



Mucosal insulin delivery systems based on complexation polymer hydrogels: effect of particle size on insulin enteral absorption

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Abstract

Insulin-loaded polymer (ILP) microparticles composed of poly(methacrylic acid) and poly(ethylene glycol), which have pH-dependent complexation and mucoadhesive properties have been thought to be potential carriers for insulin via an oral route. Nevertheless, further optimization of the polymer delivery system is required to improve clinical application. Therefore, the effect of particle size of the ILP (L-ILP: 180–230 μm , S-ILP: 43–89 μm , SS-ILP: <43 μm) on insulin absorption was studied in the in situ loop system, hypothesizing smaller particle sizes of ILP could induce bigger hypoglycemic effects due to increase mucoadhesive capacity. To verify the hypothesis, the adhesive capacities of differently sized ILPs to the mucosal tissues were evaluated. Additionally, the intestinal site-specificity of ILP for insulin absorption was investigated. Intra- and inter-cellular integrity and/or damage were also examined by lactate dehydrogenase leakage and membrane electrical resistance change to ensure the safety of ILP as a carrier for oral route. As hypothesized, the smaller sized microparticles (SS-ILP) showed a rapid burst-type insulin release and higher insulin absorption compared with the microparticles having larger sizes, resulting in greater hypoglycemic effects without detectable mucosal damage. In fact, SS-ILP demonstrated higher mucoadhesive capacity to the jejunum and the ileum than those of L-ILP. Moreover, SS-ILP's enhancement effect of insulin mucosal absorption showed a site-specificity, demonstrating maximum effect at the ileal segment. These results imply that the particle size and delivery site are very important factors for ILP with respect to increasing the bioavailability of insulin following oral administration.
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1. Introduction

A successful formulation for oral delivery of therapeutic peptide and protein drugs would have to

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overcome two main barriers against the drugs: the enzymatic barrier of the gastrointestinal tract and the physical barrier made of the intestinal epithelium. In order to overcome these barriers, various protease inhibitors [1–3], absorption promoters [4,5] and chemical modification [6,7] have been tried and evaluated as tools for developing effective formulations for mucosal delivery of the drugs. In addition to such strategies, approaches to oral delivery of the drugs have focused on encapsulation techniques and controlled-release mechanisms for their abilities to shield drug molecules from degradation in the stomach and the upper intestine that is rich in proteases [1,8,9]. Recently, several researchers have demonstrated that some types of polymers have promising properties to overcome absorption barriers of peptide and protein drugs [10–13]. It was reported that the crosslinked poly(acrylic acid) derivatives polycarboxyl and carbomer were able to inhibit the activities of the proteolytic enzymes [10]. Similarly, recent study has shown that graft copolymer networks of poly(methacrylic acid grafted with poly(ethylene glycol)) (henceforth designated P(MAA-g-EG)) have a potential to bind calcium [12], which may affect the proteolytic activity of calcium-dependent enzymes, such as trypsin. Using the polymer system, we have previously reported that the insulin incorporated into P(MAA-g-EG) microparticles (size 100–150 μm) successfully enhanced insulin absorption in both streptozotocin-induced diabetic and non-diabetic rats, achieving 4.2% bioavailability (relative to subcutaneous administration) with significant hypoglycemic effects [14]. These results strongly indicate that P(MAA-g-EG) microparticles may be potential carriers for insulin via the oral route.

The P(MAA-g-EG) system was designed to protect the insulin from the harsh acidic environment of the stomach before releasing the bioactive agent in the small intestine. In the acidic environment of the stomach these hydrogels were not swollen due to intermolecular polymer complexes [15–17]. The insulin remained in the polymer and was protected from proteolytic degradation. In the basic and neutral environment of the intestine, the complexes dissociated and resulted in rapid polymer swelling, followed by insulin release. Further, P(MAA-g-EG) was shown to possess mucoadhesive properties when the hydrogels, due to the presence

of the graft PEG chains which serve as adhesion promoters [18].

