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ORIGINAL PAPER

# DNA Aptamer Beacon Assay for C-Telopeptide and Handheld Fluorometer to Monitor Bone Resorption

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**Abstract** A novel DNA aptamer beacon is described for quantification of a 26-amino acid C-telopeptide (CTx) of human type I bone collagen. One aptamer sequence and its reverse complement dominated the aptamer pool (31.6% of sequenced clones). Secondary structures of these aptamers were examined for potential binding pockets. Three-dimensional computer models which analyzed docking topologies and binding energies were in agreement with empirical fluorescence experiments used to select one candidate loop for beacon assay development. All loop structures from the aptamer finalists were end-labeled with TYE 665 and Iowa Black quencher for comparison of beacon fluorescence levels as a function of CTx concentration. The optimal beacon, designated CTx 2R-2h yielded a low ng/ml limit of detection using a commercially available handheld fluorometer. The CTx aptamer beacon bound full-length 26-amino acid CTx peptide, but not a shorter 8-amino acid segment of CTx peptide which is a common target for commercial CTx ELISA kits. The prototype assay was shown to detect CTx peptide from human urine after creatinine and urea were removed by size-exclusion chromatography to prevent nonspecific denaturing of the aptamer beacon. This work demonstrates the potential of aptamer beacons to be utilized for rapid and sensitive bone health monitoring in a handheld or point-of-care format.

**Keywords** Aptamer · Bone · Fluorescence · Handheld · Osteoporosis

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## Introduction

Rapid, sensitive, and facile assays for bone markers or metabolites are desired by NASA to evaluate bone loss during long-term space missions, because astronauts can lose 1.0–1.5% of their bone mass per month in microgravity environments despite exercise regimens and vitamin D supplementation [1, 2]. On Earth the future medical community and patients could benefit from point-of-care (POC) assessment of osteoporosis and other bone diseases. While sensitive ELISA kits exist for quantification of bone collagen markers such as the C-terminal telopeptide (CTx) [3] of human type I bone collagen, ELISA kits are relatively time-consuming and require a skilled technician in order to ensure accurate results. Therefore, we set out to develop a facile one step (homogeneous) DNA aptamer beacon system whereby an unskilled user could simply add a urine or serum sample to lyophilized reagents, mix to equilibrate and read the results accurately within minutes using a portable fluorometer.

Preliminary results of this assay and sensor development effort have already been published [4]. However, those experiments were formatted as competitive displacement fluorescence resonance energy transfer (FRET) experiments using a “polyclonal” family of different aptamer sequences “doped” with quencher-dUTP conjugate to incorporate

quenchers into the aptamer structure [4–7]. The quencher-doped family of aptamers was then bound to fluorophore-CTx peptide conjugate and competed against various levels of unlabeled CTx peptide leading to a proportional increase in fluorescence [4].

Here, we further explore and characterize several CTx aptamer secondary loop structures derived from a dominant aptamer sequence in the population. While examining loop structures does not guarantee that one will find a target binding pocket, because induced fit at various duplex regions of the aptamer is possible, loops are logical starting points in a specific minimal length beacon search. The rationale for examining loop structures traces back to the early aptamer beacon work and the natural binding mechanism of riboswitches or other nucleic acid switches [8–11].

## Experimental

*Peptides and Other Materials* Full length 26-amino acid C-terminal telopeptide (CTx) of human type I α1 collagen having the amino acid sequence: SAGFDFSFLPQPPQE KAHDGGRYYRA [12] and type I N-terminal telopeptide (NTx α1(I)) with amino acid sequence: DEKSTGG [13] were synthesized and HPLC-purified to > 98% purity by GenScript, Inc. (Piscataway, NJ). A truncated 8-amino acid version of human CTx peptide (EKAHDGGR) was obtained from GenWay Biotech, Inc. (San Diego, CA; Cat. No. 06-271-83176). Helical peptide (HP) of human α1 (I) bone collagen from amino acids 620–633: GPPGPAG PAGERGE was purchased from Quidel Corp. (San Diego, CA; Cat. No. 8022) as were deoxypyridinoline (DPD; Cat. No. 4807) and hydroxyl-pyridinoline (H-Pyd; Cat. No. 4805). D-glucose, glucosamine, and all other reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). One cm clear polystyrene cuvettes were obtained from Thermo-Fisher Corp. (Pittsburgh, PA; Cat. No. 14955129).

