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Complexation hydrogels for intestinal delivery of interferon β and calcitonin

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Abstract

Recent studies have suggested that complexation hydrogels poly(methacrylic acid-g-ethylene glycol) (henceforth designated as P(MAA-g-EG)) exhibit high insulin incorporation efficiency, rapid insulin release in the intestine based on their pH-dependent complexation properties, enzyme-inhibiting effects and mucoadhesive characteristics. Therefore, they are promising carriers for insulin delivery via an oral route. As we designed these hydrogels as carriers suitable for oral administration of various peptide/protein drugs, in this study we aimed at investigating the applicability of P(MAA-g-EG) hydrogels to improving the intestinal absorption of various peptide/protein drugs. High incorporation efficiency into hydrogels was observed for insulin, calcitonin, and interferon β. In addition, polymer microparticles loaded with calcitonin and interferon β exhibited complexation/decomplexation and pH-sensitive release behavior. The molecular weight and chemical structure appeared to affect the efficiency of incorporation and release depending on the peptides and proteins. Furthermore, a drastic reduction of plasma calcium concentration accompanied by calcium absorption and a dose-dependent enhancement of plasma interferon β concentration were observed after the administration of particles loaded with calcitonin or interferon β into closed rat ileal segments. These findings indicate that P(MAA-g-EG) hydrogels are promising carriers for administration of various peptides and proteins via an oral route.

Keywords

interferon β; calcitonin; intestinal absorption; complexation polymers; drug delivery system

1. Introduction

Recent advances in production of bioactive peptides and proteins have enabled the use of these molecules as therapeutic agents for several diseases. Oral forms of these biomedicines are preferred by patients as they limit patient compliance problems. However, because of their inactivation in the gastrointestinal tract by enzymatic degradation and poor permeability through the mucosal epithelial membrane [1,2], these agents are presently administered via parenteral routes, such as intravenous and subcutaneous injections. To overcome these limitations, several approaches have been developed that use incorporation of absorption enhancers [3,4], use of enzyme inhibitors [4,5] as well as mixing and

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encapsulation techniques, including liposomes [6] and novel polymeric nanocarriers [7–9]. Often, the bioavailability obtained by most of these approaches has not been satisfactory or sufficient to adequately sustain oral formulations of peptides and proteins. Furthermore, some of them have exhibited untoward effects, such as disturbance of cell membrane and opening tight junctions, on the intestinal barrier to exogenous pathogens.

We have previously developed a promising carrier system for the oral delivery of insulin, based on discs, microfilms, microparticles or nanoparticles of pH-sensitive polymer and complexation hydrogels consisting of poly(methacrylic acid) grafted with poly(ethylene glycol) (henceforth designated as P(MAA-g-EG)) [10,11]. In the acidic environment of the stomach, the hydrogel carriers do not swell because of their interpolymer complexation nature which is the result of hydrogen bonding between the carboxylic and the etheric groups of this structure. Under these conditions, the network mesh size is significantly decreased, and the diffusion of insulin through the network is prevented. In the neutral and basic environment of the small intestine, the interpolymer complexes dissociate due to disruption of the hydrogen bonds, resulting in rapid polymer swelling followed by insulin release [12–15]. Moreover, it has been demonstrated that the physical and chemical integrity of insulin was maintained after incorporation into P(MAA-g-EG) hydrogels and subsequent release [16]. An additional advantage of these hydrogels is their ability to inhibit the activities of proteolytic enzymes by binding to calcium [17,18] as well as their adhesive characteristics to the mucosal membrane [19–21]. In addition, no adverse effects on mucosal membranes were observed when P(MAA-g-EG) hydrogels were administered [22,23]. Using the polymer system, we previously reported that microparticles of diameters of <53 μm (SS-ILP) composed of a 1:1 molar ratio of MAA/EG units exhibited pronounced hypoglycemic effects following oral administration to healthy rats, by achieving a 9.5% pharmacological availability in comparison to subcutaneous insulin injection [11]. Thus, P(MAA-g-EG) hydrogels may be promising carriers for unstable peptides and proteins via an oral route.

