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Research Paper

Thermosensitive Drug Delivery System of Salmon Calcitonin: *In Vitro* Release, *In Vivo* Absorption, Bioactivity and Therapeutic Efficacies

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Purpose. The purpose of this study was to develop a biodegradable triblock copolymer, mPEG-PLGA-mPEG-based delivery system for long-term controlled release of salmon calcitonin (sCT) after single subcutaneous injection.
Methods. The delivery system was prepared by dissolving sCT into polymer solution. *In vitro* release of sCT from the delivery systems was studied in phosphate buffer saline (PBS, pH 7.4) at 37°C. Stability of released sCT and sCT remaining in gel formulation was evaluated using circular dichroism, HPLC and MALDI-TOF mass spectrometry. *In vivo* absorption and therapeutic efficacy of sCT from the polymeric formulations were examined in female wistar rats and methylprednisolone acetate (MPA)-induced osteoporosis rat model, respectively.
Results. The polymeric formulations of sCT showed long term controlled release (~20 to 40 days) of sCT in its conformationally and chemically stable form. The sCT polymeric formulations controlled the release of sCT over ~20 to 40 days and prevented MPA induced osteoporosis *in vivo*. The released sCT was biologically active in terms of lowering serum calcium level.
Conclusions. The triblock copolymer delivery systems controlled the release of sCT *in vitro* and *in vivo* in chemically and conformationally stable as well as biologically active and therapeutically effective form.

KEY WORDS: controlled release; *in vivo* absorption; salmon calcitonin; stability; triblock copolymer.

INTRODUCTION

Osteoporosis is an asymptomatic disease that leads to bone fragility and an increased susceptibility to fracture, especially of the hip, spine, and wrist. In osteoporosis, the bone mineral density decreases and bone microarchitecture deteriorates. Osteoporosis causes a great burden to society in terms of mortality, morbidity, and economic costs. According to the statistics from the International Osteoporosis Foundation, osteoporosis is affecting 75 million people (data from Europe, USA and Japan) and causing millions of fractures annually (1). Osteoporosis is a major public health threat for at least 44 million Americans, or 55% of the people over 50 years old. It is estimated that 30–50% of women and 15–30% of men will suffer a fracture related to osteoporosis in their lifetime; in another words, 1 in 3 women and 1 in 5 men will experience an osteoporotic fracture (2–4).

Calcitonin is a 32-amino acid polypeptide hormone that has been used for many years in the treatment of metabolic bone diseases, particularly osteoporosis (5). This polypeptide hormone was discovered in the 1950s and was claimed to have inhibitory effects of bone resorption. Natural calcitonin is produced by the parafollicular cells of the thyroid in mammals and ultimobranchial gland in fish and birds. It has high potency in decreasing the speed of bone resoption through the inhibition of osteoclasts function (cells responsible for bone break-down and minerals release) and promoting mineralization of skeletal bone. Because of its hypocalcemic effect, calcitonin is also used in the treatment for hypercalcemia, a disorder characterized by abnormal high blood calcium level.

The most widely used calcitonin is synthetic or recombinant salmon calcitonin (sCT), as it is 40–50 times more potent and has longer duration of action than human calcitonin (6). Up to date, sCT is given parenterally, preferably by subcutaneous or intramuscular injections. sCT has a short half life of ~1 h and is primarily and almost exclusively degraded in the kidneys, forming pharmacologically inactive fragments of the molecule (7). Due to the fast clearance, daily injection of sCT is required for prevention of bone resorption. Unfortunately, the high frequency of injection decreases patient compliance, particularly for the long-term therapy of osteoporosis (1–5 years). Intranasal delivery is an alternative route for injections, but the average bioavailability of intranasal sCT is ~3%, with a range of 0.3–30.6% (8). Emisphere reported an oral tablet dosage form of sCT for the treatment of osteoporosis; however, clinical studies found that the oral delivery route required a minimum 40 times higher daily dose

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than subcutaneous injection (9). Such a high dose not only causes gastrointestinal side effects but also increases the economic burden on the society.

The frequent dosing, low bioavailability from intranasal and oral routes, and decreased patient compliance sparked research interests for developing parenteral controlled delivery system that controls the release of sCT over a long period of time after a single injection. A sustained release of sCT based on the lactide: glycolide copolymers controlled the release over 2 weeks (10). This delivery system released ~10% of loaded sCT for the first week followed by a burst release of 90% sCT during the second week. Besides the unfavorable release pattern, this delivery system requires a surgical implantation. Later on, chitosan beads were claimed to be able to control the delivery of sCT over 25 days; however, the size of the beads limited their use as injectable formulation (11). Duros implant system was reported to sustain the release of sCT over 4 months, but unfortunately, this system requires a surgical implantation and explantation (12). Thereafter, many investigations were carried out to achieve the goal of controlling the release of sCT using poly (D,L lactide-co-glycolide) or poly(D,L-lactide) polymers-based microsphere, poly(ethylene glycol)-terephthalate film, and surface-modified lipid nanoparticles (13–17). Although the investigations made improvements in the controlled delivery of sCT, these systems are still unsatisfactory because of the short release duration, large burst release, peptide degradation during manufacturing process, and incomplete release. The peptide degradation and incomplete release mainly result from the change of peptide physico-chemical structure and chemical degradation.

sCT has no tertiary conformation and even lacks well-organized secondary structure such as α-helices and β-sheets (18). Although sCT of well-organized secondary structures can bind to calcitonin receptors to initiate the biological response, it has a higher propensity for fibrilization and aggregation, which is a major reason for incomplete release of the peptide from delivery systems and decreased biological potency (19–21). sCT is also susceptible to chemical modifications, such as disulfide breakage with trisulfide bond formation, dimerization through covalent bond, backbone hydrolysis, and acylation in poly(D, L lactide-co-glycolide) polymer-based microsphere delivery systems. These chemical modifications lead to the decrease in the bioactivity of the peptide and even result into immunogenicity (22,23). Thus, a suitable controlled delivery system of sCT should be able to release incorporated sCT for a longer period in its random coil conformation and without chemical degradations such as dimmerization, hydrolysis, deamidation, acylation etc.

The importance and difficulties of long-term controlled delivery of sCT inspired our research interests in developing copolymer mPEG-PLGA-mPEG-based thermosensitive *in situ* gel forming drug delivery systems. They possess significant advantages, such as ease of manufacturing, avoidance of organic solvents, convenience of administration, and sustained release of incorporated drug (24,25). Before administration, these special drug delivery systems present as injectable solution. Upon injection, they fast transit to gel status at injection site (usually subcutaneous) in response to body temperature and served as drug delivery depot, which is due to the abrupt change in the spatial arrangement of the polymer molecules in response to the temperature change (26). Formulation procedure of the thermosensitive polymer-based drug delivery system is straightforward. Briefly, both water soluble and insoluble drugs can be incorporated into the systems by simple dissolution or suspension. These advantages make the thermosensitive polymeric systems ideal for developing controlled-release formulations for protein therapeutics.

In our previous study, the triblock mPEG-PLGA-mPEG copolymer of composition EG_{12}-$L_{35}G_{12}$-EG_{12} (Mw=4318Da) was found to be suitable for controlled release of bovine serum albumine, lysozyme and insulin over a long period while conserving proteins' stability and activity (27). Furthermore, this copolymer shows non-cytotoxicity and has excellent *in vivo* biodegradability and biocompatibility. Thus, the copolymer EG_{12}-$L_{35}G_{12}$-EG_{12}-based thermosensitive system was considered to be a potential system for long-term controlled delivery of sCT. In the present study, a parenteral controlled delivery system of sCT was developed using the copolymer (EG_{12}-$L_{35}G_{12}$-EG_{12}). The conformational and chemical stability of sCT were studied using CD for secondary structure and peptide fibrillation and reverse phase-HPLC and MALDI-TOF mass spectrometry for chemical changes. *In vivo* absorption of sCT from the delivery systems was studied in female wistar rats. The therapeutic effect of sCT delivery systems was evaluated using glucocorticoid (MPA)-induced osteoporosis in female wistar rats.

