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Optimized chemical structure of nanoparticles as carriers for oral delivery of salmon calcitonin

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Abstract

Nanoparticles having two kinds of surface hydrophilic polymeric chains were prepared by the free radical copolymerization between styrene and hydrophilic macromonomers terminating in vinylbenzyl groups. Their potential as carriers for oral peptide delivery was investigated using salmon calcitonin (sCT) in rats. After oral administration of mixtures of sCT and nanoparticles, the ionized calcium concentration in blood was measured. The absorption of sCT was significantly enhanced by nanoparticles having poly-*N*-isopropylacrylamide (PNIPAAm) chains on their surfaces. This enhancement effect was considerably increased by introducing cationic poly-vinylamine (PVAm) groups to the surface of PNIPAAm nanoparticles. The absorption enhancement depended on the ratio of NIPAAm and VAm macromonomers to styrene in the nanoparticle preparation. In contrast, the introduction of nonionic poly-vinylacetamide (PNVA) groups eliminated completely the absorption-enhancing function of PNIPAAm nanoparticles. It was suggested that this disappearance was due to the shielding of PNIPAAm groups by PNVA groups. The enhancement effect of sCT absorption by nanoparticles was greatly dominated by their chemical structure that was closely related to surface characteristics. Optimization of the chemical structure on the basis of the mechanism of the absorption enhancement resulted in the further improvement of sCT absorption.

Keywords: Nanoparticle; Macromonomer; Graft copolymer; Oral delivery; Peptide; Salmon calcitonin

1. Introduction

Nanoparticles have been studied extensively as carriers for oral peptide delivery (Sakuma et al., 2001; Kreuter, 1991; Couvreur and Puisieux, 1993). We also demonstrated previously that nanoparticles composed of novel graft copolymers

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having a hydrophobic polystyrene backbone and hydrophilic polyvinyl branches are useful as the carriers for peptide drugs (Sakuma et al., 1997a). These nanoparticles were prepared by the free radical copolymerization between hydrophilic macromonomers terminating in vinylbenzyl groups and styrene (Akashi et al., 1985, 1989a, 1990). Their surfaces were covered with hydrophilic polymeric chains (Akashi et al., 1989b) and their diversity was controlled by designing and synthesizing different functional macromonomers (Capek and Akashi, 1993).

We have already developed nanoparticles having pH sensitive anionic poly(methacrylic) acid (PMAA), cationic poly(vinylamine) (PVAm), thermosensitive nonionic poly(*N*-isopropylacrylamide) (PNIPAAm) and highly water-soluble nonionic poly(*N*-vinylacetamide) (PNVA) on their surfaces (Riza et al., 1994, 1995; Chen et al., 1996, 1999). These nanoparticles enhanced the absorption of salmon calcitonin (sCT) in rats via the gastrointestinal (GI) tract (Sakuma et al., 1997a). The absorption enhancement was affected by the chemical structure of the hydrophilic polymeric chains and sCT absorption was enhanced most strongly by nanoparticles having PNIPAAm chains on their surfaces. The absorption of sCT was also enhanced by nanoparticles having surface PMAA or PVAm chains although enhancement was weaker than that by PNIPAAm nanoparticles. PNVA nanoparticles did not show the enhancement effect of sCT absorption at all. It was concluded that the absorption enhancement of sCT by nanoparticles results from both mucoadhesion of nanoparticles incorporating sCT to the intestinal mucosa and an increase in stability of sCT against digestive enzymes (Sakuma et al., 2001, 1997a,b,c, 1999; Sakuma et al., in press).

On the other hand, the hypocalcemic effect after oral administration of a mixture of sCT and PNIPAAm nanoparticles in rats was only retained for 4 h (Sakuma et al., 1997a). This effect was independent of the nanoparticle size and molecular weight of the macromonomer. Optimization of the administration schedule of the mixture of sCT and PNIPAAm nanoparticles resulted in the stronger pharmacological activity. When the dose of the mixture was halved and one half was given orally 40 min after the other half, the sCT-induced hypocalcemic effect was markedly enhanced by PNIPAAm nanoparticles (Sakuma et al., 1997b). The area of the reduction in the blood ionized calcium concentration (Morita et al., 1994; Kobayashi et al., 1994) was about three times that after administration of a single full dose of the same mixture. There was no further enhancement of the pharmacological activity of sCT, however, when the half-doses were administered 120 min apart. It seemed that this difference depended on the mucoadhesion of PNIPAAm nanoparticles to the gastric mucosa.

In this article, we designed novel nanoparticles having two kinds of surface hydrophilic polymeric chains on the basis of their enhancement mechanism to improve the enhancement effect of sCT absorption by PNIPAAm nanoparticles.

2. Materials and methods

2.1. Materials

sCT and hydroquinone were 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 (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., 1994, 1995; Chen et al., 1996, 1999).

