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International Journal of Pharmaceutics 149 (1997) 93–106

international journal of pharmaceutics

Oral peptide delivery using nanoparticles composed of novel graft copolymers having hydrophobic backbone and hydrophilic branches

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Received 5 October 1996; received in revised form 21 November 1996; accepted 14 December 1996

Abstract

Nanoparticles composed of new graft copolymers having a hydrophobic backbone and hydrophilic branches were prepared by the dispersion copolymerization of hydrophilic polyvinyl macromonomers with styrene in a polar solvent. The potential of these nanoparticles as carriers for oral peptide delivery, was investigated using salmon calcitonin (sCT) in rats. The rate of sCT incorporated in nanoparticles was high and was affected by the macromonomer structure. Anionic nanoparticles having poly(methacrylic acid) macromonomer chains on their surfaces showed the highest incorporating activity. When the mixture of sCT and nanoparticles was administered orally, the decrease in the blood ionized calcium concentration was greater than that after oral administration of sCT aqueous solution. This hypocalcemic effect was also affected by the macromonomer structure, and the absorption of sCT was enhanced most strongly by nanoparticles having poly(*N*-isopropylacrylamide) macromonomer chains. However, the calcium concentration changed less when the nanoparticle concentration was low. On the other hand, the hypocalcemic effect was independent of the nanoparticle size and molecular weight of the macromonomers. The absorption enhancement of sCT by the nanoparticles probably results from both bioadhesion to the gastrointestinal (GI) mucosa and the increase of the stability of sCT in the GI tract. These nanoparticles were demonstrated to be useful carriers for incorporating highly water-soluble peptides and for enhancing peptide absorption via the GI tract. © 1997 Elsevier Science B.V.

Keywords: Nanoparticle; Macromonomer; Graft copolymer; Poly(*N*-isopropylacrylamide); Absorption enhancement; Peptide

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PII S0378-5173(96)04861-2

1. Introduction

It is well known that the bioavailability of peptide and protein drugs after oral administration is very low because of their instability in the gastrointestinal (GI) tract and low permeability through the intestinal mucosa (Van Hoogdalem et al., 1989; Lee and Yamamoto, 1990b). However, oral administration is the most convenient route for drug delivery, and several approaches such as chemical modification of drugs (Fujita et al., 1996), the use of an absorption enhancer (Fix, 1987; Lee, 1990a; Muranishi, 1990) and a protease inhibitor (Lee, 1990a), have been investigated to achieve oral peptide delivery. Many pharmaceutical scientists have also reported that colon delivery systems (Saffran et al., 1986; Hastewell et al., 1994; Takaya et al., 1994), mucoadhesive drug delivery systems (Gruber et al., 1987; Gu et al., 1988), and particulate drug delivery systems such as nanoparticles (Kreuter, 1991; Couvreur and Puisieux, 1993; Couvreur et al., 1995), microcapsules (Morishita et al., 1992; Couvreur and Puisieux, 1993), liposomes (Fukunaga et al., 1991) or emulsions (Matsuzaka et al., 1995), are useful in improving the absorption of peptide drugs via the GI tract.

Nanoparticles have been studied extensively as carriers for oral drug delivery, whose purpose is to improve the bioavailability of drugs with poor absorption characteristics (Florence 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 the GI mucosa irritation caused by them (Ammoury et al., 1991) and to ensure their stability in the GI tract (Roques et al., 1992). The study on absorption enhancement is the most exciting work. Maincent et al. (1986) reported that the bioavailability of vincamine was considerably greater than that after oral administration of vincamine aqueous solution when polyhexylcyanoacrylate nanoparticles carrying vincamine was administered orally to rabbits. Damgé and Michel and their respective co-workers (Damgé et al., 1988, 1990; Michel et al., 1991) found that insulin encapsulated in polyisobutylcyanoacrylate nanocapsules reduced glycemia by 50–60%, although free insulin did not affect glycemia when administered orally to diabetic rats. However, the above polyalkylcyanoacrylate is not suitable for preparing nanoparticles which incorporate hydrophilic drugs (Al Khouri Fallouh et al., 1986). Further, although many peptide and protein drugs with poor absorption characteristics are highly water-soluble (Lee and Yamamoto, 1990b), hardly any conventional nanoparticles have been suitable for them (Niwa et al., 1995).

On the other hand, we demonstrated that nanoparticles composed of new graft copolymers having a hydrophobic backbone and hydrophilic branches are useful as drug carriers (Akashi et al., 1985, 1989a). These nanoparticles are prepared by the dispersion copolymerization of hydrophilic macromonomers, which are vinylbenzyl group-terminated water-soluble polyvinyl compounds, with styrene in a polar solvent (Akashi et al., 1990). The resulting polystyrene nanoparticles have hydrophilic macromonomer chains on their surfaces (Akashi et al., 1989b). We have already reported water-dispersible nanoparticles having pH sensitive polyanionic (Riza et al., 1995), polycationic (Akashi et al., 1989b; Riza et al., 1994; Arikawa et al., 1995), or thermosensitive nonionic poly(*N*-isopropylacrylamide) chains (Chen et al., 1996) on their surfaces, in previous articles. When these nanoparticles are mixed with hydrophilic drugs in water, it is expected that the hydrophilic macromonomer chains can effectively retain these hydrophilic drugs by some kind of interaction with them.