MATERIALS AND METHODS

Materials

sCT was obtained from Bachem (Torrance, CA). Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). Methylprednisolone acetate (MPA) and Methyl cellulose (15cps) were obtained from Spectrum Chemical Mfg. Corp. (Gardena, CA). Female wistar rats were obtained from Harlan (Indianapolis, IN). Salmon calcitonin EIA kit (extract free) was obtained from Peninsula Laboratories, Inc. (San Carlos, CA). QuantiChrom™ Calcium Assay Kit was purchased from BioAssay Systems (Hayward, CA). All other chemicals used were of analytical grade (other than HPLC grade solvents).

Methods

Preparation of Delivery Systems

The triblock copolymer EG_{12}-$L_{35}G_{12}$-EG_{12} was synthesized by ring-opening polymerization following diblock condensation and characterized by ^{1}H-NMR for structural composition and GPC for molecular weight distribution of synthesized copolymers. The details about the methods of synthesis and characterization can be found in our previous publication (27).

The copolymer was dissolved in distilled water at a concentration of 40% (*w/v*). sCT, either 0.5% *w/v* (5 mg/ml) or 0.25% *w/v* (2.5 mg/ml), was added to the copolymer solution and vortexed mildly for 20 s at room temperature. sCT-loaded formulations were tested for injectability using 25G needle and suitable thermosensitivity for *in vivo*

application by tube inverting method. The detailed methods were described in our previous publications (27).

In Vitro Release of sCT

An aliquot of 0.32 ml solution formulation was injected into a 5 ml polypropylene tube followed by 10 s incubation at 37°C to form gel. Pre-warmed PBS (0.0lM, pH 7.4) containing NaN_3 (0.02% *w/v*) was added into the tube to serve as release medium for sCT from the gel. The tube was incubated in a reciprocal shaking water bath at 37°C and 35 rpm. Samples were withdrawn periodically and replaced by the same amount of fresh release medium. The amount of released sCT was determined by microBCA protein assay. An aliquot of 150 μl suitably diluted sample was mixed with 150 μl of working reagent (micro BCA reagent A, B and C in a volume ratio of 50:48:2). The mixture was incubated at 37°C for 2 h and then cooled down to room temperature. Absorbance at 570 nm was measured by Dynex MRX Revelation TC model 96-well microplate reader (Vienna, VA). The released amount of sCT was calculated by converting the absorbance into mass using the standard curve.

Stability Studies of In Vitro Released sCT

Stability of released sCT was examined using circular dichroism (CD), MALDI-TOF mass spectrometry, and HPLC methods and evaluated by comparing to the native sCT (freshly prepared sCT solution in PBS) and sCT controls (sCT solution in PBS incubated at 37°C for the same period of time as released samples).

CD measurement was performed in a quartz 0.1 cm cell, using a Jasco J-815 CD spectrophotometer (Jasco, Tokyo, Japan). CD signals were recorded from 190 to 235 nm at 25°C using a bandwidth of 1 nm and a scanning rate of 100 nm/min. All spectra were the average of 5 consecutive scans. Fresh release medium (PBS) was scanned in the same wavelength range for obtaining a baseline to eliminate the background interference. The molar ellipticity was calculated using the following equation:

$$Molar\ Ellipticity[\theta] = \theta / C \times l$$

where θ is the ellipticity in mdeg, l is the pathlength (0.1 cm) of the cell, and C is the concentration of sCT sample in mmol/L.

MALDI-TOF mass spectrometry (Bruker MALDI TOF II, Bruker Daltonics Inc., Billercia, MA) was used for confirming the primary structure stability of sCT. For sample preparation, 20 μl of released sCT sample was mixed with 90 μl of matrix solution, which is prepared by dissolving 10 mg of α-Cyano-4-hydroxycinnamic acid in 1 ml mixture of acetonitrile/1% TFA water (1:1, *v/v*). An aliquot (2 μl) of the final solution was placed on the sample plate and dried at room temperature before inserting into the mass spectrometer. Samples were run in the Positive Reflectron mode, and data was analyzed using FlexAnalysis® software provided with the instrument.

HPLC using UV detector is a technique widely used in the field of polypeptide analysis. The HPLC assay of sCT was proved to be a selective, precise and rapid method for chemical stability screening and gave excellent correlation with biological activity of the peptide (28). In this study, the HPLC analysis of sCT was performed on an Agilent 1100 system equipped with guard column (ZORBAX SB-C18, 4.6×12.5 mm, 5 μm) and analysis column (ZORBAX SB-C18, 4.6×100 mm, 3.5 μm). Gradient elution was applied using 0.1% trifluoroacetic acid (TFA) in water (phase A) and 0.1% TFA in acetonitrile (phase B) and increasing the amount of phase B from 25% to 45% over 15 min and from 45% to 90% over another 3 min at a flow rate of 0.8 ml/min. Samples were analyzed at the wavelength of 220 nm.

Stability of sCT in the Polymeric Systems During Drug Release

During the release period (weeks to months), a large amount of sCT remained inside the gel before finally releasing out. The remaining sCT in the gel is exposed to various stresses, as an example, acylation by degradation products of the PLGA. Therefore, it is important to check the stability of sCT remaining in the gel depot during the *in vitro* release. In this study, prepared sCT polymeric solution (0.32 ml) was injected into a polypropylene tube at room temperature and then transferred to a water bath at 37°C to form a gel. Pre-warmed PBS (0.01 M, 4 ml) was added into the tube and replaced periodically (same as in release study) for a total of 35 days to mimic the *in vitro* release study procedure. At predetermined time points, PBS was decanted, and the gel depot was collected. sCT remaining inside the gel was extracted out by dissolving the gel in a 1:1 acetonitrile/PBS solution with a gentle shaking for 20 min followed by centrifugation at 5,000 rpm for 15 min. The supernatant containing sCT was collected and examined by MALDI-TOF mass spectrometer and HPLC for chemical stability.

In Vivo Absorption of sCT from the Polymeric Systems

Female Wistar rats with body weight 200±10 g were randomly divided into different groups. Each group consisted of 6 animals. Rats in polymeric formulation groups were injected subcutaneously in the back of the neck with the delivery system at dose of sCT 2,000 μg/kg (total ~2000 IU, a 20-days dose) for formulation A group and sCT 4,000 μg/kg (total ~4000 IU, a 40-days dose) for formulation B group, assuming a 100 μg/kg (total 100 IU/day) daily dose for each formulation. Rats in sCT solution group were treated with sCT solution in PBS at the dose of 100 μg/kg (total 100 IU). Control groups were injected with blank polymeric delivery system without sCT. Blood samples (400 μl) were withdrawn from the tail vein at predetermined time points and centrifuged at 4°C and 10,000 rpm for 5 min. Serum was collected and stored at −80°C until further analysis. The sCT in serum was measured using sCT EIA kit (Peninsula Laboratories, Inc. San Carlos, CA). Serum calcium level was determined by QuantiChrom™ Calcium Assay Kit (BioAssay Systems, Hayward, CA) and described as the percentage of initial serum calcium level.