2.2. Preparation of nanoparticles

2.2.1. Macromonomer

Nonionic NIPAAm, NVA, and BMA macromonomers were prepared by using the procedures reported in our earlier article (Sakuma et al., 1997a). Oligomers whose molecular weight was of the magnitude of 10^3–10^4 were prepared by the free radical polymerization of monomers (NIPAAm, NVA and BMA) by using 2,2′-azobi-

sisobutyronitrile (AIBN) as an initiator in the presence of 2-mercaptoethanol as a chain transfer agent. The resulting hydroxyl group-terminated oligomers were reacted with *p*-CMSt to introduce a vinylbenzyl group in alkali solution with tetrabutylphosphonium bromide as a phase transfer catalyst. The respective vinylbenzyl group-terminated NIPAAm, NVA and BMA macomonomers were obtained. After dialysis of the macromonomers, they were lyophilized.

Ionic VAm and MAA macromonomers were prepared by hydrolysis of the NVA and BMA macromonomers, respectively. NVA macromonomers (10 mg/ml) were dissolved in 4 N hydrochloric acid (HCl) containing an amount of hydroquinone two times the molar quantity of the macromonomer to prevent them from polymerizing. The NVA macromonomers were hydrolyzed at 80 °C for 24 h so as to replace NVA with VAm. BMA macromonomers (10 mg/ml) were dissolved in ethanol containing 0.5 N HCl and an amount of hydroquinone two times the molar quantity of the macromonomer. After hydrolysis of the BMA macromonomers at 80 °C for 12 h, the normality of the solution was changed to 2 N by adding HCl. Subsequent hydrolysis was carried out at 80 °C for 24 h. The respective VAm and MAA macromonomers obtained were dialyzed in purified water and then lyophilized.

2.2.2. Nanoparticles

2.2.2.1. Nonionic nanoparticles having one kind of surface polymeric chains. PNIPAAm, PNVA and PBMA nanoparticles were prepared using the procedures reported in our previous article (Sakuma et al., 1997a). The respective macromonomers were polymerized with styrene in ethanol including AIBN. The molar ratio of the macromonomer to styrene was 1 to 40. Only PNIPAAm nanoparticles were also prepared in the ratio of 1:5. After the dispersion copolymerization, the resulting nonionic PNIPAAm, PNVA and PBMA nanoparticles were dialyzed to remove unreacted substances, and then lyophilized.

2.2.2.2. Ionic nanoparticles having one kind of surface polymeric chains. Two synthesis routes were adopted to prepare ionic PVAm and PMAA nanoparticles. One route was a modification of the hydrolysis of nonionic nanoparticles described in our earlier article (Sakuma et al., 1997a). PNVA nanoparticles prepared in the previous section were hydrolyzed in 4 N HCl at 80 °C for 24 h. After the resulting PVAm nanoparticles were dialyzed in purified water, they were lyophilized. PBMA nanoparticles were first hydrolyzed in ethanol containing 0.5 N HCl at 80 °C for 12 h. After this reaction, the normality of the solution was changed to 2 N by adding HCl. Subsequent hydrolysis was carried out at 80 °C for 24 h. The resulting PMAA nanoparticles were dialyzed in purified water and then lyophilized.

The other route was dispersion copolymerization between ionic macromonomers and styrene. The corresponding ionic macromonomers, styrene and AIBN were dissolved in 5 ml of ethanol containing 20 v/v% of purified water. The molar ratio of the macromonomer to styrene was 1 to 40 or 1 to 5. The total weight of monomers was adjusted to 1.0 g. AIBN was less than 1 mol.% of the total monomers. The copolymerization was carried out at 60 °C for 24 h in a vacuum. Ionic nanoparticles obtained were dialyzed in purified water and then lyophilized.

2.2.2.3. Nanoparticles having two kinds of surface polymeric chains. Nanoparticles having two kinds of surface polymeric chains were prepared by the dispersion copolymerization between the corresponding macromonomers and styrene initiated by AIBN as shown in Fig. 1.

When nanoparticles having both PNIPAAm and PNVA chains on their surfaces were prepared, NIPAAm macromonomers, NVA macromonomers, styrene and AIBN were first dissolved in 5 ml of ethanol. They were in the molar ratio of 1:1:80:0.08 (NIPAAm macromonomer:NVA macromonomer:styrene:AIBN). The total weight of monomers was adjusted to 1.0 g. The copolymerization was carried out at 60 °C for 24 h in a vacuum. The resulting PNIPAAm–PNVA nanoparticles (NIPAAm:NVA:styrene = 1:1:80) were dialyzed in purified water and then lyophilized.