In a previous *in vivo* investigation, oral P(MAA-g-EG) microparticles showed a good bioavailability of insulin [14]; nevertheless, further improvement of the delivery system is required for their clinical application. If the size of P(MAA-g-EG) microparticles is decreased, the mucoadhesiveness would be increased due to an increase of surface area contact with the mucosa. If so, significant quantities insulin could be released closer to the surface of intestine, which might result in a decrease of protease attack and improved uptake of intact drug. Therefore, we hypothesized that smaller particle sizes of the polymer carrier could induce greater hypoglycemic effects. In the present study, the effect of particle size of P(MAA-g-EG) on insulin absorption was studied in the *in situ* absorption system. The mucoadhesion on the mucosal tissues was compared among differently sized P(MAA-g-EG) microparticles to verify this hypothesis. Additionally, insulin release profiles from differently sized microparticles were evaluated. Furthermore, insulin absorption using the most effective P(MAA-g-EG) microparticles were compared at various intestinal segments to determine their site-specific effects. Finally, to ensure the safety of P(MAA-g-EG) microparticles as a carrier via the oral route, intra- and inter-cellular integrity and/or damage were examined by lactate dehydrogenase (LDH) leakage and membrane electrical resistance (R_m) changes.

2. Materials and methods

2.1. Materials

Methacrylic acid (MAA), dimethoxy propyl acetophenone (DMPA) and lactate dehydrogenase reagent were purchased from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) (PEG) monomethacrylate (PEGMA, with PEG of molecular weight 1000) and tetraethylene glycol dimethacrylate (TEGDMA) were obtained from Polysciences (Warrington, PA, USA). Crystalline human insulin (26.7 units/mg) was kindly supplied by Aventis Research and Technologies (Frankfurt, DE). All other chemicals were of analytical grade and commercially available.

2.2. Hydrogel synthesis

Microparticles of P(MAA-g-EG) were prepared by a free-radical solution polymerization of MAA and PEGMA with PEG of molecular weight 1000. The monomers were mixed to yield solutions with a 1:1 ratio of MAA/EG in the gels. As a crosslinking agent, TEGDMA was added in the amount of $X=0.075$ mol TEGDMA per mole MAA. Following the complete dissolution of the monomer, nitrogen was bubbled through the well-mixed solutions for 30 min to remove dissolved oxygen, a free radical scavenger, which would act as an inhibitor for polymerization.

The initiator, DMPA, was added in the amount of 1% weight of the monomers in a nitrogen atmosphere. The reaction mixtures were pipetted between flat plates to form films of 0.9 mm thickness. The monomer films were exposed to UV light (Ultracure 100, Efos, Buffalo, NY, USA) at 1 mW/cm^2 at 365 nm and allowed to react for 30 min. The ensuing hydrogels were rinsed for a week in deionized water to remove unreacted monomer and uncrosslinked oligomer chains, then dried under vacuum and ground into powders with diameters of <43 (SS-ILP), 43–89 (S-ILP) and 180–230 (L-ILP) μm .

2.3. Preparation of insulin loading polymer (ILP)

Insulin loading was performed by equilibrium partitioning in pH 7.4 phosphate-buffered saline (PBS) as described previously [19]. Briefly, specific amounts of crystalline human insulin were dissolved in 0.1 ml of 0.1 M HCl. The insulin solution was diluted with 19.8 ml of PBS and normalized with 0.1 ml of 0.1 M NaOH. Loading was accomplished by soaking 140 mg of each set of the dried P(MAA-g-EG) microparticles for 6 h in the insulin solution. The microparticles were collapsed with 20 ml of 0.1 M HCl and filtered using cellulose acetate/cellulose nitrate filter with 1 μm pores (Fisher Scientific International, Hanover Park, IL, USA). The insulin loading polymers (ILP) were dried under vacuum and stored at 4 °C until use. The degree of loading was determined using HPLC as described previously [19]. Insulin incorporation efficiency was unaffected by the size of ILP, and reached more than 90% of the initial amount.

