Application of Surface-Coated Liposomes for Oral Delivery of Peptide: Effects of Coating the Liposome's Surface on the GI Transit of Insulin

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Abstract □ We prepared two kinds of surface-coated liposomes and investigated their potencies as oral dosage forms for peptide drugs by focusing on their effects on the gastrointestinal (GI) transit of drugs. The surface of the liposomes was coated with poly(ethylene glycol) 2000 (PEG-Lip) or the sugar chain of mucin (Mucin-Lip). As a model peptide drug, insulin was encapsulated in these liposomes. Coating the surface with poly(ethylene glycol) was found to reduce the transit rate of liposomes in the small intestine after oral administration to rats in vivo. Mucin-Lip was retained in the stomach longer than PEG-Lip or uncoated liposomes. The effect of surface coating on the intestinal transit of liposomes was determined by means of in situ single pass perfusion in the rat small intestine. Statistical moment analysis was applied to the outflow pattern of both liposomes and encapsulated insulin. The mean transit time (MTT) and deviation of transit time (DTT) in the intestinal tract were calculated. The MTT of PEG-Lip was much longer than those of uncoated liposomes and Mucin-Lip and was significantly shortened after removal of the intestinal mucous layer. These results indicated that PEG-Lip interacts strongly with the intestinal mucous layer, leading to its slow transit in the intestine. In contrast, coating the liposome's surface with mucin did not affect either the MTT or DTT of liposomes in the intestine. This result is in accordance with the in vivo observation that Mucin-Lip was highly retained in the stomach, but not in any region of the small intestine in vivo. Both the MTT and DTT values of insulin encapsulated in PEG-Lip and Mucin-Lip were almost the same as those of liposomes themselves, suggesting that surface-coated liposomes retained insulin in the intestinal tract. However, MTT and DTT of insulin were significantly shorter than those of uncoated liposomes because these liposomes degraded and released significant amounts of insulin during single pass perfusion. The ability of surface-coated liposomes, especially of PEG-Lip, to interact with the mucus layer and slow the transit rate in the GI tract is considered desirable for oral delivery of peptide drugs. Modification of the liposomal surface with appropriate materials, therefore, should be an effective method by which to achieve the oral delivery of peptide drugs.

A number of peptide and protein drugs with high therapeutic potency have been developed. Most of these, however, can be administered only by injection due to their instability in the gastrointestinal (GI) tract and poor absorption. Recently, particulate systems such as liposomes, emulsions, and micro- or nanosized polymer particles have attracted a great deal of attention as possible oral dosage forms for such peptide drugs.[1−3] Among these particulate systems, liposomes possess the advantage that they are composed of physiological materials, e.g. phospholipids. Since Patel and Ryman reported the significant hypoglycemic effect of orally administered insulin encapsulated in liposomes,[4] many studies have been carried out to evaluate the potency of liposomes. However, the degradation of liposomes in the GI tract through the interaction with bile salts sometimes obscured the effects of liposomes on drug absorption. To circumvent this problem, we reported previously that liposomes coated with poly(ethylene glycol) 2000 (PEG-Lip) or the sugar chain of mucin (Mucin-Lip) became resistant to digestion by bile salts and were useful for oral delivery of peptide drugs. The oral administration of insulin encapsulated in PEG-Lip, in particular, showed a greatly enhanced and sustained hypoglycemic effect in rats in comparison to insulin encapsulated in normal liposomes.[5] It was revealed that the increased resistance of surface-coated liposomes against digestion by bile salts leads to the increased stability of insulin in the GI tract.

However, it is not possible to explain the enhanced and prolonged hypoglycemic effects of insulin only by the increase in stability because many other factors affect intestinal absorption of peptides. Kimura et al.[6] reported that the oral administration of insulin incorporated in poly(vinyl alcohol)-gel spheres significantly increased its bioavailability due to the decreased gastrointestinal transit rate of insulin. In this study, therefore, the effects of surface-coated liposomes on the oral absorption of peptide drugs were analyzed from the viewpoint of their effects on the gastrointestinal transit of drugs.