In Vivo Therapeutic Effects of sCT Polymeric Systems

Before treatments, 48 female wistar rats (9 weeks old) from Harlan (Indianapolis, Indiana) were allowed to acclimatize to feeding conditions on a normal diet and water *ad*

libitum for 2 weeks and then randomly divided into 8 groups. Six groups (Group 1, 2, 3, 4, 5, 6) of the rats (11 weeks old) were induced with the disease model of osteopenia (early state of osteoporosis) by injecting with methylprednisolone acetate solution (MPA in methyl cellulose solution) intraperitoneally (i.p.), at the dose of 0.2 mg/kg, three times a week during the study (29). At the beginning of the MPA treatment, four (Group 1, 2, 3, 4) of the six groups were injected subcutaneously a bolus dose at the back of the neck with temperature-sensitive polymeric formulations of sCT at the dose of 2,000 μg/kg (total ~2000 IU, a 20-days dose) for formulation A (Group 1, 2) and 4,000 μg/kg (total ~4000 IU, a 40-days dose) for formulation B (Group 3, 4), assuming a daily dose of 100 IU for each formulation. The other two (Group 5, 6) of the six groups were treated with blank polymeric formulation without sCT. Rats in the two control groups (Group 7, 8) received a bolus subcutaneous injection of the blank polymeric delivery system and blank methylcellulose solution without MPA (i.p. injection, three times/week). The timeline of treatments is described in Table I. Blood samples were withdrawn from the tail vein after 2, 4, 6 and 8 weeks of treatments and centrifuged immediately at 4°C and 10,000 rpm. Serum samples were collected and stored at −80°C for analysis of serum osteocalcin level using Rat Osteocalcin ELISA kit. At indicated time points, rats were euthanized, and their left tibia were immediately fixed in 10% neutral buffered formalin at 4°C for 48 h. Subsequently, the left tibia were decalcified in 10% EDTA-2Na at 4°C for 20 days, dehydrated with increased concentrations of alcohol and embedded in paraffin. Midsagittal longitudinal sections (5 μm thick) were prepared from the proximal tibial metaphysis and stained by tartrate-resistant acid phosphatase (TRAP) kit to identify osteoclasts followed by counterstaining with Alcian Blue and Hematoxylin. Osteoclasts in specified area were identified and counted following Wang's method (30).

Data Analysis

The data are presented as mean ± standard deviation. Analysis of variance (ANOVA) and student t-test were used for statistical comparison. A probability value less than 0.05 was considered significant.

The bioavailability of calcitonin was calculated based on the area under the curve (AUC) of serum sCT level *versus* time. AUC was estimated using trapezoidal rule (24) and the following equations:

$$AUC_{0-n} = \sum_{i=0}^{n} \left\{ \frac{C_i + C_{i+1}}{2} \times (t_{i+1} - t_i) \right\} \quad (1)$$

$$AUC_{n-\infty} = \frac{C_n}{K_{el}} \quad (2)$$

$$\text{Relative bioavailability} = \frac{AUC_{polymeric\ formulation}/Dose}{AUC_{sCTsolution}/Dose} \quad (3)$$

where C_i is the serum sCT concentration at time point t_i, n is the number of sampling time intervals, C_n is the concentration at the last time point, and K_{el} is the elimination constant calculated from the slop of the final segment of serum sCT concentration *versus* time. The maximum serum concentration (C_{max}) and the time to reach maximum serum concentration (T_{max}) were calculated.

RESULTS

In Vitro Release of sCT

sCT-loaded polymeric formulations showed ease of injection via 25G needle and fast sol-gel transition at 37°C (Fig. 1). *In vitro* release profiles of sCT from delivery formulations are shown in Fig. 2. Formulation A containing 0.5% (*w/v*) of sCT exhibited an initial burst release of 10.93± 0.83% on day 1, followed by a sustained release of the peptide for ~20 days. Compared to formulation A, the formulation B containing 0.25% (*w/v*) of sCT had significantly lower ($p<0.05$) initial burst release (6.05±1.31%) and longer release period over ~42 days. Additionally, release of

Table I. Grouping and Treatment Schedule for the Study of *In Vivo* Prevention of Glucocorticoid-Induced Osteoporosis by sCT from the Polymeric Delivery System

Group No.	Treatment	Time of Euthanization	
		1 month	2 months
1	MPA/Vehicle 1+sCT Formulation A	X	
2	MPA/Vehicle 1+sCT Formulation A		X
3	MPA/Vehicle 1+sCT Formulation B	X	
4	MPA/Vehicle 1+sCT Formulation B		X
5	MPA/Vehicle 1+Blank polymer formulation	X	
6	MPA/Vehicle 1+Blank polymer formulation		X
7	Vehicle 1+Blank Polymer formulation	X	
8	Vehicle 1+Blank Polymer formulation		X

X: Day of euthanization; MPA: Methylprednisolone; sCT: Salmon calcitonin; Vehicle 1: Methyl cellulose solution; sCT solution formulation: sCT in PBS

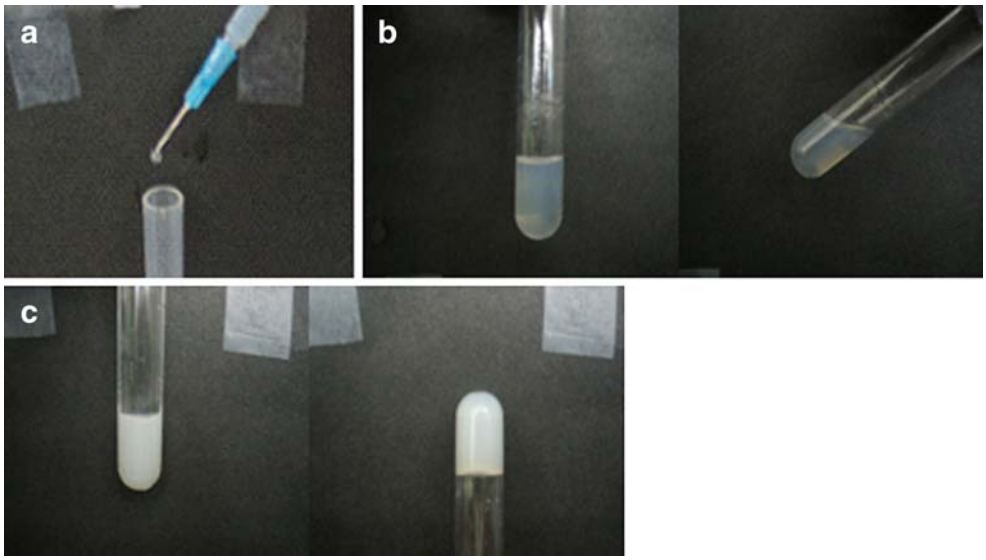

Fig. 1. mPEG-PLGA-mPEG polymer-based formulations of sCT are injectable solution at room temperature and turn to gel after incubation at 37°C for 10 s. **a** sCT polymeric formulations injected through 25G needle at room temperature; **b** sCT polymeric formulations are free-flow solution at room temperature; **c** sCT polymeric formulations turn into gel after incubation at 37°C for 10 s.

the peptide was complete from both polymeric formulations, as ~100% release of total loaded sCT was observed at the end of release duration. Table II summarizes the release kinetics of sCT from formulations. The correlation coefficients (r^2) for the release profile of sCT from the formulation A were 0.9895 for zero-order, 0.9837 for Higuchi model and 0.8715 for first-order release kinetics. The r^2 values for release kinetics for formulation B were 0.9845 for Higuchi, 0.976 for zero-order and 0.7536 for first-order kinetic models. Thus, the release profile can be attributed to follow both zero-order and Higuchi kinetic models.

Stability of *In Vitro* Released sCT

Fig. 3 presents the CD spectra of native and *in vitro* released sCT. Native sCT showed a typical negative band CD spectrum with single minimum at ~201 nm, indicating a major random coil structure with a small part of α-helix (~16%). The released sCT from formulation A presented similar CD spectra as native sCT. The CD spectra of sCT released from formulation B are also comparable to the native sCT with a little enhanced absorbance at the minimum of ~200 and concurrently decreased percentage of α-helix to ~15%.

