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Synthesis, characterization and *in vitro* evaluation of a bone targeting delivery system for salmon Calcitonin

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ABSTRACT

Synthetic salmon Calcitonin (sCT) is currently used to treat and manage conditions associated with low bone mass, and elicits its antiresorptive effect by acting upon Calcitonin receptors (CTRs) located on bone-resorbing osteoclast cells. However, CTRs are also widely distributed in many non-skeletal tissues (such as kidney, brain, and lung), and the competitive uptake of available sCT amongst such CTRs likely reduces sCT availability for bone resident osteoclast cells, particularly if the drug is administered systemically and not specifically targeted to bone. Hence, the objective of this study was to synthesize and characterize a bisphosphonate (BP)-mediated bone targeting delivery system for sCT and to determine whether the bioactivity of sCT was retained after BP conjugation. BP–sCT conjugates were synthesized by initially reacting sCT with sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) in dimethyl formamide in the presence of triethylamine (TEA) at room temperature. Thiolated (Thiol)-BP was then reacted with the sCT–sulfo-SMCC conjugates to generate sCT–BP conjugates, which were purified by dialysis and assayed using the micro-BCA protein assay. Non-BP containing control sCT–Cysteine conjugates were also synthesized using the same procedure. Reactions were monitored and characterized using matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF) analysis and Tris–Tricine SDS-PAGE. Conjugates were evaluated for *in vitro* bone mineral affinity using a hydroxyapatite binding test, for bone mineral specificity using different calcium salt binding affinity assays, and for continued sCT bioactivity after conjugation using an intracellular cAMP stimulation in human T47D breast cancer cells. Our results confirmed that BP-conjugated sCT exhibited significantly greater affinity and specificity for bone mineral over unmodified sCT, and that sCT–BP conjugates retained strong CT bioactivity after conjugation. Our conjugation strategy holds the promise of facilitating the delivery of sCT preferentially to skeletal bony tissues, thereby increasing its local concentration to bone surfaces. This peptide hormone–bisphosphonate drug system represents a new class of antiresorptive drug that has not previously been attempted, nor has a bone targeting formulation of sCT been reported.

1. Introduction

Synthetic salmon Calcitonin (sCT) is synthesized as a 32 amino acid peptide hormone, and is currently indicated and used in the treatment of Paget's disease of bone, osteolytic bone cancers (Lee and Sinko, 2000; Torres-Lugo and Peppas, 2000) and osteoporosis, a disease of low bone mass and the micro-architectural deterioration of bone structure, which in combination, predispose the patient to enhanced bone fragility and fracture risk (Raisz, 2005; Chambers and Magnus, 1982; Chambers, 1982). Calcitonin therapy inhibits or slows osteoclast-mediated resorptive bone loss and positively influences osteogenesis and bone formation (Okubo et al., 2000; Farley et al., 2000). It has also recently been shown to attenuate cartilage degradation and stimulate new cartilage formation in osteoarthritis (Karsdal et al., 2007). Calcitonin has been reported to slow the progression of osteoarthritis (OA) and to improve the clinical signs and symptoms of rheumatoid arthritis (Aida et al., 1994).

However, the therapeutic use of exogenously administered Calcitonin is severely hampered by its rapid elimination from the body and short half-life (~43 min), which in combination contribute to its poor and variable systemic bioavailability (Lee et al., 2003; Shin et al., 2004; Youn et al., 2006). Furthermore, as the human skeleton comprises only 4–5% of total body mass (Heymsfield et al., 2005), only a small proportion of available sCT from conventional delivery systems would reach bone in order to elicit its therapeutic effect.

sCT elicits its antiresorptive effect by acting on Calcitonin receptor (CTR) found on bone-resorbing osteoclast cells (Naot and

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Cornish, 2008). However, CTRs are widely distributed in non-skeletal tissues as well, even though CT function has been well defined in osteoclasts. CTRs have been identified and high affinity Calcitonin binding has been demonstrated in tissues such as kidney (Warshawsky et al., 1980), brain (Goltzman, 1985), lung (Fouchereau-Peron et al., 1981), placenta (Nicholson et al., 1988), ovaries (Gorn et al., 1992), testes (Chausmer et al., 1980), and spermatozoa (Silvestroni et al., 1987). Accordingly, the competitive uptake of available sCT amongst such CTRs likely further reduces sCT availability to bone osteoclast cells, particularly if the drug is administered systemically, and not specifically targeted to bone. Thus, we attempted to develop a delivery system capable of improving sCT targeting, localization and retention to bone with the potential to positively impact sCT therapy, whilst reducing the drug concentration in non-bone loci containing the CTR (Pierce and Waite, 1987; Kasugai et al., 2000; Yokogawa et al., 2001; Orme and Labroo, 1994; Fujisaki et al., 1997; Cenni et al., 2008).

One potential method to impart bone mineral affinity is the chemical coupling of therapeutic candidate drugs to bone seeking moieties such as bisphosphonate (BP) drugs, due to their high affinity for hydroxyapatite (HA), the mineral phase of bone which is not present in other tissues. In previous studies it was shown that replacement of $Lysine_{11}$ and $Lysine_{18}$ amino acid residues by other amino acids or N-terminus acetylation of the parent molecule did not affect sCT bioactivity (D'Santos et al., 1988; Rittel et al., 1976). Thus, we hypothesized that chemical coupling of a bone targeting BP moiety with sCT utilizing these sites in reaction would be a feasible approach in order to develop a bone targeting delivery system for sCT without the loss of sCT bioactivity.

2. Experimental

2.1. Materials

Salmon Calcitonin was purchased from Calbiochem, USA, and the sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) was from Molecular Biosciences, USA. Thiol functionalized bisphosphonate (Thiol-BP) was purchased from Surfactis Technologies Inc., France. HPLC grade water, dimethyl formamide (DMF) and triethylamine (TEA) were from Sigma–Aldrich (Saint Louis, MO, USA).

2.2. Generation of free thiol reactive functional group in sCT and reaction condition optimization

30 μl sCT in DMF (6.66 mg/ml) was mixed with 5 μl sulfo-SMCC in DMF (26.182 mg/ml) and 5 μl of 0.8% TEA in DMF (final concentration of TEA 0.1%, v/v) was then added. Reaction between the primary $–NH_2$ in sCT and NHS group of sulfo-SMCC was allowed to proceed at room temperature with constant stirring for 30 min.