2.4. In situ absorption studies

The animal experiments in this work complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals at Thomas Jefferson and Hoshi Universities. Male Sprague–Dawley rats weighing 180–200 g fasted for 24 h prior to the experiments and were anesthetized by an i.p. injection of 50 mg/kg sodium pentobarbital. The rats were restrained in a supine position and kept at body temperature of 37 °C using warming lamps. The jejunum, the ileum or the colon was exposed following small midline incision carefully made in the abdomen, and each segment (length=10 cm) was cannulated at both ends using polypropylene tubings (4 mm o.d., 2 mm i.d., Cole-Parmer Instrument, Vernon Hills, IL, USA). These were securely ligated to prevent fluid loss and subsequently, carefully returned to their original location inside the peritoneal cavity. In order to wash the intestinal content, phosphate-buffered saline (PBS; pH 7.4) at 37 °C were singly circulated through the cannula at 1.0 ml/min for 20 min using a peristaltic pump (MasterFlex® tubing pumps, Cole-Parmer Instrument). Subsequently, the segments were tightly closed following removal of cannulation tubings; approximately 0.5 ml of perfusion solutions remained left in the segments. Rats were further left on the board at 37 °C for 1 h to be recovered from the elevated blood glucose levels due to surgical operations described above. Following 1 h resting, pure insulin or each ILP (approximately 3 mg) with 0.5 ml of PBS solution was directly administered into the loops (6 cm) made from the pretreated segment (10 cm); the jejunal loop was made at 5 cm away from the ligament of Treitz; the ileal loop was made at the end of the small intestine, just proximal to the ileo-cecal junction; the colonic loop was made at the ascending colon. Insulin PBS solution (0.5 ml) was used as a control. The dose for all samples was fixed at 25 IU/kg body weight. During the experiment, a 0.2 ml blood aliquot was taken from the jugular vein at $t=0, 5, 10, 15, 30, 60, 120, 180$ and 240 min after dosing. The relative bioavailability of the enterally administered insulin was calculated relative to the subcutaneous (s.c.) route, using methods described by Matsuzawa et al. [20]. Briefly, insulin solutions were prepared by dissolving an appropriate amount of

crystalline human insulin in PBS. The insulin s.c. doses were 0.25, 0.5, 1.0 and 2.0 IU/kg body weight. Blood samples (0.2 ml) were collected from jugular vein before and 5, 10, 15, 30, 60, 120, 180 and 240 min after dosing. Animals receiving s.c. insulin were given the same surgical operation (the ileal loop) as in the intestinal administration study, in order to set the same physical condition on the rats. Tuberculin syringes (1 ml) were pre-heparinized in the usual fashion consisting of coating of the syringe wall through aspiration of heparin and expelling all heparin by depressing plunger down to needle hub. Plasma was separated by centrifugation at 13,000 rpm for 1 min. The blood glucose levels were determined using HemoCue® B-glucose analyzer (HemoCue, CA, USA). The plasma insulin levels were determined using an enzyme immunoassay (Alpco Research, Windham, NH, USA).

The total area under the insulin concentration curve (AUC) from time 0 to 240 min was estimated from the sum of successive trapezoids between each data point. The bioavailability was calculated relative to s.c. injection as described above. The plasma peak level (C_{max}) and the time taken to reach the plasma peak level (T_{max}) were determined from the plasma insulin level–time curves. Mean residence time (MRT) was calculated by dividing AUMC by AUC, where AUMC is the area under the first moment curve for insulin from 0 to 240 min point.

2.5. *In vitro* release studies

The formulations (10 mg) were placed into beakers containing 20 ml of PBS at 37 °C under constant stirring. Samples (0.1 ml) were taken at discrete intervals using filtered syringes (pore size: 10 μ m; Ishikawa Manufactory). Insulin concentration in each sample was determined by using HPLC method reported previously [19]. The fractional release of insulin from the formulations, defined here as the ratio of the amount released at any time (M_t) to the total amount released after 1 h (M_∞) was calculated.

2.6. Mucoadhesion studies

The jejunal and the ileal loops from the pretreated segment following the in situ experiments as de-

scribed above were used. Following washing of the remaining PBS in the loop with air, L-ILP or SS-ILP (each 30 mg) with 0.5 ml of PBS was administered to each loop and remained left in the segments for 1 h. Then, the loops were washed by circulating PBS at 1.0 ml/min for 1 min using a peristaltic pump. The detached microparticles were collected, dried under vacuum and weighed. The mucoadhesive capacity (%) was calculated as (the initial amount (30 mg) – the collected amount) \times 100/the initial amount (30 mg).

2.7. Biochemical evaluation of intestinal damage

The ileal loop from the pretreated segment following the in situ experiments as described above was used here. The ileum with treated with 20 ml of PBS (warmed to 37 °C) and then flushed out with air. One milliliter of PBS, 1% (w/v) sodium caprate, 1% (w/v) sodium glycocholate or SS-ILP (5 mg) PBS suspension was administered to the ileum and incubated in the segments for 2 h. Then, the ileal loop was washed with 1.0 ml of PBS, and the intestinal fluid was collected. The concentration of lactate dehydrogenase (LDH) in the fluid was determined using a LDH-UV kit (Sigma).