Experimental Section

Materials—Dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL) and distearoylphosphatidylethanolamine−poly(ethylene glycol) 2000 (DSPE-PEG), were gifts from Nippon Fine Chemical Co., Ltd. (Tokyo, Japan). Bovine insulin (25.7 IU/mg) and stearylamine (SA) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]DPPC (113.4 mCi/mmol) was obtained from New England Nuclear (Boston, MA). All other chemicals used were of analytical grade. Cetyl-mucin, one of the surface-coating materials, was synthesized as previously reported.[5] Briefly, mucin from pig stomach was trypsinized for 5 h at 37 °C, treated with proteinase K for another 40 h after adjusting to pH 8.0, and centrifuged and filtered to obtain the sugar portion of mucin. Further purification was carried out using Sepharose CL-2B column chromatography. The cetyl group was grafted to the sugar chain domain of mucin using cetyl bromide and triethylamine.

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Table 1—Lipid Composition of Liposomes Used in This Study[a]

	DPPC	Chol	SA	cetyl-mucin (mg/mL)	DSPE-PEG
(+)-Lip	10	10	1	–	–
mucin-Lip	10	10	1	1	–
PEG-Lip	10	10	–	–	1

[a] Each value was expressed as a molar ratio.

Preparation of Surface-Coated Liposomes—The sugar chain portion of mucin and poly(ethylene glycol) (PEG) were used as surface-coating materials. The compositions of liposomes are listed in Table 1. Liposomes were prepared according to the method of Bangham[7] with some modifications. Briefly, a mixture of DPPC, cholesterol, and surface-coating material (cetyl-mucin or DSPE-PEG) dissolved in chloroform was evaporated to dryness in a rotary evaporator. The lipid film was further dried in vacuo for 8 h to remove the solvent completely. Then the lipid film was hydrated with phosphate-buffered saline (PBS, pH 7.4) containing insulin. After three cycles of freeze−thawing, the liposomal suspension was diluted with PBS to adjust the lipid concentration. Just before the experiment, the liposomal suspension was centrifuged three times at 15000 rpm for 10 min to remove untrapped drugs. The entrapment efficiency of insulin in uncoated liposome, Mucin-Lip, and PEG-Lip was 31.4 ± 6.0%, 35.3 ± 5.9%, 37.7 ± 4.8%, respectively. The size distribution of liposomes was measured by electrophoretic light scattering photometer, ELS-800 (Otsuka Electronics Co. Ltd., Japan). The mean particle size of uncoated liposome, Mucin-Lip, and PEG-Lip was 348 ± 148 nm, 453 ± 165 nm, 479 ± 187 nm, respectively.

In Vivo Oral Administration Experiment—Male Wistar rats, weighing 250−300 g, were obtained from Japan SLC, Inc. (Shizuoka, Japan). Suspensions of liposomes in which the membranes were labeled with [^{14}C]DPPC were orally administered to rats (1 mL/rat). At predetermined time points, rats were anesthetized with sodium pentobarbital, and then the gastrointestinal tract was excised and divided into eight regions, i.e., stomach, duodenum, proximal jejunum, middle jejunum, distal jejunum, ileum, cecum, and colon. The contents of each region were washed with 10 mL of saline, and aliquots of each sample were placed in scintillation vials. Then, 10 mL of Clearsol (Nacalai Tesque, Kyoto, Japan) was added, and the radioactivity of each sample was measured using a liquid scintillation analyzer, LSA 1600 CA (Packard). The remaining amount of liposomes in each region was calculated and expressed as the mean % against the total recovery. The total recovery of radioactivity was more than 91% of the dose applied for each experiment.

In Situ Single Pass Perfusion Experiment—In situ local intestinal perfusion was carried out according to the method of Kakutani[8] with some modifications. The rats were fasted for 12 h and then anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). An abdominal incision was made, and both the duodenum and proximal jejunum (20 cm below the duodenum) were cannulated and ligated tightly. After preperfusion with PBS (pH7.4) for 10 min, 0.15 mL of test solutions including 1 mg/mL insulin, insulin in PBS (Solution; pH7.4), or liposomal suspensions were injected into the line of perfusion flow as a pulse using a three-position valve. The outflow perfusate was collected into preweighed tubes. In the early stages of the experiment, the samples were collected every 15 s, while at later stages sampling was performed every 30 s. After weighing each sample, the concentration of intact insulin was determined by HPLC. Bovine serum albumin labeled with Evans' blue (EB-BSA) was used as a nonabsorbable marker.[9] The recovery ratio of EB-BSA in outflow perfusate was more than 95% of the dose applied, and the coefficient of variability was 3.3%. When determining the transit of liposomes, diphenylhexatriene (DPH) was used as a liposomal membrane marker, and the concentration of DPH in the perfusate was measured spectrofluorometrically.