Several types of PNIPAAm–PVAm nanoparticles were prepared. NIPAAm macromonomer, VAm macromonomer and styrene were combined in the ratios of 1.5:0.5:80, 1.5:0.5:10, 1:1:80 and 0.5:1.5:80. AIBN was less than 1 mol.% of the total monomers, and the total weight of monomers was adjusted to 1.0 g. After all materials were dissolved in ethanol containing 20 v/v% of purified water, the copolymerization was carried out at 60 °C for 24 h in a vacuum. The resulting PNIPAAm–PVAm nanoparticles were dialyzed in purified water and then lyophilized. The other ionic PVAm–PMAA nanoparticles and PNVA–PMAA nanoparticles were prepared in the same manner as that described for PNIPAAm–PVAm nanoparticles.

2.3. Characterization of nanoparticles

The oligomers, macromonomers and nanoparticles were characterized by using the method described in earlier articles (Sakuma et al., 1997a; Riza et al., 1994, 1995; Chen et al., 1996, 1999). Briefly, the number-average molecular weights (Mn) of oligomers and macromonomers were determined by gel permeation chromatography. Hydrolysis of macromonomers and nanoparticles was estimated by IR spectra and elemental analysis. The nanoparticle size was measured by dynamic light-scattering spectrophotometry (DLS-700, Otsuka Electronics Co., Japan). The zeta potential of ionic nanoparticles was measured by electrophoretic light-scattering spectrophotometry in phosphate buffered solution (pH: 7.4; ionic strength: 0.15) and NaCl–HCl aqueous solution (pH: 2.0; ionic strength: 0.15) at 25 °C (ELS-800, Otsuka Electronics Co., Japan).

2.4. In vivo studies

2.4.1. Dosing solution

The lyophilized nanoparticles were re-dispersed

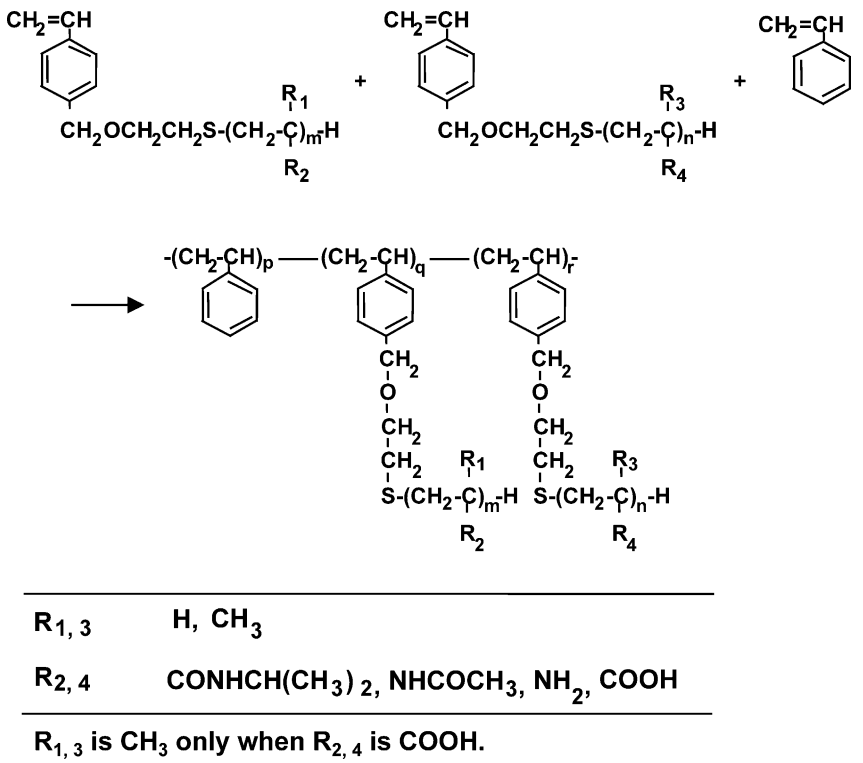

Fig. 1. Synthesis of nanoparticles having two kinds of polymeric chains on their surfaces.

in purified water at a concentration of 20 mg/ml. sCT was dissolved in purified water at a concentration of 0.2 mg/ml. Nanoparticle dispersion was mixed with the equivalent volume of sCT aqueous solution.

2.4.2. Rate of sCT incorporated in nanoparticles

The milky dosing solution, which was comprised of nanoparticles incorporating sCT and free non-incorporated sCT, was centrifuged at 15000 rpm for 30 min at 25 °C. The concentration of sCT in the supernatant was determined by the HPLC reported in our previous study (Sakuma et al., 1997a). After measurement, the rate of sCT incorporated in nanoparticles was calculated from the difference in sCT concentration between the supernatant and the original mixture.