In the present study, we evaluated the usefulness of these nanoparticles as carriers for hydrophilic drugs, and their potential as carriers that enhance peptide absorption via the GI tract, using salmon calcitonin as a model drug.

2. Materials and methods

2.1. Materials

Salmon calcitonin (sCT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-Vinylacetamide (NVA) monomer was supplied by

Showa Denko Co. (Tokyo, Japan). *N*-Isopropylacrylamide (NIPAAm) and *t*-butyl methacrylate (*t*-BMA) monomers were obtained from Kohjin Co. (Tokyo, Japan). *p*-Chloromethyl styrene (*p*-CMSt) was furnished by Nippon Oil and Fats Co. (Tokyo, Japan). t-BMA monomer was purified by distillation under reduced pressure in a nitrogen atmosphere. NIPAAm and NVA monomers and *p*-CMSt were used without further purification. All other chemicals were commercial products of reagent grade and were purified in the usual manner previously described (Riza et al., 1994; Chen et al., 1996).

2.2. Preparation of nanoparticles

2.2.1. Poly(methacrylic acid) (PMAA) nanoparticles

Nanoparticles having PMAA chains on their surfaces were prepared by the procedure of Riza et al. (1995), as follows. *t*-BMA oligomers were prepared by the free radical oligomerization of *t*-BMA monomer using 2,2′-azobisisobutyronitrile (AIBN, less than 1 mol.% to monomer) as an initiator in the presence of 2-mercaptoethanol (2MEtOH) as a chain transfer agent in tetrahydrofuran (THF) at 60°C in a nitrogen atmosphere for 6 h. The hydroxyl group-terminated *t*-BMA oligomers obtained were precipitated in a methanol/water mixture (1:1, v/v), reprecipitated from 2-propanol in the methanol/water mixture, and then dried under reduced pressure. *t*-BMA oligomers were reacted with *p*-CMSt in *N*,*N*-dimethylformamide (DMF) containing 50 w/v.% potassium hydroxide (KOH) aqueous solution at 30°C for 48 h in the presence of tetra-butylphosphonium bromide (TBPB) as a phase transfer catalyst. *p*-CMSt, KOH and TBPB were used in amounts 20 times, 5 times and equal to the molar quantity of *t*-BMA oligomers. The precipitated potassium chloride (KCl) was removed by filtration. The vinylbenzyl group-terminated *t*-BMA macromonomers obtained were recovered by precipitation in the methanol/water mixture, dialyzed in methanol using a cellulose dialyzed tube to remove unreacted substances, reprecipitated in the methanol/water mixture, and then dried under reduced pressure. Nanoparticles having poly(*t*-BMA) chains on their surfaces were prepared by the dispersion copolymerization of *t*-BMA macromonomers with styrene using AIBN in ethanol at 60°C in a vacuum for 48 h. The resulting poly(*t*-BMA) nanoparticles were dialyzed in methanol, and isolated by centrifugation. Further, poly(*t*-BMA) nanoparticles were hydrolyzed in ethanol containing 2 N hydrochloric acid (HCl) at 75°C for 12 h so as to replace *t*-BMA with MAA on the nanoparticle surface. After hydrolysis, PMAA nanoparticles were dialyzed in water.

2.2.2. Poly(N-isopropylacrylamide) (PNIPAAm) nanoparticles

PNIPAAm nanoparticles were prepared by the procedure of Chen et al. (1996). Briefly, NIPAAm monomer was oligomerized by using AIBN in the presence of 2MEtOH in ethanol at 60°C under nitrogen for 7 h. After oligomerization, the solvent was removed by evaporation. The resulting NIPAAm oligomers were dissolved in water, heated so that only these oligomers were deposited, isolated by centrifugation at more than 50°C, and then lyophilized. NIPAAm oligomers were reacted with *p*-CMSt in DMF containing 50 w/v% KOH solution at 30°C for 72 h in the presence of TBPB. Amounts of *p*-CMSt, KOH and TBPB 10, 5 and 0.5 times the molar quantity of the NIPAAm oligomers were used. After the removal of KCl, NIPAAm macromonomers obtained were dialyzed in water and lyophilized. These macromonomers were polymerized with styrene using AIBN in ethanol at 60°C under vacuum for 24 h. The resulting PNIPAAm nanoparticles were dialyzed in water, lyophilized, and then redispersed in water.

2.2.3. Poly(N-vinylacetamide) (PNVA) nanoparticles

PNVA nanoparticles were prepared by the procedure of Arikawa et al. (1995). Briefly, NVA monomer was oligomerized under the same conditions as the oligomerization of NIPAAm. The resulting NVA oligomers were precipitated in diethylether, dissolved in ethanol, reprecipitated in diethylether, and then dried under reduced pressure. The condensation of NVA oligomers and

p-CMSt was carried out under the same conditions as the preparation of MAA macromonomers. *p*-CMSt, KOH and TBPB were used in amounts 20, 10 and 5 times the molar quantity of NVA oligomers. After the removal of KCl, the purification of NVA macromonomers obtained were carried out under the same conditions as that of NVA oligomers. NVA macromonomers were polymerized with styrene using AIBN in ethanol at 60°C under vacuum for 48 h. The resulting PNVA nanoparticles were dialyzed in water, dried under reduced pressure, and then redispersed in water.