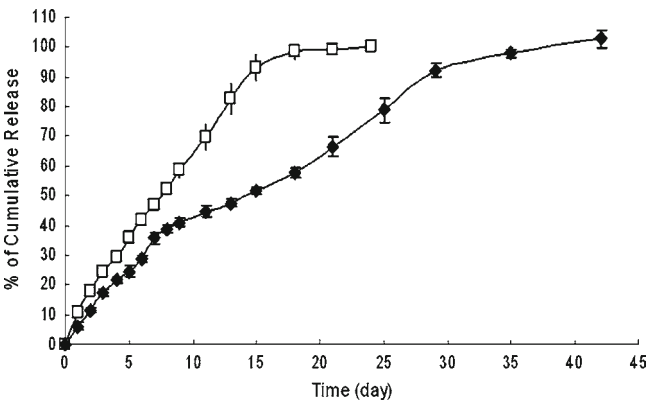

Fig. 2. *In vitro* release of sCT from thermosensitive formulations. Keys (□) Formulation A containing sCT of 0.5% *w/v*; (♦) Formulation B containing sCT of 0.25% *w/v*.

The chemical stability of released sCT was evaluated using MALDI-TOF mass spectrometry and compared to sCT control (sCT solution in PBS incubated at 37°C) (Fig. 4a). Native sCT showed a strong $(M+H)^+$ signal at the m/z ratio of 3432. After 7 days incubation, small peaks at the m/z ratio of 2329 and 2742 observed on the spectrum of sCT control were considered to be possibly corresponding to (Leu12-Pro32) and (Cys1-Arg24+Na), the back bone hydrolysis products of sCT. However, most of the peptide still kept its chemical integrity. Subsequently, sCT control withdrawn at day 15 showed a major peak at the m/z ratio of 2329 for degradation products and a smaller peak at 3432 for intact peptide, indicating the hydrolysis of most of the peptide. At the end of the incubation (day 35), the peak corresponding to intact sCT was not observed on the mass spectrum, suggesting that all the peptide was degraded.

Compared to mass spectra of the sCT control, the spectra of released sCT from the polymeric formulation A showed major signal corresponding to the molecular weight of 3432 for chemically intact peptide, except the appearance of small peak of degradation product at the m/z ratio of 2329 for sample withdrawn at day 15 (Fig. 4b). Mass spectra of 15-day released sCT from polymeric formulation B showed single peak at the m/z ratio of 3432, implying the chemical integrity of the released peptide. Although a peak for degradation product was observed for the sample withdrawn at day 35, the peak corresponding to the molecular weight of 3432 is still the major peak on the spectra, indicating most of the peptide retained its chemical integrity (Fig. 4c).

HPLC assay was used to further assess the chemical stability of released sCT and the control samples. The chromatography of chemically intact sCT showed single peak at the time point of 12.3 min (Fig. 5a). In contrast to the intact

Table II. Release Kinetics of sCT from Polymeric Formulations

Formulation	Copolymer (*w/v*)	Salmon calcitonin (*w/v*)	Zero order r^2	First order r^2	Higuchi r^2
A	40	0.5%	0.9895	0.8715	0.9837
B	40	0.25%	0.976	0.7536	0.9845

sCT, released sample from the blank polymeric formulation did not result in visible absorbance peak at the same time point on the chromatograph. Incubation of sCT control sample for 7 days showed the peak at 9.7 min, indicating a degradation product of the peptide (Fig. 5b). Along with the prolonged incubation time, increase in the relative height of degradation peak was observed with concurrently decreased height of the peak corresponding to chemically intact sCT. The peak for chemically intact sCT disappeared from the chromatograph in the 35 day incubated sample, indicating complete degradation of the peptide.

Fig. 5c shows the HPLC chromatographs of sCT released from the polymeric formulation A. The released sCT resulted in a major absorbance peak at the time point of 12.3 min corresponding to the chemically intact peptide. No major peaks for peptide degradation products were observed. The chromatographs of released sCT from polymeric formulation B are shown in Fig. 5d. Same as observed for formulation A, the released samples from formulation B withdrawn at day 15 and day 35 showed major peak at the time point of 12.3 min corresponding to chemically intact peptide. Although a relative small absorbance signal at the time point of 9.7 min was observed on the HPLC chromatogram of released sCT from formulation B withdrawn at day 35, the main absorbance peak is still at the time point of 12.3 min, suggesting that calcitonin retained its chemical integrity.

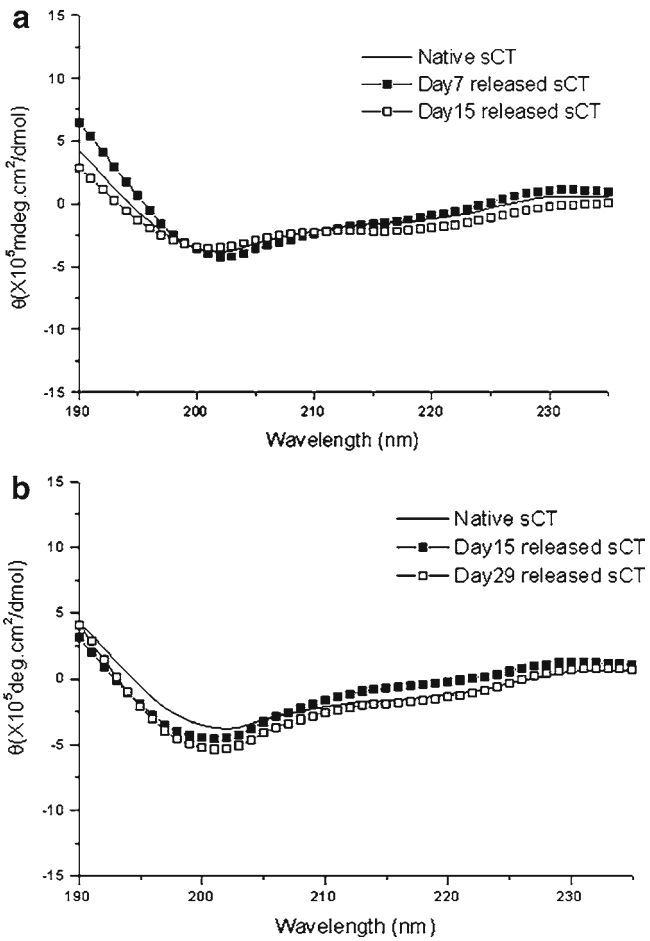

Fig. 3. CD spectra of native sCT and released sCT from the polymeric formulations. Keys: **a** Native sCT and released sCT from formulation A withdawn at day 7 and day 15, **b** Native sCT and released sCT from formulation B withdrawn at day 15 and day 29.

Stability of sCT in the Gel During the Peptide Release

sCT remaining in the polymeric gel was extracted, and its chemical stability was examined using MALDI-TOF mass spectroscopy and HPLC techniques. Fig. 6a shows the mass spectra of the peptide extracted from the gel after 7, 15 and 35 days incubation. All the samples showed a main peak at the m/z ratio of 3432 corresponding to chemically intact sCT without the appearance of peaks for any degradation products. Furthermore, HPLC chromatographs of the extracted peptide demonstrated a main peak at the time point of 12.3 min showing the peptide in the gel retained its chemical stability (Fig. 6b).

In Vivo Absorption of sCT from Polymeric Formulations

Fig. 7 shows the serum sCT concentration and calcium level after subcutaneous administration of the sCT solution in PBS (pH 7.4). After subcutaneous administration, serum sCT concentration increased rapidly and reached to the peak concentration (C_{max}) of 1.22 ng/ml in 30 min (T_{max}) post-administration, and declined afterwards to the background level within one day. Concurrently, the serum calcium level decreased to ~67% of initial calcium level during the first 3 h and was restored to basal level gradually within 24 h post-administration. Meanwhile, no noticeable decrease of serum calcium level was observed for blank control group.

