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Stabilization of salmon calcitonin by polystyrene nanoparticles having surface hydrophilic polymeric chains, against enzymatic degradation

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Abstract

The effect of polystyrene nanoparticles having surface hydrophilic polymeric chains on the stability of salmon calcitonin (sCT) in the presence of digestive enzymes was investigated in vitro. sCT was protected against pepsin- or trypsin-catalyzed degradation by nanoparticles other than those with surface poly(*N*-vinylacetamide) chains, which do not enhance sCT absorption via the gastrointestinal tract in vivo. This stabilizing effect was affected by the structure of the polymeric chains. Nanoparticles whose surface was covered by poly(*N*-isopropylacrylamide) inhibited completely sCT degradation by pepsin. However, they did not increase sCT stability in the presence of trypsin. The degradation of sCT by trypsin was inhibited totally by nanoparticles with surface poly(methacrylic acid) chains, even though sCT stability in the presence of pepsin was only slightly improved by them. Nanoparticles having poly(vinylamine) chains on their surfaces stabilized sCT in the presence of either enzyme. It is probable that the stabilizing effect results mainly from the physicochemical interaction between the enzyme and the nanoparticles. These results demonstrated that nanoparticles have the property of stabilizing peptide drugs in the gastrointestinal tract, and that this property affects the absorption enhancement of orally administered sCT. © 1997 Elsevier Science B.V.

Keywords: Nanoparticle; Absorption enhancement; Stabilization; Salmon calcitonin; Pepsin; Trypsin

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1. Introduction

Nanoparticles have been studied extensively as carriers for oral drug delivery, whose purposes are to improve the bioavailability of drugs with poor absorption characteristics (Kreuter, 1991; Couvreur and Puisieux, 1993; Couvreur et al., 1995), to deliver vaccine antigens to the gut associated lymphoid tissues (Eldridge et al., 1990), to control the release of drugs (Hubert et al., 1991), to reduce drug-caused gastrointestinal (GI) mucosal irritation (Ammoury et al., 1991), and to ensure drug stability in the GI tract (Roques et al., 1992). We also demonstrated previously that nanoparticles composed of novel graft copolymers having a hydrophobic backbone and hydrophilic branches, are very useful as drug carriers (Akashi et al., 1985, 1989a). These nanoparticles are prepared by the free radical copolymerization between macromonomers, which are hydrophilic polyvinyl compounds terminating in vinylbenzyl groups, and hydrophobic styrene (Akashi et al., 1990). The surface of the nanoparticles obtained is covered by hydrophilic polymeric chains (Akashi et al., 1989b). By designing and synthesizing different functional macromonomers, a variety of nanoparticles having functional polymeric chains on their surfaces can be obtained (Capek and Akashi, 1993).

In our previous study (Sakuma et al., 1997a), we investigated the potential of these nanoparticles as carriers for oral peptide delivery using salmon calcitonin (sCT) in rats, and found that sCT absorption via the GI tract was enhanced by these nanoparticles. This absorption enhancement was affected by the macromonomer structure, and sCT absorption was enhanced most strongly by nanoparticles having poly(*N*-isopropylacrylamide) (PNIPAAm) chains on their surfaces. The absorption of sCT was also enhanced by nanoparticles with surface poly(vinylamine) (PVAm) or poly(-methacrylic acid) (PMAA) chains, although these effects were weaker than that of PNIPAAm nanoparticles. However, there was no absorption enhancement of sCT by poly(*N*-vinylacetamide) (PNVA) nanoparticles. We consider that the absorption enhancement of sCT by nanoparticles results mainly from both bioadhesion of nanoparticles incorporating sCT to the GI mucosa and an increase in the stability of sCT in the GI tract.

In this study, the stabilizing effect of nanoparticles on the enzymatic degradation of sCT was evaluated as one of the mechanism of absorption enhancement.