2.2.4. Poly(N-vinylacetamide-co-vinylamine) (PNVA-co-VAm) nanoparticles

The PNVA nanoparticles obtained as described above were hydrolyzed in 2 N HCl at 95°C for 6 h in order to partially replace NVA on the nanoparticle surface with VAm. After hydrolysis, PNVA-co-VAm nanoparticles were dialyzed in water.

2.2.5. Poly(vinylamine) (PVAm) nanoparticles

PNVA nanoparticles were hydrolyzed in 2 N HCl at 95°C for 12 h in order to completely replace NVA with VAm. After hydrolysis, PVAm nanoparticles were dialyzed in water.

2.3. Characterization of nanoparticles

The oligomers, macromonomers and nanoparticles were characterized using the method described in earlier articles (Riza et al., 1994, 1995; Chen et al., 1996). Briefly, the number-average molecular weight (M_n) of oligomers and macromonomers was determined by gel permeation chromatography, and the functionality of the macromonomers was obtained from ^{1}H-NMR spectra. The particle size of the nanoparticles was next measured by dynamic light-scattering spectrophotometry. Finally, the IR spectra were used to determine the degree of hydrolysis on nanoparticle surface.

2.4. Preparation of nanoparticles incorporating sCT

2.4.1. Dosing solution for animal experiment

An aqueous dispersion of nanoparticles was mixed with sCT dissolved in water prior to animal experiments. The final concentrations of sCT and nanoparticles in this mixture for use as a dosing solution, were adjusted to 0.1 mg/ml and 1–100 mg/ml, respectively.

2.4.2. Rate of sCT incorporated in nanoparticles

The mixture of sCT and nanoparticles was centrifuged to separate the sCT that was not associated with the nanoparticles, from the sCT incorporated in nanoparticles. The concentration of sCT in the supernatant was determined using a modification of the HPLC procedure reported by Lee et al. (1991), which was carried out as follows. Mobile phase A consisted of 0.1 w/v% trifluoroacetic acid (TFA) in acetonitrile/water mixture (3/1, v/v), and 0.1 w/v% TFA aqueous solution was used as mobile phase B. A linear gradient was run from 40 v/v% A to 60 v/v% A in 15 min. A column of 150 mm × 4.6 mm filled with octadecylsilyl silica gel of 5 μm mean particle size was used. The column temperature was set to 40°C. The injection volume was 0.1 ml, and the flow rate was 1.3 ml/min. sCT was detected by UV absorption at 215 nm. After measurement, the rate of sCT incorporated in the nanoparticles was calculated from the difference in sCT concentration between the supernatant and the original mixture.

2.5. Animal experiments

Male Sprague–Dawley (SD) strain rats weighing 200–230 g were used. The rats (five per group) were kept under normal conditions, however, fasted overnight with free access to water before the in vivo study. The mixture of sCT and nanoparticles (containing both incorporated sCT and non-incorporated sCT) was given orally in a single administration at a dose of 0.25 mg sCT/2.5 ml mixture/kg body weight, to rats. As a control, an aqueous solution of sCT was administered to rats under the same conditions. Blood samples

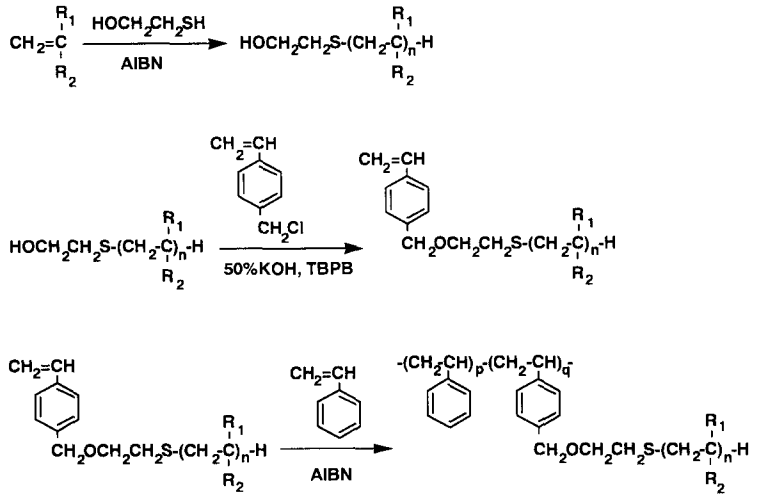


Fig. 1. Synthesis of nanoparticles.

(approx. 0.1 ml) were obtained from the tail vein. The blood ionized calcium concentration was measured with an analyzer using the calcium electrodes (634 automated Ca^{++}/pH analyzer, Ciba Corning Diagnostics Co, Tokyo, Japan).

2.6. Statistical analysis

The change in the blood ionized calcium concentration from before to after the oral administration of the dosing solution was calculated, and the means and standard errors for all values were determined. Statistical significance was assessed with Student's t-test, and P values of 0.05 or less were considered significant.