The effect of reaction time was studied by carrying the above reaction separately for 10, 20, 30, 40, 50, and 60 min. The effect of sulfo-SMCC concentration was also studied using sCT:sulfo-SMCC at 1:3, 1:5, 1:7 and 1:10 mol/mol ratios in the above manner for 30 min. Finally, the effect of TEA concentration was evaluated using 0.05, 0.1, 0.2, 0.3 and 0.4% (v/v) final concentration of TEA and carrying the reaction for 30 min using sCT:sulfo-SMCC at 1:5 mol/mol ratios.

2.3. Coupling of functionalized sCT with free thiol containing BP

Before proceeding with the reaction, the amount of available reactive free thiol groups present in Thiol-BP was calculated by Ellman's thiol assay. Briefly, 50 μl of 4 mg/ml Ellman's reagent solution in 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA was added to 250 μl of Thiol-BP solution, mixed and incubated at room temperature for 15 min. The yellow color developed was then measured at 412 nm. The amount of free thiol group was obtained using a calibration curve obtained with L-Cysteine as a control.

Similarly, the amount of total phosphate present was determined using an organic phosphate assay. Briefly, 50 μl of Thiol-BP sample of different concentration was mixed with 30 μl of 10% $Mg(NO_3)_2$ in 95% ethanol in glass tubes and ashed over a flame. After boiling in 0.3 ml of 0.5N HCl for 15 min, 0.1 ml of ascorbic acid (10%, w/v) and 0.6 ml of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.42% (w/v) in 1N H_2SO_4) were added to the tubes and the samples were incubated at 37 °C for 1 h. The absorbance of the blue color developed were then determined at 820 nm. The amount of phosphate in Thiol-BP was calculated using the calibration curve obtained for Alendronate sodium.

Concentration of free thiol group was about 47% compared to L-Cysteine and concentration of phosphate group was about 70% compared to Alendronate. Accordingly, the amount of Thiol-BP used was calculated based on these assays.

Thiol-BP was dissolved in 10 mM PBS, pH 6.55, at a concentration of 4.225 mg/ml. 960 μl of this solution (6 μmol free –SH, 4.056 mg BP) was reacted with functionalized sCT in 20:1 mol/mol ratio for 2 h at room temperature in dark. The reaction mixture was then incubated at 4 °C overnight.

2.4. Synthesis of control non-BP–sCT conjugates

Control conjugates were synthesized L-Cysteine. Functionalized sCT synthesized as above was reacted with L-Cysteine dissolved in 10 mM PBS, pH 6.55, in 1:20 mol/mol ratio for 2 h at room temperature in dark and the reaction mixture was then incubated at 4 °C overnight.

2.5. Conjugate characterization

In all cases, conjugation reactions were monitored by MALDI-TOF analysis of unpurified reaction mixtures. Conjugates were also analyzed by Tris–Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Briefly, 10 μl of sample solution was mixed with 20 μl of loading buffer, premixed protein sample buffer for peptides and small proteins (Bio-Rad, USA, Cat# 161-0739) and loaded in wells of the 16.5% Tris–Tricine/Peptide precast gel (Bio-Rad, USA, Cat# 161-1107). Reference Polypeptide SDS-PAGE Standards (Bio-Rad, USA, Cat# 161-0326) was diluted with sample buffer at 1:20 (v/v) ratio, heated at 95 °C for 5 min, cooled and loaded at 5 μl/well. After electrophoresis at 100 V using Tris/Tricine/SDS buffer (Biorad, Cat# 161-0744), the gel was fixed with a solution of 40% methanol, 50% water and 10% acetic acid for 30 min. Fixing solution was then removed and the gel was stained for 1 h with Coomassie brilliant blue R-250 (0.008% in 10% acetic acid) at 95 °C for 20 s, followed by staining at room temperature for 15 min. Gels were destained in water overnight, scanned and saved as an image using gel documentation software.

2.6. Conjugate purification and determination of the amount of sCT and phosphates

Conjugates were purified by dialysis (MWCO 1000 D, Spectrum Laboratory, USA) to remove unconjugated BPs and the amount of sCT in sCT–BP conjugates was determined by micro-BCA protein assay. Briefly, an aliquot of 100 μl suitably diluted sample was mixed with 100 μl of working reagent (micro-BCA reagents 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 562 nm was measured using 96-well microplate reader. The

amount of sCT was calculated by converting the absorbance into mass using the standard curve.

2.7. Determination of bone mineral affinity

100 μl of purified conjugate solution containing approximately 60 μg of sCT was mixed with 5 mg hydroxyapatite (HA) in 100 μl of 10 mM phosphate buffer (pH 7.0) in microcentrifuge tubes in duplicate. As a reference, control samples were incubated in tubes without HA (i.e., 0% of binding), and the effect of sCT modification (due to chemical conjugation) upon mineral affinity was also analyzed by HA binding assay of sCT–Cysteine conjugates.

The tubes were incubated at room temperature on a shaker for 3 h, and centrifuged at 5000 × *g* for 5 min to separate the HA from the supernatant. sCT concentration in the supernatant was determined using the BCA microprotein assay (Pierce, USA). HA centrifugate remaining after removal of supernatant was washed five times using 1 ml water each time and the last washing after cen-