2.8. Ileal membrane electrical resistance (R_m)

The ileal loop from the pretreated segment following the in situ experiments as described above was used in this experiment. Following washing the ileum with warmed 20 ml of PBS, the remaining PBS in the loop was then washed out with air. Then, 10 mg of SS-ILP with 0.5 ml of PBS was administered to the ileal loop and remained left for 5 and 30 min. Similarly, 0.5 ml of control (PBS) or 20 mM Na₂EDTA (ethylenediaminetetraacetic acid, disodium salt, dihydrate), as a positive control, was administered to the ileal loop for 5 and 30 min. Immediately after each pretreatment, the ileal segments were quickly opened along the mesenteric border to prepare the flat sheet membranes. They were carefully washed with ice-cold calcium free Krebs–Ringer’s bicarbonate-buffered solution (pH 7.4) [21]. The solution (in mM) was composed of 125.0 NaCl, 11.1 D-glucose, 10.0 NaHCO₃, 5.0 KCl, 1.2 NaH₂PO₄, 4.2 mannitol. The membranes were

mounted in an Ussing chamber system (CEZ-9100, Nihon-Kohden Tokyo, Tokyo, Japan) equipped with diffusion cells of 1 cm² as the effective area maintained at 37 °C. Apical and basal compartments were filled with 5.0 ml of isotonic calcium free Krebs–Ringer’s solution and stirred with oxygenation (95% O₂/5% CO₂) for 20 min prior to monitoring electrophysiological parameters. The spontaneous transmucosal potential difference (PD, mV) and the short circuit current (Isc, μ A) were recorded simultaneously at 20 min, and Rm (Ω) calculated by PD/Isc, based on the Ohm’s law. These were corrected by eliminating the offset voltage between the electrodes and series fluid resistance, which was determined prior to each experiment using the identical bathing solutions, yet in the absence of ileal membranes mounted in the chamber.

2.9. Statistical analysis

Each value was expressed as the mean \pm S.D. For group comparisons, the one-way layout ANOVA with duplication was applied. Significant differences in the mean values were evaluated by the Student’s unpaired *t*-test. A *p* value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Effect of particle size of ILP on insulin absorption from the ileum

Fig. 1 shows the effect of particle size of ILP on insulin absorption (A) and resultant hypoglycemic effect (B). No apparent hypoglycemic response was observed in the control group, demonstrating no insulin absorption from the ileal segments. Similarly, L-ILP demonstrated the absence of appreciable insulin absorption from the ileal segments although plasma glucose levels seemed to be lower than those of control. In contrast, S- and SS-ILP significantly enhanced insulin ileal absorption. SS-ILP showed more predominant enhancement effect on insulin absorption than that of S-ILP, implying that the smaller size of ILP induced the bigger insulin absorption from the ileum.

Table 1 summarizes pharmacokinetic parameters derived from the insulin concentration–time profiles following in situ administration of ILPs to the ileal segments. In this study, the relative bioavailability of insulin was calculated based on the s.c. injection study (Fig. 2). Plotting AUC of insulin vs. dose, there is the linear relationship having $r=0.934$ ($n=17$, $p<0.01$). While there is no significant difference in pharmaco-

