Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 303 (2005) 160–170
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs

Hirofumi Takeuchi *, Yuji Matsui, Hikaru Sugihara, Hiromitsu Yamamoto, Yoshiaki Kawashima

Gifu Pharmaceutical University, 5-6-1 Mitahora Higashi, Gifu 502-8585, Japan

Received 23 May 2004; accepted 22 June 2005
Available online 24 August 2005

Abstract

The mucoadhesive behavior of chitosan-coated liposomes in the intestinal tract of the rat was examined to elucidate their particle size effects on the absorption of an entrapped drug, calcitonin. The intestine was removed from rats after oral administration of liposomes containing a fluorescent dye, and its various parts were observed with confocal laser scanning microscopy. Penetration of submicron-sized liposomes (ssLip) or chitosan-coated ssLip (ssCS-Lip) into the mucosa was observed, while such behavior was not observed for the multilamellar liposomes, even when coated with chitosan (CS-Lip). The retentive property of ssCS-Lip was confirmed by measuring the amount of dye in each part of the intestine. The pharmacologic effects of calcitonin-loaded liposomes of different particle size were measured after oral administration in rats. The pharmacologic effect of oral administration of ssLip coated with chitosan was detected up to 120 h after administration. The extensive pharmacologic effect of ssCS-Lip was attributed to their prolonged retention in the intestinal mucosa, partly owing to their penetrative property into the intestinal mucosa. The chitosan-coated ssLip, with their higher retentive property in the intestinal tract, are much more effective than ssLip and CS-Lip in improving the enteral absorption of peptide drugs.

Keywords: Liposomes; Chitosan; Mucoadhesion; Calcitonin; Nanoparticle

1. Introduction

The bioadhesion of the oral dosage forms of poorly absorbable drugs has received much attention, as have transdermal and buccal systems. Bioadhesion to mucosa in the gastrointestinal tract can be described in terms of mucoadhesion. Since mucoadhesion can prolong the resident time of drug carriers at absorption sites, improved drug absorption is expected with a combination of mucoadhesiveness and controlled drug release from devices.

Longer et al. (1985) first showed that delayed gastrointestinal transit induced by bioadhesive polymers could lead to increased oral bioavailability

* Corresponding author. Tel.: +81 58 237 8574; fax: +81 58 237 6524.
E-mail address: takeuchi@gifu-pu.ac.jp (H. Takeuchi).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.06.028

of a drug. A multi-unit bioadhesive system was also prepared by coating microspheres of polyhydroxyethyl-methacrylate with mucoadhesive polymers using laboratory-scale equipment (Lehr et al., 1990, 1992). Akiyama et al. (1995) prepared a polyglycerol ester of fatty acid-based microspheres coated with Carbopol934P (CP) and CP-dispersing microspheres to evaluate their mucoadhesive properties. In developing colloidal drug delivery systems, Lenaerts et al. (1990) demonstrated the mucoadhesive property of polyalkylcyanoacrylate nanoparticles with autoradiographic studies and confirmed that the bioavailability of vincamine was improved with the nanoparticulate systems. Pimienta et al. (1992) investigated the bioadhesion of hydroxypropylmethacrylate nanoparticles or isohexylcyanoacrylate nanocapsules coated with poloxamers and poloxamine on rat ileal segments in vitro using a labeled compound.

We developed mucoadhesive liposomes by coating the anionic liposomal surface with a cationic mucoadhesive polymer, chitosan, to observe their mucoadhesive properties both in vitro and in vivo (Takeuchi et al., 1994, 1996). A similar technique was applied successfully to the preparation of Carbopol-coated liposomes (Takeuchi et al., 2003).

One of the attractive applications of mucoadhesive carrier systems is in the oral administration of peptide drugs. Peptide drugs are intrinsically poorly absorbed owing to their high molecular weight and hydrophilicity, and they are also susceptible to enzymatic degradation in the gastrointestinal tract. Drug carriers are expected to remain in the gastrointestinal tract, with protecting the entrapped peptide drugs from enzymatic degradation so that they may be absorbed as a released or intact particulate form. In previous studies, we have demonstrated improved absorption of calcitonin as well as insulin using chitosan-coated liposomes (Takeuchi et al., 2001, 2003).

In this study, the effects of particle size on the mucoadhesive properties of chitosan-coated liposomes and on the absorption of entrapped calcitonin were evaluated. The mucoadhesive properties were observed using confocal laser scanning microscopy (CLSM). The absorption of calcitonin contained within the liposomes was monitored by measuring the blood calcium concentration after oral administration of liposomal calcitonin.

2. Materials and methods

2.1. Materials

L-α-Distearoylphosphatidylcholine (DSPC, Nippon Oil and Fats Co.), dicetyl phosphate (DCP, Sigma), cholesterol (Chol, Sigma) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo carbocyanine perchlorate (DiI, LAMBDA, Austria) were used as received. Chitosan (CS), which is a deacetylated chitin (poly *N*-deacetylglucosamine) industrially prepared by hydrolyzing the aminoacetyl groups of chitin in aqueous alkaline solution, was a gift from Katakurachikkarin Co., Japan. The molecular weight and the deacetylation percentage of the CS used were ca. 150,000 and 85%, respectively. Calcitonin (elcatonin) was kindly supplied by Asahi Chemical Co., Japan. All other reagents were of analytical grade.