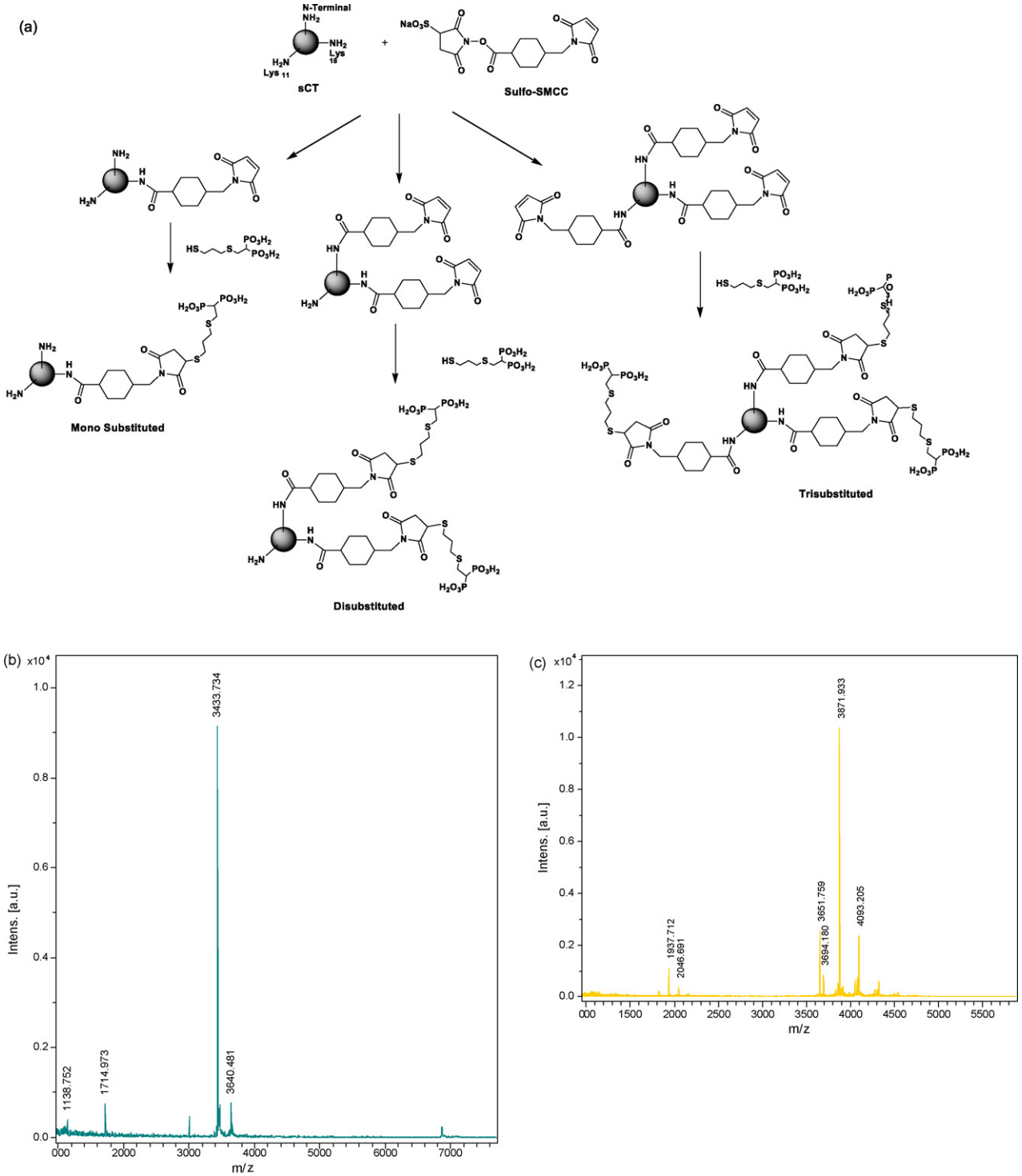

Fig. 1. (a) Probable products when sulfo-SMCC is reacted with sCT followed by the reaction of sCT–SMCC intermediate with Thiol-BP. (b) MALDI-TOF spectra of sCT. (c) MALDI-TOF spectra of the products of sCT and sulfo-SMCC reaction.

trifugation at 5000 × *g* for 5 min was collected. Both the washings and the HA centrifugate were then analyzed for sCT as described above. The amount of sCT in the supernatant, washings and HA centrifugate was then inferred by measuring the absorbance at 562 nm, with a value of 100% for control samples without HA.

2.8. Determination of bone mineral specificity

100 μl of purified conjugate or control sCT solution containing approximately 60 μg of sCT was mixed with 5 mg each of HA, calcium carbonate, calcium pyrophosphate, tricalcium phosphate and calcium citrate in 100 μl of 10 mM phosphate buffer (pH 7.0), incubated at room temperature on a shaker for 3 h, centrifuged at 5000 × *g* for 5 min, and centrifugates washed with water and analyzed for sCT as above.

2.9. In vitro bioactivity assay: intracellular cAMP stimulation in human T47D cells by bisphosphonate (BP)-conjugated salmon Calcitonin

T47D cells (ATCC, VA, USA) were cultured in RPMI-1640 culture medium containing 1% penicillin–streptomycin, 10% fetal bovine serum, and insulin (0.2 IU/ml). Cells were seeded on 48-well plates at an initial density of 5×10^4 cells/well and incubated in 95% air and 5% CO_2 at 37 °C for 2 days. Cells were then washed with HBSS and pre-incubated in RPMI-1640 culture medium devoid of FBS, insulin and antibiotics. Cells were then dosed with phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, 0.2 mM) and incubated at 37 °C for 30 min. 100 nM of sCT, BP-conjugated sCT, or Cysteine-conjugated sCT (conjugation control) was then added to the cells and incubated for 20 min at 37 °C (21).

After removing the supernatant, cells were rinsed three times in cold PBS and resuspended in 500 μl of Cell Lysis Buffer. Cells were frozen at −20 °C and thawed with gentle mixing. The freeze/thaw cycle was repeated three times and the mixture was centrifuged at 600 × *g* for 10 min at 2–8 °C to remove cellular debris. The supernatant was collected and stored at −20 °C. cAMP concentrations were then measured using the cAMP Enzyme Immuno-Assay (EIA) kit (KGE002B, R&D Systems, USA). Increased cAMP production in response to the different forms of sCT was calculated using a calibration curve as per the manufacturer's protocol.