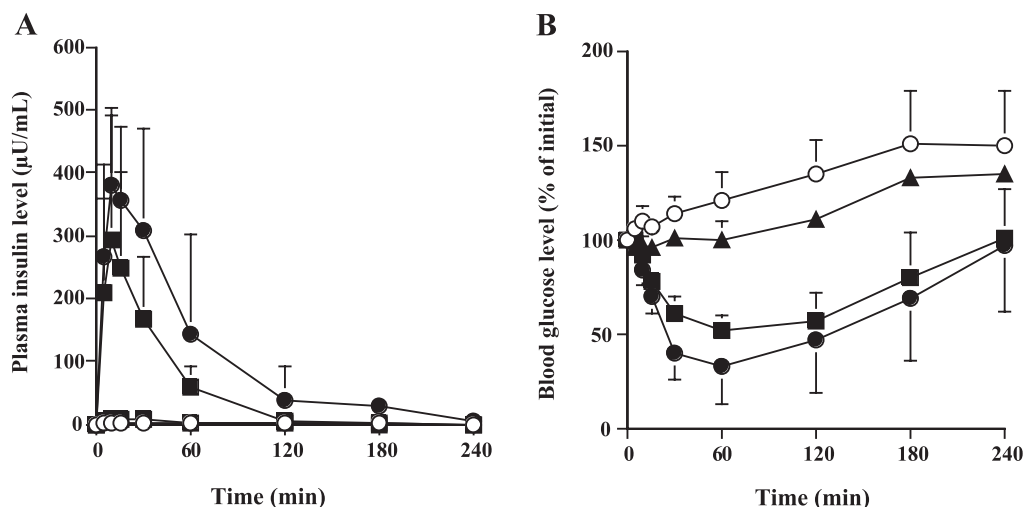


Fig. 1. Plasma insulin (A) and blood glucose (B) levels versus time profiles following in situ administration of insulin loaded P(MAA-g-EG) microparticles (25 IU/kg) into the ileal segments. Each data represents the mean \pm S.D. from $n=3-4$. Key: (○) insulin PBS solution (control); (▲) L-ILP; (■) S-ILP; (●) SS-ILP.

Table 1

Pharmacokinetic parameters derived from the plasma level vs. time profiles for insulin (25 IU/kg), following in situ administration into ileal segments

Preparation	Cmax [$\mu\text{g}/\text{ml}$]	Tmax [h]	MRT [h]	AUC [$\mu\text{g h}/\text{ml}$]	F [%]
Control	3.9 ± 4.4	0.20 ± 0.39	0.38 ± 0.42	2.45 ± 3.27	0.5 ± 0.1
L-ILP	7.8 ± 7.9	0.17 ± 0.28	0.48 ± 0.41	6.14 ± 7.16	0.7 ± 0.2
S-ILP	$299.7 \pm 202.5^*$	0.08 ± 0.12	0.35 ± 0.20	$149.4 \pm 90.5^*$	$5.2 \pm 2.8^*$
SS-ILP	$411.6 \pm 82.0^*$	0.14 ± 0.24	0.76 ± 0.21	$391.4 \pm 263.7^*$	$12.8 \pm 8.3^*$

Data: mean \pm S.D. ($n=3-4$).

Cmax: the maximum concentration; Tmax: the time to reach the Cmax; MRT: the mean residence time; AUC: the area under the curve; F: the bioavailability relative to s.c. injection.

* $p < 0.05$ against control.

kinetic parameters between control and L-ILP, S- and SS-ILP induced the significant increase in parameters, Cmax, AUC and F, which are related to the extent of absorption. On the other hand, the absorption rate related parameters, such as Tmax and MRT appeared to be unaffected by the reduction of the size of ILP.

It should be noted that the ileal insulin absorption from ILPs rapidly occurred, and this rapid insulin absorption rate was compared to that of s.c. insulin injection (Fig. 2). The P(MAA-g-EG) microparticles are not swollen in acidic surrounding, but are quickly swellable and, thereby, instantly release drug in neutral and basic condition because of the reversible complex-

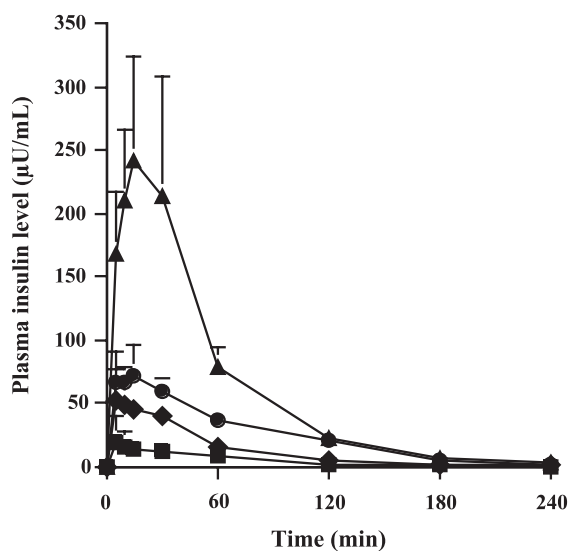


Fig. 2. Plasma insulin levels versus time profiles following s.c. administration of insulin solution. Each data represents the mean \pm S.D. from $n=4$. Key: (■) 0.25 IU/kg; (◆) 0.5 IU/kg; (●) 1.0 IU/kg; (▲) 2.0 IU/kg.

ation/uncomplexation nature responding to the surrounding medium [15,19]. In fact, both polymers swelled rapidly and a rapid burst-type release of insulin occurred in PBS solution (Fig. 3). However, during the initial 15 min of the release study, insulin was far more rapidly released from SS-ILP than that from L-ILP. This implies that the smaller particle size can provide for near instant insulin release at the absorption site resulting in a higher local concentration, thereby more amount of insulin can be absorbed. This may greatly contribute to the significant hypoglycemic effects induced by the ILP of smaller particle size.