2.4.3. Animal experiment

The animal experiments were carried out in the same manner as described in our previous study (Sakuma et al., 1997a). Seven week-old male Sprague–Dawley strain rats (ca. 220 g of body weight) were fasted overnight with free access to water before experiments ($n = 5$). The dosing solution (a mixture of sCT and nanoparticles) was administered orally to rats at a dose of 0.25 mg of sCT and 25 mg of nanoparticles in 2.5 ml of mixture/kg of body weight. As a control, an aqueous solution of sCT was administered to rats under the same conditions. The ionized calcium concentration in blood (approx. 0.1 ml) obtained from the tail vein was measured with an analyzer using the calcium electrodes.

The change in calcium concentration from before to after oral administration of the dosing solution was calculated, and the means and standard errors were determined. Each value was plotted as a function of time. The area between the curve for the change in the ionized calcium concentration versus time and a horizontal line representing zero change was also calculated by the trapezoidal method until the ionized calcium concentration returned to the initial value. The value obtained (area of ionized calcium reduction) was used as an index of the biological effect of sCT, along with a minimum ionized calcium concentration level (Sakuma et al., 1997b; Morita et al., 1994; Kobayashi et al., 1994). Statistical significance was assessed with Student's t-test, and P-values of 0.05 or less were considered significant.

3. Results

3.1. Characteristics of nanoparticles

Table 1 shows the characteristics of nanoparticles. The number-average molecular weights of macromonomers on the nanoparticle surface were adjusted to the order of 10^3–10^4. IR spectra and elemental analysis supported that ionic VAm and MAA macromonomers were obtained by hydrolysis of NVA and BMA macromonomers, respectively (data not shown). The weight-average diameters of nanoparticles were less than 1000 nm. The size of PNIPAAm nanoparticles decreased with the decreasing ratio of NIPAAm macromonomer to styrene as was also shown in the previous research (Chen et al., 1996). A similar phenomenon was observed when PNIPAAm–PVAm nanoparticles were prepared.

Details of the zeta potential of nanoparticles were examined. The electric charges of nanoparticles having one kind of surface polymeric chains were consistent with the charges of the corresponding macromonomers. Nonionic PNIPAAm and PNVA nanoparticles rarely showed the surface electric charge. The zeta potential of anionic PMAA nanoparticles prepared by the hydrolysis of PBMA nanoparticles was -26.0 mV at pH 7.4, which was very close to that of PMAA nanoparticles prepared by the copolymerization of MAA macromonomers with styrene. They did not possess a surface electric charge at pH 2.0 because the carboxyl group on the nanoparticle surface was not dissociated. On the other hand, the zeta potential of cationic PVAm nanoparticles increased with decreasing pH. The preparation methods of PVAm nanoparticles did not affect the magnitude of the zeta potential. When the ratio of VAm macromonomer to styrene was increased, the zeta potential of PVAm nanoparticles at pH 7.4 increased by 5 mV to 16 mV.

Table 1
Physicochemical properties of nanoparticles

	Ratio of monomer	Mn[g]	Particle size (nm)	Zeta potential (mV)		sCT incorporation (%)
				pH 2.0	pH 7.4	
PNIPAAm NPs	1:40[e]	3.5	895	n.t.[h]	−3.2	15
PNIPAAm NPs	1:5[e]	3.5	315	n.t.	0.7	28
PNVA NPs	1:40[e]	15	154	n.t.	−1.6	80
PVAm NPs[a]	1:40[e]	15	220	23.6	12.7	52
PVAm NPs[b]	1:40[e]	19	645	18.5	11.1	n.t.
PVAm NPs[b]	1:5[e]	19	296	n.t.	16.2	n.t.
PMAA NPs[c]	1:40[e]	26	372	n.t.	−26.0	100
PMAA NPs[d]	1:40[e]	14	197	−1.4	−27.8	n.t.
PNIPAAm–PVAm NPs	1.5:0.5:80[f]	3.5/19	804	16.9	7.1	n.t.
PNIPAAm–PVAm NPs	1.5:0.5:10[f]	3.5/19	386	14.5	3.4	30
PNIPAAm–PVAm NPs	1:1:80[f]	3.5/19	851	17.4	9.1	n.t.
PNIPAAm–PVAm NPs	0.5:1.5:80[f]	3.5/19	751	15.8	5.1	n.t.
PNIPAAm–PMAA NPs	1:1:80[f]	3.5/14	540	n.t.	−9.9	100
PNIPAAm–PNVA NPs	1:1:80[f]	3.5/15	736	n.t.	0.0	n.t.
PVAm–PMAA NPs	1:1:80[f]	19/14	318	24.9	−13.8	92
PNVA–PMAA NPs	1.5:0.5:80[f]	15/14	189	n.t.	−0.7	n.t.
PNVA–PMAA NPs	1:1:80[f]	15/14	189	n.t.	−0.3	n.t.
PNVA–PMAA NPs	0.5:1.5:80[f]	15/14	148	n.t.	−2.2	n.t.