The present study was aimed at further examining the applicability of P(MAA-g-EG) hydrogels to the oral delivery of various peptide and protein drugs. The properties of the incorporation and release of the peptides and proteins using P(MAA-g-EG) hydrogels were examined using calcitonin, interferon β, and bovine serum albumin (BSA). In addition, the usefulness of this hydrogel carrier in the intestinal absorption of these molecules was evaluated.

2. Materials and methods

2.1. Materials

Recombinant human insulin (26 IU/mg) and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human interferon β (0.6×10^7 IU/vial) was kindly supplied by Toray Industries, Inc. (Kanagawa, Japan). Crystalline salmon calcitonin was purchased from Calbiochem, an affiliate of Merck KGaA (Darmstadt, Germany). FITC-labeled BSA, methacrylic acid (MAA) and dimethoxy propyl acetophenone (DMPA) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Tetraethylene glycol dimethacrylate (TEGDMA) and poly (ethylene glycol) monomethylether monomethacrylate (PEGMA) were purchased from Polysciences Inc. (Warrington, PA, USA). All other chemicals were of analytical grade and commercially available.

2.2. Synthesis of complexation hydrogels

Microparticles of P(MAA-g-EG) were synthesized by a UV-initiated free-radical solution polymerization of MAA and PEGMA containing PEG tethers of molecular weight of 1000. The monomers were mixed to yield solutions with a 1:1 feed ratio of MAA/EG in the reactants of the polymerization reactions that produced the ensuing gels. TEGDMA, a crosslinking agent, was added in the amount of 0.075 mol% of the total monomers. Following the complete dissolution of the monomer, nitrogen was bubbled through the well-mixed solutions for 30 min to remove dissolved oxygen, which could inhibit polymerization by a free radical scavenger. The photoinitiator DMPA was added in the amount of 1wt% of the monomers in a nitrogen atmosphere. The reaction mixtures were pipetted between flat glass plates separated by Teflon spacers of 0.9 mm thickness, and exposed to UV light (Ultracure 100, Efos Inc., Buffalo, NY, USA) at 1 mW/cm^2 at 365 nm for 30 min. The ensuing hydrogel films were rinsed for a week in deionized water to remove the unreacted monomers and uncrosslinked oligomer chains. Then the copolymers were dried under vacuum at room temperature for two days and ground into powders with diameters of <53 μm.

2.3. Incorporation of peptides and proteins

Incorporation of peptides and proteins into samples of the above prepared and purified complexation hydrogels was performed by equilibrium partitioning as described previously [24]. In the case of insulin, 10 mg of powder was dissolved in 200 μL of 0.1 M HCl, and this solution was diluted with 19.6 mL of phosphate-buffered saline (PBS, pH 7.4). Furthermore, 200 μL of 0.1 M NaOH was added into this solution to adjust the pH to 7.4. In the case of calcitonin and BSA, 10 mg of calcitonin or BSA was dissolved in 20 mL of PBS. An IFN-β solution was prepared to 4.0×10^4 IU/mL. Loading was accomplished by imbibing 140 mg of dried microparticles for 2 h in each solution. The particles were collapsed with 20 mL of 0.1 M HCl and filtered using cellulose acetate/cellulose nitrate filter paper with 1.0μm pores. These loaded polymers were dried under vacuum and stored at 4°C prior to use in further studies.

The incorporation efficiencies of insulin, calcitonin and BSA were determined by high-performance liquid chromatography (HPLC). The HPLC was composed of a pump (LC-10AS, Shimadzu Co. Ltd., Kyoto, Japan), a UV detector (SPD-10A, Shimadzu), a system controller (SCL-10A), an autoinjector (SIL-10A, Shimadzu), and an integrator (C-R3A, Shimadzu). The HPLC measurement conditions used for each peptide and protein are described in Table 1. Meanwhile, the incorporation efficiency of IFN-β was determined using a human IFN-β EIA kit (Toray Industries, Inc.).