3. Results

3.1. Preparation of nanoparticles

Fig. 1 shows the synthesis of nanoparticles. This method is divided into three processes that is the radical oligomerization of monomers initiated by AIBN in the presence of 2MEtOH to give oligomers terminating in hydroxyl groups, the condensation of the oligomers and p-CMSt in the presence of TBPB to give macromonomers terminating in vinylbenzyl groups, and the dispersion copolymerization initiated by AIBN between hydrophilic macromonomers and styrene. Polyanionic or polycationic nanoparticles are prepared by the hydrolysis of esters or acid-amides, respectively, located on the nanoparticle surface. The conditions and the results in each of these processes are tabulated in Tables 1–3. All results were in good agreement with those obtained in previous studies (Akashi et al., 1989a,b; Riza et al., 1994, 1995; Arikawa et al., 1995; Chen et al., 1996). Briefly, molecular weights of oligomers and macromonomers were adjusted to the magnitude of 10^3. All monodispersed nanoparticles possessed good water-dispersibility because hydrophilic macromonomer chains were located on the surface of hydrophobic polystyrene nanoparticles (Akashi et al., 1989b). The particle size of PNIPAAm nanoparticles decreased with increasing the molecular weight or concentration of macromonomers. Further, when PNVA-co-VAm and PVAm nanoparticles were prepared by the hydrolysis of PNVA nanoparticles, the NVA/VAm ratio was controlled by changing the duration of the hydrolysis.

3.2. Preparation of nanoparticles incorporating sCT

Fig. 2 shows the chemical structures of the

Table 1
Oligomerization of monomers

	Monomer (mmol)	2MEtOH[a] (mmol)	Solvent (ml)	M_n^b
t-BMA oligomers	176	5.3	60	3.0
NIPAAm oligomers	177	2.6	80	3.4
NIPAAm oligomers	177	6.1	80	2.2
NIPAAm oligomers	177	25.6	80	1.3
NVA oligomers	222	111	50	3.4

[a] 2-Mercaptoethanol.
[b] Number-avernge molecular weight ($\times 10^{-3}$).

nanoparticles used in this study. The rate of sCT incorporated in each of these nanoparticles is shown in Table 4. There was no aggregation when sCT was mixed with the nanoparticles. The rate of sCT incorporation was affected by the chemical structure of the macromonomers. Anionic PMAA nanoparticles showed the highest sCT incorporation rate (100%). Cationic PNVA-co-VAm and PVAm nanoparticles also showed relatively high sCT incorporation rate. On the other hand, sCT incorporation in cationic nanoparticles increased when VAm was substituted for NVA, and nonionic PNVA nanoparticles showed a fairly high incorporation rate. However, the amount of sCT incorporated in PNIPAAm nanoparticles was much smaller than that in PNVA nanoparticles, although both nanoparticles carried no charge. These low rates of incorporation were hardly affected by particle size or by the molecular weights of the macromonomers, although they increased in proportion to the nanoparticle concentration.

3.3. Absorption enhancement of sCT by nanoparticles

3.3.1. Effect of macromonomer structure

We next examined the potential of these nanoparticles as carriers that enhance the absorption of sCT via the GI tract. Figs. 3 and 4 show the changes in blood ionized calcium concentration after oral administration of mixtures of sCT and nanoparticles. In this study, when nanoparticles without sCT or water were administered orally, the blood ionized calcium concentration did not decrease at all (data not shown). As is obvious from Figs. 3 and 4, when a mixture of sCT and nanoparticles was administered orally, the decrease in the blood ionized calcium concentration was greater than that after oral administration of sCT in water, except for the case of PNVA nanoparticles. This decrease was affected by the macromonomer structure, and the strongest hypocalcemic effect was obtained after oral administration of the mixture of sCT and PNIPAAm nanoparticles. In this case, the changes in blood ionized calcium concentration at 1.3 and 2 h after administration were significant ($P < 0.01$). There was no the histological change in the GI tract 8 h after oral administration of this mixture, with the naked eye. On the other hand, the hypocalcemic effect by the mixture of sCT and PNVA nanoparticles was slightly increased by using PNVA-co-VAm or PVAm nanoparticles obtained by the hydrolysis of PNVA nanoparticles. There was a tendency toward enhanced absorption of sCT by cationic nanoparticles as the VAm fraction increased. These results suggest that PNIPAAm nanoparticles enhance most strongly the absorption of sCT via the GI tract.

3.3.2. Effect of characteristics of PNIPAAm nanoparticles

Fig. 5 shows the effect of nanoparticle concentration on sCT absorption. After oral administration of the mixture of sCT and PNIPAAm nanoparticles, a hypocalcemic effect was observed and the calcium concentration at 2 h was significant ($P < 0.01$) even at a low concentration (1 mg/ml) of PNIPAAm nanoparticles. Although

Table 2
Condensation of oligomers and *p*-CMSt

	Oligomers		*p*-CMSt[b] (mmol)	Solvent (ml)	Yield[c] (%)	Functionality[d]	M_n^a
	M_n^a	(mmol)					
t-BMA macromonomers	3.0	1.6	32.2	100	72	77	3.1
NIPAAm macromonomers	3.4	2.9	29.8	50	82	76	3.5
NIPAAm macromonomers	2.2	2.5	22.4	40	77	72	2.3
NIPAAm macromonomers	1.3	8.5	120	80	24	80	1.6
NVA macromonomers	3.4	1.4	27.7	50	74	100	3.5