[a] Prepared by hydrolysis of PNVA NPs.
[b] Prepared by copolymerization of VAm macromonomer with styrene.
[c] Prepared by hydrolysis of PBMA NPs.
[d] Prepared by copolymerization of MAA macromonomer with styrene.
[e] Ratio of macromonomer to styrene.
[f] Ratio of the respective macromonomers to styrene.
[g] Number-average molecular weight of macromonomer ($\times 10^3$).
[h] Not tested.

When NIPAAm macromonomers were copolymerized with ionic macromonomers and styrene, the electric charge on the nanoparticle surface was consistent with that of the corresponding ionic macromonomer. However, the magnitude of the zeta potential of PNIPAAm–PVAm and PNIPAAm–PMAA nanoparticles was decreased by the addition of nonionic NIPAAm macromonomers in the copolymerization. There was no correlation between the zeta potential of PNIPAAm–PVAm nanoparticles and the amount of the cationic VAm macromonomer that was used in their preparation. On the other hand, when anionic MAA macromonomers were copolymerized with nonionic NVA macromonomers and styrene, the zeta potential of the resulting PNVA–PMAA nanoparticles was almost 0 mV, irrespective of the ratio of each macromonomer. In the case of PVAm–PMAA nanoparticles (VAm macromonomer:MAA macromonomer:styrene = 1:1:80), they possessed a positive charge (24.9 mV) at pH 2.0 and a negative charge (−13.8 mV) at pH 7.4.

The rates of sCT incorporated in PNIPAAm, PNVA, PVA and PMAA nanoparticles (macromonomer:styrene = 1:40) were 15, 80, 52 and 100%, respectively. They almost coincided with those obtained in our past research (Sakuma et al., 1997a). The rate of sCT incorporation in PNIPAAm nanoparticles increased with increasing the macromonomer ratio to styrene. It also increased when cationic VAm macromonomers were added in the copolymerization to prepare PNIPAAm–PVAm nanoparticles. Overall, sCT was strongly interacted with anionic nanoparticles because it is a basic peptide.

3.2. Oral absorption enhancement of sCT by nanoparticles

Table 2 shows the hypocalcemic effect after oral administration of sCT alone and mixtures of sCT and nanoparticles. When a mixture of sCT and PVAm nanoparticles prepared by the hydrolysis of PNVA nanoparticles was administered orally, the minimum ionized calcium concentration level and area of ionized calcium reduction were 88.5% and 0.49 mM·h, respectively. PVAm nanoparticles which were prepared by copolymerization of VAm macromonomer and styrene possessed the equivalent enhancement effect (minimum ionized calcium concentration level: 85.4%; area of ionized calcium reduction: 0.39 mM·h). The enhancement effect of sCT absorption by PMAA nanoparticles was also not affected by their preparation methods. The absorption of sCT was enhanced significantly by PNIPAAm nanoparticles (minimum ionized calcium concentration level: 82.3%; area of ionized calcium reduction: 0.52 mM·h; $P < 0.05$) as shown in Fig. 2. Furthermore, the area of ionized calcium reduction doubled when the ratio of NIPAAm macromonomer to styrene was increased.

The absorption of sCT was also enhanced by PNIPAAm–PVAm nanoparticles as shown in Fig. 3 and Table 2. The degree of this enhancement effect depended on the ratio of each macromonomer. The area of ionized calcium reduction after oral administration of a mixture of sCT and PNIPAAm–PVAm nanoparticles (NIPAAm macromonomer:VAm macromonomer: styerene = 1.5:0.5:80) was 1.18 mM · h. It was twice the area of ionized calcium reduction by the mixture of sCT and PNIPAAm nanoparticles (macromonomer:styerene = 1:40).

Table 2
Hypocalcemic effect after oral administration of sCT alone and mixtures of sCT and nanoparticles