Determination of Insulin—Insulin was assayed by reverse phase HPLC on a LiChrospher 300 RP-8 column (250 × 4.0 mm, 10 μm). The HPLC consisted of a Shimadzu model LC-10As pump, SPD-6A UV spectrophotometric detector, and C-R6A integrator. The mobile phase was a mixture of 0.1% trifluoroacetic acid/acetonitrile = 60/40% (v/v) and was run at a flow rate of 1 mL/min. The UV detector was set at 210 nm.

Interaction between Liposomes and Intestinal Mucous Layer—To remove mucous fluid,[10] dithiothreitol solution (20 mM in PBS, pH 7.4) was injected into the perfused region of the rat jejunum. Thirty minutes after injection, dithiothreitol was washed out with PBS. Thereafter, in situ perfusion was carried out as described above. It was confirmed that 20 mM of dithiothreitol solution did not induce damage to the intestinal epithelial (data not shown).

Moment Analysis—Statistical moment analysis[8] was applied to the data obtained from the single pass perfusion experiment. The mean transit time (MTT) and the deviation of transit time (DTT) of insulin or liposomes were calculated from their outflow patterns. Briefly, the zero order moment (S_0) was calculated from the area under the time versus amount (% of dose) in the perfusate curve. Similarly, the first-order moment (S_1) and the second-order moment (S_2) were calculated from the area under the time versus time × amount curve and the time versus time × amount^2 curve, respectively. Then, MTT and DTT were calculated from the following equations.

$$\mathrm{MTT} = S_1/S_0$$

$$\mathrm{VTT} = (S_2/S_0) + (\mathrm{MRT})^2$$

$$\mathrm{DTT} = (\mathrm{VTT})^{1/2}$$

where VTT is variance of transit time. DTT, the square root of VTT, was used here, since VTT was too large.

Results

GI Transit of Liposomes in Vivo—Figure 1 shows the time course of remaining radioactivity (% of recovered) of [^{14}C]DPPC in each region of the GI tract after oral administration of liposomes to rats. Positively charged liposomes ((+)-Lip) were used as a control because among uncoated liposomes only (+)-Lip enhanced the absorption of insulin.[5] Although all kinds of liposomes examined reached the distal jejunum at 0.5 h postadministration, their transit patterns in the GI tract were markedly different. In the case of (+)-Lip, about 20% of liposomes were detected in the ileum at 1 h, and most were in the cecum at 2 h post-administration. On the other hand, Mucin-Lip was retained in the stomach for a longer time and then spread throughout the whole jejunum. When PEG-Lip was administered, about 30% remained in the distal jejunum even at 2 h post-administration, and 25% was still detected in the ileum at 3 h. The retention of PEG-Lip in both the distal-jejunum and ileum was remarkable compared to those in other regions.

Intestinal Transit of Insulin and Liposomes in Situ—The outflow pattern of insulin administered in liposomal form is shown in Figure 2. Both (+)-Lip and Mucin-Lip showed sharp peaks of insulin outflow, whereas a broad peak was observed after administration of PEG-Lip. In (+)-Lip and Mucin-Lip, insulin was detectable up to 250 and 495 s after administration, respectively. However insulin was continuously detected until the last sampling time point in PEG-Lip. Moment analysis was applied to these data to compare the differences in the intestinal transit patterns of insulin incorporated in the three kinds of liposomes. As summarized in Table 2, the recovery ratio of insulin was the highest in Mucin-Lip and the lowest in (+)-Lip. PEG-Lip showed 2-fold longer MTT than (+)-Lip or Mucin-Lip, reflecting the slow transit of insulin encapsulated in PEG-Lip. From the comparison of DTT, it was demonstrated that insulin encapsulated in PEG-Lip spread widely in the intestinal tract.