2.4. In vitro release study

Peptide and protein release experiments were performed following the Japanese Pharmacopoeia (JP) method. The first (pH 1.2) and the second (pH 6.8) fluids of JP were used for the release experiments, and the solution (37°C) was constantly stirred. Peptide or protein-loaded polymer particles (10 mg) were treated for 2 h with the first or second fluid (50 mL). Samples (0.2 mL) were taken at discrete intervals using filtered syringes (pore size: 10 μm; Ishikawa Manufactory). The concentration of insulin and calcitonin in each sample was determined by HPLC. The HPLC conditions are described in Table 1.

The concentration of IFN-β was determined using a human IFN-β EIA kit (Toray Industries, Inc.). In the release study, FITC-BSA was also used and its concentration was detected by using a microplate luminometer (Mithras LB940, Berthold Japan, Tokyo, Japan) at excitation and emission wavelengths of 485 and 535 nm, respectively. The fractional release

of peptides and proteins from the formulations was represented as the ratio of measured release amount to the theoretical maximum release amount.

2.5. In situ loop absorption experiments

The animal experiments in this work complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals at Hoshi University. Male Sprague–Dawley rats weighing 170–200 g fasted for 24 h prior to the experiments and were anesthetized by an i.p. injection of 50 mg/kg sodium pentobarbital (Dainippon Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan). The rats were restrained in a supine position and kept at a body temperature of 37°C using warming lamps. The ileum was exposed after a small midline incision was carefully made in the abdomen, and each segment (length = 10 cm) was cannulated at both ends using polypropylene tubing (4 mm o.d., 2 mm i.d.). These were securely ligated to prevent fluid loss and then carefully returned to their original location in the peritoneal cavity. To wash the intestinal content, phosphate-buffered saline (PBS; pH 7.4) at 37°C was singly circulated through the cannula at 1.0 ml/min for 20 min using an infusion pump (KD scientific Inc., Holliston, MA, USA). Subsequently, the segments were tightly closed following the removal of the cannulation tubing; approximately 0.5 ml of the perfusion solutions remained in the segments. Rats were left on the board at 37°C for 1 h to recover from the elevated blood glucose levels due to the surgical operations described above. Following 1 h of rest, pure calcitonin, calcitonin loaded P(MAA-g-EG) microparticles, pure interferon or interferon β loaded P(MAA-g-EG) microparticles (approximately 3 mg) with 0.5 ml of PBS solution was directly administered into loops (6 cm) made from the pretreated segment (10 cm). The ileal loop was made at the end of the small intestine, just proximal to the ileocecal junction. A calcitonin or interferon β PBS solution (0.5 ml) was used as a control. The dose for calcitonin was fixed at 200μg/kg body weight. The dose for interferon β was fixed at 0.15×10^6, 0.4×10^6 or 1.2×10^6μg/rat. During the experiment, a 0.25 ml blood aliquot was taken from the jugular vein at t=0, 5, 10, 15, 30, 60, 120, 180, 240 and 360 min after dosing. Tuberculin syringes (1 ml) were pre-heparinized in the usual fashion consisting of coating the syringe wall through the aspiration of heparin and then expelling all heparin by depressing the plunger to the needle hub. The plasma was separated by centrifugation at 13,000 rpm for 1 min. The plasma calcium levels were measured using a calcium test Wako (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and interferon β levels were determined using a human IFN-β EIA kit (Toray Industries, Inc.). The total area under the interferon β concentration curve (AUC) from time 0 to 360 min was estimated from the sum of successive trapezoids between each data point.