Fig. 8 shows the serum sCT concentration and calcium level in the rats after subcutaneous injection of sCT-loaded polymeric formulation A. The serum sCT concentration and calcium level obtained from blank control group was used for presenting background interference. In contrast to the solution group, the increased serum sCT concentration was prolonged up to ~21 days post subcutaneous administration of the formulation A accompanied with a significant ($p<0.05$) declined serum calcium level for ~20 days. Rats administrated with formulation B showed an increased sCT concentration over ~40 days and a significantly ($p<0.05$) lower serum calcium level for more than a month in comparison to the control (Fig. 9). Pharmacokinetic parameters of sCT are summarized in Table III. The relative bioavailability was calculated using AUC values after dose correction. Both polymeric formulations showed similar bioavailability compared to the subcutaneous solution formulation of sCT.

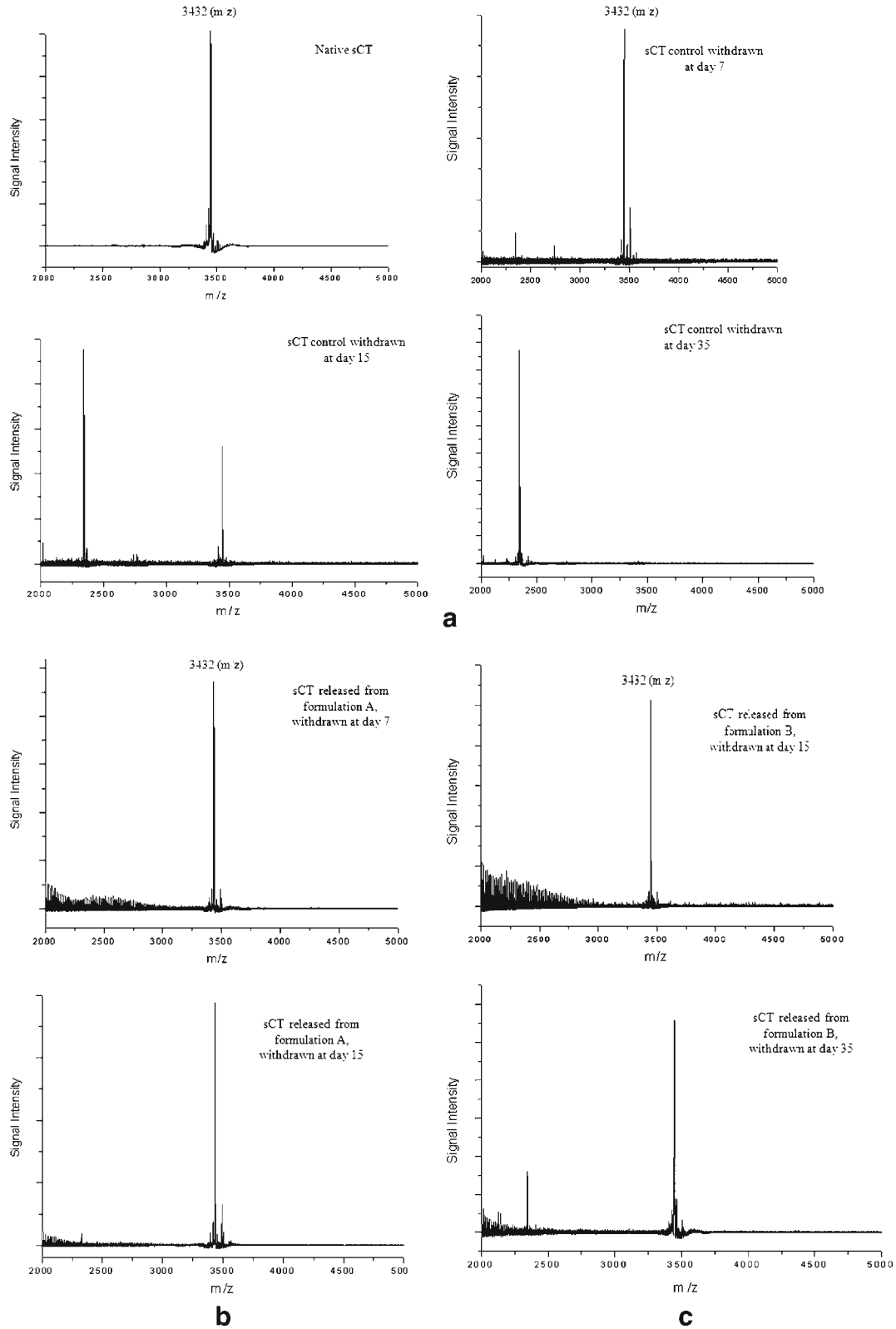

Fig. 4. sCT stability by MALDI-TOF MS. Keys: **a** mass spectra of native sCT, sCT control withdrawn at day 7, sCT control withdrawn at day 15, and sCT control withdrawn at day 35; **b** mass spectra of released sCT from polymeric formulation A withdrawn at day 7 and released sCT from polymeric formulation A withdrawn at day 15; **c** mass spectra of released sCT from polymeric formulation B withdrawn at day 15 and released sCT from polymeric formulation B withdrawn at day 35.