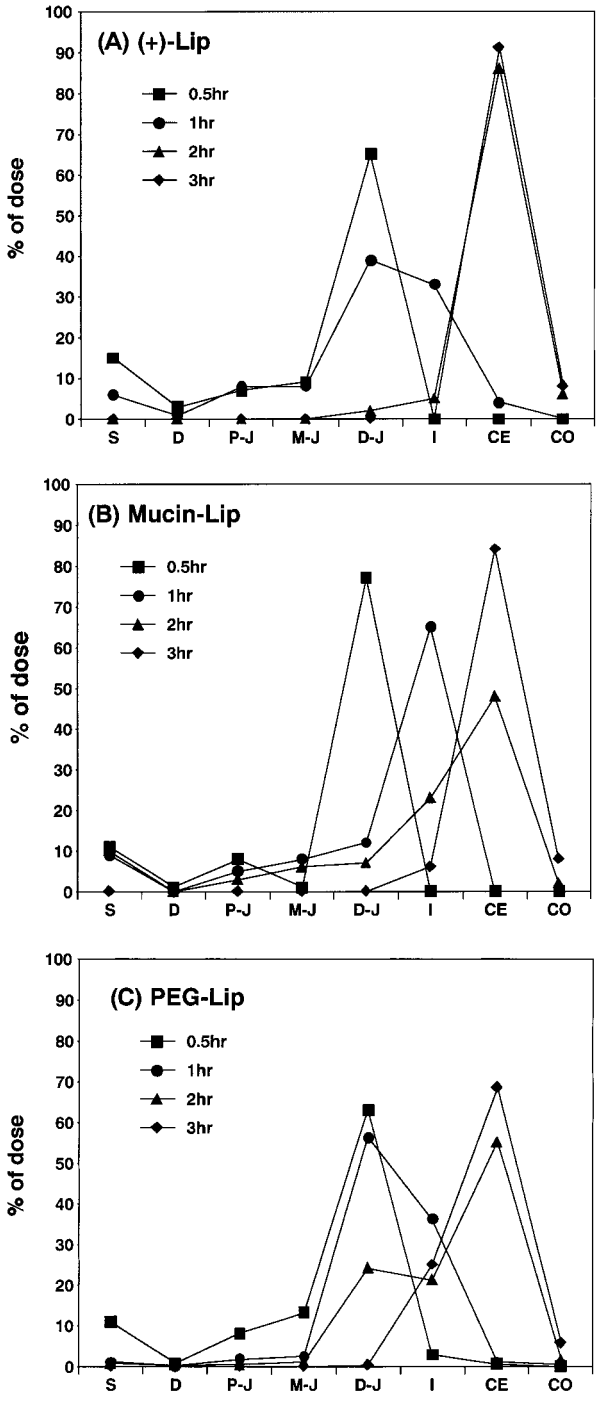

Figure 1—Time course of remaining radioactivity (% of recovered) of [^{14}C]-DPPC in the GI tract (A) (+)-Lip, (B) Mucin-Lip, (C) PEG-Lip. S, stomach; D, duodenum; P-J, proximal jejunum; M-J, middle jejunum; D-J, distal jejunum; I, ileum; CE, cecum; CO, colon. Data are expressed as means of 4–6 experiments.

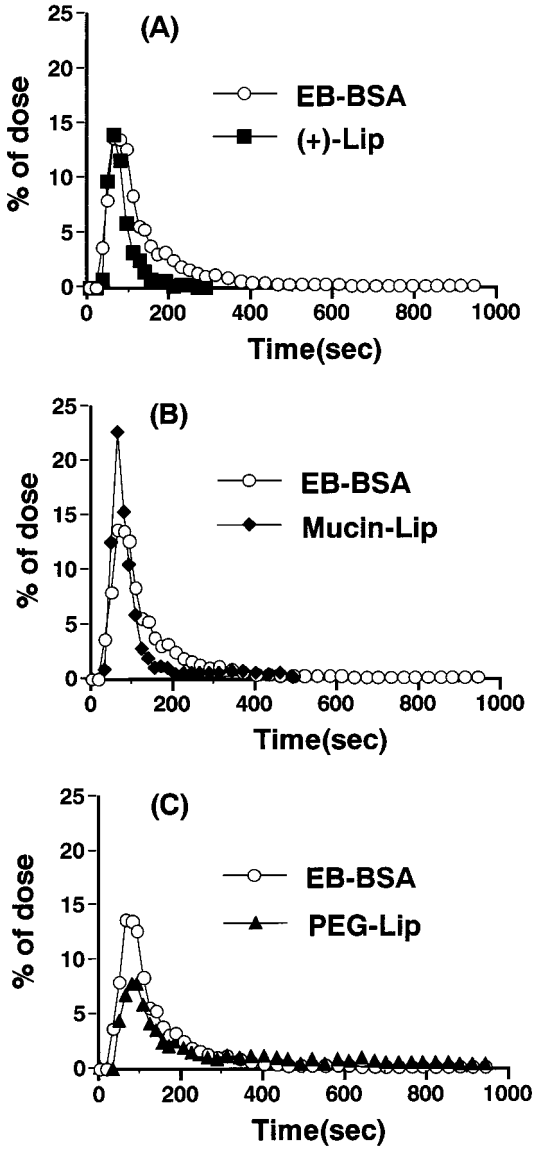

Figure 2—Typical outflow pattern of insulin obtained from in situ single pass perfusion experiment: (A) (+)-Lip, (B) Mucin-Lip, (C) PEG-Lip.