2.6. Statistical analysis

Each value is expressed as the mean ± S.E. of 3–8 determinations. For group comparisons, analysis of variance (ANOVA) with a one-way layout 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 discussions

3.1. Incorporation efficiency of peptides and proteins into polymer hydrogels

Previously, the studies in our laboratory revealed that P(MAA-g-EG) hydrogels had a high affinity to the peptide drug insulin, and that insulin was also efficiently incorporated into these hydrogels [24]. Therefore, it is possible that other peptides and proteins could also be efficiently incorporated into the hydrogels. In the present study, we evaluated the incorporation efficiency of peptides and proteins such as calcitonin, interferon β and BSA into hydrogels. As shown in Table 2, insulin was significantly incorporated into hydorogels (90.1 ± 3.1%) as well as the results in our previous report. Calcitonin was also effectively

incorporated and retained into hydrogels (51.3 ± 8.4%), however, this value was lower than that of insulin and interferon β. The relatively lower incorporation efficiency of calcitonin might be due to its lower molecular weight (insulin: 5.8 kDa and interferon β: 23 kDa vs. calcitonin: 3.4 kDa). On the other hand, in our previous report, fluorescein isothiocyanate-labeled dextran 4,400 (FD-4) having similar molecular weight (average Mw 4.4 kDa) to calcitonin showed low incorporation efficiency (7.0 ± 0.3%). In addition, the incorporation efficiency of other non-peptide compounds, such as theophylline, and dextrans, FD-10 (Mw 10 kDa) and FD-20 (Mw 20 kDa) into these hydrogels was not high (1.1 ± 0.5, 5.2 ± 0.2 and 5.2 ± 0.7%, respectively) [24]. These results suggest that P(MAA-g-EG) hydrogels have a specifically high affinity to peptide drugs. While non-peptide/protein drugs have simple linear structures, peptides and proteins may have more bulky structures. Therefore, the difference of incorporation efficiency between peptides and non-peptides might be attributed to such structural differences.

Since interferon β (Mw: 23 kDa) was more efficiently incorporated into hydrogels than calcitonin (Table 2), it is considered that P(MAA-g-EG) can be applied for large molecular weight proteins. However, the incorporation efficiency of a larger protein, BSA (Mw: 69 kDa), into hydrogels was lower than that of other peptides and proteins. In addition, the incorporation efficiency of BSA prior to acid treatment was also lower than that of other peptides and proteins (data not shown), suggesting that incorporation of BSA was limited by its high molecular weight and that the incorporation efficiency of peptides and proteins is dependent on the molecular weight.

3.2. Release of peptides and proteins from polymer hydrogels

Previously it was reported that P(MAA-g-EG) hydrogels have the ability to control the insulin release from hydrogels in response to changes of the environmental pH [12–14]. In the present study, the release characteristics of various peptide and protein drugs incorporated in hydrogels were examined. Insulin release from hydrogels is shown in Fig. 1A. Insulin was retained in the polymer hydrogels at pH 1.2, whereas insulin was immediately released from the hydrogels at pH 6.8. A steady state was achieved in approximately 10 min, implying that P(MAA-g-EG) hydrogels are ideal for oral insulin absorption.

Based on this result, the release of other peptide and protein drugs from P(MAA-g-EG) hydrogels was also estimated. As shown in Fig. 1B, the release of calcitonin from hydrogels at the pH 1.2 condition was approximately 40%, which is higher than the release efficiency of insulin at the same condition. The relatively higher efficiency of calcitonin release at pH 1.2 is maybe because of its lower molecular weight than insulin, as small molecules are easily leaked from the hydrogels. In contrast at pH 6.8, the efficiency of calcitonin release from the hydrogels was similar to that of insulin. On the other hand, the release of interferon β from the P(MAA-g-EG) hydrogels at pH 1.2 was as low as that of insulin, as shown in Fig. 1C. However, the release of interferon β at pH 6.8 was lower than that of insulin. The low release of interferon β at acidic condition was due to small mesh size of P(MAA-g-EG) hydrogels, meanwhile sufficient release of interferon β was not observed at neutral condition contrary to our expectation. Interferon β has much higher molecular weight than insulin (23 kDa vs. 5.8 kDa) and also it has bulky sugar chains. This might be a hindrance factor for the release from the polymer network. In addition, interferon β has positive charge in the neutral solution, therefore it may interact with carboxylic acid of the polymer. This may also be the reason for the unexpected lower release of interferon β. Furthermore, the low release of interferon β from the hydrogels under neutral pH conditions can be attributed to insufficient diffusion of interferon β due to its low content (insulin: 6.7% vs. interferon β: 0.057%). Since we have already demonstrated that the release of drug from P(MAA-g-EG) hydrogels at neutral condition was improved by increasing of drug loading amount in hydrogels [16],