2. Materials and methods

2.1. Materials

Pepsin (2100 IU/mg), trypsin (11 000 BAEE IU/mg) and sCT were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-vinylacetamide (NVA) monomer was supplied by Showa Denko Co. (Tokyo, Japan). *N*-isopropylacrylamide (NIPAAm) and *t*-butyl methacrylate (BMA) monomers were obtained from Kohjin Co. (Tokyo, Japan). *p*-Chloromethyl styrene (*p*-CMSt) was furnished by Nippon Oil and Fats Co. (Tokyo, Japan). All other chemicals were commercial products of reagent grade. These materials were purified in the usual manner when necessary (Riza et al., 1995; Chen et al., 1996).

2.2. Preparation of nanoparticles

2.2.1. Preparation

PNIPAAm, PNVA, PVAm and PMAA nanoparticles were prepared by the procedures reported in earlier articles (Riza et al., 1995; Chen et al., 1996; Sakuma et al., 1997a). Oligomerization of each monomer initiated by 2,2′-azobisisobutyronitrile (AIBN) was carried out in the presence of 2-mercaptoethanol to give oligomers terminating in hydroxyl groups. These oligomers were condensed with *p*-CMSt to obtain macromonomers terminating in vinylbenzyl groups. After dispersion copolymerization initiated by AIBN between the macromonomers and styrene, PNIPAAm, PNVA and polyBMA nanoparticles were obtained. The molar concentration of styrene was 40 times that of macromonomers. PVAm and PMAA nanoparticles were prepared by the hydrolysis of PNVA and polyBMA nanoparticles, respectively. The resulting nanoparticles were dialyzed to remove unreacted substances, and then lyophilized.

2.2.2. Characterization

The nanoparticles were characterized using the method described in an earlier article (Sakuma et al., 1997a). Briefly, the number-average molecular weight (Mn) and the weight-average molecular weight (Mw) of macromonomers on the nanoparticle surface were determined by gel permeation chromatography. The particle size of the nanoparticles was measured by dynamic light-scattering spectrophotometry. The IR spectra were used to confirm the presence of amino and carboxyl groups on the surface of PVAm and PMAA nanoparticles, respectively.

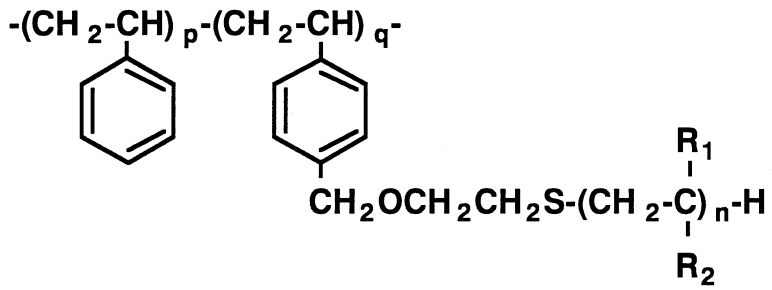
2.3. Stability of sCT in the presence of nanoparticles

2.3.1. Effect of nanoparticles on enzymatic degradation of sCT

Twenty mg of the lyophilized nanoparticles were redispersed in 0.9 ml of purified water (22.2 mg/ml). sCT was dissolved in purified water and the concentration was adjusted to 0.222 mg/ml. The aqueous solution of sCT was mixed with the same volume of the nanoparticle dispersion and with purified water. Separately, pepsin was dissolved in a hydrochloric acid–sodium chloride (HCl–NaCl) buffered solution of which pH was 1.2 and ionic strength was 0.1, and the concentration was adjusted to 10 000 IU/ml. This pepsin solution (0.1 ml) was mixed with 0.9 ml of the mixture of sCT with nanoparticles and with 0.9 ml of sCT aqueous solution (0.111 mg/ml), respectively, and the incubation was carried out at 37°C. The initial concentrations of sCT, nanoparticles and pepsin in the sample solution (1 ml) were 0.1 mg/ml, 10 mg/ml and 1000 IU/ml, respectively. At given time intervals, the enzymatic reaction was stopped by the addition of 1 ml of an ice-cold acetonitrile/purified water mixture (3/1, v/v) containing trifluoroacetic acid (1 mg/ml) and urea (1.6 mg/ml). Each sample (2 ml) was subsequently cooled at 4°C for 20 min, and was left at 40°C for 2 h to liberate the sCT incorporated in the nanoparticles. After the sample was centrifuged at 15 000 rpm for 30 min at 40°C, the sCT concentration in the supernatant, which included the total amount of sCT both incorporated and not incorporated in the nanoparticles, was determined by the HPLC procedure reported in our previous study (Sakuma et al., 1997a). The effect of nanoparticles on sCT degradation by trypsin was also examined under the same conditions except that a phosphate buffered solution (pH: 6.8; ionic strength: 0.1) was used as a solvent of trypsin end the trypsin concentration just before incubation was 3 BAEE IU/ml.