2.2. Preparation of multilamellar and submicron-sized CS-coated liposomes

The multilamellar liposomes composed of DSPC, DCP, and Chol in a molar ratio of 8:2:1 and containing DiI were prepared by the hydration method. In a typical procedure, 161 mg (204 μmol) of DSPC, 27.9 mg (51 μmol) of DCP, 9.7 mg (25 μmol) of Chol, and 1.68 mg of DiI were dissolved in a small amount of chloroform, and the solution was rotary evaporated at 40 °C to obtain a thin lipid film. The thin lipid film was dried in a vacuum oven overnight to ensure complete removal of the solvent, and hydration was carried out with 5 mL of acetate buffer solution (pH 4.4, 100 mM) by vortexing, followed by incubation at 10 °C for 30 min.

The submicron-sized liposomes (ssLip) were prepared by sonicating (Sonifier250, Branson) the multilamellar liposomes. The particle size of ssLip was controlled by controlling the sonication time. For example, ssLip of ca. 200 nm were prepared by sonicating three times for 3 min. The particle size of the resultant liposomes was confirmed by dynamic light scattering analysis with LPA-300 (Otsuka Electronics). To measure the liposomal diameter, an aliquot of each liposomal suspension was diluted with a large amount of purified water.

For preparation of chitosan-coated liposomes (CS-Lip), an aliquot of the liposome suspension was mixed

with the same volume of acetate buffer solution (pH 4.4) of CS (0.6%), followed by incubation at 10 °C for 1 h. Thus, the final concentrations of the lipids and polymers were half of those of the original solutions. The noncoated liposomes were mixed with the corresponding buffer solutions to adjust the liposomal concentrations to those of the coated liposomes. The submicron-sized, CS-coated liposomes (ssCS-Lip) were prepared in the same manner. The average particle size of the resultant ssCS-Lip was in the range of 300–400 nm; that of the original noncoated liposomes was 200 nm.

2.3. Preparation of calcitonin-loaded liposomes

Calcitonin-loaded liposomes were prepared with the hydration method, in which the calcitonin solution was used instead of the buffer solution. The lipid films composed of 129.0 mg (163.2 μmol) of DSPC, 22.3 mg (40.8 μmol) of DCP, and 7.9 mg (20.4 μmol) of Chol in a molar ratio of 8:2:1 were hydrated with 1 mL of phosphate buffer solution (pH 6.8) of calcitonin (160 μg/mL). The resultant liposomal suspension was diluted with 3 mL of the same phosphate buffer solution to obtain the calcitonin-loaded multilamellar liposomes. The particle size of the liposomes was controlled via sonication.

Polymer coating of the calcitonin-loaded liposomes was carried out after separating the liposomal pellet by ultracentrifuging the liposomal suspension to minimize the aggregation of liposomal particles. The conditions of ultracentrifugation for the submicron-sized liposomes were 75,000 rpm (231000 × g) and 40 min. The chitosan solution (0.3%)was added the liposomal pellet and subsequently resuspended by vortexing. The calcitonin-loaded, submicron-sized liposomes were coated with chitosan in the same manner.

To measure the encapsulation % of calcitonin, the liposomal pellet was obtained by ultracetrifuging the liposomal suspension under the same conditions described above. The calcitonin content in the liposomal pellet was determined by measuring the calcitonin concentration after destroying the liposomal structure of the pellet by adding an appropriate amount of Tween 20 solution (20%, w/v). The calcitonin concentration was determined by HPLC carried out under the following conditions—column: ODS-2 (GL Science); mobile phase: acetonitrile:0.1% TFA solution = 35:65; wavelength for spectrophotometry: 220 nm.

2.4. Observation of the mucoadhesive behavior and retentive profile of liposomes

The liposomes containing DiI were administered intragastrically into male Wistar rats (13 weeks old) that had been fasted for 48 h before administration. The fasted conditions were set to minimize the contents in the intestines, which disturbed the washing process for the following confocal scanning microscopic observation. The dose was determined based on the amount of DiI formulated to the liposomes, 0.168 mg DiI/rat.

The small intestine was excised from rats sacrificed 2 h after administration and then washed with a large amount of saline solution (ca. 20 mL). The each part of the rat intestine (duodenum, upper jejunum, lower jejunum, upper ileum, and lower ileum) was sliced with Cryostat (LEICA) to generate sections for confocal laser scanning microscopic observation. The thickness of the sections was 10 μm. Each sample was placed on the confocal laser scanning microscope (LSM510, Zeiss) and observed at an excitation wavelength of 550 nm and an emission wavelength of 570 nm.

For quantitative evaluation of the retentive profile of the liposomes, the each part of small intestine excised was homogenised with 3 mL of saline solution without washing. DiI in the homogenate (0.3 mL) was extracted with 6 mL of a mixed organic solvent of chloroform and methanol (1:1) by shaking for 40 min. DiI concentration in the organic solvent separated from the mixture by centrifugation (3500 rpm, 10 min) was measured with fluorometry (F3010, Hitachi) at an excitation wavelength of 550 nm and an emission wavelength of 570 nm

2.5. Administration of calcitonin-loaded liposomes to rats

The absorption test was carried out using male Wistar rats (9 weeks old) fasted for 12 h before intragastric administrationa calcitonin-loaded liposome suspension (500 IU/kg). The dose was determined based on our previous work with PLGA nanoparticles containing elcatonin, where the pharmacologic effect calculated with oral administration of PLGA-elcatonin was increased with increasing the dose from 125 to

500 IU/kg (Kawashima et al., 2000). The liposomes were administered as liposomal suspensions. As a reference, an equivalent amount of calcitonin solution was administered. The rats had free access to water during the experiment. The fasted conditions were kept up to 48 h after administration of the drug. A 200-μL blood sample was obtained from the jugular vein at an appropriate interval to determine the calcium level. The plasma calcium level was measured using a commercially available kit (Calcium C-test WAKO, Wako Pure Chemical, Japan).

