These effects should be related to the slow process of drug release from microspheres that was described in our pervious study (Malaekeh-Nikouei et al., 2006). Because lymphocytes don't have phagocytic properties, only that part of drug which released during cell culture studies is effective to inhibit T cell proliferation.

In conclusion, the loading of CyA into chitosan-coated microspheres was performed. According to the mucoadhesive properties of these delivery systems, they would increase the prospects of their usefulness as oral drug delivery systems for CyA.

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