[a] Number-average molecular weight ($\times 10^{-3}$).
[b] *p*-Chloromethyl styrene.
[c] Calculated from the weight of the lyophilized macromonomers.
[d] Introduction rate of vinyl group at the end of the macromonomers.

this effect was somewhat less than that at higher concentrations (10 mg/ml and over) of PNIPAAm nanoparticles, it was constant even if the concentration of PNIPAAm nanoparticles was more than 10 mg/ml. Figs. 6 and 7 show the effect of the particle size and molecular weights of the macromonomers, respectively. The concentration-time profile of ionized calcium in blood was independent of particle size. Molecular weights of the macromonomers between 1600 and 3500 also did not affect the hypocalcemic effect. These results indicate that the sCT/nanoparticle ratio is one of the factors which affect the absorption of sCT.

4. Discussion

sCT has been often studied as a model drug for oral drug delivery (Lee and Yamamoto, 1990b). It is highly water-soluble peptide that consists of 32 amino acid residues and has a molecular weight of approximately 3430. sCT absorption via the GI tract is very low because of its extreme instability in the GI tract and its high water solubility (Lee and Yamamoto, 1990b).

In our study on the rate of sCT incorporated in nanoparticles, charged nanoparticles exhibited high incorporating activity (Table 4). sCT has an intramolecular carboxyl group arising from glutamic acid, although sCT is a basic peptide. The high degree of sCT incorporated in these charged nanoparticles probably results from an electrostatic interaction between sCT and the macromonomer chains on the nanoparticle surface. Nevertheless, sCT was also associated with non-charged nanoparticles. These data suggest that there is not only electrostatic interaction but also other interactions such as hydrogen bond between sCT and the hydrophilic macromonomer chains on the nanoparticle surface. Further, the intramolecular non-polar amino acid residues of sCT may interact with the hydrophobic polystyrene core which is incompletely covered by macromonomer chains, and also the difference in the flexibility of the macromonomer chains may affect the degree of sCT incorporation. However, there is insufficient data to identify all the interactions between sCT and nanoparticles. This problem will be addressed in future reports.

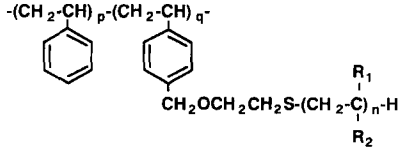
Next, we examined the potential of these nanoparticles as carriers that improve the bioavailability of sCT, by assessing the hypocalcemic effects after oral administration of the mixture of sCT and nanoparticles. The blood ionized calcium concentration decreased slightly after oral administration of sCT aqueous solution. This suggests that a small amount of intact sCT is absorbed via the GI tract (Hastewell et al., 1994), although the majority of sCT is degraded by the proteases and the other metabolic enzymes in the GI tract, mucosa, brush-border membrane or cytosol, and the membrane permeability of the residual intact sCT is very low. The bioavailability of sCT is estimated at less than 1% when it is

Table 3
Dispersion copolymerization of hydrophilic macromonomers with styrene

	Macromonomers		Styrene (mmol)	Diameter[b] (nm)
	M_n^a	(mmol)		
PMAA nanoparticles	3.1	0.1	1	590
PNIPAAm nanoparticles	3.5	0.15	5	440
PNIPAAm nanoparticles	3.5	0.1	5	610
PNIPAAm nanoparticles	3.5	0.05	5	750
PNIPAAm nanoparticles	2.3	0.05	5	1220
PNIPAAm nanoparticles	1.6	0.1	13	1250
PNVA nanoparticles	3.5	0.074	0.43	420
PNVA-co-VAm nanoparticles[c]	3.5	0.074	0.43	410
PVAm nanoparticles	3.5	0.074	0.43	400

[a] Number-average molecular weight ($\times 10^{-3}$).
[b] Weight-average diameter.
[c] Molar ratio of NVA and VAm was 1:1, as estimated using IR spectra.

administered orally without any added substances (Humphrey, 1986). The low bioavailability was improved considerably by PNIPAAm nanoparticles (Figs. 3 and 4). There was no remarkable contribution of the characteristics of PNIPAAm nanoparticles on the absorption enhancement of sCT (Figs. 5–7). Further, nanoparticles having anionic PMAA or cationic PVAm chains on their surfaces enhanced sCT absorption, although this effect was weaker than that of PNIPAAm nanoparticles. However, these hypocalcemic effects after oral administration of mixtures of sCT and nanoparticles were only retained for 4 h, irrespective of the macromonomer structure. This



	R_1	R_2
PMAA nanoparticles	CH_3	COOH
PNIPAAm nanoparticles	H	$CONHCH(CH_3)_2$
PNVA nanopartcles	H	$NHCOCH_3$
PNVA-co-VAm nanoparticles	H	$NHCOCH_3$, NH_2
PVAm nanoparticles	H	NH_2

Fig. 2. Chemical structure of nanoparticles.

suggests that the absorption amount of sCT was not sufficient for inducing the substantial hypocalcemic effect. Further improvements of nanoparticles will be necessary for achieving oral peptide delivery by this technology.