	Ratio of monomer	Minimum Ca^{2+} level[g] (% of initial)	Area of Ca^{2+} reduction[h] (mM·h)
sCT alone		89.3 ± 1.8	0.21 ± 0.09
sCT and PNIPAAm NPs	1:40[e]	82.3 ± 2.3 (0.05[i])	0.52 ± 0.10 (0.05[i])
sCT and PNIPAAm NPs	1:5[e]	76.3 ± 1.8 (0.001[i], n.s.[j,k])	0.91 ± 0.16 (0.01[i], n.s.[k])
sCT and PNVA NPs	1:40[e]	92.0 ± 1.5	0.26 ± 0.03
sCT and PVAm NPs[a]	1:40[e]	88.5 ± 2.3	0.49 ± 0.11
sCT and PVAm NPs[b]	1:40[e]	85.4 ± 2.4	0.39 ± 0.08
sCT and PMAA NPs[c]	1:40[e]	88.5 ± 3.1	0.34 ± 0.13
sCT and PMAA NPs[d]	1:40[e]	86.9 ± 2.3	0.31 ± 0.14
sCT and PNIPAAm–PVAm NPs	1.5:0.5:80[f]	72.0 ± 1.0 (0.001[i], 0.005[k])	1.18 ± 0.09 (0.001[i,k])
sCT and PNIPAAm–PVAm NPs	1.5:0.5:10[f]	71.6 ± 1.0 (0.001[i], 0.005[k])	1.53 ± 0.09 (0.001[i,k])
sCT and PNIPAAm–PVAm NPs	1:1:80[f]	83.7 ± 3.1	0.40 ± 0.19
sCT and PNIPAAm–PVAm NPs	0.5:1.5:80[f]	81.6 ± 1.7 (0.05[i], n.s.[k])	0.67 ± 0.09 (0.05[i], n.s.[k])
sCT and PNIPAAm–PMAA NPs	1:1:80[f]	83.7 ± 3.7	0.46 ± 0.16
sCT and PNIPAAm–PNVA NPs	1:1:80[f]	89.8 ± 2.4	0.24 ± 0.08
sCT and PVAm–PMAA NPs	1:1:80[f]	78.3 ± 1.9 (0.005[i], n.s.[k])	0.69 ± 0.11 (0.05[i], n.s.[k])

[a] Prepared by hydrolysis of PNVA NPs.
[b] Prepared by copolymerization of VAm macromonomer with styrene.
[c] Prepared by hydrolysis of PBMA NPs.
[d] Prepared by copolymerization of MAA macromonomer with styrene.
[e] Ratio of macromonomer to styrene.
[f] Ratio of the respective macromonomers to styrene.
[g] Minimum blood ionized calcium concentration level (mean ± S.E.).
[h] Area between blood ionized calcium concentration level versus time curve and horizontal 0 at change (mean ± S.E.).
[i] Statistically significant difference from sCT alone.
[j] Not significant ($P > 0.05$).
[k] Statistically significant difference from sCT and PNIPAAm NPs (1:40).

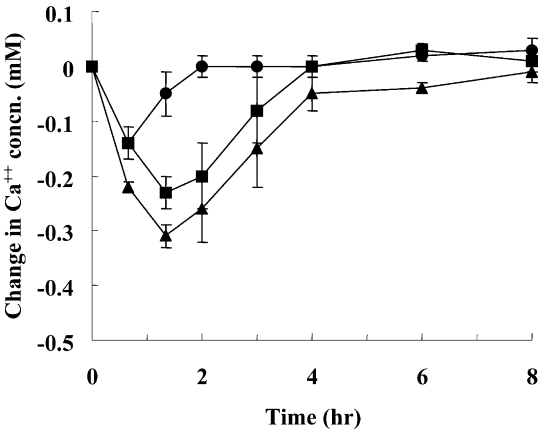

Fig. 2. Concentration–time profiles of ionized calcium in blood after oral administration of sCT aqueous solution (●), a mixture of sCT and PNIPAAm nanoparticles (macromonomer:styrene = 1:40) (■) and a mixture of sCT and PNIPAAm nanoparticles (1:5) (▲) in rats (0.25 mg of sCT with 25 mg of nanoparticles/2.5 ml/kg). Each value represents the mean ± S.E. of five experiments.

This hypocalcemic effect after oral administration of sCT with the PNIPAAm–PVAm nanoparticles was further improved when the ratio of the respective macromonomers to styrene was increased.

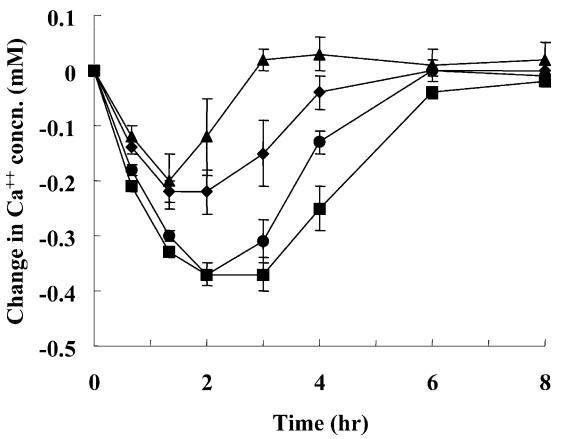

Fig. 3. Concentration–time profiles of ionized calcium in blood after oral administration of a mixture of sCT and PNIPAAm–PVAm nanoparticles (NIPAAm macromonomer:VAm macromonomer:styrene = 1.5:0.5:80) (●), a mixture of sCT and PNIPAAm–PVAm nanoparticles (1.5:0.5:10) (■), a mixture of sCT and PNIPAAm–PVAm nanoparticles (1:1:80) (▲) and a mixture of sCT and PNIPAAm–PVAm nanoparticles (0.5:1.5:80) (◆) in rats (0.25 mg of sCT with 25 mg of nanoparticles/2.5 ml/kg). Each value represents the mean ± S.E. of five experiments.