2.3.2. Effect of nanoparticles on enzymatic activity

Ten mg of the lyophilized nanoparticles were redispersed in 0.9 ml of purified water (11.1 mg/ml). This nanoparticle dispersion (0.9 ml) was mixed with 0.1 ml of the pepsin solution (10 000 IU/ml) and with 0.1 ml of a trypsin solution whose concentration was 3000 BAEE IU/ml, respectively, and these mixtures were incubated at 37°C for 20 min. The concentrations of nanoparticles and pepsin in the sample solution were the same as those in Section 2.3.1. The trypsin concentration, just before incubation, was changed to 300 BAEE IU/ml to evaluate precisely how much its activity changed. After the sample was centrifuged at 15 000 rpm for 30 min at 37°C, the enzymatic activity in the supernatant was determined by the conventional methods. Namely, pepsin activity was calculated from the change of the absorbance at 280 nm/min at a pH of 2.0 and a temperature of 37°C using hemoglobin as the substrate. Trypsin activity was calculated from that at 253 nm/min, pH 7.6 and 25°C using BAEE (N-α-benzoyl-L-arginine ethyl ester) as the substrate. The effect of oligomers, which are located on the nanoparticle surface as hydrophilic polymeric chains, on the enzymatic activity was also examined as well as that of nanoparticles. Ten mg of oligomers were dissolved in 0.9 ml of purified water, and this solution was mixed with 0.1 ml of the pepsin solution (10 000 IU/ml) and with 0.1 ml of the trypsin solution (3000 BAEE IU/ml), respectively. After the incubation at 37°C for 20 min, the assay of the enzymatic activity in these samples was carried out. In addition, the enzymatic activity in the absence of nanoparticles or oligomers was measured under the same conditions, taking account of the decrease of the activity due to this experimental procedure.



	R_1	R_2
PNIPAAm nanoparticles	H	$CONHCH(CH_3)_2$
PNVA nanoparticles	H	$NHCOCH_3$
PVAm nanoparticles	H	NH_2
PMAA nanoparticles	CH_3	COOH

Fig. 1. Chemical structures of nanoparticles.

2.3.3. Stability of sCT incorporated or not incorporated in nanoparticles

Mixtures of sCT and enzyme with or without nanoparticles were incubated for 20 min under the conditions described in Section 2.3.1. The concentrations of sCT, nanoparticles, pepsin and trypsin in the sample solution (1 ml) just before incubation were adjusted to 0.1 mg/ml, 10 mg/ml, 1000 IU/ml and 3 BAEE IU/ml, respectively. After incubation, 0.5 ml of the sample solution was mixed with 0.5 ml of ice-cold purified water. This mixture was centrifuged immediately at 15 000 rpm for 30 min at 4°C and 0.5 ml of the supernatant was mixed with 0.5 ml of the acetonitrile/purified water mixture (3/1, v/v) containing trifluoroacetic acid (1 mg/ml) and urea (1.6 mg/ml) (method 1). The other 0.5 ml of the sample solution was mixed with the acetonitrile/water mixture. After this mixture was treated by the same procedure as that in Section 2.3.1, 0.5 ml of the supernatant was mixed with 0.5 ml of purified water (method 2). The concentrations of sCT in each supernatant obtained from the above two methods were determined by the HPLC procedure. The concentrations of sCT not incorporated in nanoparticles, incorporated in them, and degraded by enzyme were defined, respectively, as the concentration obtained by the method 1, the difference in concentration between two methods, and the difference in concentration between method 2 and the original mixture just before incubation.