3. Results

3.1. Mucoadhesion profile of submicron-sized CS-Lip

To evaluate the particle size effect of chitosan-coated liposomes on their mucoadhesive property, submicron-sized liposomes were prepared by sonicating multilamellar liposomes (Lip). The submicron-sized, chitosan-coated liposomes were prepared by mixing the ssLip with a chitosan solution. The resultant liposomes showed zeta potential values similar to those of CS-Lip, suggesting formation of the same chitosan coating layer as for CS-Lip (Table 1). Although the particle size of ssCS-Lip increased slightly in the coating process partly because of aggregation, we used them as obtained. The fluorescence marker DiI was incorporated into each liposome for measuring the mucoadhesive property with confocal laser scanning microscopy.

The liposomes containing DiI were administered intragastrically to rats, and the intestine was removed 2 h after administration. The mucoadhesive profiles of the liposomes in the intestinal tube were evaluated by observing the residual liposomes on the mucosa with confocal laser scanning microscopy. The higher mucoadhesive tendency of ssCS-Lip compared with CS-Lip was confirmed by comparing the resultant photographs in Fig. 1. At the jejunum, small amounts of particles were observed in the case of CS-Lip administration, while large amounts of ssCS-Lip were detected there. In the ileum, the retention of the liposomal particles seemed almost the same, but ssCS-Lip tended to penetrate into the mucosal part of the intestine.

In measuring the mucoadhesive property of the noncoated liposomes with different particle sizes, Lip and ssLip, a similar size-dependency was observed (Fig. 2). Although the retained amount of the noncoated liposomes was relatively lower than the chitosan-coated liposomes, ssLip showed a penetrative behavior similar to that of ssCS-Lip. The lower retention of ssLip compared with that of ssCS-Lip was confirmed, since a small amount of ssLip was observed at the jejunum (Fig. 2).

The mucoadhesive property of ssCS-Lip was measured for all parts of the intestinal tract, i.e., duodenum, upper and lower jejunum, and upper and lower ileum, and at both the mucosal and basolateral side of the intestinal membrane (Fig. 3). The four photographs on the top side of the figure show the mucosal side and the bottomed four corresponds to the basolateral side. The white lines in each photograph show the edges of the sliced intestinal membrane. Looking at the mucous layer side, considerable amounts of liposomes were observed throughout the intestinal tract, from the upper part of the jejunum to the

Table 1
Characterization of chitosan-coated liposomes

Desired particle size of samples	Chitosan concentration (%)	Zeta potential (mV) in pH 4.4 A.B.	Actual particle Size (nm)
4 μm (MLVs)	0	−22.6	3810
	0.3	32.5	4130[a]
	0.75	33.6	4640[a]
400 nm	0	−24.6	446.6
	0.3	34.8	769.9[b]
200 nm	0	−25.1	207.4
	0.3	30.3	406.2[b]

[a] CS-Lip.
[b] ssCS-Lip.

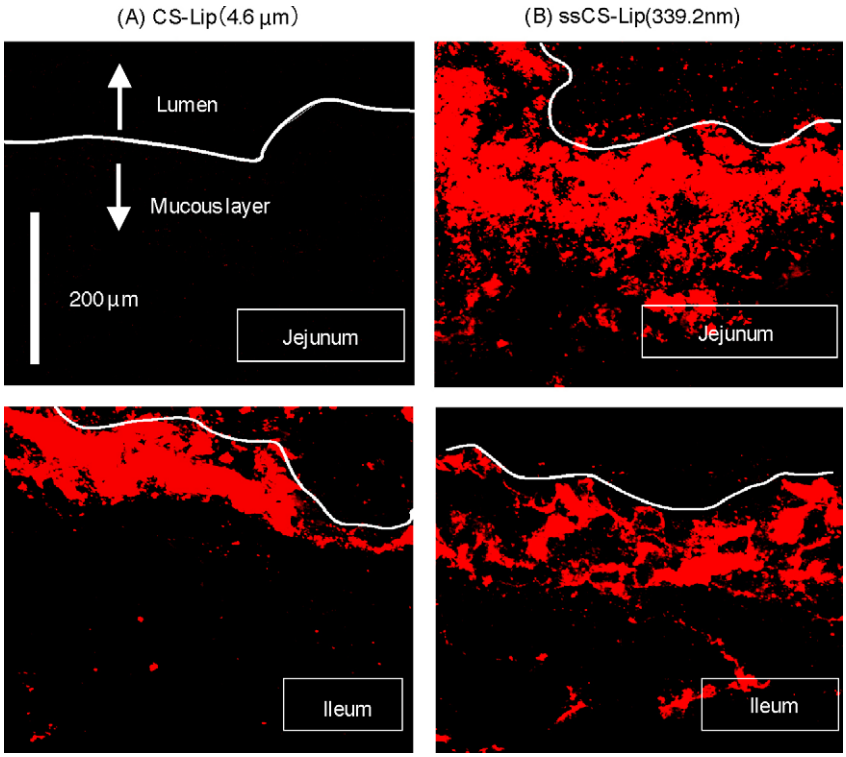

Fig. 1. Confocal laser scanning microscopy photographs of the jejunum and ileum of the rat intestine 2 h after intragastric administration of chitosan-coated liposomes: (A) CS-Lip and (B) ssCS-Lip. The measured mean diameters of the liposomes are 4.6 μm (A) and 339.2 nm (B), respectively. The formulation of liposomes: DSPC:DCP:Chol = 8:2:1. The concentration of chitosan in the coating is 0.3%.

lower part of the ileum, which suggested their excellent mucoadhesive property. It was characteristic that the red parts suggesting the existence of liposomes were observed in the basolateral side of the intestinal membrane.