low release efficiency of interferon β under neutral pH conditions might be improved by increasing the content of interferon β in the hydrogels. Because the analytical sensitivity of BSA by HPLC was relatively limited, the release efficiency of BSA from the P(MAA-g-EG) hydrogels was measured by using FITC-labeled BSA, which has an incorporation efficiency almost identical to that of non-labeled BSA (data not shown). As shown in Fig. 1D, the release of FITC-BSA from the hydrogels at pH 1.2 was relatively higher than that of insulin. High release efficiency of BSA is derived from the fraction adsorbed to the hydrogel surface rather than from the incorporated fraction due to the bulky conformation of BSA, which also has a much greater molecular weight. The release of FITC-BSA from the hydrogels was readily observed at pH 6.8, as with other peptides and proteins. As mentioned above, although the release efficiency of peptides and proteins from the P(MAA-g-EG) hydrogels under a neutral pH condition was dependent on their content in the hydrogels, it appears that the P(MAA-g-EG) hydrogels have the ability to control the release profiles of peptides and proteins in a manner that is dependent on the environmental pH. Therefore, these polymer hydrogels are likely feasible devices for delivering the various peptide and protein drugs following oral administration.

3.3. Improvement of ileal peptide and protein absorption using polymer hydrogels

The absorption of orally administered peptides and proteins is significantly limited by several factors, such as digestion in the stomach, enzymatic degradation in the intestine, and low permeability across the epithelial membranes [1,2]. Based on the P(MAA-g-EG) hydrogels controlling the release efficiency as a function of the environmental pH, we hypothesized that the absorption of orally administered peptides and proteins can be improved by using these polymer hydrogels. Moreover, P(MAA-g-EG) hydrogels improve the intestinal absorption of peptide and protein drugs through, for example, the adsorption to intestinal mucosa [19–21] and inhibition of enzymatic degradation [17,18]. Because the hydrogels may facilitate the intestinal absorption of peptides and proteins, the absorption of calcitonin and interferon β incorporated in P(MAA-g-EG) hydrogels was examined. Figure 2 shows plasma calcitonin concentration, which was measured as the index of calcitonin absorption, versus time profiles following ileal administration of the calcitonin-loaded P(MAA-g-EG). A slight decrease in plasma calcium concentration was observed during the administration of the pure calcitonin solution. In contrast, the administration of the calcitonin-loaded P(MAA-gEG) decreased the plasma calcium concentration compared with that of the pure calcitonin solution, and a decreased calcitonin concentration was kept at the end of experiments. Figure 3 shows the plasma interferon β concentration versus time profiles following ileal administration of various doses of the interferon β-loaded P(MAA-g-EG). No significant absorption was observed with the pure interferon β solution at any dose. In contrast, the higher absorption of interferon β was observed by the administration of the interferon β-loaded P(MAA-g-EG) compared with that of pure interferon β. In addition, as shown in Fig. 3 and Table 3, interferon β absorption increased with the dose of interferon β-loaded P(MAA-g-EG). Therefore, the application of P(MAA-g-EG) hydrogels enables not only improvement of absorption of peptides and proteins but also control of their plasma concentration by adjusting the dose of hydrogels.

The advantages of the P(MAA-g-EG) include the absorption-enhancing mechanisms, such as the adsorption of hydrogels to the intestinal mucosa [19–21], and the protease inhibitory effect in the intestinal tract [17,18], as well as the sufficient incorporation affinity of peptides and proteins into the hydrogels and their controlled release in response to environmental pH. Namely, it is speculated that the anchoring effect of the PEG chain on P(MAA-g-EG) leads to their release beside the epithelial membrane. Furthermore, a calcium-adsorptive effect may be due to the depolymerized carboxyl functional groups on

methacrylic acids, which circumvent the enzymatic degradation of peptides and proteins within the mucus/glycocalyx layer.