The mechanism of the absorption enhancement of sCT by these nanoparticles has not yet been sufficiently explicated. However, we can speculate about this mechanism on the basis of past studies on oral colloidal drug carriers (Kreuter, 1991; Couvreur and Puisieux, 1993; Couvreur et al., 1995; Florence et al., 1995). Ch'ng et al. (1985) and Longer et al. (1985), having a series of cross-linked swellable polymers, measured the force of bioadhesion of these polymers to the GI mucosa of the rat, and they found that poly(acrylic acid) or poly(methacrylic acid) cross-linked by divinylglycol have a strong bioadhesion to the mucous layer, and that the oral absorption of chlorothiazide encapsulated in albumin microspheres was improved by the addition of cross-linked poly(acrylic acid). Lehr et al. (1990, 1992) also reported that the in vitro absorption of a vasopressin derivative from the lumen of intestinal segments of the rat was enhanced by encapsulating the vasopressin derivative in poly(2-hydroxyethyl methacrylate) microspheres being coated with the same cross-linked poly(acrylic acid). It is accepted generally that a bioadhesive polymer can improve drug absorption in two ways (Kreuter et al., 1989;

Table 4
Characterization of nanoparticles incorporating sCT

	Macromonomers (M_n^a)	Diameter[b] (nm)	Concentration[c] (mg/ml)	sCT Incorporation[d] (%)
PMAA nanoparticles	3.1	590	10	100
PNIPAAm nanoparticles	3.5	440	1	10
PNIPAAm nanoparticles	3.5	440	10	15
PNIPAAm nanoparticles	3.5	440	30	22
PNIPAAm nanoparticles	3.5	440	100	38
PNIPAAm nanoparticles	3.5	610	10	14
PNIPAAm nanoparticles	3.5	750	10	19
PNIPAAm nanoparticles	2.3	1220	10	11
PNIPAAm nanoparticles	1.6	1250	10	9
PNVA nanoparticles	3.5	420	10	84
PNVA-co-VAm nanoparticles[e]	3.5	410	10	62
PVAm nanoparticles	3.5	400	10	60

[a] Number-average molecular weight ($\times 10^{-3}$).
[b] Weight-average diameter.
[c] Concentration of nanoparticles.
[d] Rate of sCT incorporated in nanoparticles (mean of three experiments).
[e] Molar ratio of NVA and VAm was 1:1, as estimated using IR spectra.

Pimienta et al., 1990; Mikos et al., 1991; Durrer et al., 1994). One is by increasing the residence time of the drugs at the absorption site through prolonging their GI transit time, and the other is by increasing the drug concentration in the vicinity of the epithelial cells through intensifying the contact of the drug with the intestinal mucous

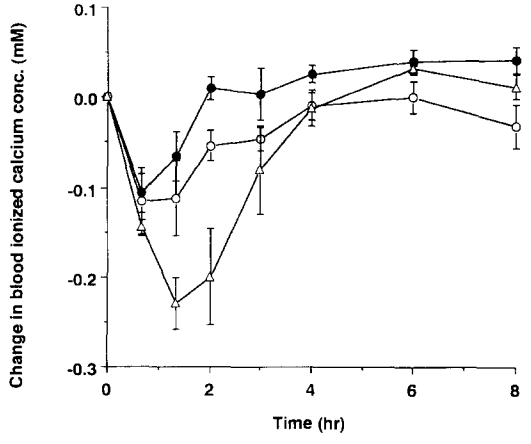


Fig. 3. Concentration-time profiles of ionized calcium in blood after oral administration of sCT aqueous solution (●), a mixture of sCT and PMAA nanoparticles (○) and a mixture of sCT and PNIPAAm nanoparticles (△) in rats (0.25 mg sCT/2.5 ml dosing solution/kg rat). The nanoparticle concentration in the dosing solution was 10 mg/ml. The PNIPAAm nanoparticle size and molecular weight of macromonomers were 750 nm and 3500, respectively. Each value represents the mean ± S.E.

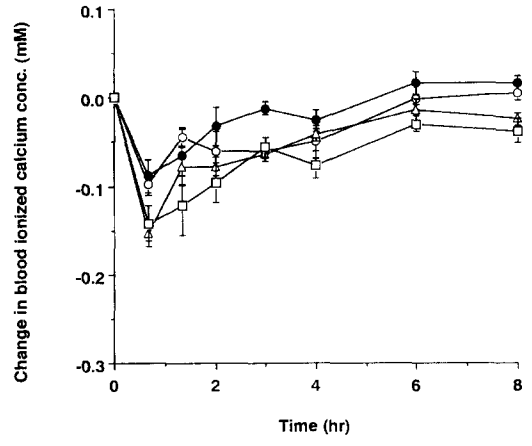


Fig. 4. Concentration-time profiles of ionized calcium in blood after oral administration of sCT aqueous solution (●), a mixture of sCT and PNVA nanoparticles (○), a mixture of sCT and PNVA-co-VAm nanoparticles (△), and a mixture of sCT and PVAm nanoparticles (□) in rats (0.25 mg sCT/2.5 ml dosing solution/kg rat). The nanoparticle concentration in the dosing solution was 10 mg/ml. Each value represents the mean ± S.E.