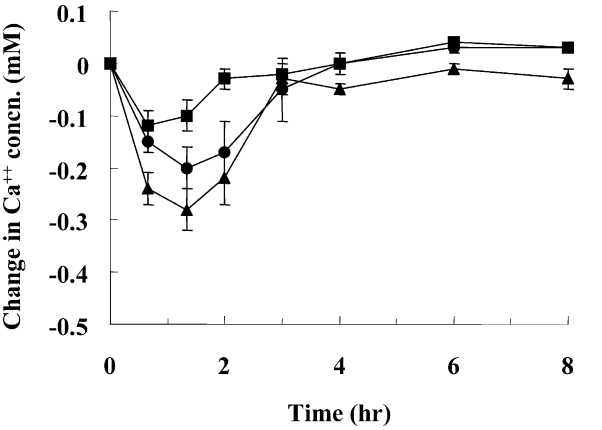

Fig. 4. Concentration–time profiles of ionized calcium in blood after oral administration of a mixture of sCT and PNIPAAm–PMAA nanoparticles (1:1:80) (●), a mixture of sCT and PNIPAAm–PNVA nanoparticles (NIPAAm macromonomer:NVA macromonomer:styrene = 1:1:80) (■) and a mixture of sCT and PVAm–PMAA nanoparticles (1:1:80) (▲) in rats (0.25 mg of sCT with 25 mg of nanoparticles/2.5 ml/kg). Each value represents the mean ± S.E. of five experiments.

Fig. 4 shows the concentration–time profiles of ionized calcium in blood after oral administration of mixtures of sCT and the residual nanoparticles. The area of ionized calcium reduction by a mixture of sCT and PNIPAAm–PMAA nanoparticles was 0.46 mM·h. It was almost equal to that by the mixture of sCT and PNIPAAm nanoparticles (1:40). The hypocalcemic effect by a mixture of sCT and PVAm–PMAA nanoparticles (minimum ionized calcium concentration level: 78.3%; area of ionized calcium reduction: 0.69 mM·h) was larger than that by the mixture of sCT and PNIPAAm nanoparticles (1:40) although there was no statistical significant difference between both biological effect. There was no enhancement effect of sCT absorption by PNIPAAm–PNVA nanoparticles.

4. Discussion

It is essential to design rationally the chemical structure of carriers that dominates their physico-

chemical properties and behavior on exposure to physiological media. New chemical substances are often used to create promising drug carriers. We demonstrated that nanoparticles composed of novel graft copolymers having a hydrophobic backbone and hydrophilic branches are useful as carriers for oral peptide delivery. sCT, which was used as a model drug, were incorporated in nanoparticles through not only the electrostatic interaction between sCT and the surface hydrophilic polymeric branches but other interaction such as hydrogen bonding between them (Sakuma et al., 1997a, 2001). The decrease in blood ionized calcium level induced by sCT in the presence of nanoparticles depended on the chemical structure of hydrophilic branches located on the nanoparticle surface. This hypocalcemic effect was enhanced in decreasing order by PNIPAAm, PVAm and PMAA nanoparticles (Sakuma et al., 1997a), and the area under the plasma sCT concentration–time curve (AUC) after its oral administration with PNIPPAm nanoparticles, for instance, was 10 times that after oral administration of sCT alone (data not published). Our past research (Sakuma et al., 1997c, 1999; Sakuma et al., in press) concluded that both the protection of sCT against digestive enzyme-catalyzed degradation by nanoparticles and the delivery of sCT to the vicinity of the intestinal mucous layer through the mucoadhesive properties of nanoparticles incorporating sCT mainly contribute to the absorption enhancement.

PNIPAAm nanoparticles possessed the strongest mucoadhesive property and completely inhibited the degradation of sCT by pepsin (Sakuma et al., 1997b,c, 1999). This character of PNIPAAm nanoparticles was related to the strong enhancement effect of sCT absorption although the hypocalcemic effect after oral administration of sCT (250 μg/kg) with them was weaker than that after subcutaneous administration of sCT at a dose of 3 μg/kg. The absorption of sCT in the presence of PNIPAAm nanoparticles was further improved when the ratio of NIPAAm macromonomer to styrene was increased. In this situation, it is probable that a larger amount of NIPAAm macromonomer was located on the nanoparticle surface. Our speculation was supported by the zeta potential of PVAm nanoparticles (VAm macromonomer:styrene = 1:5) which was higher than that of PVAm nanoparticles (VAm macromonomer:styrene = 1:40). These findings pointed out that an increase in the amount of macromonomers, which induce the absorption enhancement, is effective for improving the absorption enhancement.