Table 2—Moment Parameters for Intestinal Transit of Insulin and Liposomes

		recovery[c] (%)	MTT[c] (s)	DTT[c] (s)
(+)-Lip	liposome	65.36 ± 4.56	111.96 ± 8.79	107.02 ± 13.41
	insulin	52.57 ± 4.67[b]	86.38 ± 2.63[b]	34.96 ± 1.32[b]
mucin-Lip	liposome	70.43 ± 5.07	99.22 ± 5.90	83.64 ± 17.42
	insulin	80.71 ± 5.29[a]	103.54 ± 12.52[a]	81.11 ± 14.02[a]
PEG-Lip	liposome	77.92 ± 2.93[a]	198.57 ± 22.16[a]	179.85 ± 22.54[a]
	insulin	64.54 ± 1.19[a,b]	201.58 ± 19.44[a]	165.48 ± 8.06[a]

[a] Significant at $p < 0.05$ vs (+)-Lip. [b] Significant at $p < 0.05$ between liposome and insulin. [c] Results were expressed as the mean ± SE of 5–8 experiments.

The intestinal transit of liposomes themselves was also investigated by labeling the liposomal membrane with diphenylhexatriene (DPH). Moment parameters for outflow patterns of liposomes are also listed in Table 2. After administration of Mucin-Lip and PEG-Lip, there were no significant differences in MTT or DTT between liposomes and insulin. In contrast, both MTT and DTT of liposomes were significantly longer than those of insulin in (+)-Lip. These results suggested that most of the insulin passed through the intestinal tract in liposomal form in the surface-coated liposomes, while in (+)-Lip significant amounts of insulin were released from liposomes in the intestinal loop.

Effect of Removal of the Mucous Layer on the Intestinal Transit of Liposomes—The effect of pretreatment of the intestine with dithiothreitol, which can remove the mucous layer from the epithelial surface, on the intestinal transit of surface-coated liposomes was investigated. As listed in Table 3, this pretreatment did not cause any significant changes in the recovery ratio of either type of liposomes. However, a marked decrease was observed

Table 3—Effects of Intestinal Mucous Layer on the Transit of Liposomes

		recovery[b] (%)	MTT[b] (s)	DTT[b] (s)
mucin-Lip	control	70.43 ± 5.07	99.22 ± 5.90	83.64 ± 17.42
	treated	61.20 ± 3.72	81.27 ± 3.97	97.41 ± 16.49
PEG-Lip	control	77.92 ± 2.93	198.57 ± 22.16	179.85 ± 22.54
	treated	76.25 ± 5.07	127.59 ± 18.70[a]	128.38 ± 15.28[a]

[a] Significant at $p < 0.05$ between control and treated. [b] Results were expressed as the mean ± SE of 5–8 experiments.

in MTT and DTT of PEG-Lip following pretreatment with dithiothreitol.

Discussion

We have reported that coating the surface of liposomes with poly(ethylene glycol) or the sugar chain of mucin increased the potency of liposomes as a tool for oral delivery of peptide drugs.[5] Insulin encapsulated in surface-coated liposomes showed enhanced and sustained hypoglycemic effects after oral administration. It was revealed that surface coating resulted in liposomes that resisted digestion by bile salts, leading to the stabilization and slow release of insulin in the GI tract. However, many factors other than drug stability affect the absorption profile of peptide drugs. Surface coating with these materials may also affect the intestinal transit of liposomes through interaction with the intestinal wall. Therefore, we investigated the behavior of surface coated liposomes in the GI tract in vivo and also in situ by focusing on the effect on liposomal transit.

When liposomes were orally administered to rats in vivo, uncoated liposomes, (+)-Lip, showed rapid transit to distal parts of the intestine, suggesting the weak interaction of liposomes themselves with the intestinal wall. Although surface coating reduced the transit rate of liposomes, PEG-Lip and Mucin-Lip showed different behavior in the GI tract (Figure 1). Mucin-Lip was retained in the stomach longer than other liposomes. As the surface of the stomach is completely covered with a mucin layer, this may be caused by the interaction between mucin on the surface of the stomach and that of the liposomes. In contrast, PEG-Lip was markedly retained in the lower region of the intestine rather than in the stomach, suggesting the higher affinity of poly(ethylene glycol) to the surface of the small intestine. These results indicated that the behavior of liposomes in the GI tract depends on the features of the surface-coating materials.