*DNA Aptamer Development, Cloning, Sequencing and Beacon Synthesis* Systematic Evolution of Ligands by Exponential enrichment (SELEX) was carried out by first dissolving full-length CTx peptide (26 amino acids) in Dulbecco's calcium and magnesium-free phosphate buffered saline (PBS) and immobilizing the peptide on 2.8 micron Dynal tosyl-magnetic beads (MBs, Invitrogen Corp., Carlsbad, CA) for 2 h at 37 °C. CTx-coated MBs were then collected using a Dynal MPC-S magnetic rack and washed three times in 1 ml of 1X binding buffer (1XBB; 0.5 M NaCl, 10 mM Tris-HCl, and 1 mM $MgCl_2$, pH 7.5–7.6). The beads were then blocked for 2 h at 37 °C in 1XBB plus 2% ethanolamine and washed three times as before. CTx-conjugated MBs were subjected to five rounds of MB-SELEX as previously described [4–7, 14], followed by cloning into chemically competent *E. coli* using a Lucigen GC kit (Middleton, WI) and proprietary DNA sequencing methodology for GC-rich sequences at Sequetech, Inc. (Mountain View, CA). All DNA aptamer loops (candidate beacons from Fig. 1) were synthesized with 5′-TYE 665 fluorophore and 3′-Iowa Black RQ quencher labels and HPLC-purified at Integrated DNA Technologies, Inc. (Coralville, IA).

*Computer Modeling Software and Techniques* Two-dimensional modeling was achieved by means of internet-based Vienna RNA software [15] using DNA parameters [16] at 25 °C. Loop structures were designated by their relative positions on a 12-h clock as 2 O'clock (2 h), 6 O'clock (6 h) and 10 O'clock (10 h). GRAMM [17] and Forcite Plus® software (Accelerys, Inc., San Diego, CA) were used to calculate binding energies and to produce 3-dimensional renderings of the 26-amino acid CTx peptide and the CTx 2R aptamer in the absence of water and salts or other ionic influences (i.e., *in vacuo*). Geometry minimization as provided by Accelrys Discovery Studio was used to create initial (nearly 3-D) structures of the aptamer and peptide. Low resolution macromolecuar docking using GRAMM

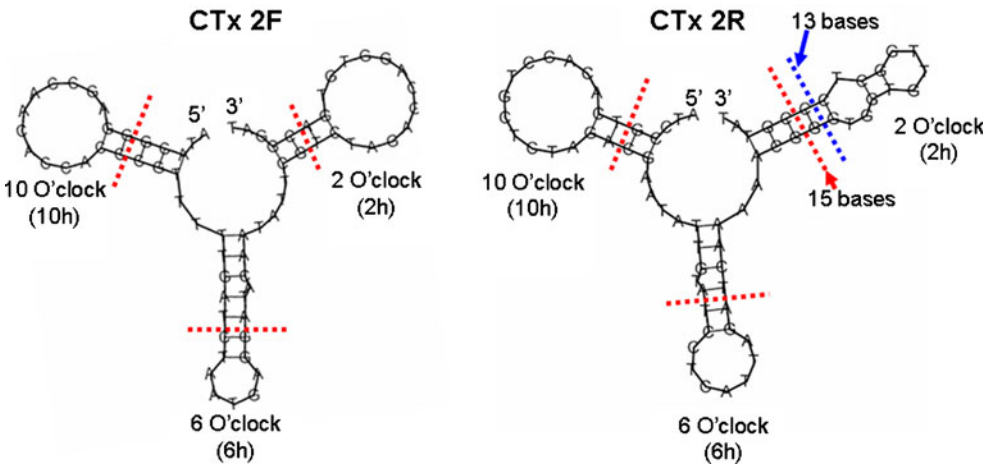


**Fig. 1** Secondary structures of the CTx 2F and 2R aptamers as determined by Vienna RNA software using DNA parameters at 25 °C. The 5′ and 3′ ends are indicated as well as the 12-h clock orientation scheme and demarcation of each beacon by dotted lines. The physical limits of the 13-base and 15-base CTx 2R 2h beacons are indicated as well

was carried out to search for any global peptide-aptamer binding arrangements. Low resolution docking parameters were used as described for GRAMM [17].