3. Results

3.1. Preparation of nanoparticles

Fig. 1 and Table 1 show the chemical structures and the characteristics of the nanoparticles, respectively. These characteristics were the same as those described in the previous studies (Riza et al., 1995; Chen et al., 1996; Sakuma et al., 1997a), as follows. The number-average molecular weight of macromonomers on the nanoparticle surface was adjusted to the order of 10^3 and the weight-

Table 1
Characteristics of nanoparticles

	Mn[a]	Mw/Mn[b]	Diameter (nm)[c]
PNIPAAm nanoparticles	5.2	2.2	750
PNVA nanoparticles	8.3	2.1	670
PVAm nanoparticles	8.5	2.1	720
PMAA nanoparticles	3.9	2.0	500

[a] Number-average molecular weight of macromonomers ($\times 10^{-3}$).
[b] Weight-average molecular weight (Mw)/number-average molecular weight (Mn) ratio.
[c] Weight-average diameter.

average diameter of nanoparticles was less than 1000 nm. The presence of the amino and carboxyl groups on the surfaces of PVAm and PMAA nanoparticles, respectively, were confirmed by the IR spectra (data not shown). All monodispersed nanoparticles possessed good water-dispersibility because hydrophilic polymeric chains were located on the surface of hydrophobic polystyrene nanoparticles as shown in Fig. 2 (Akashi et al., 1989b).

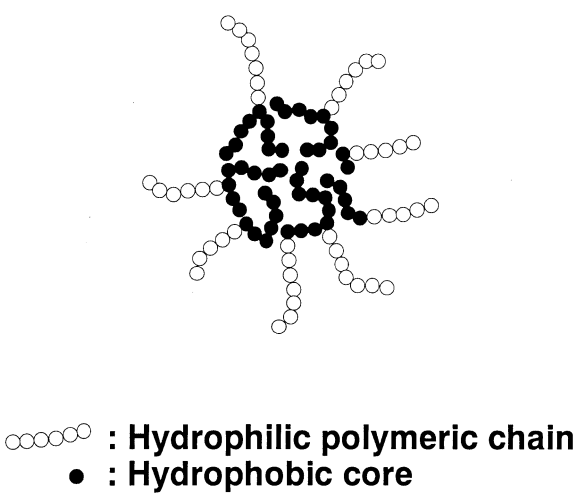


Fig. 2. Schematic representation of nanoparticle form.

3.2. Stability of sCT in the presence of nanoparticles

3.2.1. Effect of nanoparticles on enzymatic degradation of sCT

Fig. 3 shows the residual amounts of sCT after incubation with enzyme in the presence or absence of nanoparticles. In this experiment, sCT was not degraded at all when no enzyme was present (data not shown) and more than 95% of sCT incorporated in the nanoparticles was released from them by the addition of the acetonitrile/water mixture, irrespective of the nanoparticle type. PNIPAAm and PVAm nanoparticles completely protected sCT against degradation by pepsin. The stability of sCT in the presence of pepsin was also improved slightly by PNVA or PMAA nanoparticles. On the other hand, sCT degradation by trypsin was completely inhibited by PMAA nanoparticles and was somewhat reduced by PVAm nanoparticles. However, PNIPAAm and PNVA nanoparticles did not exhibit at all the stabilizing effect on sCT degradation by trypsin.

3.2.2. Effect of nanoparticles on enzymatic activity

We next examined the effect of nanoparticles on the activity of pepsin and trypsin as shown in Fig. 4. After pepsin was incubated with PNIPAAm or PVAm nanoparticles at 37°C for 20 min in the HCl–NaCl buffered solution, the pepsin activity in the supernatants decreased to 100 or 150 IU/ml, respectively. In the case of PNVA and PMAA nanoparticles, there was no marked decrease of pepsin activity, as compared with the control solution that contained no nanoparticles. Trypsin activity in the supernatant was reduced by PMAA or PVAm nanoparticles to less than 50 BAEE IU/ml. However, PNIPAAm and PNVA nanoparticles did not affect the trypsin activity. On the other hand, when the respective enzymes were incubated with oligomers, there was no decrease in the enzymatic activity, irrespective of oligomer type (data not shown).