3. Results

3.1. Generation of free thiol reactive functional group in sCT

Synthetic sCT is a polypeptide of 32 amino acids with a molecular weight of ~3430 kDa. Fig. 1(b) represents MALDI-TOF spectra of sCT with a peak at 3433.7. Other peaks represent either impurities or degradation products during analysis. As shown in Fig. 1(a), when the primary amines in sCT viz Lys 11, Lys 18 and N-terminal are reacted with the NHS functional group of sulfo-SMCC, there exists the probability of formation of three intermediate conjugates: mono-, di- and tri-substituted products with respective molecular weights of 3651.7, 3871.9 and 4093.2. These peaks are shown in Fig. 1(c). DMF was chosen as the reaction medium because

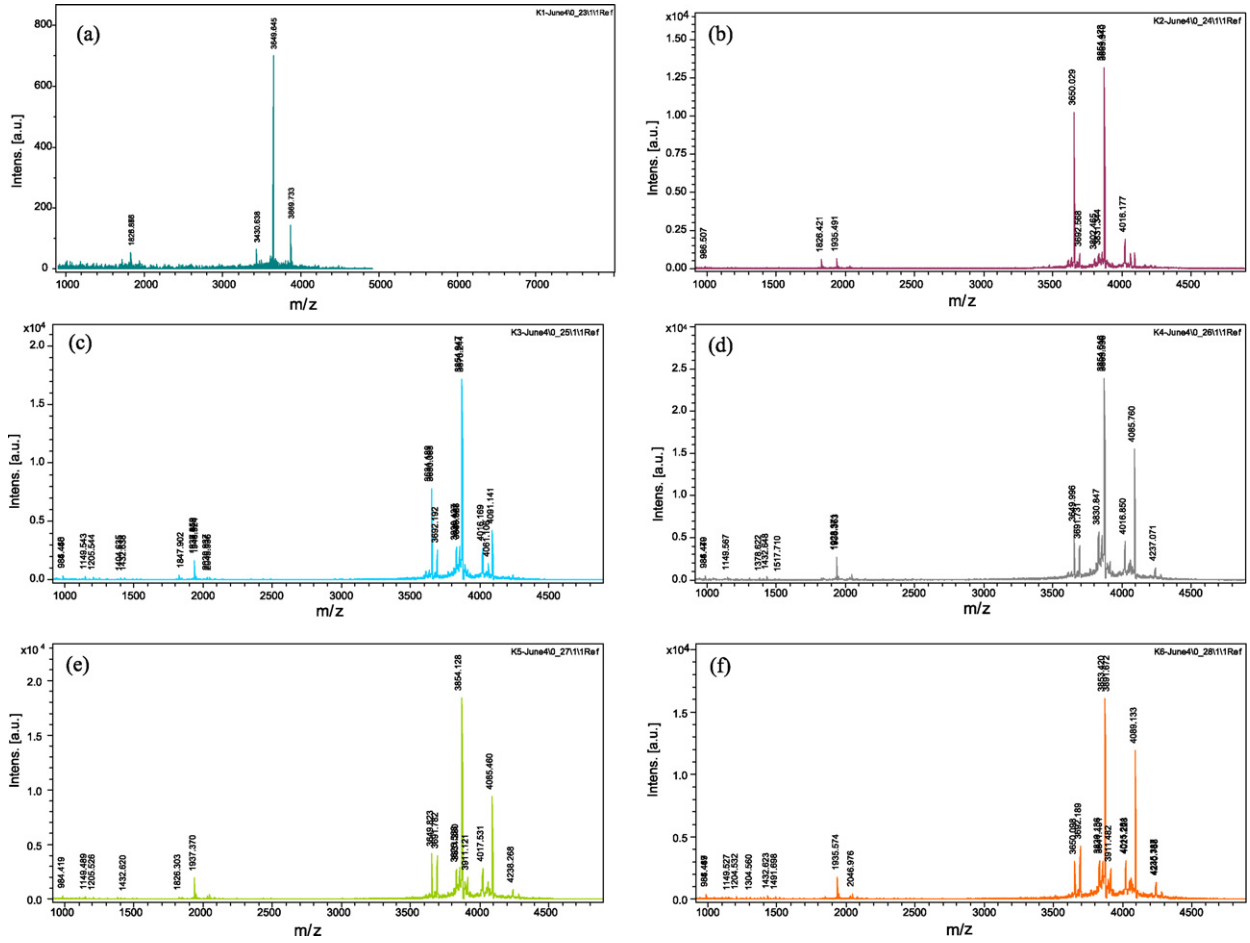

Fig. 2. (a–f) Optimization of reaction time when reacting sCT with sulfo-SMCC. (a) Effect of reaction after 10 min. (b) Effect of reaction after 20 min. (c) Effect of reaction after 30 min. (d) Effect of reaction after 40 min. (e) Effect of reaction after 50 min. (f) Effect of reaction after 60 min.

of the hydrolytic instability of NHS in aqueous solution and also due to the instability of sCT in aqueous solution. TEA was added for its favorable effect in the reaction of $-NH_2$ with the NHS group in sulfo-SMCC.

3.2. Effect of reaction time

Effect of reaction time was studied to optimize the duration of reaction. Results are presented in Fig. 2(a)–(f) for 10, 20, 30, 40, 50, and 60 min, respectively. Although there was formation of mono- and di-substituted products, the reaction was incomplete at 10 min as a peak at 3430 representing sCT remained. However, the reaction was complete (in terms of consuming the sCT peak at 3430) at or after 20 min. The formation of tri-substituted products might be considered favorable in terms of bone targeting potential due to the presence of more BP molecules per sCT molecule. However, in the literature, it has been reported that an increased protein substitution may substantially alter its secondary structure, receptor binding potential and hence its activity. Accordingly, for this initial report of sCT–BP synthesis, we elected to use a reaction time of 30 min so as to utilize all the sCT reactant whilst minimizing tri-substituted product. Collectively, our results presented in Fig. 2 suggested that the reaction time could have an impact on the ratio of mono-, di- and tri-substituted products. Thus, a prolonged reaction time may further result in the alteration of the BP substitution ratio.

3.3. Effect of sulfo-SMCC concentration

Since sCT has three available reaction sites for the NHS group in sulfo-SMCC, the effect of sulfo-SMCC concentration in the substitution was studied using sCT:sulfo-SMCC at 1:3, 1:5, 1:7 and 1:10 mol/mol ratios. Results are shown in Fig. 3(a)–(d), respectively. Although the formation of tri-substituted products was seen for all chosen ratios, the reaction was complete after a 1:5 mol/mol ratio. Since the number of SMCC substitution in sCT will be proportional with the Thiol-BP substitution in later reactions and the number of Thiol-BP would increase the bone targeting and localization of conjugates, a molar ratio of 1:5 was chosen for the reactions.