In order to estimate binding interactions and intramolecular distances, more accurate 3-D models were determined by molecular dynamics (MD) simulations. Accelrys Forcite® Dynamics was used to find individual local energy minima for the isolated aptamer and isolated CTx peptide. The unbound CTx peptide model was also subjected to a more accurate and explicit energy minimization procedure, whereas, a 500 ps dynamics simulation was used on the free aptamer. The polymer-consistent force field (PCFF) was used in all cases.

Next, GRAMM was used to find new docking poses of the 3-D relaxed aptamer model and relaxed CTx peptide. Two docking poses, representing energy minima for binding to the 2 h and 10 h loops, respectively, were selected for further simulation. Finally, these two relaxed models representing different binding site locations, were subjected to a more extensive conformational search using a temperature annealing algorithm (Accelerys Forcite Plus®). The temperature was varied between 300 and 800 K over at least 5 cycles for a total simulation time of 500 ps for each model. The resolution setting on energy evaluation was set to "fine." The simulations achieved an apparent local energy minimum for each docking site.

*Aptamer Beacon Assays, Spectrofluorometry and Handheld Fluorometry* Serial two-fold dilutions of CTx peptides or other analytes were typically prepared in 1 ml of 1XBB or PBS as indicated per plastic cuvette at the concentrations shown in the figures. One ml of aptamer beacon was added per cuvette at a final concentration of 1.25 or 2.5 μg/ml as indicated in each figure and cuvettes were gently mixed for a minimum of 30 min at RT prior to obtaining fluorescence spectra or intensity readings. Spectra were obtained on a Varian Cary Eclipse spectrofluorometer with excitation at 645 nm for the TYE 665-labeled aptamer beacons with emission scanning from 650 nm to 720 nm using 5 nm excitation and emission slit widths with photomultiplier tube (PMT) settings of 700 to 1,000 V as indicated in each figure. This spectral emission range (650–720 nm) was