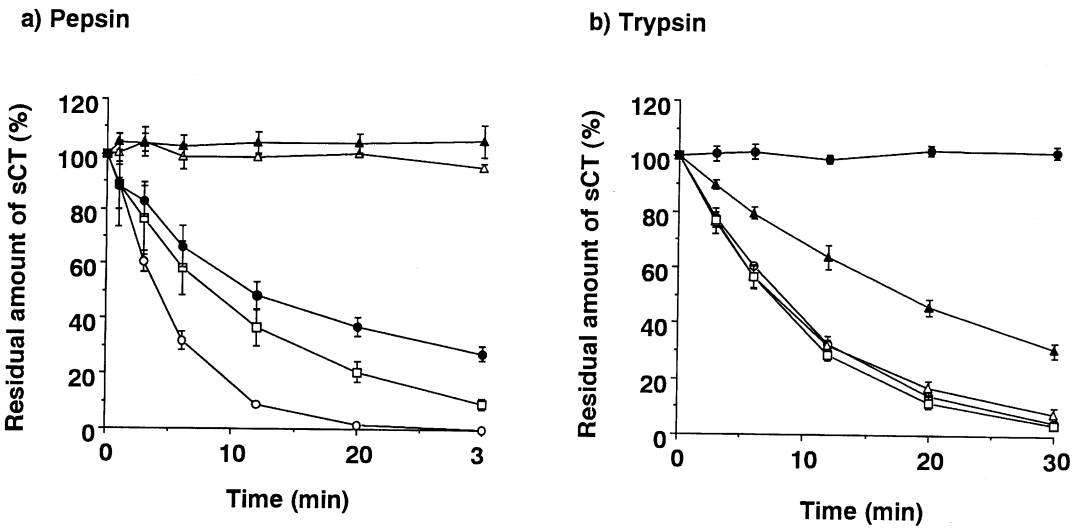


Fig. 3. Residual amount of sCT after incubation of sCT alone (○), a mixture of sCT and PNIPAAm nanoparticles (△), a mixture of sCT and PNVA nanoparticles (□), a mixture of sCT and PVAm nanoparticles (▲) and a mixture of sCT and PMAA nanoparticles (●) with pepsin and with trypsin in a hydrochloric acid–sodium chloride buffered solution of pH 1.2 and a phosphate buffered solution of pH 6.8, respectively, at 37°C. The initial concentrations of sCT, nanoparticles, pepsin and trypsin were 0.1 mg/ml, 10 mg/ml, 1000 IU/ml and 3 BAEE IU/ml, respectively. Each value represents the mean + S.D. of three experiments.

3.2.3. Stability of sCT incorporated or not incorporated in nanoparticles

Fig. 5 shows the respective amounts of the sCT that was incorporated or not incorporated in nanoparticles after incubation of the mixture of sCT and nanoparticles with each enzyme for 20 min at 37°C in adequate buffered solution. When the mixture was incubated with pepsin, most of the residual sCT was free from nanoparticles, whereas about 70% of the sCT stabilized by PVAm or PMAA nanoparticles in the presence of trypsin was incorporated in them. The amounts of free sCT in the mixtures including these ionic nanoparticles were consequently lower than those in the mixtures including nonionic PNIPAAm or PNVA nanoparticles which did not stabilize sCT in the presence of trypsin.

4. Discussion

It has been reported that some of those phospholipids, cyclodextrins and polyacrylates that are used as excipients in medical products protect peptide and protein drugs against degradation by the digestive enzymes such as pepsin, trypsin, chymotrypsin, aminopeptidase and carboxypeptidase (Weingarten et al., 1985; Haeberlin et al., 1996; Lueßen et al., 1996). Yamamoto et al. (1994) have also studied the effect of protease inhibitors on the intestinal absorption of insulin in rats, and found that the reduction in the proteolytic rate of insulin was related to the decrease in plasma glucose concentration after insulin was administered into the intestinal loop with these protease inhibitors.

In the present study, we also demonstrated that polystyrene nanoparticles having hydrophilic polymeric chains on their surfaces can stabilize peptide drugs in the GI tract. This stabilization of sCT was affected by the structure of the hydrophilic polymeric chains and the most suitable structure depended on the type of enzymes. No degradation of sCT by pepsin occurred at all in the presence of PNIPAAm nanoparticles. PMAA nanoparticles completely inhibited sCT degradation by trypsin. PVAm nanoparticles protected sCT against degradation both by pepsin and by trypsin.