3.4. Effect of TEA concentration

Since the presence of TEA favors the reaction of $-NH_2$ with the NHS group in sulfo-SMCC, the effect of TEA concentration was studied. Fig. 4(a)–(e) represents the effect of TEA concentration of 0.05, 0.1, 0.2, 0.3 and 0.4% (v/v). The reaction was complete for all chosen ratios and all three products were formed, and the 0.1% (v/v) concentration was chosen arbitrarily for further investigation.

3.5. sCT–BP conjugate characterization

The expected reaction products after conjugation of functionalized sCT with Thiol-BP are shown in Fig. 1(a). Since many possible substitution sites are possible, and the Thiol-BP used was a disodium salt, there exist many compounds. However, they only differ in BP substitution and Na addition. MALDI-TOF results of conjugates are shown in Fig. 5(a). The peak at 3989.848 was of sCT–SMCC–BP + 2Na and at 4167.271 for sCT–2SMCC–1BP. sCT–2SMCC–1BP–1BP on disulfide bond appeared at 4459.757 and sCT–2SMCC–2BP was found at 4463.845. Similarly, the peak at 4501.735 was of sCT–2SMCC–2BP + 4Na (2Na each on BP) and at 4814.287 of sCT–2SMCC–3BP + 3Na. Another tri-substituted product, sCT–3SMCC–3BP, appeared at 4979.743.

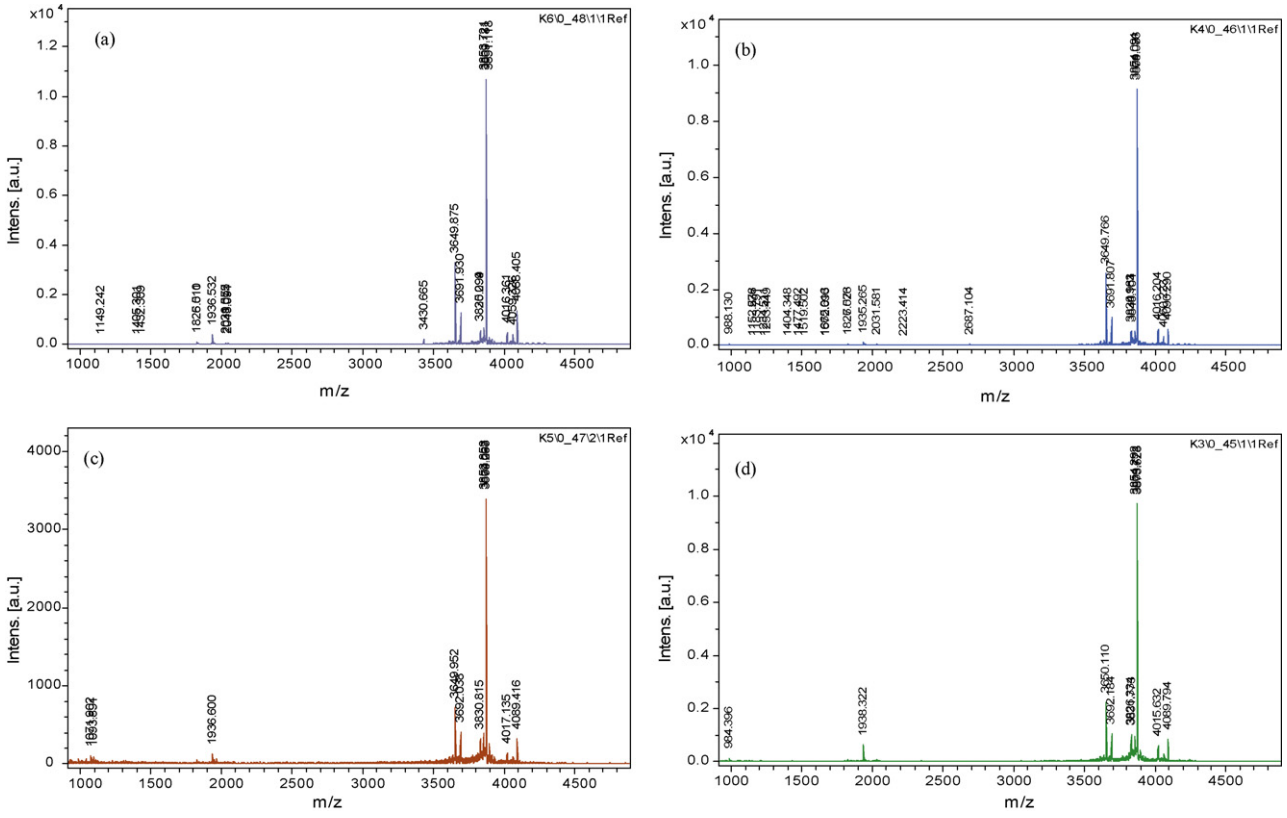

Fig. 3. (a–d) Effects of varying sulfo-SMCC concentration when reacting sulfo-SMCC with sCT. (a) 1:3 mol/mol ratio of sCT to sulfo-SMCC, respectively. (b) 1:5 mol/mol ratio of sCT to sulfo-SMCC, respectively. (c) 1:7 mol/mol ratio of sCT to sulfo-SMCC, respectively. (d) 1:10 mol/mol ratio of sCT to sulfo-SMCC, respectively.