**Table 1** CTx aptamer DNA sequences

| | |
|---|---|
| CTx 1F | ATACGGGAGCCAACACCACTAACTTGTTGCTGATCTTATCCAGAGCAGGTGTGACGGAT (59) |
| CTx 1R | ATCCGTCACACCTGCTCTGGATAAGATCAGCAACAAGTTAGTGGTGTTGGCTCCCGTAT (59) |
| CTx 2, 13, 19, 20, 25, 32F | ATACGGGAGCCAACACCACCCGTTTTTGATCTAATGAGGATACAATATTCGTCTAGAGCAGGTGTGACGGAT |
| CTx 2, 13, 19, 20, 25, 32R | ATCCGTCACACCTGCTCTAGACGAATATTGTATCCTCATTAGATCAAAAACGGG**TGGTGTTGGCTCCCGTAT** |
| CTx 3F | ATACGGGAGCCAACACCAATCGATGGTTAGACTATTACACTAGATGGAATTCATAGAGCAGGTGTGACGGAT |
| CTx 3R | ATCCGTCACACCTGCTCTATGAATTCCATCTAGTGTAATAGTCTAACCATCGATTGGTGTTGGCTCCCGTAT |
| CTx 6F | ATACGGGAGCCAACACCAATCTGCCGACTAGGCCAAGTAATTATATTCAGCTGGAGAGCAGGTGTGACGGAT |
| CTx 6R | ATCCGTCACACCTGCTCTCCAGCTGAATATAATTACTTGGCCTAGTCGGCAGATTGGTGTTGGCTCCCGTAT |
| CTx 7, 8F | ATACGGGAGCCAACACCACAGCTGATATTGGATGGTCCGGCAGAGCAGGTGTGACGGAT (59) |
| CTx 7, 8R | ATCCGTCACACCTGCTCTGCCGGACCATCCAATATCAGCTGTGGTGTTGGCTCCCGTAT (59) |
| CTx 11F | ATACGGGAGCCAACACCACATTACAATAGATGTATTGACATATCCGGACAGTCGAGAGCAGGTGTGACGGAT |
| CTx 11R | ATCCGTCACACCTGCTCTCGACTGTCCGGATATGTCAATACATCTATTGTAATGTGGTGTTGGCTCCCGTAT |
| CTx 14F | ATACGGGAGCCAACACCACTCGTGTAGTGCTGTCTTTGTGGAATCCTTGCATCGAGAGCAGGTGTGACGGAT |
| CTx 14R | ATCCGTCACACCTGCTCTCGATGCAAGGATTCCACAAAGACAGCACTACACGAGTGGTGTTGGCTCCCGTAT |
| CTx 15F | ATACGGGAGCCAACACCACCACGTGACCCATACGATACAACAAATAATTGCTCAAGAGCAGGTGTGACGGAT |
| CTx 15R | ATCCGTCACACCTGCTCTTGAGCAATTATTTGTTGTATCGTATGGGTCACGTGG**TGGTGTTGGCTCCCGTAT** |
| CTx 16F | ATACGGGAGCCAACACCATCCATAGCTCATCTATACCCTCTTCCGAGTCCCACCAGAGCAGGTGTGACGGAT |
| CTx 16R | ATCCGTCACACCTGCTCTGGTGGGACTCGGAAGAGGGTATAGATGAGCTATGGATGGTGTTGGCTCCCGTAT |
| CTx 17F | ATACGGGAGCCAACACCAGACGCGGAACGACTCATCGCAAAATGTCGTGATGCAAGAGCAGGTGTGACGGAT |
| CTx 17R | ATCCGTCACACCTGCTCTTGCATCACGACATTTTGCGATGAGTCGTTCCGCGTCTGGTGTTGGCTCCCGTAT |
| CTx 18F | ATACGGGAGCCAACACCATGGTTAGGCTGCTCCATATATTCCCGCCCCGCACGTAGAGCAGGTGTGACGGAT |
| CTx 18R | ATCCGTCACACCTGCTCTACGTGCGGGGCGGGAATATATGGAGCAGCCTAACCATGGTGTTGGCTCCCGTAT |
| CTx 38, 40F | ATACGGGAGCCAACACCATAGTGTTGGGCCAATACGGTAACGTGTCCTTGGAGAGCAGGTGTGACGGAT (69) |
| CTx 38, 40R | ATCCGTCACACCTGCTCTCCAAGGACACGTTACCGTATTGGCCCAACACTATGGTGTTGGCTCCCGTAT (69) |

All sequences are presented 5′ to 3′ from left to right. Sequence numbers demarcated by a comma, indicate multiple copies of identical sequences in the cloned population. Numbers in parentheses indicate the length in bases of truncated aptamers. The underlined and highlighted region of the CTx 2R and 15R aptamers indicates the 15-base CTx aptamer beacon sequence. The 18 bolded bases near the 3′ end indicate one of the fixed primer regions common to all or the reverse-primed aptamers.

selected to match the bandpass emission filter of a customized Quantifluor™-P handheld fluorometer (Promega Corp., formerly the Picofluor™ by Turner Biosystems prior to acquisition by Promega) which excited samples with a light-emitting diode (LED) centered at 650 nm. The customized Quantifluor™-P unit was used to emulate projected portable diagnostics for bone loss markers by astronauts in outer space or POC medical applications on Earth with limited laboratory or fluorescence measurement capabilities and to obtain simple integrated fluorescence intensity values for plotting. The STD VAL button of the Quantifluor™-P unit was set to the highest (999.0) setting to maximize sensitivity.

*Isolation of CTx Peptide from Human Urine* The first author contributed a clean-catch urine sample which was cooled to RT and spiked with full-length CTx peptide at 100 μg/ml. Because urea and creatinine in urine were found to denature aptamer secondary structure leading to high fluorescence background during early experiments, the CTx-spiked urine sample was first purified through a 1XBB-equilibrated D-Salt™ polyacrylamide column (Pierce Chemical Co., Cat. No. 43426) having a molecular weight cut off (MWCO) of 1,800 daltons (MW of CTx= 2,942 daltons) and a reported 95% analyte recovery efficiency. One ml fractions were collected in 1XBB and 20 μl samples of each fraction were run on an 8% SDS-