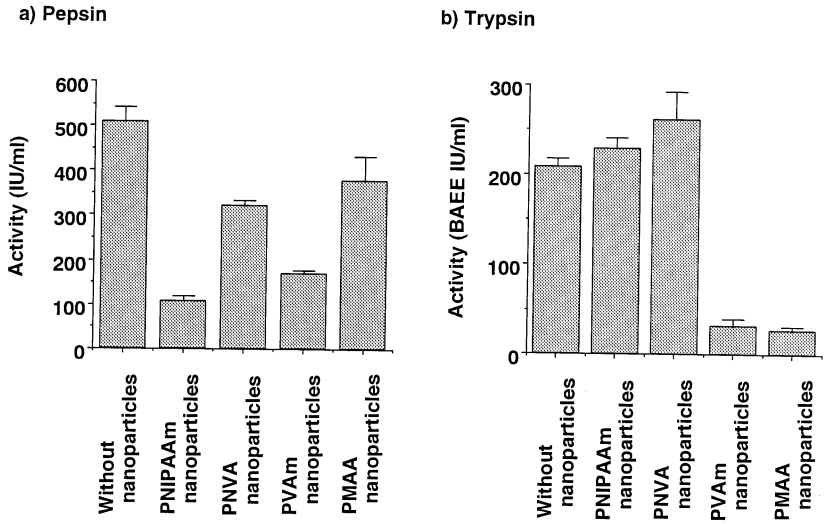


Fig. 4. Enzymatic activity after pepsin and trypsin were incubated in a hydrochloric acid-sodium chloride buffered solution of pH 1.2 and a phosphate buffered solution of pH 6.8, respectively, in the presence or absence of nanoparticles at 37°C for 20 min. The initial concentrations of sCT, nanoparticles, pepsin and trypsin were 0.1 mg/ml, 10 mg/ml, 1000 IU/ml and 300 BAEE IU/ml, respectively. Each value represents the mean ± S.D. of three experiments.

We consider this stabilizing effect results from the interactions among sCT, enzyme and hydrophilic polymeric chains on the nanoparticle surface. When pepsin and trypsin were incubated with nanoparticles that protected sCT against degradation by these enzymes, the activity of each enzyme in the supernatants of the mixtures decreased, as shown in Fig. 4. There was a good correlation between this decrease and the degree of the stabilizing effect. On the other hand, oligomers had no effect on the enzymatic activity. These results indicate that the enzyme is not inactivated chemically but is captured physicochemically by the surface polymeric chains of nanoparticles so that the amounts of free enzymes decrease.

On the other hand, as is obvious from Fig. 5(a), sCT was almost all released from PNIPAAm or PVAm nanoparticles in the presence of pepsin. This suggests that most sCT is attacked by pepsin which is not captured by nanoparticles. Indeed, the half-life of sCT degradation was 25 min when sCT alone was incubated with pepsin whose concentration was 200 IU/ml (data not shown). Nevertheless, PNIPAAm and PVAm nanoparticles protected sCT fully against degradation by pepsin. The acidamide binding site of sCT that is hydrolyzed by pepsin may be protected by these hydrophilic polymeric chains from attack by pepsin, even though there is no direct interaction between sCT and the nanoparticles. The details of the above mechanism will be described in future reports.

On the other hand, sCT stability in the presence of trypsin also depended on the incorporation rate of sCT in nanoparticles (Fig. 5(b)), differing from the case of pepsin. The interaction between sCT and nanoparticles probably contributes to sCT stabilization in the presence of trypsin as well as the interaction between trypsin and nanoparticles. The stabilization of sCT by PMAA nanoparticles appears to have been stronger than that of PVAm nanoparticles because the amount of sCT incorporated in the former nanoparticles was larger than that in the latter. However, it is considered that sCT incorporation in nanoparticles is not good for enhancing sCT absorption via the intestinal tract. The amount of sCT not incorporated in PVAm nanoparticles, which was absorbed via the intestinal membrane, was lower than that in PNIPAAm nanoparticles so that the absorption enhancement of sCT by PNIPAAm nanoparticles