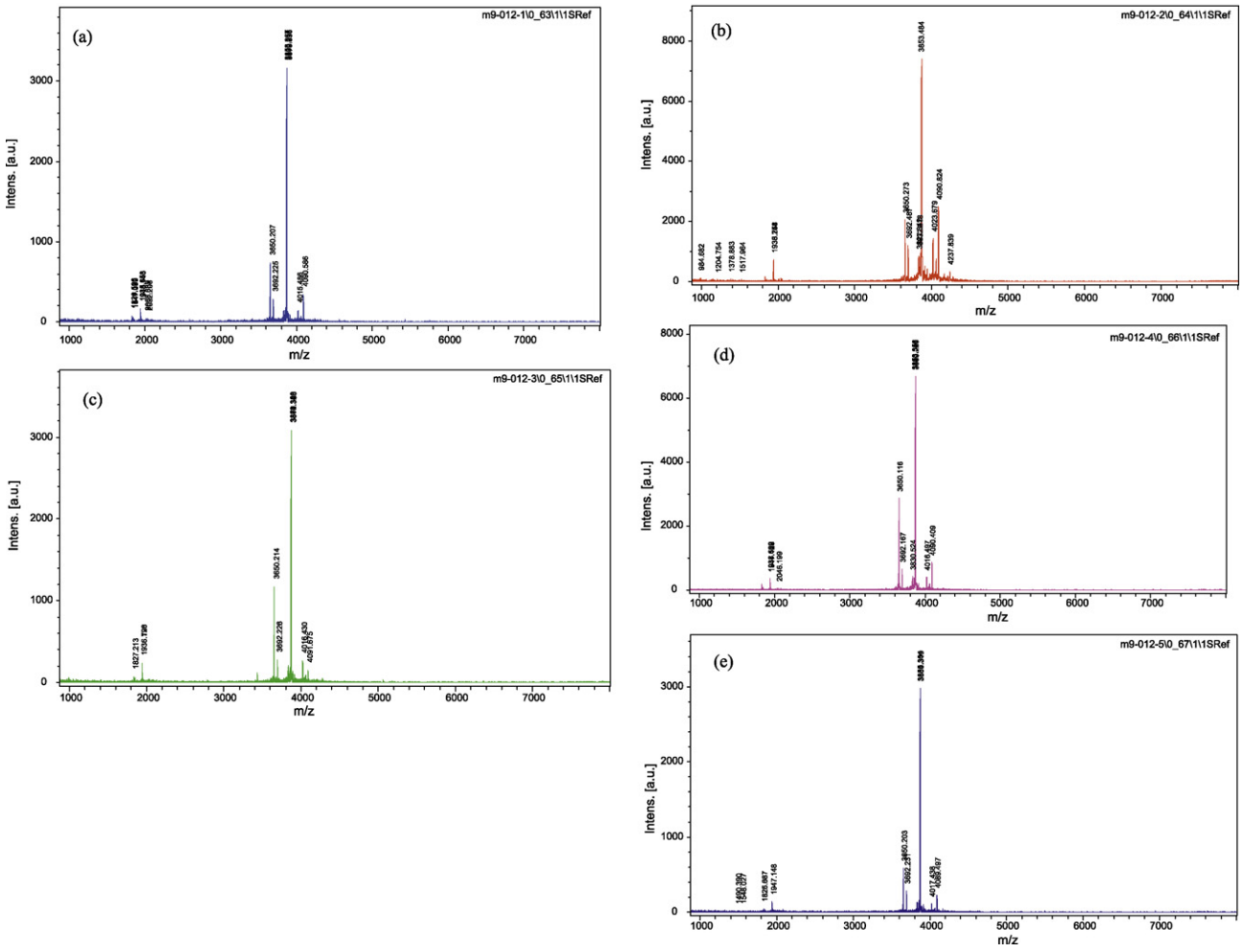

Fig. 4. (a–e) Effect of a TEA concentration when reacting sCT with sulfo-SMCC. (a) 0.05% (v/v) TEA. (b) 0.1% (v/v) TEA. (c) 0.2% (v/v) TEA. (d) 0.3% (v/v) TEA. (e) 0.4% (v/v) TEA.

In liquid chromatography mass spectroscopy (LC–MS/MS), the Thiol-BP appeared at 294.96 *m/z* (without sodium) and at 316.94 (with +1Na; data not shown). Hence, with MALDI, sCT–SMCC–BP appeared at masses corresponding to with (or without) sodium. For the sCT–2SMCC–1BP–1BP, two amines in sCT were substituted by 2SMCC – with one of the SMCC further reacting with 1BP whilst the other did not. However 1BP may have undergone a thiol-substitution reaction with the 1,7-disulfide bond in sCT to give that product. Similarly, for the sCT–2SMCC–3BP + 3Na, two BP with sodium reacted with 2 SMCC and one may have undergone sulfide exchange.

Conjugation reaction mixtures were run in Tris–Tricine SDS-PAGE. However, because of the small differences in their molecular weights, they appeared as a single band and did not resolve distinctly under these conditions on SDS-PAGE gels. However, the band position was altered in comparison to native sCT, as shown in Fig. 5(c).

3.6. Synthesis and characterization of control sCT–Cysteine conjugates

Possible products were confirmed by MALDI-TOF (Fig. 5(b)) and Tris–Tricine SDS-PAGE (Fig. 5(c)). Since the mono-, di- and tri-substituted sCT–SMCC had respective molecular weights of 3651.7, 3871.9 and 4093.2, coupling of L-Cysteine (MW 121.16) with these functionalized sCT resulted into the formation of mono-, di- and tri-Cysteine substituted sCT–Cysteine conjugates as represented, respectively by the peaks at 3770.219, 4113.367 and 4453.437.

3.7. Bone mineral affinity and specificity

When all compounds were assayed directly (i.e., without HA), the absorbance of protein in those samples was readily detected in the supernatant. The incubation of sCT–BP with HA substantially reduced the sCT concentration in the supernatant, due to its BP-mediated binding to the HA (Fig. 6(a)). The alteration of primary amines in sCT alone (by the cross-linker conjugation chemistry) did not improve HA binding, as represented by the lack of sCT–Cysteine control conjugate HA binding. To further confirm the HA binding of conjugates, the samples were washed until the washings were free of sCT, then centrifuged, and the HA pellet obtained then analyzed for sCT. As shown in Fig. 6(a), sCT was substantially bound to HA. The small absorbance seen for native sCT, and for the sCT–Cysteine conjugate likely represent the non-specific surface adsorption of sCT on HA. The conjugates were also assayed for their specificity to bone mineral using different calcium salts. As shown in Fig. 6(b), sCT was substantially bound by HA over that of other calcium salts.