To characterize the penetrative properties of various liposomal particles into the intestinal mucosa, the same experiment was carried out for the other liposomal particles – Lip, CS-Lip and ssLip – in the same manner. The photographs taken of the jejunum and lower part of the intestine are shown in Fig. 4. No particles of Lip and CS-Lip were observed on the basolateral side of the intestine. In the case of ssLip, the similar penetrative property as observed for ssCS-Lip was confirmed, although its retention profile was lower than ssCS-Lip, as shown in Fig. 2. These tendencies were confirmed at other parts of the intestine (data not shown). The penetrative property of the particles seemed to be highly dependent on their size.

3.2. Retentive profile of liposomal particles in the intestine

The retentive profile of these liposomal particles in the gastrointestinal tract was evaluated by measuring the amount of particles after oral administration of these particles. As shown in Fig. 5, the chitosan-coated liposomes (CS-Lip and ssCS-Lip) tended to remain in the upper part of the gastrointestinal tract 1 h after administration. Their mucoadhesive properties may play an important role in retarding their moving rate in the gastrointestinal tract.

In comparing ssCS-Lip and CS-Lip, ssCS-Lip was more retentive than CS-Lip. The penetrative property of ssCS-Lip seemed to be effective for prolonging their retention in the intestinal tract. However, ssLip showed the comparable retention as observed for Lip, although the penetrative tendency was observed for ssLip with CLSM analysis. Based on these observations, we can

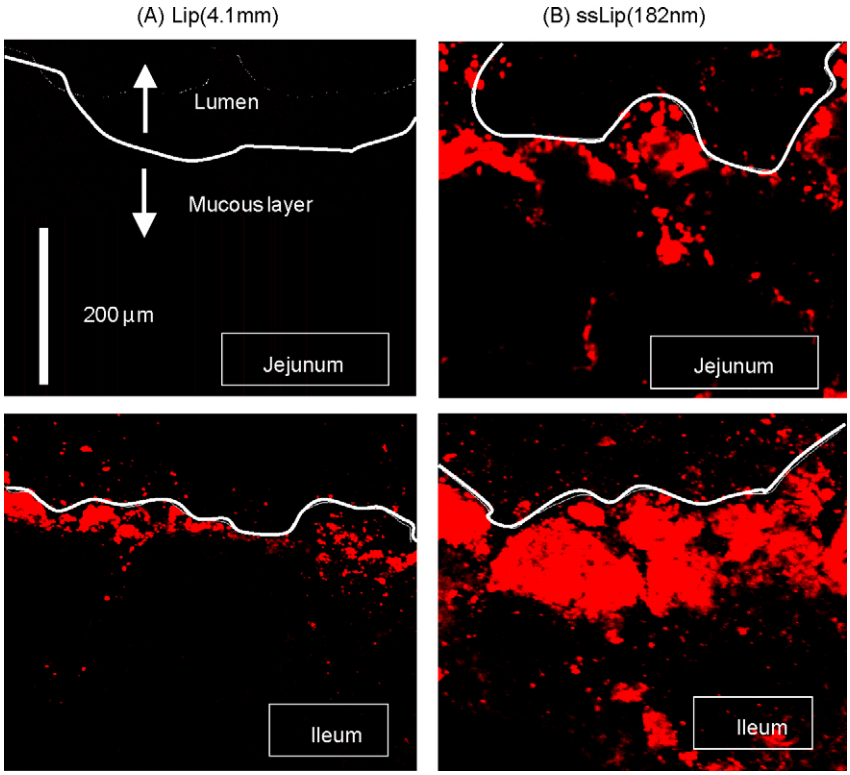

Fig. 2. Confocal laser scanning microscopy photographs of the jejunum and ileum of the rat intestine 2 h after intragastric administration of noncoated liposomes: (A) Lip and (B) ssLip. The measured mean diameters of the liposomes are 4.1 μm (A) and 181.9 nm (B), respectively. The formulation of liposomes: DSPC:DCP:Chol = 8:2:1.

estimate that the surface property of the particle is extensively important to improve its retentive profile in the intestinal tract for drug absorption.

To confirm the retentive profile of ssCS-Lip, its residual amount was observed at the different time interval after oral administration in rats. Comparing the three stages shown in Fig. 6, we can confirm that the liposomal particles move slowly through the gastrointestinal tract. The profile of ssCS-Lip at 2 h after (Fig. 6) was similar to that observed for ssLip at 1 h after administration (Fig. 5).

3.3. Coating of calcitonin-loaded, submicron-sized liposomes with chitosan

When the calcitonin-loaded ssLip suspension was mixed with a chitosan solution for coating, aggregation of the liposomal particles was observed. As the aggregation tendency was considerably stronger than that observed for the empty liposomes, the aggregation was attributed to the calcitonin molecules in the solution or the surface of liposomal particles. Therefore, chitosan coating of ssLip containing calcitonin was carried out after separating the free calcitonin in the solution by ultracentrifugation. The ultracentrifuged pellet of ssLip was redispersed in a chitosan solution with vortexing. By applying a slight sonication to the redispersed liposomal suspension, ssCS-Lip were obtained. The encapsulation of calcitonin into Lip and ssLip was more than 90%, as previously reported (Takeuchi et al., 2003).