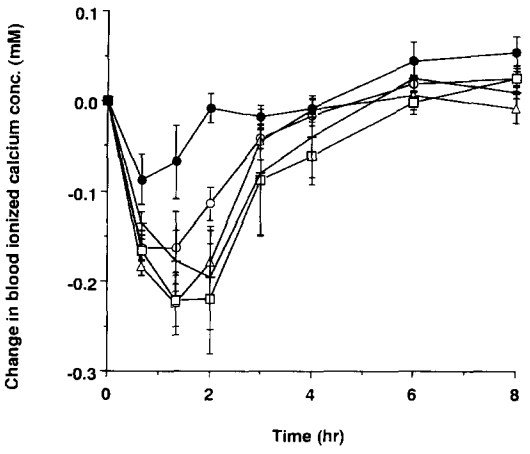


Fig. 5. Concentration-time profiles of ionized calcium in blood after oral administration of sCT aqueous solution (●) and a mixture of sCT and PNIPAAm nanoparticles in rats (0.25 mg sCT/2.5 ml dosing solution/kg rat). The nanoparticle concentrations in the dosing solution were 1 mg/ml (○), 10 mg/ml (△), 30 mg/ml (□) and 100 mg/ml (+). The nanoparticle size and molecular weight of macromonomers were 440 nm and 3500, respectively. Each value represents the mean ± S.E.

layer. The latter of these most likely helps to overcome both the diffusional and the enzymatic barriers which normally restrict the absorption of peptide and protein drugs. The absorption enhancement of sCT by PMAA nanoparticles also probably results from bioadhesion of PMAA chains. On the other hand, the basic component of the mucous layer is glycoprotein, and sialic acid is located at the ends of the oligosaccharide chains of glycoprotein (Gu et al., 1988). It is considered that a cationic compound is preferable to a nonionic or anionic compound for binding to the mucous layer having negative charge. Indeed, the absorption enhancement of sCT by nanoparticles increased with the replacement of nonionic NVA by cationic VAm on the nanoparticle surface. This suggests that stronger interactions between the mucous layer and nanoparticles make possible the achievement of further absorption enhancement of sCT.

The absorption enhancement effect of sCT by nonionic PNIPAAm nanoparticles was considerably higher than that by charged nanoparticles. Any scientists have never reported that thermosensitive PNIPAAm shows bioadhesion. The surface of PNIPAAm nanoparticles becomes hydrophobic at body temperature because the phase transition of PNIPAAm macromonomer chains occurs about 35°C (Chen et al., 1996). The mucous glycoprotein is hydrophilic (Gu et al., 1988) and it seems that the interaction between the mucous layer and hydrophobic PNIPAAm nanoparticles is not very strong. However, we confirm that the phase transition of PNIPAAm nanoparticles at 40°C occurs slowly, and that these nanoparticles do not aggregate immediately even at 50°C (Chen et al., 1996). These facts suggest that PNIPAAm nanoparticles possess not only hydrophobicity but also some hydrophilicity above the phase transition temperature. It appears that this delicate balance between hydrophobicity and hydrophilicity at body temperature is suitable for causing bioadhesion.

On the other hand, the improvement of sCT stability in the GI tract is also important for achieving absorption enhancement because sCT is extremely unstable in the GI tract. It is expected that these nanoparticles will offer some assurance of the stability of sCT in the GI tract, since they protect sCT against enzyme degradation by sur-

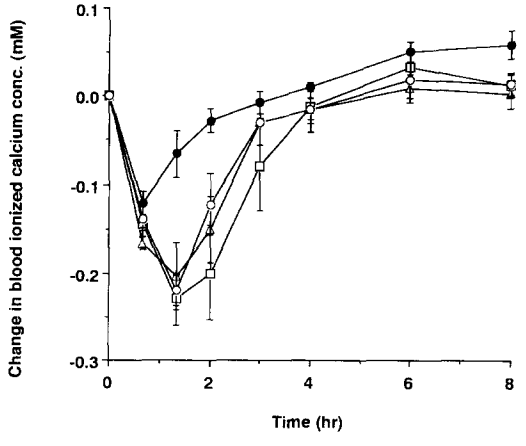


Fig. 6. Concentration-time profiles of ionized calcium in blood after oral administration of sCT aqueous solution (●) and a mixture of sCT and PNIPAAm nanoparticles in rats (0.25 mg sCT/2.5 ml dosing solution/kg rat). The nanoparticle size in the dosing solution is 440 nm (○), 610 nm (△) and 750 nm (□). The nanoparticle concentration and molecular weight of macromonomers were 10 mg/ml and 3500, respectively. Each value represents the mean ± S.E.