Generally, when insulin solutions were administered given to the small intestine, insulin was subjected to extensive degradation by proteolytic enzymes and, thereby, insulin absorption occurred negligibly like control group of this study. It has been shown that insulin was degraded by pancreatic proteases like trypsin and chymotrypsin, which are be-

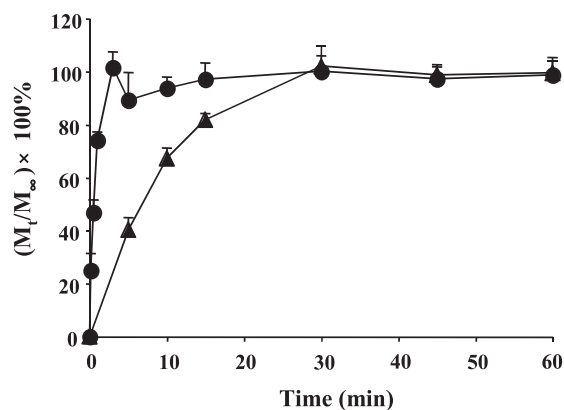


Fig. 3. Release profiles of insulin from P(MAA-g-EG) microparticles. Each data represents the mean \pm S.D. from $n=3-6$. Key: (▲) L-ILP; (●) SS-ILP.

lieved to reside in both intestinal fluids and the mucous/glycocalyx layers [22,23]. In this study, enzymes in the former compartment could be removed by washing pretreatment with PBS in the *in situ* experiments. Nevertheless, the mucous/glycocalyx layers might remain and in fact, the layers had significant roles in insulin's metabolic loss [24]. Moreover, the importance of ectopeptidases contributing to the metabolic loss of certain polypeptides including insulin has been reported [25,26]. Regardless of such multi-compartment enzymatic barriers, however, the ileal insulin absorption clearly occurred in the small intestine following the application of S- and SS-ILP. One possibility of the increased insulin's absorption may be associated with the reduced proteolytic activity in the presence of the polymer. It has been already reported that P(MAA-g-EG) microparticles may inhibit the metal-dependent enzymes via the binding of Ca^{2+} with carboxylic group on the backbone chain [12]. Indeed, a range of proteases can be inhibited by chelating essential metal ions from the enzyme structure, resulting in protein denaturation and loss of activity [27]. Therefore, this may play the crucial role for the enhancing absorption of insulin following ILP administration.

3.2. Effect of particle size of ILP on its adhesive capacity to the mucosal tissues

Hydrogels of P(MAA-g-EG) exhibit excellent mucoadhesive characteristics for delivery of drugs to the small intestine due to the presence of the graft PEG chains which serve as adhesion promoters [18]. However, the mucoadhesive capacity of differently sized P(MAA-g-EG) microparticles have not been compared. A size-dependent particle deposition in the gastrointestinal tract of rats has been suggested recently [28,29]. The authors reported an increased adherence for smaller particles in the whole gut. We observed the similar phenomenon as shown in Fig. 4. SS-ILP demonstrated that more than 60% of administered microparticles were tightly adhered to both the jejunum and the ileum tissues following 1-h administration. Likewise, L-ILP showed a high mucoadhesive capacity at both intestinal regions, however, the values were significantly lower than those of SS-ILP. Similar trends were observed following 5-min administration (data not shown). Therefore, these results can support