3.4. Oral administration of calcitonin-loaded liposomes

In a previous study (Takeuchi et al., 2003), we confirmed the effectiveness of mucoadhesive property of liposomes in oral administration of liposomal

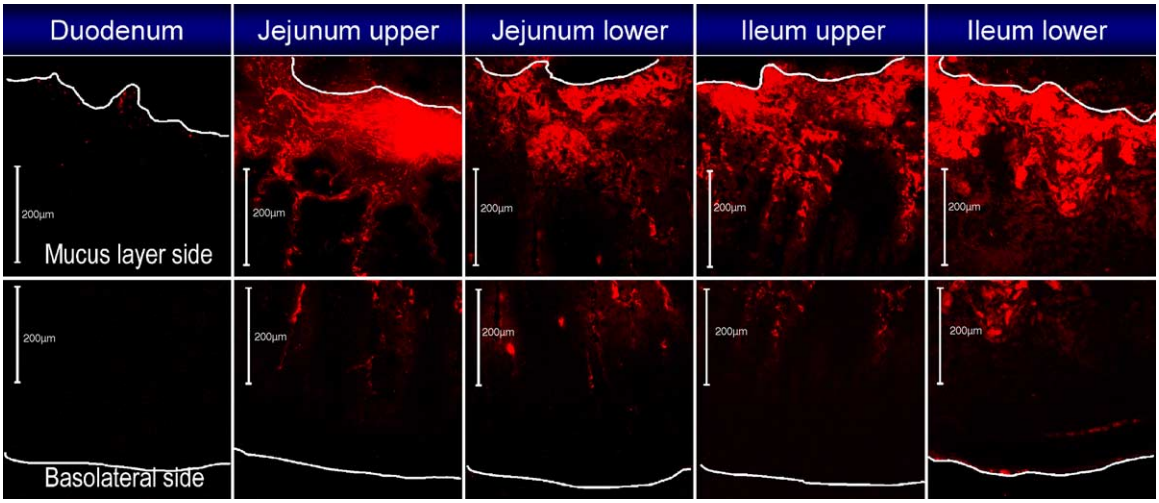

Fig. 3. Confocal laser scanning microscopy photographs of various parts of the intestinal tract of the rat 2 h after intragastric administration of ssCS-Lip. The measured mean diameter is 281.2 nm. The formulation of liposomes: DSPC:DCP:Chol = 8:2:1.

calcitonin to rats. As the mucoadhesive property of CS-Lip was extensively improved by reducing their particle size to a submicron order (ssCS-Lip), we expected to improve the enteral absorption of peptide drugs by using ssCS-Lip as carriers.

The same amount of calcitonin (500 IU/kg rat) entrapped in the liposomal formulations was intragastrically administered to rats. Administration of calcitonin-loaded ssCS-Lip led to a sustained decrease in calcium concentration in the blood up to 120 h after

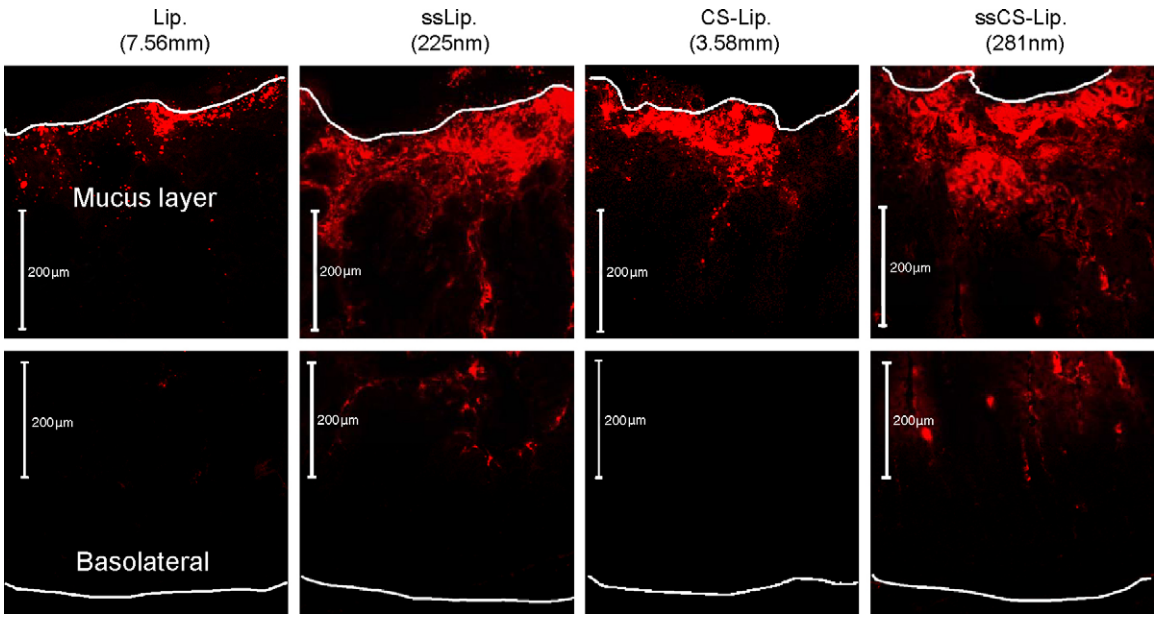

Fig. 4. Confocal laser scanning microscopy photographs of the lower part of the jejunum 2 h after intragastric administration of various types of liposomes. The measured mean diameters of each liposome are shown in brackets.