Here, the low incorporation rate and lack of protection against trypsin-catalyzed degradation were disadvantages of PNIPAAm nanoparticles. It has already been confirmed that both ionic PVAm and PMAA nanoparticles can incorporate a sufficient amount of sCT and inhibit sCT degradation by trypsin (Sakuma et al., 1997a,c). Then, PVAm and PMAA chains were introduced onto the surfaces of PNIPAAm nanoparticles in order to compensate for the disadvantage of the nanoparticles. The introduction of ionic polymeric chains was verified by measurement of the zeta potential. The decrease in the magnitude of the zeta potential suggested that nonionic PNIPAAm chains partially shielded electric charges of the PNIPAAm–PVAm and PNIPAAm–PMAA nanoparticles (Sakuma et al., 1989, 1990). This shielding effect resulted in no tendency for the zeta potential to increase in proportion to the amount of VAm macromonomer. These nanoparticles having both surface PNIPAAm and ionic polymeric chains enhanced sCT absorption as well as did PNIPAAm nanoparticles. In the case of PNIPAAm–PVAm nanoparticles, the enhancement effect depended on the ratio of the respective macromonomers. PNIPAAm–PVAm nanoparticles (NIPAAm macromonomer:VAm macromonomer:styrene = 1.5:0.5:80) showed two times the enhancement effect of PNIPAAm nanoparticles (NIPAAm macromonomer: styrene = 1:40). This enhancement effect was further improved by increasing the ratio of the respective NIPAAm and VAm macromonomers to styrene. However, there was no difference in the enhancement effect between the PNIPAAm nanoparticles and other PNIPAAm–PVAm nanoparticles. The proper ratio of NIPAAm macromonomer to VAm macromonomer may be 3:1 for strongly enhancing sCT absorption. The function of PNIPAAm chains is both the reten-

tion of sCT in the vicinity of intestinal mucosa through the strong mucoadhesion and the stabilization of sCT in the stomach through the protection of sCT against pepsin-catalyzed degradation (Sakuma et al., 1997b,c; Sakuma et al., in press). Additional PVAm chains probably assisted PNIPAAm chains in terms of protecting sCT against trypsin-catalyzed degradation in the small intestine and delivery of a larger amount of sCT to the vicinity of the mucous layer through the relatively high incorporation of sCT as shown in Table 1.

On the contrary, the absorption-enhancing function of PNIPAAm nanoparticles vanished without a trace when PNVA chains were introduced onto their surfaces. As shown in the zeta potential of PNVA–PMAA nanoparticles, nonionic PNVA chains also shielded the electric charge of anionic PMAA chains. It seemed that this shielding effect was stronger than that of PNIPAAm chains, because the anionic charge of PNVA–PMAA nanoparticles disappeared completely, irrespective of the ratio of MAA macromonomer to NVA macromonomer. The interference by PNVA chains on the PNIPAAm–PNVA nanoparticle surface probably took away the ability of PNIPAAm chains to enhance sCT absorption.

This research is a guide to optimize the chemical structure of nanoparticles as carriers for oral peptide delivery. In the case of sCT, its absorption was significantly enhanced by nanoparticles composed of a hydrophobic polystyrene backbone and hydrophilic PNIPAAm branches. This enhancement effect was further improved by increasing the ratio of PNIPAAm to styrene. The introduction of cationic PVAm chains also facilitated the enhancement effect of sCT absorption synergistically although the ratio of PVAm chains to PNIPAAm chains greatly affected the enhancement effect. The optimal chemical structure of a nanoparticle probably varies according to characteristics of peptide drugs. We are certain, therefore, that research on the optimization of nanoparticles for other peptide drugs will make the field of nanoparticle technology fertile. The optimization of the chemical structure of nanoparticles for human calcitonin will be discussed with quantitative data such as bioavailability in the next report.

5. Conclusions

Novel nanoparticles having two kinds of surface hydrophilic polymeric chains were synthesized to improve the enhancement effect of sCT absorption by PNIPAAm nanoparticles. The enhancement effect was considerably increased by introducing cationic PVAm groups onto the surface of PNIPAAm nanoparticles. The absorption enhancement depended on the ratio of NIPAAm and VAm macromonomers to styrene in the nanoparticle preparation. In contrast, the absorption-enhancing function of PNIPAAm nanoparticles vanished without a trace after the introduction of nonionic PNVA groups. It was suggested that this disappearance was due to the shielding of PNIPAAm groups by PNVA groups. The enhancement effect of sCT absorption by nanoparticles was greatly dominated by their chemical structure that was closely related to surface characteristics. Optimization of the chemical structure of nanoparticles resulted in the further improvement of sCT absorption.

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