3.8. In vitro bioactivity assay: intracellular cAMP stimulation in human T47D cells

The ability for native sCT, BP-conjugated sCT, and Cysteine-conjugated sCT to generate increased intracellular cAMP activity, after binding to the CTR, was tested using a cAMP assay on T47D cells, which contain the CTR. Incubation of T47D cells with 100 nM equivalent of each sCT compound, in the presence of a phosphodiesterase inhibitor, stimulated intracellular cAMP release. Fig. 6(c)

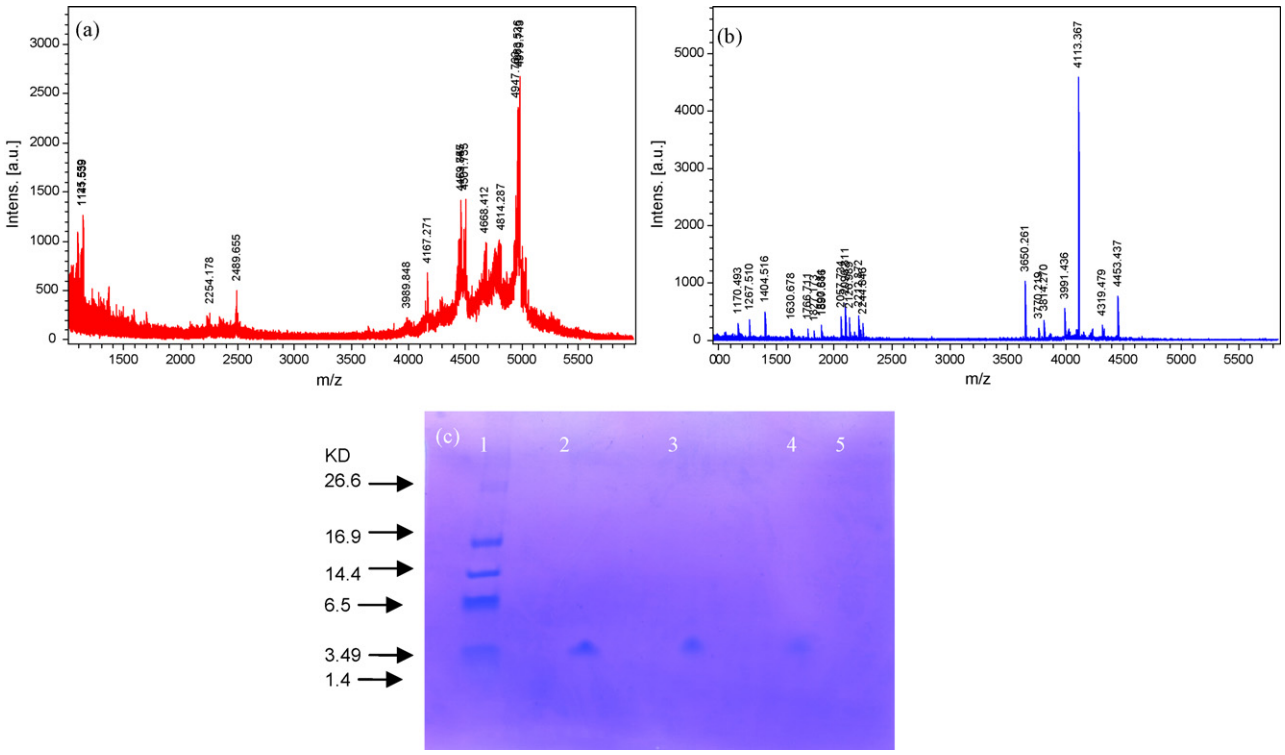

Fig. 5. (a) MALDI-TOF spectra of the products of sCT–SMCC intermediate and Thiol-BP reaction. (b) MALDI-TOF spectra of the products of sCT–SMCC intermediate and L-Cysteine reaction. (c) Tris–Tricine SDS-PAGE of reaction mixtures. Lane 1, peptide SDS-PAGE molecular weight standards. Lane 2, salmon Calcitonin: MW: 3.43 kDa. Lane 3, sCT–SMCC conjugate. Lane 4, sCT–SMCC–BP conjugate. Lane 5, loading dye.