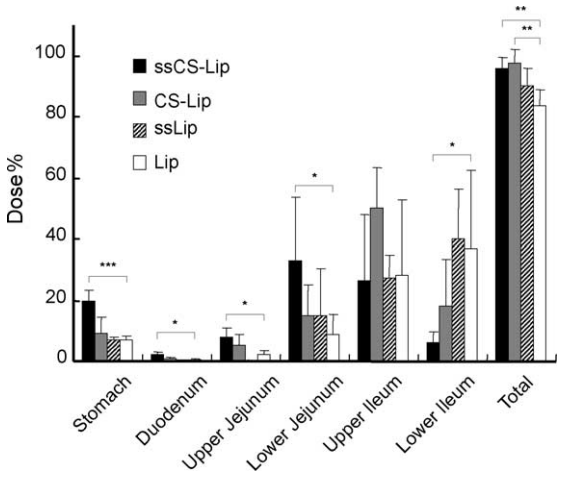

Fig. 5. Retentive profile of various types of liposomes in the gastrointestinal tract at 1 h after intragastrical administration. The measured mean particles sizes of Lip, ssLip, CS-Lip, and ssCS-Lip are 3.90 μm, 182.9 nm, 7.46 μm, and 304.7 nm, respectively. The formulation of liposomes is DSPC:DCP:Chol = 8:2:1. The concentration of chitosan for coating is 0.3%. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$: significantly different from Lip ($n = 4$ in each case).

administration (Fig. 7). The period of reduced calcium was much longer than that observed when CS-Lip were used as carriers of calcitonin under the same conditions (Takeuchi et al., 2003). Comparing these profiles, the particle size effect on calcitonin absorption was confirmed. The ssLip were also found to be effective in reducing the calcium level under the same experimental conditions, although the extent was much less than that of ssCS-Lip, as shown in Fig. 5.

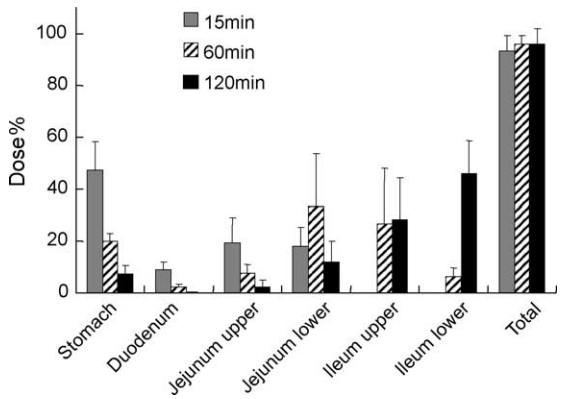

Fig. 6. Retentive profile of ssCS-Lip in the gastrointestinal tract up to 2 h after intragastrical administration. The measured mean particles sizes of ssCS-Lip are in the range of 300–400 nm. The formulation of liposomes is DSPC:DCP:Chol = 8:2:1. The concentration of chitosan for coating is 0.3%.

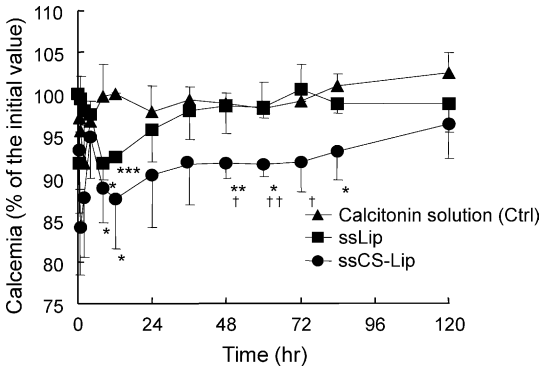

Fig. 7. Profiles of plasma calcium levels after intragastric administration of submicron-sized liposomes, ssLip and ssCS-Lip, containing calcitonin. The measured mean particles sizes of ssLip and ssCS-Lip are 196.4 and 473.4 nm, respectively. The formulation of liposomes is DSPC:DCP:Chol = 8:2:1. The concentration of chitosan for coating is 0.3%. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$: significantly different from the level for calcitonin solution; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$: significantly different from the level for ssLip ($n = 3$ in each case).

The pharmacologic effects of the various liposomal calcitonins tested were evaluated by calculating the area above the blood calcium level (AAC) by use of a trapezoidal method. In Fig. 8, AAC values for the three types of liposomes of different particle size are shown. The particle sizes of the noncoated liposomes were 4.0 μm, 400 nm, and 200 nm. The chitosan-coated liposomes were prepared by using these noncoated liposomes, and their particle sizes were 4.1 μm, 660.8 nm, and 473.4 nm, respectively. This evaluation clearly showed that the pharmacologic effect increased with decreasing particle size of the liposomes. The effect of chitosan coating was also confirmed by comparing that value for noncoated and CS-coated liposomes of similar particle size.

4. Discussion

It is well known that one of the functions of mucosa is to restrict the penetration of large molecules, including particles. Generally, it has been believed that the particles hardly passed through the absorptive enterocytes of mucosa.