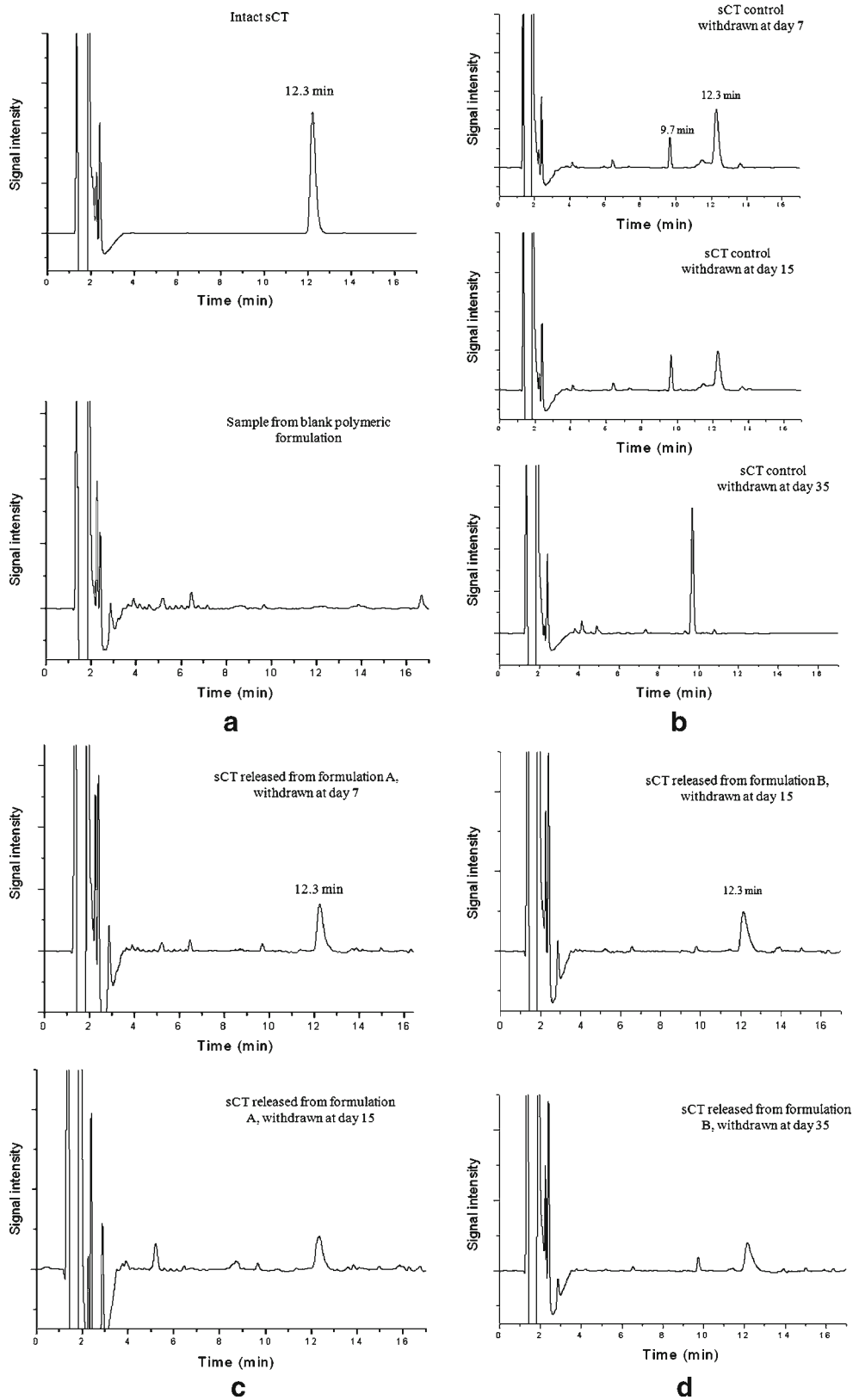

Fig. 5. sCT stability by HPLC chromatography. Keys: **a** HPLC graphs of chemically intact salmon calcitonin (freshly prepared salmon calcitonin solution in PBS) and released sample from blank polymeric formulation; **b** HPLC graphs of sCT control (sCT solution in PBS incubated at 37°C) withdrawn at day 7, sCT control withdrawn at day 15, and sCT control withdrawn at day 35; **c** HPLC graphs of released sCT form polymeric formulation A withdrawn at day 7 and released sCT from polymeric formulation A withdrawn at day 15; **d** HPLC graphs of released sCT from polymeric formulation B withdrawn at day 15 and released sCT from polymeric formulation B withdrawn at day 35.

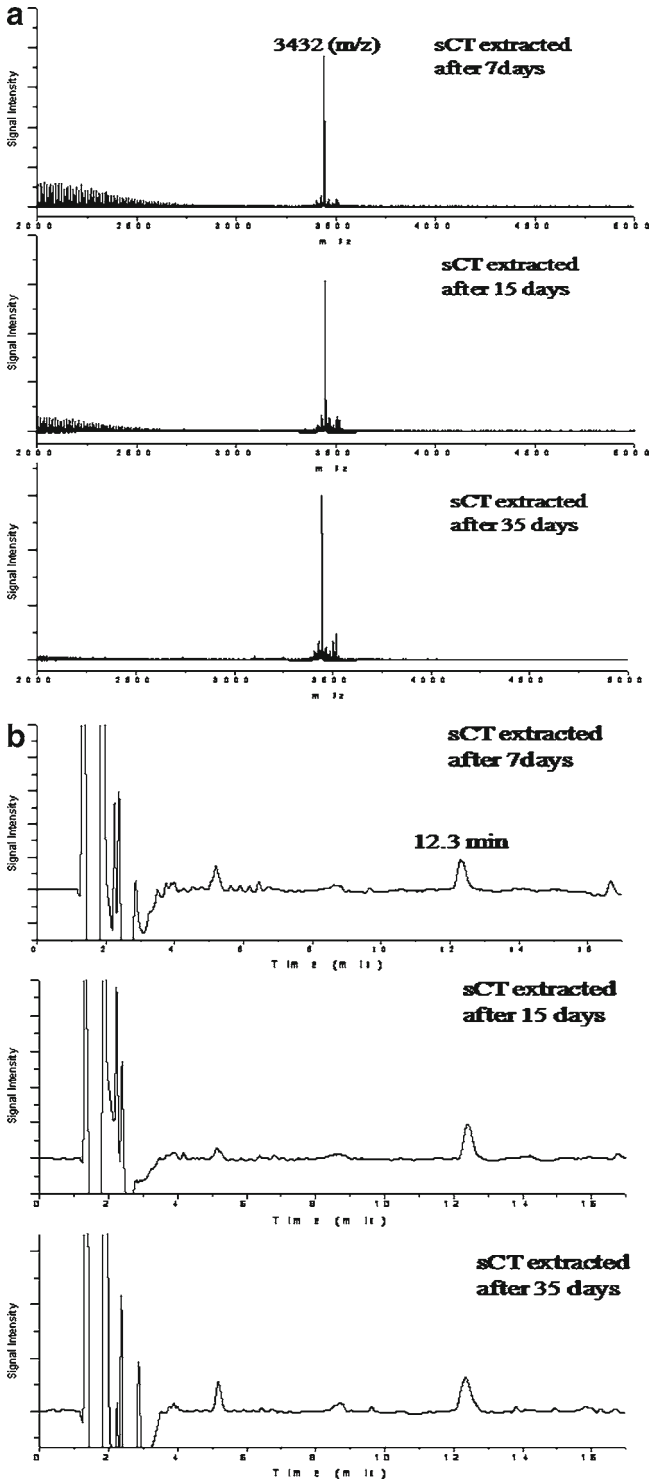

Fig. 6. Stability of sCT extracted from *in situ* forming polymeric gels. Keys: **a** MALDI-TOF mass spectra of sCT extracted from polymeric gel after 7 days incubation, sCT extracted from polymeric gel after 15 days incubation, and sCT extracted from polymeric gel after 35 days incubation; **b** HPLC chromatographs of sCT extracted from polymeric gel after 7 days incubation, sCT extracted from polymeric gel after 15 days incubation, and sCT extracted from polymeric gel after 35 days incubation.

In Vivo Therapeutic Effect of sCT Polymeric Formulations

The effect of sCT from polymeric formulations on the prevention of methylprednisolone acetate (MPA)-induced osteopenia (early stage of osteoporosis) was evaluated *in vivo* in rats. The serum osteocalcin level (a marker for bone formation) and the number of osteoclast (a marker for bone resorption) were evaluated. As shown in Fig. 10, MPA treatment significantly decreased ($p<0.05$) serum osteocalcin level in rats compared to the blank control group. In contrast, rats accepting a bolus dose of polymeric formulations of sCT in the beginning of the MPA treatment retained its normal serum osteocalcin level for 4 weeks from the formulation A and for 6 weeks from the formulation B. However, after 8-week treatment, rats receiving the formulations of sCT and MPA did not show significant ($p<0.05$) difference with the rats treated with MPA only.

The number of osteoclast in rat tibia was used as a marker for bone turnover. MPA treatment resulted in a significantly ($p<0.05$) increased number of osteoclast, compared to the blank control group. The formulation A remarkably ($p<0.05$) prevented the MPA-induced increase in the number of osteoclasts in rat tibia for the first 4 weeks but failed to inhibit the increase of osteoclasts after 8 weeks treatment of MPA (Fig. 11). The formulation B significantly ($p<0.05$) restrained the MPA-induced increase in the number of osteoclasts in rat tibia for 8 weeks.

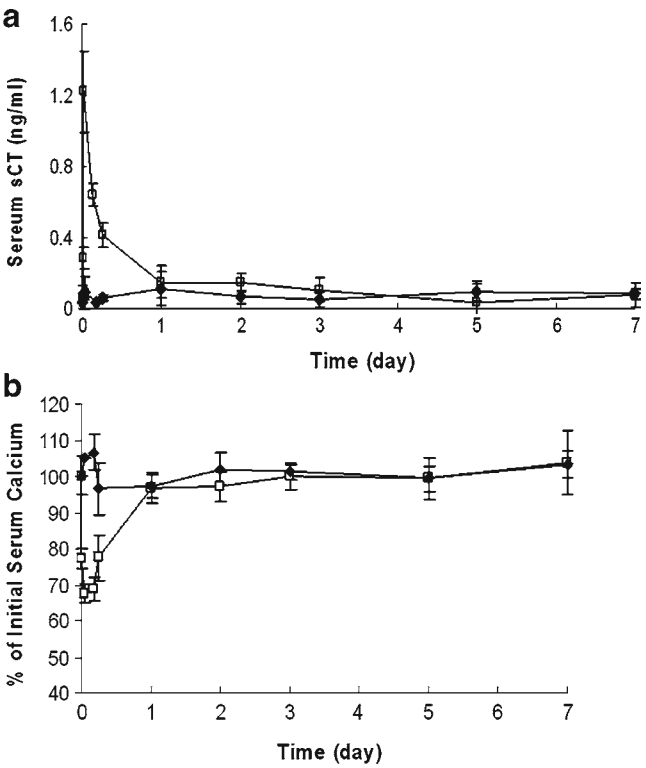

Fig. 7. Serum sCT concentration (**a**) and serum calcium level (**b**) after administration of sCT solution. ($n=6$) Key: (□) rats administrated with sCT solution formulation, (♦) rats administrated with blank polymeric formulation.