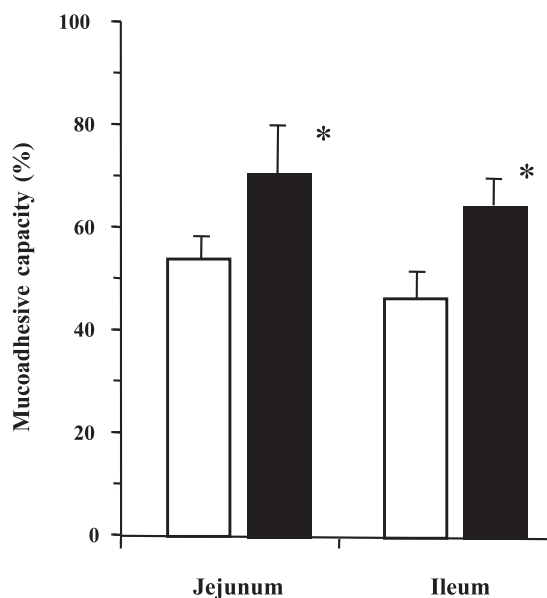


Fig. 4. Mucoadhesive capacities of insulin loaded P(MAA-g-EG) microparticles at jejunal and the ileal regions. Each data represents the mean \pm S.D. from $n=3$. Key: (□) L-ILP; (■) SS-ILP. Statistically significant difference from L-ILP: $p < 0.05$, *.

our hypothesis that the reduction of particle size may give the stronger bioadhesive competence, resulting in closer insulin release to the membrane epithelium and, thereby, in an increase of the number of insulin molecules those can escape from proteolysis in the lumen. Moreover, proteolytic inhibitory effects of the polymer may act more effectively on the insulin absorption when insulin could be released from microparticles to the closer surface of intestine.

3.3. Comparison of hypoglycemic effects following administration of SS-ILP to various intestinal segments

Although SS-ILP successfully increased insulin absorption from the ileal segment and therefore, induced significant hypoglycemic effects, the intestinal site-specificity of P(MAA-g-EG) microparticles for insulin absorption has not been determined. In fact, it was reported that the apparent insulin permeability varies with different intestinal regions [4,30]. Thus, SS-ILP was administered to the jejunal, the ileal and the colonic segments, and their site-specific hypoglycemic effects were examined. As shown in Fig. 5, hypoglycemic effects induced by SS-ILP were

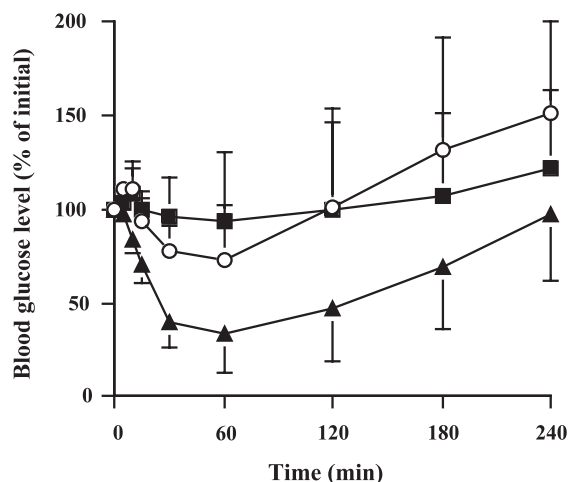


Fig. 5. Blood glucose level versus time profiles following in situ administration of SS-ILP into various site of intestine. Each data represents the mean \pm S.D. from $n=4-5$. Key: (○) jejunum; (▲) ileum; (■) colon.

different at three intestinal regions. In the jejunal segment, relatively weak hypoglycemic effects were observed following SS-ILP administration, while SS-ILP induced the dramatic reduction of blood glucose levels in the ileal segment (Fig. 5).

The possible explanation of the results is due to the difference in proteolytic activity in the intestine. Comparing the pancreatic enzyme activity, it was much stronger in the upper small intestine than in the descending small intestine like the ileal segments [4]. Assuming that amount of released insulin from SS-ILP at each region would be the same, indeed, pH value in the intestinal fluid is thought to be enough for SS-ILP to allow swelling, the degree of insulin degradation was much larger in the jejunum than in the ileum. As a consequence, the net insulin amount capable of being absorbed through the small intestine is much smaller in the jejunum than in the ileum and, therefore, it was likely that SS-ILP administration to the jejunal segment revealed small hypoglycemic effect.