To elucidate this in further detail, we investigated the effect of coating the surface of liposomes on the intestinal transit by means of in situ perfusion experiments. The recovery ratio of insulin after administration of (+)-Lip was significantly lower than that after administration of surface-coated liposomes (Table 2). Furthermore, both the MTT and DTT of insulin encapsulated in (+)-Lip were different from those of the liposomes themselves. On the other hand, there were no significant differences in MTT or DTT between liposomes and insulin after administration of surface-coated liposomes. Therefore, it is obvious that (+)-Lip degraded and released insulin during transit through the intestinal tract, while surface-coated liposomes retained insulin in the intestine. These results are consistent with our observation that insulin encapsulated in surface-coated liposomes was much more stable than that in uncoated liposomes in the intestinal fluid in vitro.[5] Shegal and Rogers et al.[11] reported that coating the surface of liposomes with *O*-palmitoylpullulan was an effective way to avoid the interaction of the liposomal lipid membrane with bile salts. Also, Zeisig et al.[12] reported that the thickness of the fixed aqueous layer on the surface of liposomes was increased by coating the surface with poly(ethylene glycol). This fixed aqueous layer on PEG-Lip could protect the lipid membrane against digestion by bile salts. Since the surface of Mucin-Lip was covered with the long sugar chain, this also prevented the digestion of liposomes in the intestinal tract.

Neither the MTT nor DTT of Mucin-Lip were significantly different from those of (+)-Lip, indicating that surface coating with mucin did not affect the transit rate of liposomes in the small intestine. This result was supported by the observation that Mucin-Lip was highly retained in the stomach rather than in the intestine after oral administration in vivo.

In contrast, it is apparent from the much longer values of MTT and DTT that PEG-Lip strongly interacts with the intestinal wall. The viscosity and mucoadhesiveness of the liposomal formulation may be the most important factors affecting intestinal transit. Since coating the liposome's surface with poly(ethylene glycol) only slightly affected their viscosity (data not shown), it could be speculated that increased mucoadhesiveness decreased the intestinal transit rate and caused the wide spread distribution patterns of PEG-Lip observed in vivo. Hassan et al.[13] reported that the adhesive force of poly(ethylene glycol) to mucin is comparable to that of other neutral polymers. Also, Ascentiis et al.[14] reported that the mucoadhesiveness of methacrylate polymer microparticles was increased by the copolymerization of methacrylate with poly(ethylene glycol). The increased mucoadhesiveness of PEG-Lip was also confirmed by in situ perfusion experiments using rat intestine in which the mucous layer had been removed. Both the MTT and DTT of PEG-Lip were significantly shortened by removal of the intestinal mucous layer, whereas those of Mucin-Lip were changed only slightly (Table 3). This result clearly indicated that poly(ethylene glycol) interacts with intestinal wall through adhesion to the mucous layer. Rao et al. reported that the mucoadhesiveness of glass beads coated with hydrophilic polymers decreased following removal of the mucous layer.[15] Furthermore, since the amounts of mucous fluid in both the distal-jejunum and ileum are richer than those in other regions, the marked retention of PEG-Lip in these regions in vivo (Figure 1) may reflect its strong interaction with the mucous layer. Ilan et al.[16] reported that the intestinal absorption of desmopressin was enhanced by using mucoadhesive submicron emulsion. They speculated that coating the surface of the emulsion with Carbopol-940 caused a strong interaction between the emulsion and the mucous layer and increased the retention of the emulsion in the intestinal tract, resulting in the enhanced absorption of desmopressin. Lehr et al.[17] also reported that the intestinal absorption of 9-des-glycinamide, 8 arginine vasopressin (DGAVP) was enhanced by using mucoadhesive microspheres in the rat intestine in vitro. Thus, for oral delivery of peptide drugs, the slow transit of the formulation in the intestinal tract, such as PEG-Lip in this study, should be desirable.

In conclusion, Mucin-Lip adhered preferentially to the surface of the stomach. PEG-Lip moved along the GI tract slowly and spread widely in the small intestine because of the strong interaction with the intestinal mucous layer, leading to the enhanced and prolonged hypoglycemic effects of insulin. These findings clearly demonstrated that the surface coating of liposomes is a useful method for oral delivery of peptide drugs.

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