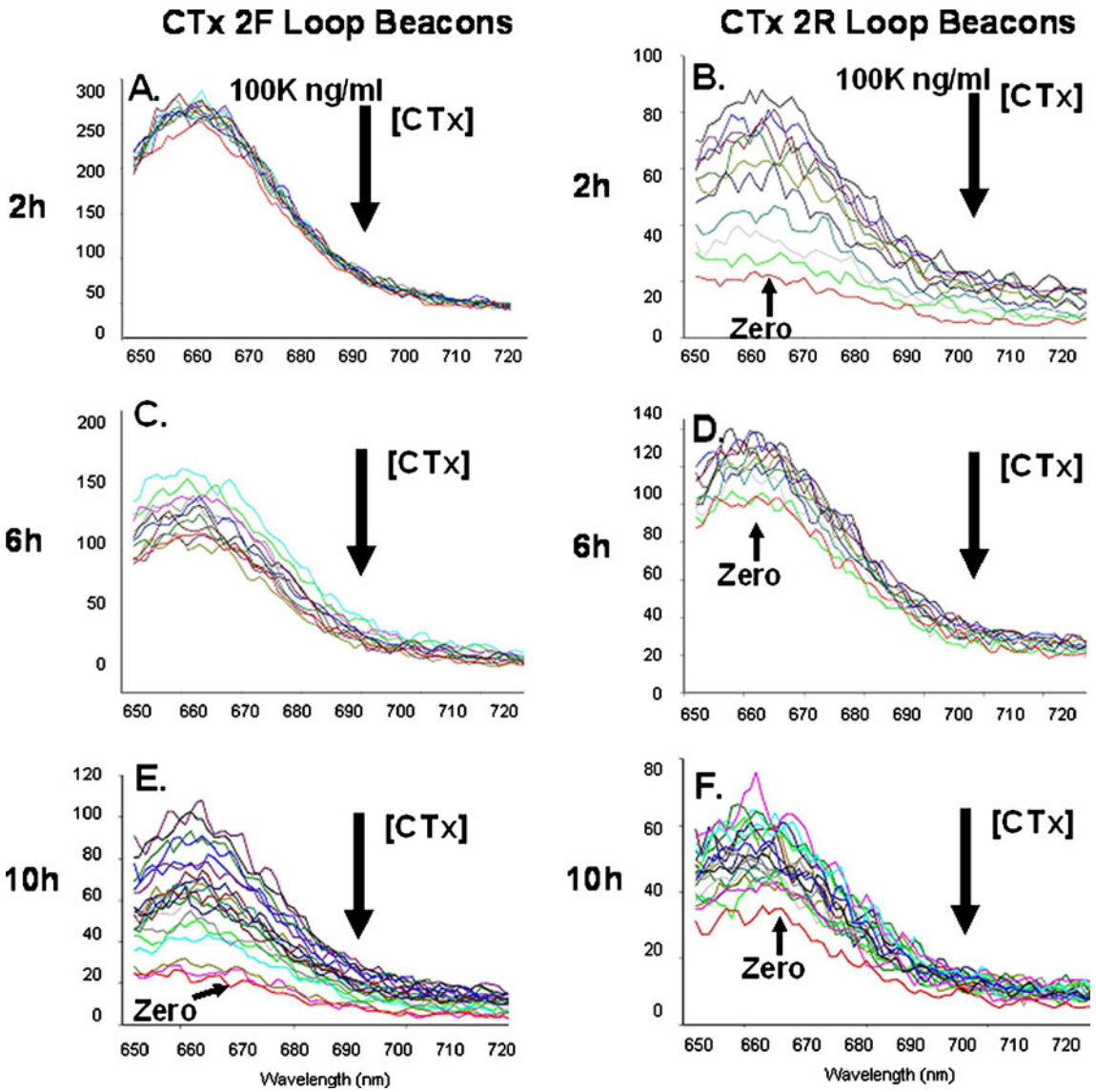


**Fig. 2** Comparison of candidate CTx aptamer beacon (2.5 μg/ml) fluorescence responses for the six truncated loop structures defined in Fig. 1. Serial two-fold dilutions of CTx in 1XBB were performed beginning with 100,000 ng/ml of CTx and shown as decreasing in concentration ([CTx]) by the arrows. Zero background control fluorescence spectra of the beacon only in 1XBB are represented by the red traces (typically lowest traces) in each case. The 15-base version of the CTx 2R 2h beacon candidate is shown (panel B). PMT voltage was set at 700 V