On the other hand, SS-ILP administration to the colonic segment, where the proteolytic activity may be much smaller than those in the small intestine, induced little or no hypoglycemic effects. The result is presumably due to the thickness of mucus layers [31] and tightness of tight junctions of the colon [32]. It is well known that the presence of the mucous layers

was one of the permeation barriers for peptide and protein drug absorption [33–35]. It was demonstrated that the diminishment of mucous/glycocalyx layers showed the significant increase of insulin permeability [24]. Furthermore, the tight junction is more rigid in the large intestine than in the small intestine, so the permeability of macromolecular drugs from the large intestine might be lowered compared with the small intestine [32]. Although PEG chain grafted in PMAA was penetrated into mucous layers [18], and P(MAA-g-EG) may be adhered to the mucosal membrane, because of having thicker mucus layers and tightness of mucosal membrane in the colonic region, insulin absorption in the colon may be decreased, even though the insulin was released to the same extent in the colonic segment as in the small intestine. These results suggest the necessity for oral P(MAA-g-EG) microparticles to be delivered into the lower small intestine to attain higher insulin absorption.

3.4. Biochemical and electrophysiological examination of the ileal membranes following SS-ILP administration

In this study, we demonstrated that the reduction of particle size of P(MAA-g-EG) microparticles made ileal insulin absorption significantly increase. However, in some cases, coadministration of macromolecular drugs with penetration enhancers or polymer carriers was accompanied with various adverse side effects. In fact, some penetration enhancers are known to induce extensive damage on the epithelial of intestinal membrane [36,37]. Therefore, this study investigated whether ILP induce the mucosal damage at the applied site. Table 2 shows the LDH leakage following 2-

Table 2

Lactate dehydrogenase (LDH) leakage following PBS (control), SS-ILP, 1.0% (w/v) sodium caprate and 1.0% (w/v) sodium glycocholate

Preparation	LDH leakage [U]
Control	0.39 \pm 0.17
SS-ILP	0.47 \pm 0.11
Sodium caprate	1.13 \pm 0.39*
Sodium glycocholate	2.35 \pm 1.19**

Data: mean \pm S.D. ($n=4-6$).

* $p < 0.05$ against control.

** $p < 0.01$ against control.

Table 3

Membrane electrical resistance (Rm), following application of PBS (control), SS-ILP and Na₂EDTA into the ileal segment

Preparation	Rm [Ω cm ²]	
	5 min	30 min
Control	42.7 ± 5.0	42.3 ± 2.9
SS-ILP	40.2 ± 4.3	36.1 ± 2.0
Na ₂ EDTA	24.6 ± 5.2*	25.4 ± 2.9*

Data: mean ± S.D. ($n=3-4$).

* $p < 0.05$ against control.

h administration of SS-ILP to the ileal region. Lactate dehydrogenase was negligibly leaked into the ileal loop, which was similar to the leakage seen in the control group. In contrast, the leakage was dramatically induced by administration of typical penetration enhancers, 1.0% (w/v) sodium caprate and 1.0% (w/v) sodium glycocholate. Likewise, the values for Rm were unchanged following application of SS-ILP to the ileal region, compared to those in the PBS-treated (control) counterparts (Table 3). This was quite contrastive to active control, Na₂EDTA-treated segment which induced significant decrease in the values of Rm. These data suggested that the ileal membrane integrity and cellular tight junctions remain mostly unaltered by the administration of SS-ILP. The mechanisms of the increased insulin's absorption were therefore, not associated with facilitation of intra- and inter-cellular diffusion, but could be with the increased mucoadhesion and the reduced protease inhibition.

4. Conclusions

Smaller sized P(MAA-g-EG) microparticles (SS-ILP) showed the higher insulin absorption compared with the microparticles having larger size (L-ILP), resulting in greater hypoglycemic effects without detectable mucosal damage such as LDH leakage and Rm reduction. Furthermore, SS-ILP demonstrated higher mucoadhesive capacity to the jejunum and the ileum than those of L-ILP. The high mucoadhesive capacity may reduce insulin degradation between microparticles and intestinal absorbing membrane, thereby increasing insulin absorption from the small intestine. A rapid burst-type release of insulin was observed with the smaller sized ILP which was

believed to have contributed to an increase relative bioavailability of insulin. Moreover, SS-ILP's enhancement effect of insulin mucosal absorption showed a site-specificity, demonstrating maximum effect at the ileal segment. These results imply that the use of smaller size of P(MAA-g-EG) microparticles, and the delivery them to the lower small intestine holds promise for increasing the bioavailability of insulin following oral administration.

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