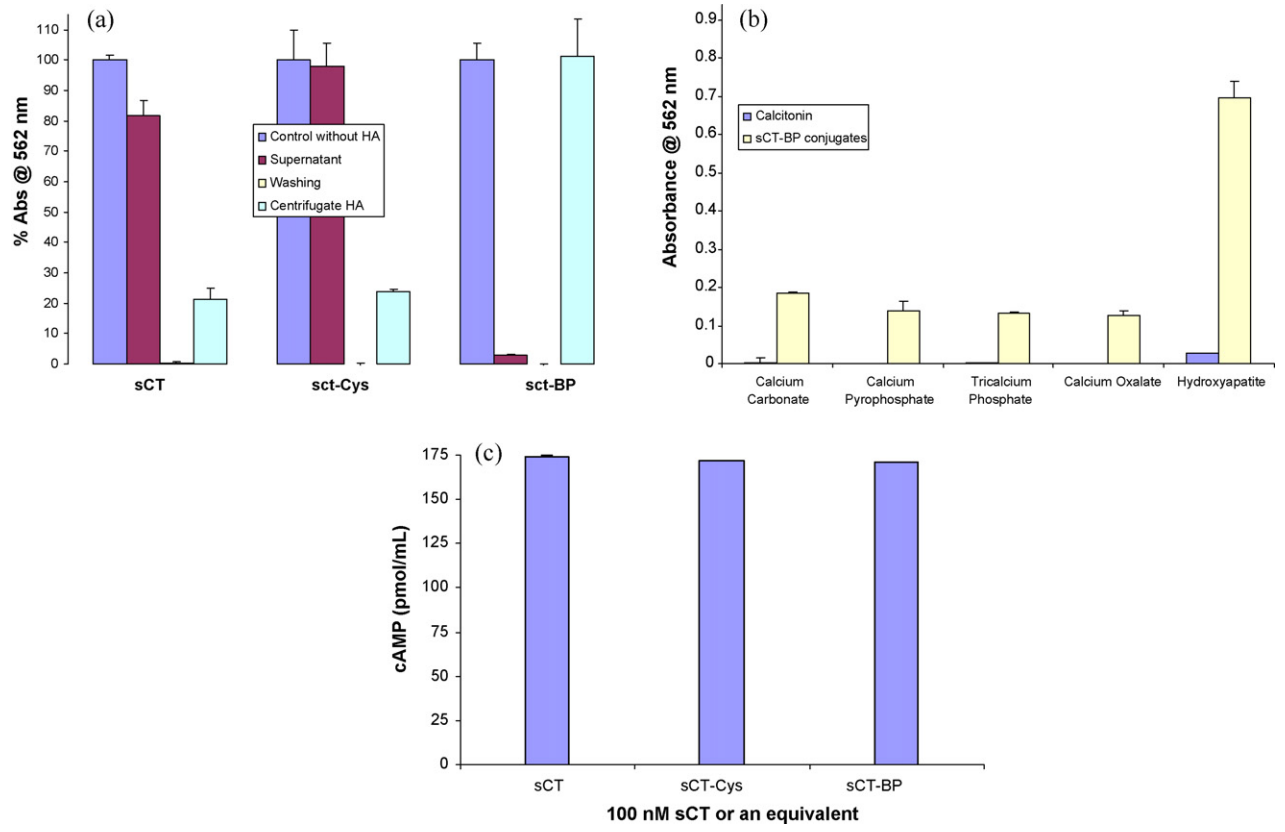

Fig. 6. (a) Hydroxyapatite (HA) binding assay of conjugates to determine their bone mineral affinity. (b) Calcium salts binding assay of conjugates to determine their bone mineral specificity. (c) *In vitro* bioactivity assay: intracellular cAMP stimulation in human T47D cells by bisphosphonate (BP)-conjugated salmon Calcitonin ($n = 3$).

shows the cAMP generated as 173.98 ± 1.18 pmol/ml for native sCT, 171.98 ± 0.24 pmol/ml for sCT–Cys, and 170.9 ± 0.59 pmol/ml for sCT–BP.

4. Discussion

Antiresorptive therapy of bone disease utilizing conventional sCT is hampered by the poor systemic uptake of sCT and its short half-life, contributing to its poor and variable bioavailability and reported antiresorptive efficacy. In response, investigators have attempted to increase the duration of sCT circulation by conjugation to linear polyethylene glycol (PEG). Compared to native sCT, the PEGylated conjugates showed reduced systemic clearance due to altered tissue distribution, with enhanced *in vivo* hypocalcaemic efficacy versus native sCT for both the intra-intestinal and pulmonary delivery routes (Lee et al., 2003; Shin et al., 2004; Youn et al., 2006; Fujisaki et al., 1997; Cenni et al., 2008; Cheng et al., 2007). However, increasing the circulation time of non-targeting formulations of sCT would not necessarily translate into optimal bone-based therapeutic effects, as the competitive uptake of sCT by non-bone tissue resident CTRs would still remain despite PEGylation. Thus, a delivery system increasing sCT targeting, localization and retention to bone is justified and it has the potential to positively impact sCT therapy, whilst reducing the drug concentration in non-bone loci containing the CTR.

In our study, the BP moiety was utilized as a model bone targeting ligand due to its proven high affinity for hydroxyapatite (HA), the mineral phase of bone which is not present in other tissues (Rogers et al., 1999). Nonetheless, our experience with BP conjugative strategies has amply demonstrated that each protein or peptide will behave differently during the conjugation process, with unique solubility, stability, and bioactivity issues (Doschak et al., 2009; Uludag et al., 2000a,b; Gittens et al., 2003). Accordingly, one "generalized" conjugation procedure will not work as a universal reaction scheme for all therapeutic peptides and proteins and individualized strategies will need to be considered and employed on a "case-by-case" basis for individual peptide or protein candidates, as previously hypothesized (Hirabayashi and Fujisaki, 2003).

The conjugation of BP to sCT proved challenging due to their contradictory chemical and physical properties. CT is highly unstable in aqueous buffered reaction conditions that are generally suitable for protein or peptide coupling. In aqueous solutions, CT has a pronounced tendency to aggregate into long, thin fibrillar aggregates, yielding a viscous and turbid dispersion (Cholewinski et al., 1996; Bauer et al., 1995). Thus, the reaction methods utilized for larger model proteins that are soluble and stable in buffered solution could not be applied to sCT. sCT–BP conjugation was further hampered by the physical and chemical properties of the BP. Although CT is highly stable in organic solvents, the insolubility of BP in organic solvents and the low reactivity of the functional group in Thiol-BP further complicate the conjugation. Thus, we avoided aqueous reaction conditions in the initial reaction step to minimize the instability of CT and used mild aqueous conditions in subsequent reaction steps to minimize BP solubility issues. A lower concentration buffer than those generally used was also employed to minimize the aggregation and precipitation of sCT in the buffer, in order to proceed with the second step reaction. We also confirmed the conjugate's chemical identity in detail using MALDI-TOF, and optimized the reaction conditions for various key parameters so that they were reproducible and consistent.

The bone mineral affinity and specificity of this sCT delivery system were subsequently investigated using an HA binding test, followed by different calcium salt binding affinity assays. If the sCT was targeted to HA as a result of BP conjugation, then after centrifugation and complete removal of unbound sCT, the majority of remaining sCT should be detected in the pelleted HA centrifugate compared to other calcium salts. As a control, native sCT was reacted with HA under identical conditions. To ensure that there was no direct binding of the cross-linking chemistry employed with HA, we also tested a non-BP containing conjugate (sCT–Cysteine) that was reacted with HA under identical conditions. In the aggregate, those *in vitro* assays confirmed that the sCT–BP conjugates were specifically bound to HA. As HA is the principal mineral found in bone matrix, the *in vivo* administration of sCT–BP conjugates should lead to improved bone accumulation of sCT compared to free sCT or sCT conjugates without BP.

The binding of CT with its receptor on osteoclasts in bone inhibits osteoclast-mediated resorptive activity by activating adenylyl cyclase, an enzyme responsible for generation of cyclic adenosine monophosphate (cAMP). The induction of cAMP activity by sCT, BP-conjugated sCT and Cysteine-conjugated sCT was examined using an *in vitro* cAMP assay in the T47D human breast cancer cell line, which is known to possess CTRs. Our cAMP assay results confirmed that the modification of sCT peptide using the BP conjugation strategy described above did not adversely affect the ability for sCT to bind the CTR and trigger its biological activity, wherein cAMP levels are increased.

In conclusion, significant bone mineral affinity and HA specificity was imparted to sCT using a BP conjugation strategy in order to develop a biologically active bone-targeted delivery system for sCT. That system holds promise for the rapid uptake of sCT by the skeletal bony tissues, which in turn would increase the local concentration of sCT in bone, whilst maintaining sCT bioactivity. This sCT delivery system represents a new class of bone targeting antiresorptive compound, which could find clinical utility in the treatment of osteopenic bone disease and other related indications.

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