Thus, the increase of peptide and protein concentrations beside the absorptive region leads to an augmentation of the concentration gradient and subsequently to efficient absorption of the peptides and proteins from the intestinal tract. Meanwhile, it has been demonstrated that the improved absorption using P(MAA-g-EG) hydrogels was accomplished regardless of the paracellular pathway [21]. Therefore, the efficient absorption of calcitonin and interferon β using the P(MAA-g-EG) hydrogels in the present study led to the high concentration of intact peptides and proteins in the absorptive region and subsequent permeation of them across the intestinal epithelium via transcellular pathways.

4. Conclusions

In this study, we demonstrated that P(MAA-g-EG) hydrogels have high incorporation properties not only for insulin but also for calcitonin and interferon β, and the peptides and proteins incorporated into these hydrogel are released in response to environmental pH. Furthermore, we found that the intestinal administration of calcitonin and interferon β using these hydrogels significantly improved absorption of these drugs. These findings suggest that P(MAA-g-EG) hydrogels are likely potential carriers to enable the administration of various peptides and proteins via the oral route.

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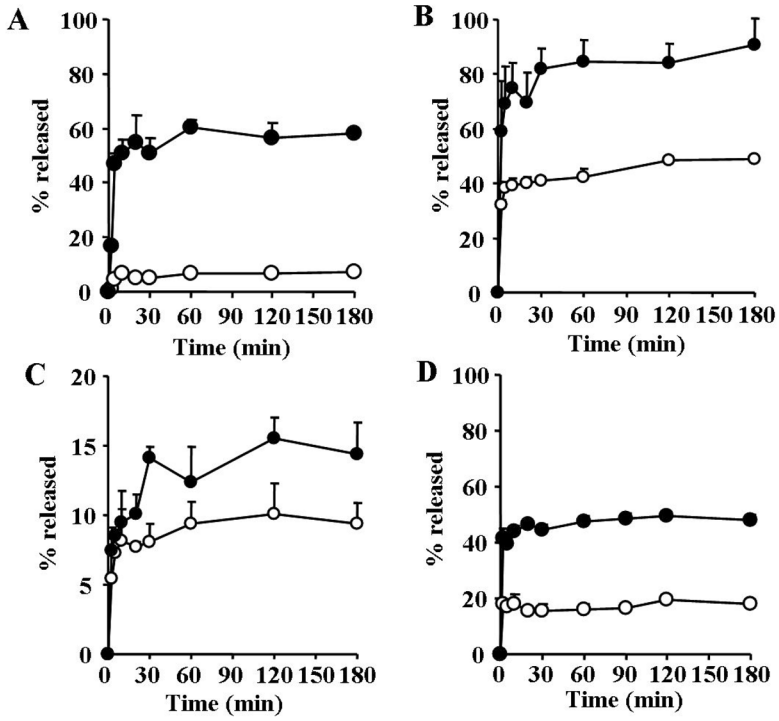

Fig. 1.

Release profiles of insulin (A), calcitonin (B), interferon β (C) and BSA (D) from P(MAA-g-EG) microparticles in the 1st (pH 1.2) (open circles) or 2nd (pH 6.8) (closed circles) fluids of Japanese Pharmacopoeia. Each data point represents the mean ± S.E. from n=3–4.