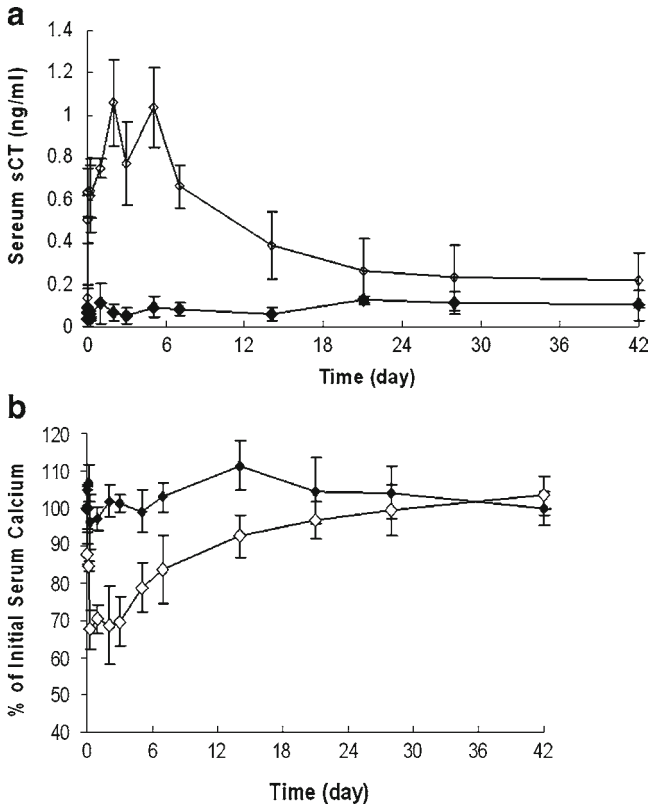

Fig. 8. Serum sCT concentration (**a**) and serum calcium level (**b**) after administration of sCT-loaded polymeric formulation A. (n=6). Key: (◊) rats administrated with sCT polymeric formulation A, (♦) rats administrated with blank polymeric formulation.

DISCUSSION

Various implant drug delivery systems have been studied for controlled delivery of calcitonin in order to avoid frequent injection (10,11,30). Unfortunately, administration of these implants surgically restrained their use as drug delivery systems, due to the unpleasant dosing process and accompanied complications. Our study found that these difficulties in controlled delivery of calcitonin can be resolved by the thermosensitive polymer-based *in situ* gel forming drug delivery system.

sCT-loaded thermosensitive polymeric solution formulations was injectable through 25 G needle and exhibited a rapid sol-gel transition at 37°C, indicating the ease in formulation administration and the avoidance of surgical implantation process. Both polymeric formulations showed a relatively low initial burst release and controlled the release of loaded sCT over a long period (~20 to 40 days). Polymeric formulation A with a higher loading concentration (0.5% *w/v*) of sCT showed a higher initial burst release and controlled the release of the peptide for a shorter duration in comparison to formulation B (0.25% *w/v*). The higher initial burst release resulting from an increased loading concentration of the peptide is attributed to the release of sCT located in the hydrophilic zone of the *in situ* formed gel and the push-out effect of the system during the gel formation (31). Consequently, the initial faster release produced more pores and channels in the polymeric matrix of the gel, thereby shortening the total release period of loaded peptide (32,33). The ideal release model for long-term controlled release is zero-order release, which means the incorporated therapeutic agent should be released at a constant rate to minimize the fluctuation of drug blood level. Both polymeric formulations showed high correlation coefficients (r^2) for zero-order release kinetics (Table II).

As discussed in the introduction, sCT lacks well-organized physico-chemical structure (18). sCT mainly adopts an un-organized random coil structure in aqueous environment with small components of α-helix and β-turns. The random coil components and well-organized secondary structures (α-helix, β-turns, and β-strands) are in a dynamic balance in the aqueous environment, which can be shifted corresponding to the presence of stimuli (34–36). The well-organized secondary structure of sCT results in incomplete release from controlled delivery systems due to higher tendency of peptide fibrillation and aggregation in formulations (19–21). Hence, in this study, the secondary structure of sCT released from the formulations was monitored using CD and compared with the native sCT. The spectra of the released sCT from the two polymeric formulations were similar to the native sCT in aqueous release medium, showing random coil conformation. Thus, CD examination revealed that the copolymer-based thermosensitive formulations do not affect the conformational stability of sCT.

As a small polypeptide, sCT is susceptible to chemical modifications, causing the decrease of bioactivity and producing immunogenicity. These chemical modifications include disulfide breakage with trisulfide bone formation, dimerization through covalent bond, backbone hydrolysis, and acylation. Hence, it is important for the polymeric formulation to release the loaded sCT in its chemically intact form. MALDI-

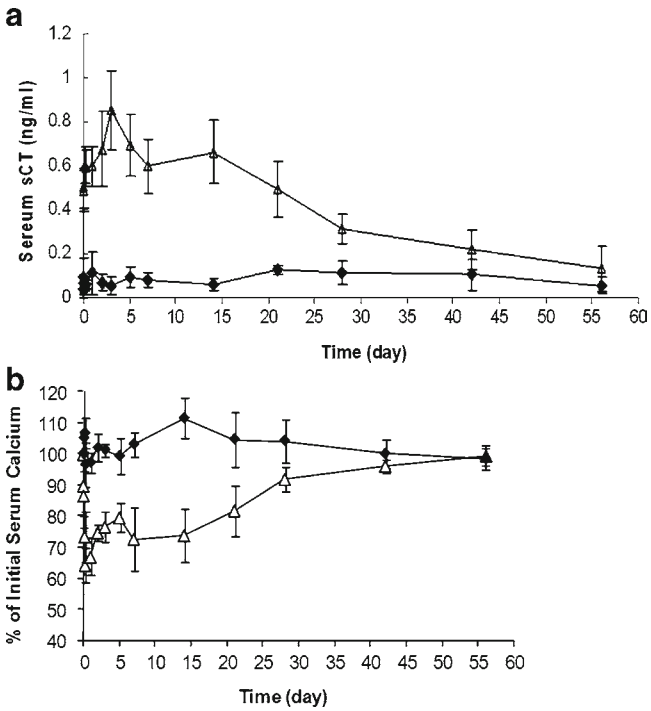

Fig. 9. Serum sCT concentration (**a**) and serum calcium level (**b**) after administration of sCT-loaded polymeric formulation B. (n=6) Key: (Δ) rats administrated with sCT polymeric formulation B, (♦) rats administrated with blank polymeric formulation.

Table III. *In Vivo* Pharmacokinetic Parameters of sCT Delivery Systems in Rats. (n=6)

Group	C_{max} (ng/ml)	T_{max} (hour)	AUC (ng*day/ml)	Relative bioavailability (compared to s.c.)
S.C	1.22±0.23	0.5	0.507±0.017	\
Formulation A	\	\	9.974±3.123	98.41±25.58%
Formulation B	\	\	19.787±4.709	97.48±12.15%

S.C.: sCT solution group
Formulation A: sCT polymeric formulation A group
Formulation B: sCT polymeric formulation B group

TOF MS and HPLC techniques were used to monitor the possible chemical modification of sCT. As shown in the results, sCT gradually lost its chemical stability in PBS at 37°C, evidenced by the hydrolysis of the peptide backbone. Remarkably, *in vitro*-released sCT from both polymeric formulations conserved its chemical integrity, which means the copolymer-based thermosensitive formulations protect sCT from chemical modification by the aqueous environment. A minor peak corresponding to peptide degradation products was observed for released sCT from polymeric formulation B. This may be due to the released sCT degraded in the release medium during the longer sampling interval.