Contrary to the general consideration, confocal laser scanning microscopic photographs (Figs. 3 and 4)

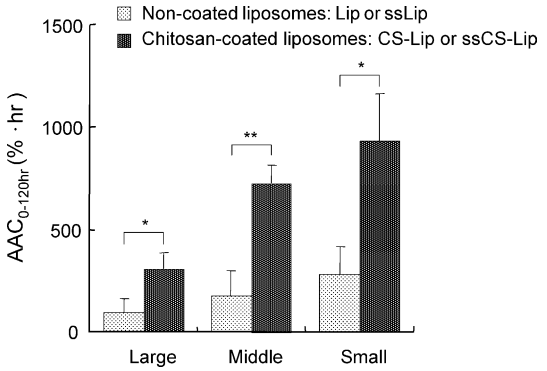

Fig. 8. Area above the plasma calcium level curve (AAC) for liposomes, Lip, ssLip, CS-Lip, and ssCS-Lip, having different particle size and containing calcitonin calculated for 120 h after intragastric administration. The formulation of liposomes is DSPC:DCP:Chol = 8:2:1. The concentration of CS for coating is 0.3%. The actual particle sizes of large, middle, and small liposomes are 4.0 μm, 400 nm, and 196.4 nm for noncoated liposomes and 4.1 μm, 660.8 nm, and 473.4 nm for chitosan-coated liposomes, respectively. $^{*}p < 0.05$ and $^{**}p < 0.01$: significant differences for the noncoated and chitosan-coated liposomes of similar particle size ($n = 3$).

suggested the penetration of the submicron-sized liposomal particles into the mucosa. This penetration seemed to occur at the absorptive enterocytes of the intestine, because the lymphoid tissues (Peyer's patches) were excluded in preparation of the sample for CLSM observation. It was characteristic that penetration was observed only for the submicron-sized particles (ssSC-Lip and ssLip).

There are some reports that demonstrate particle uptake in the gastrointestinal tract or Caco-2 cells. Jani and colleagues have shown the absorption of polystyrene nanoparticles and confirmed their uptake occur through both the gut associated lymphoid tissue (GALT) and, to a lesser extent, normal intestinal tissue (Jani et al., 1990; Hillery et al., 1994; Florence, 1997). Desai et al. (1996) examined the gastrointestinal uptake of biodegradable nano- and microparticles and demonstrated its particle size dependency. Histologic evaluation of tissue sections demonstrated that 100 nm particles diffused throughout the submucosal layers, while the larger-sized particles were predominantly localized in the epithelial lining of the tissue. It also confirmed the similar particle size-dependent uptake in an experiment with Caco-2 cells (Desai et al., 1997).

The particle size dependency observed in our experiment corresponds to that observed with these polymeric nanoparticles. One of the possible mechanisms explaining the uptake of the submicron size liposomes is adsorptive endocytosis, because the GALT (Peyer's patches) was excluded in preparation of the sample for CLSM observation, as mentioned above. The particles are required to pass through the mucous layer before reaching the surface of enterocytes. An interaction of chitosan on the surface of ssCS-Lip with mucin can contribute it. Although more experiments are needed to confirm the absorption mechanism of the liposomal particles, adsorptive endocytosis is tentatively the most possible mechanism.

The penetrative behavior of ssCS-Lip contributed to their improved retentive property in the intestinal tract after administration as shown in Figs. 5 and 6. In comparing the results shown in Fig. 5 with those observed in Figs. 1 and 2, there is some inconsistency for the retention of the liposomal particles. For example, while strong fluorescence of the liposomes adsorbed to Jejunum is observed for the ssCS-Lip (Fig. 1) and essentially no fluorescence is observed for the CS-Lip, Fig. 5 indicates that the amount of the liposomes adsorbed onto Jejunum for the ssCS-Lip is about a half of that of CS-Lip; the retention of ssLip was as low as that of Lip having larger particle size (Fig. 5), although penetrative property into the intestinal mucosa was observed for ssLip (Fig. 2). These inconsistencies may be related to the adhesion part of liposomes in the intestinal tracts. In measuring the retentive profile, the intestine was homogenized without washing to collect all liposomal particles in the intestines, while it was washed with enough amount of saline solution for CLSM observation as described in the experimental section. The liposomal particles on the surface of mucous layer of intestine with weak adhesive interaction may be washed out in the case of CLSM observation.

Based on these results we considered that the mucous layer on the surface of enterocytes controlled the retention of the particulate systems. As the mucous layer is a highly viscous gel, it may restrict the amount of the ssLip particles reaching to the surface of enterocytes. Although CS-Lip having a good affinity to the mucous layer can reach to the surface of enterocytes, they may remain there without being entrapped into the enterocytes. It is well known that the mucosa tends

to turn over in a relatively short time. Therefore, the residence of CS-Lip in the intestinal tract may be shortened. On the other hand, once the particles pass through the mucous layer and/or are taken up into the enterocytes, they can remain there for a relatively longer time. Then, the retention time of ssCS-Lip may be much inproved than that of CS-Lip.

The liposomal formulation of calcitonin retained in the mucous layer can enhance the drug absorption by increasing the drug concentration there. The passive diffusion may be responsible for its absorption phenomena. The prolonged pharmacological effect after oral administration of calcitonin entrapped in the ssCS-Lip was attributed to their longer retentive properties in the intestinal tract owing to their penetrative properties into the mucosa. So far it is not clear if the drug entrapped in the liposomes is released in the cell or if it is carried to the basolateral side of the mucosa as the entrapped form. However, it is certain that part of the drug reaches the bloodstream. There is also a claim that enterocytes are sloughed off within a few days. In these situations the particulate systems cannot remain for such a long time as a few days. In our experiment, the fasted conditions for 48 h after administration of the liposomal calcitonin may affect the turn over period of enterocytes. The more precise experiments considering these physiological conditions of mucosa are being continued.