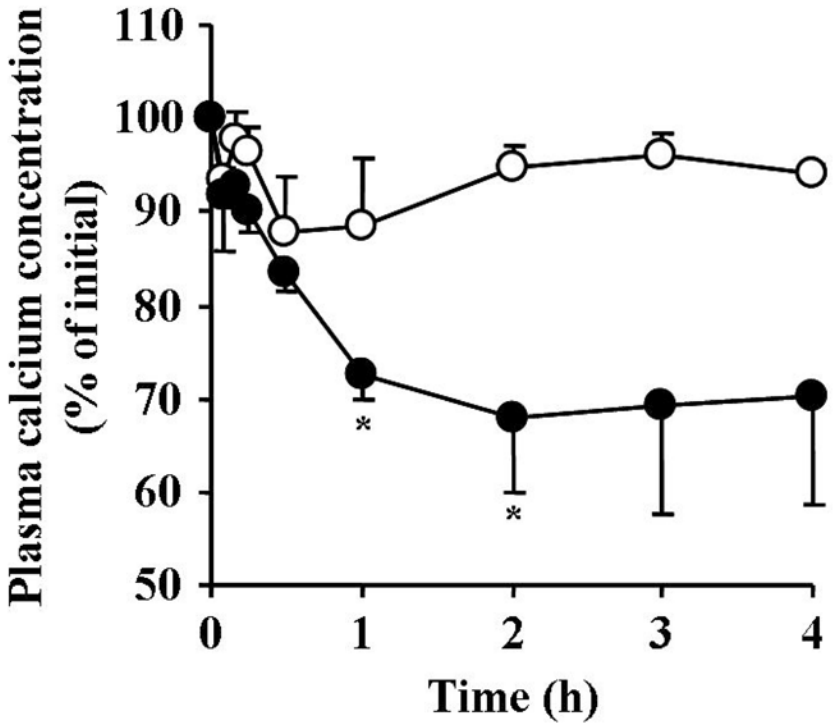

Fig. 2.

Plasma calcium concentration vs. time profiles following in situ administration of calcitonin-loaded P(MAA-g-EG) microparticles (closed circles) or calcitonin solution (open circles) into the ileal segments. Each data point represents the mean ± S.E. from n=3–5. *$p <$ 0.05, significant difference compared with “calcitonin solution”.

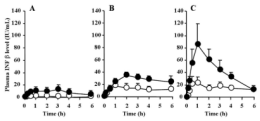

Fig. 3.

Plasma interferon β concentration vs. time profiles following in situ administration of interferon β loaded P(MAA-g-EG) microparticles (closed circles) or interferon b solution (open circles) into the ileal segments. A: 0.75×10^6 IU/kg, B: 2.0×10^6 IU/kg, C: 6.0×10^6 IU/kg of interferon β. Each data point represents the mean ± S.E. from n=3–8.

Table 1

Chromatographic conditions

	Mobile phase	Flow (mL/min)	Wavelength (nm)	Column
Insulin	Acetonitrile-0.1% trifluoroacetic acid-sodium chloride (31: 69: 0.58, v/v/w)	1.0	220	GL-Pack Nucleosil 100-5C18 (150 × 4.6mm i.d.)
Calcitonin	Acetonitrile-0.1% trifluoroacetic acid-sodium chloride (35: 65: 0.58, v/v/w)	1.0	220	GL-Pack Nucleosil 100-5C18 (150 × 4.6mm i.d.)
BSA	0.1M Sodium dihydrogen phosphate-0.1M Sodium hydrogen phosphate	0.6	280	TSKgel G3000SWXI (300 × 7.8mm i.d.)

Table 2

Drug incorporation ratio

	Incorporation ratio (%)
Insulin	90.1 ± 3.1
Calcitonin	51.3 ± 8.4
Interferon β	76.8 ± 5.3
BSA	17.0 ± 4.6

Each value represents the mean ± S.E. from n=3–4.

Table 3

AUC values following in situ administration of interferon β loaded P(MAA-g-EG) microparticles into the ileal segments

	AUC	
Dose (× 10^6 IU/kg)	**Interferon β solution**	**Interferon β loaded P(MAA-g-EG)**
0.75	6.7 ± 6.0	48.4 ± 6.7**
2.0	74.6 ± 14.2	154.4 ± 29.0
	#	#
6.0	84.8 ± 32.1	257.9 ± 80.7

AUC: the area under the curve.

Each value represents the mean ± S.E. from n=3–8.

** $p < 0.01$, significant difference compared with the corresponding “interferon β solution”.

# $p < 0.05$, significant difference among doses of “interferon β solution” or “interferon β loaded P(MAA-g-EG)”.