Along with the degradation of PLGA part of the copolymers, the amount of lactic acid, glycolic acid and small oligomer increases and possibly results in the acylation of sCT (37). This unfavorable chemical modification was observed significantly for microsphere formulation using PLGA polymers as the amount of acylated peptide increased along with the process of polymer degradation. In this study, the released sCT from the copolymer-based thermosensitive formulations did not show characteristic signals pertaining to acylated peptide (m/z of 3491 by glycolic acid and 3549 by lactic acid). The small peak observed on the mass spectra at the m/z ratio of 3508 was considered to be due to interference from solvent, because it occurred on the spectra of both sCT controls (no copolymer added).

The results from HPLC examination of sCT controls are in agreement with results from MALDI-TOF study. sCT gradually degraded in PBS with increasing incubation period, evidenced by the appearance of the peaks corresponding to degradation products with progressively increased signal intensity and the relatively decreased signal intensity of peak for chemically intact sCT. In contrast to the sCT controls, released sCT from polymeric formulations showed the major peak at 12.3 min on the chromatographs corresponding to native sCT, denoting the conserved chemical stability of the peptide.

Both polymeric formulations showed controlled release for sCT at 37°C. Although the released sCT conserved its structural and chemical stability, the stability of peptide remained in the *in situ* formed gel had not been studied. Many studies reported that peptide remaining in biodegradable polymer matrices (containing PLGA block) is vulnerable to backbone hydrolysis, deamidation and acylation caused by the acidic environment with abundant lactic acids and glycolic acids in the delivery system. Therefore, sCT remaining in the polymeric delivery system was extracted and examined for its chemical stability using MALDI-TOF and HPLC. We found that the sCT remaining in the gel showed major signals for chemically intact peptide, denoting the chemical degradation of the peptide in the polymeric formulation during release was negligible.

sCT is mainly administered through subcutaneous injection because of fast and efficient absorption from injection site and rapid onset of its physiological activity (38). The half-life of absorption following subcutaneous injection is ~10 min, and the peak serum concentration (C_{max}) is reached ~30 min post administration. This peptide is primarily and almost exclusively metabolized into pharmacologically inactive molecules in kidney. Therefore, the bioavailability of the subcutaneous delivery route is very high (more than 71%).

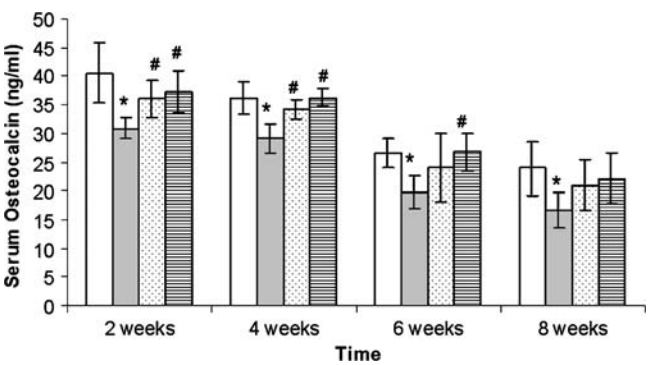

Fig. 10. The effect of polymeric formulations of sCT on MPA-induced changes of rat serum osteocalcin. Key: (□) blank control, (■) MPA, (▩) MPA + sCT-loaded polymeric formulation A, (▤) MPA + sCT-loaded polymeric formulation B. * P<0.05, compared to blank control, # P<0.05, compared to MPA group

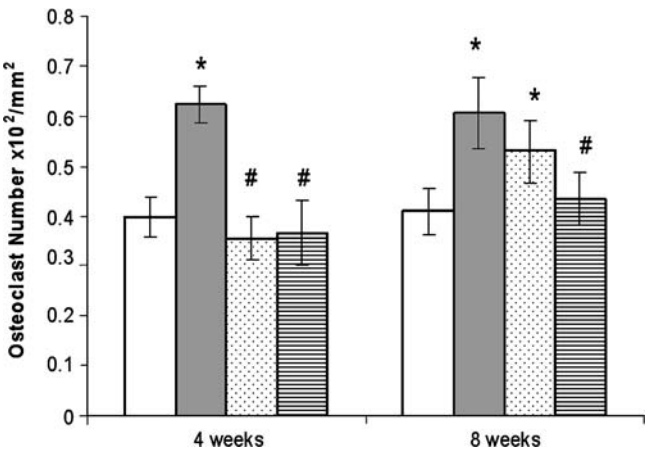

Fig. 11. The effect of polymeric formulations of sCT on MPA-induced changes of rat osteoclast number in tibia. Key: (□) blank control, (■) MPA, (▩) MPA + sCT-loaded polymeric formulation A, (▤) MPA + sCT-loaded polymeric formulation B. * P<0.05, compared to blank control, # P<0.05, compared to MPA group

In this study, rats dosed subcutaneously with sCT solution showed similar pharmacokinetic parameters as reported in the literature (38). The peak serum concentration is observed at 30 min (T_{max}) post dosing, and the drug blood level decreased gradually to basal level within one day. The administration of sCT solution formulation induced the transient drop of serum calcium level, which was restored to the baseline.

Subcutaneous administration of the sCT polymeric formulations resulted in a rapid increase in concentration of sCT but at a lower level than the solution formulation. The increased serum concentration of sCT lasted for ~20 days and 40 days from formulation A and B, respectively. Such findings indicate the controlled release of sCT from the polymeric formulations *in vivo*. The serum calcium level in rats remained significantly lower ($p<0.05$) for the same period of time, which means the released sCT retained its biological activity. Also, increased serum concentration of sCT lasted for a longer period for polymeric formulation B compared to the polymeric formulation A, which is in accord with the *in vitro* release data.

Incomplete release of peptide is a major difficulty for controlled delivery systems. In this study, both polymeric formulations A and B showed a high relative bioavailability (~100%) compared to the control solution, indicating the complete release of sCT from the formulations. No significant difference in bioavailability was observed between the two formulations.

In order to test the preventive and therapeutic effect of the sCT-loaded polymeric formulations on osteoporosis, female rats were treated with MPA to induce osteopenia (initial stage of osteoporosis) following the Furuichi's method (28,39). MPA-induced osteopenia is characterized by a significant drop in rat serum osteocalcin level and increase in the number of osteoclasts in tibia. As shown in Figs. 10 and 11, administration of the sCT polymeric formulations in the beginning of MPA treatment remarkably inhibited the changes in rat serum osteocalcin level and the osteoclast counts in rat tibia. The difference in the protecting effect of the two polymeric formulations was also appreciable. Administration of sCT-loaded polymeric formulation A protected rats from the MPA-induced osteopenia for the first 4 weeks, but the formulation B protected rats from osteopenia for more than 6 weeks. This difference was considered to be related to the difference in controlled release duration of sCT from the two formulations, as the formulation A controlled the release of the peptide for ~20 days and formulation B for ~40 days.

CONCLUSIONS

The mPEG-PLGA-mPEG copolymer (40%*w/v* of EG_{12}-$L_{35}G_{12}$-EG_{12})-based thermosensitive *in situ* gel forming drug delivery system possesses good injectability, controls the release of sCT in its structurally stable and biologically active form over a long period of time, and shows significant prevention of osteoporosis. Therefore, the EG_{12}-$L_{35}G_{12}$-EG_{12} (40%, *w/v*)-based thermosensitive solution formulations of sCT can be applied for prevention and management of osteoporosis to improve quality of life of the osteoporotic patients.

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