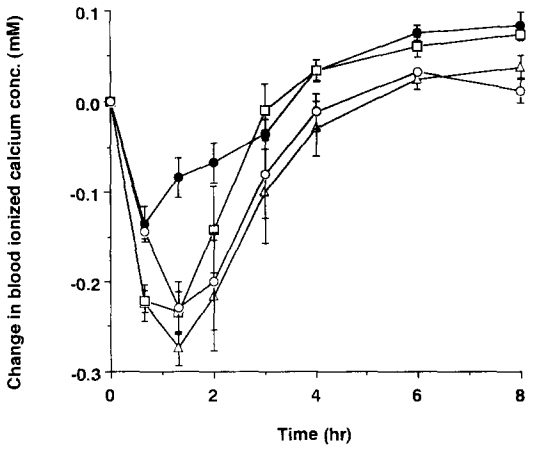


Fig. 7. Concentration-time profiles of ionized calcium in blood after oral administration of sCT aqueous solution (●) and a mixture of sCT and PNIPAAm nanoparticles in rats (0.25 mg sCT/2.5 ml dosing solution/kg rat). The molecular weight of macromonomers on the surface of nanoparticles in the dosing solution was 3500 (○), 2300 (△) and 1600 (□). Nanoparticle concentration and size were 10 mg/ml and 750–1250 nm, respectively. Each value represents the mean ± S.E.

rounding it with macromonomer chains. From the present data, it seems that the force that stabilizes sCT depends mainly on the macromonomer structure, and that the stabilization of sCT in the GI tract is perhaps independent of the characteristics of the nanoparticles such as their ability to incorporate sCT. The absorption enhancement of sCT by PNIPAAm nanoparticles is probably higher than that by charged nanoparticles, because PNIPAAm nanoparticles can give substantial stability of sCT in the GI tract, apart from their bioadhesive properties. However, when the absorption enhancement of sCT by PNIPAAm nanoparticles is discussed, it is also necessary to take account of the low incorporation rate. In this study, the measurement of sCT incorporation was carried out in purified water at room temperature. As mentioned before, sCT has both of positive and negative charges, and PNIPAAm nanoparticles have thermosensitivity. It seems that the interaction between sCT and PNIPAAm nanoparticles depended on pH, ionic strength and temperature of the solvent. Enzymes in the GI tract may also affect the interaction. This problem will be described in future reports with the desorption of sCT from PNIPAAm nanoparticles.

We consider that these nanoparticles have not only the property of bioadhesion to the GI mucosa but also the stabilization effect of peptide and protein drugs in the GI tract as shown in Fig. 8, and that the balance of these actions is determined by the macromonomer structure located on the nanoparticle surface. It seems that the macromonomer chains have to be selected appropriately in response to the characteristics of the peptide drugs, because the proper balance is important for inducing the excellent absorption enhancement. Nanoparticle technology will, it is very likely, facilitate an even higher oral bioavailability of peptide and protein drugs, including sCT, when the absorption enhancement mechanism and the interaction have been explained. Further work will be successively discussed future reports.

5. Conclusions

Nanoparticles composed of new graft copolymers having a hydrophobic backbone and hydrophilic branches were synthesized, and the usefulness of these nanoparticles as carriers for oral peptide delivery were evaluated. The rate of sCT incorporated in these nanoparticles was high, although it was depended on the structure of hydrophilic branches. When the mixture of sCT and these nanoparticles was administered orally, the hypocalcemic effect was greater than that after oral administration of sCT aqueous solution. This effect was also affected by the hydrophilic chain structure, and the absorption of sCT via the GI tract was considerably enhanced by nanoparticles having PNIPAAm chains on their surfaces. The absorption enhancement of sCT by these nanoparticles probably results from both bioadhesion to the GI mucosa and the increase of the stability of sCT in the GI tract. The nanoparticles that we developed were demonstrated to be useful carriers for incorporating highly water-soluble peptides and for enhancing peptide absorption via the GI tract.

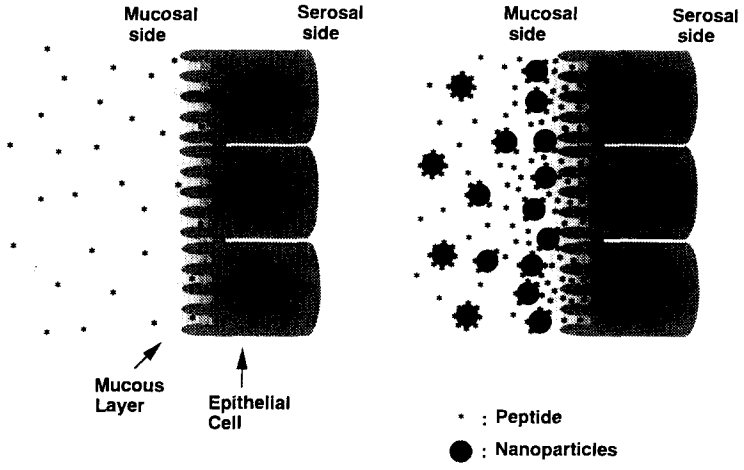


Fig. 8. Schematic representation of the mechanism of absorption enhancement of sCT by nanoparticles composed of graft copolymers having a hydrophobic backbone and hydrophilic branches.

Acknowledgements

We thank Dr. Julie K. Rhie at Professor Gordon L. Amidon's laboratory in the University of Michigan, for her support in this work. This work was financially supported in part by a Grant-in-Aid for Scientific Research in Priority Areas of 'New Polymers and Their Nano-Organized Systems' (No.277/08246243) from the Ministry of Education, Science, Sports and Culture, Japan.

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