The extensively prolonged pharmacological effect such as for 20 days after oral administration of the particulate system was reported by Damge et al. (1988) in administration of insulin-loaded polyalkylcyanoacrylate nanocapsules for diabetic rats. We have also demonstrated a long pharmacological effect (up to 48 h) after oral administration of calcitonin entrapped into the mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan (Kawashima et al., 2000). It is important to characterize the behavior of the particles including these polymeric particles in the intestinal tract in order to clarify the mechanism of peptide absorption with these fine particulate systems.

Our observation with CLSM could partly clarify the longer residence time of ssCS-Lip in the gastrointestinal tract after oral administration. However, it was a trace amount of particles that was detected with the same observation manner and conditions 24 or 48 h after administration. Further experiments are required to understand completely the reason why the particulate systems can contribute the extended pharmacological effect up to 120 h after oral administration.

5. Conclusion

It was found that the ssCS-Lip possess an excellent penetrative property in to the intestinal mucosa. They showed the highest retentive property in the intestinal tract after oral administration owing to the penetrative property. The prolonged pharmacologic effect of calcitonin with ssCS-Lip was attributed to their excellent retentive property. The noncoated ssLip did not show such a prolonged pharmacologic effect, probably because the number of particles taken up into the enterocytes was lower than that of ssCS-Lip, owing to the resistance of mucous layer. The mucoadhesive property of ssCS-Lip provided by the CS coating layer is essential for their long retention in the intestinal tract. They show an excellent peptide carrier function for oral administration.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (B-14370731) from Japan Society for the Promotion of Science. We are also grateful to Katakura-Chikkarin Co., Japan, and Asahi Chemical Co., Japan, for the gifts of chitosan and elcatonin, respectively.

References

Akiyama, Y., Nagahara, N., Kashihara, T., Hirai, S., Toguchi, H., 1995. In vitro and in vivo evaluation of mucoadhesive microspheres prepared for the gastrointestinal tract using polyglycerol esters of fatty acids and a poly(acrylic acid) derivatives. Pharm. Res. 12, 397–405.

Desai, M.P., Labhasetwar, V., Walter, E., Amidon, G.L., Levy, R.J., 1996. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm. Res. 13, 1838–1845.

Desai, M.P., Labhasetwar, V., Walter, E., Levy, R.J., Amidon, G.L., 1997. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. Pharm. Res. 14, 1568–1573.

Damge, C., Michel, C., Aprahamian, M., Couvereur, P., 1998. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. Diabetes 37, 246–251.

Florence, A.T., 1997. The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual. Pharm. Res. 14, 259–266.

Hillery, A.M., Jani, P.U., Florence, A.T., 1994. Comparative, quantitative study of lymphoid and non-lymphoid uptake of 60 nm polystyrene particles. J. Drug. Target. 2, 151–156.

Jani, P.U., Halbert, G.W., Langridge, J., Florence, A.T., 1990. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J. Pharm. Pharmacol. 42, 821–826.

Kawashima, Y., Yamamoto, H., Takeuchi, H., Kuno, Y., 2000. Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin. Pharm. Dev. Technol. 5, 77–85.

Longer, M.A., Ch'ng, H.S., Robinson, J.R., 1985. Bioadhesive polymers as platforms for oral controlled drug delivery. III. Oral delivery of chlorothiazide using a bioadhesive polymer. J. Pharm. Sci. 74, 406–411.

Lehr, C.M., Bouwstra, J.A., Tukker, J.J., Junginger, H.E., 1990. Intestinal transit of bioadhesive microspheres in an in situ loop in the rat-A comparative study with copolymers and blends based on poly acrylic (acid). J. Control. Release 13, 51–62.

Lehr, C.M., Bouwstra, J.A., Kok, W., De Boer, A.G., Tukker, J.J., Verhoef, J.C., Breimer, D.D., Junginger, H.E., 1992. Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat. J. Pharm. Pharmacol. 44, 402–407.

Lenaerts, V., Couvreur, P., Grislain, L., Maincent, P., 1990. Nanoparticles as a gastroadhesive drug delivery system. In: Lenaerts, V., Gurny, R. (Eds.), Bioadhesive Drug Delivery Systems. CRC Press, FL, pp. 93–104.

Pimienta, C., Chouinard, F., Labib, A., Lenaerts, V., 1992. Effect of various poloxamer coatings on in vitro adhesion of isohexylcyanoacrylate nanospheres to rat ileal segments under liquid flow. Int. J. Pharm. 80, 1–8.

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., Kawashima, Y., 1994. Mucoadhesion of polymer-coated liposomes to rat intestine in vitro. Chem. Pharm. Bull. 42, 1954–1956.

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., Kawashima, Y., 1996. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm. Res. 13, 896–901.

Takeuchi, H., Matsui, Y., Yamamoto, H., Kawashima, Y., 2003. Mucoadhesive properties of Carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. J. Control. Release 86, 235–242.

Takeuchi, H., Yamamoto, H., Kawashima, Y., 2001. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv. Drug Deliv. Rev. 47, 39–54.