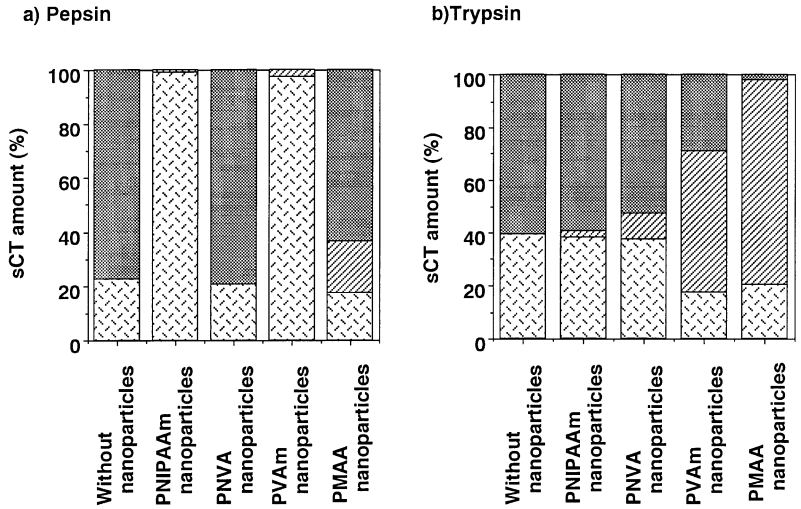


Fig. 5. Amounts of sCT degraded by enzyme (▩), incorporated in nanoparticles (▨), and not incorporated in them (⊠) after sCT was incubated with pepsin and with trypsin in a hydrochloric acid–sodium chloride buffered solution of pH 1.2 and a phosphate buffered solution of pH 6.8, respectively, in the presence or absence of nanoparticles at 37°C for 20 min. The initial concentrations of sCT, nanoparticles, pepsin and trypsin were 0.1 mg/ml, 10 mg/ml, 1000 IU/ml and 3 BAEE IU/ml, respectively. Each value represents the mean of three experiments.

was probably greater than that by PVAm nanoparticles. It seems that PMAA nanoparticles can not enhance sCT absorption sufficiently because of the tight interaction between them and sCT, and because of the lack of the stabilizing effect on sCT degradation by pepsin.

The above interpretation apparently corresponds to the ranking of the effectiveness of nanoparticles for enhancing sCT absorption via the GI tract (Sakuma et al., 1997a). However, the present study was carried out using a simple in vitro system consisting of sCT, enzyme and nanoparticles. The interactions among these substances are probably affected by the other components such as mucin. The molecular weight, the distribution of the molecular weight and the density of the hydrophilic polymeric chains on the nanoparticle surface perhaps affect the stabilizing effect. It is also necessary to examine the correlation between in vitro and in vivo. On the other hand, we consider that the mucoadhesion of nanoparticles affects the absorption enhancement of sCT. It has been already suggested that PNIPAAm nanoparticles have the property of adhering to the GI mucosa (Sakuma et al., 1997b). As additional effects of nanoparticles, we have to take account of the uptake of nanoparticles by Peyer's patches (Tomizawa et al., 1993; Florence et al., 1995), or the opening of the tight junction between the intestinal epithelial cells by nanoparticles (Borchard et al., 1996). Further work will be successively discussed in future reports.

5. Conclusions

We investigated the effect of nanoparticles having surface hydrophilic polymeric chains on the stability of sCT in the presence of digestive enzymes. It was demonstrated that these nanoparticles stabilize sCT in the GI tract. This stabilization of sCT was affected by the structure of the surface polymeric chains and the most suitable structure depended on the type of enzymes. PNIPAAm and PVAm nanoparticles completely protected sCT against degradation by pepsin. The degradation of sCT by trypsin was totally inhibited by PMAA nanoparticles and was somewhat reduced by PVAm nanoparticles. PNIPAAm nanoparticles did not substantially stabilize sCT in the presence of trypsin, nor did PMAA nanoparticles when pepsin was present. It was considered that the stabilizing effect of nanoparticles on the enzymatic degradation of sCT results mainly from the physicochemical interaction between the enzyme and the hydrophilic polymeric chains on the nanoparticle surface.

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