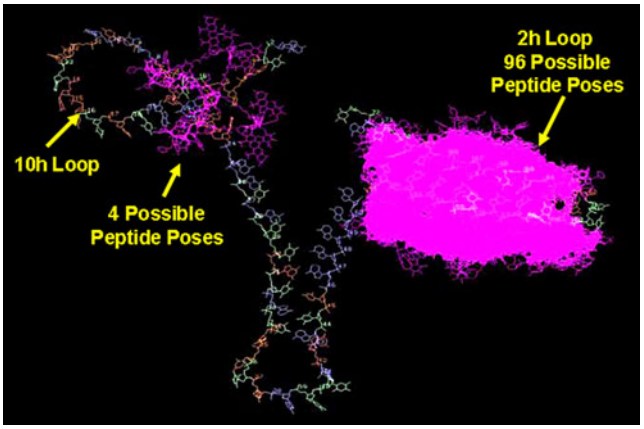


**Fig. 3** Superposition of the top 100 most favorable binding poses of 26-amino acid CTx peptide (magenta) on CTx-2R aptamer as determined by GRAMM. The vast majority (96/100) of favorable configurations of the CTx-2R aptamer with the peptide occur within the 2 O'clock (2 h) loop

HEPES-polyacrylamide mini gel (Pierce Chemical Co.) for 45 min at 100 V in cold HEPES running buffer followed by Coomassie blue staining to verify that the CTx peptide eluted from the column. Fractions 3 and 4 (2 ml total volume) were pooled for serial two-fold dilution beacon fluorescence experiments.

## Results

Examination of the CTx aptamer sequences from Table 1 revealed that one forward (F) and one reverse (R) sequence dominated the aptamer population. These two sequences comprised 31.6% of the sequenced aptamers (12 of 38 total sequenced aptamers presented in Table 1) and are named CTx 2F and CTx 2R for their first occurrence in clone number 2. The secondary structures of these two candidate aptamers as determined by Vienna RNA software [15] using DNA parameters [16] at 25 °C are revealed in Fig. 1. Candidate aptamer beacons were derived from these secondary structures as demarcated by the dotted lines in Fig. 1 and designated according to a 12-h clock scheme shown in the figure. Six different 5′-TYE 665 and 3′-Iowa Black quencher-labeled candidate beacons (outer loop structures beyond the dotted lines in Fig. 1) were then synthesized and HPLC-purified by Integrated DNA Technologies, Inc.

The six candidate aptamer beacons (loops) were subjected to fluorescence titration analysis against serial two-fold dilutions of full-length CTx peptide in a final volume of 2 ml of 1XBB beginning at 100,000 ng/ml. Results of this series of experiments are given in Fig. 2 which clearly showed that the best fluorescence response (i.e., greatest separation of spectra as a function of CTx peptide concentration; [CTx]) belonged to the CTx 2R-2 O'clock or second hour (2 h) aptamer beacon (Fig. 2, panel B). It is of interest to note that the CTx 2R-2h loop (putative binding pocket) sequence segment also occurs as part of clone CTx 15R (Table 1) further enhancing the dominance of this pocket in the library.

The CTx 2F and 2R aptamers also exhibited some FRET (separation of spectra as a function of CTx peptide concentration) in both of their 10 O'clock loops (Fig. 2, panels E and F). However, the combination of low background and superior concentration-dependent spectral separation in the 15-base CTx 2R-2h aptamer beacon (5′-GGTGGTGTTGGCTCC-3′) as a function of CTx peptide concentration suggested it was the beacon of choice for further experiments.

The empirical FRET data obtained in Fig. 2 were also well correlated to theoretical predictions for where CTx peptide would bind the CTx 2R aptamer as shown in Fig. 3. Despite the slight energetic favorability advantage in

**Table 2** Binding energy calculations demonstrating energy preference for CTx peptide binding to the 10 O'clock (10 h) versus 2 O'clock (2 h) loops

| | | Aptamer binding energy (Enthalpy) | | |
|---|---|---|---|---|
| 10 O'clock Loop | 10 O'clock Loop Complex | Aptamer only | Ligand Only | Net Binding Energy |
| Van der Waals | 626 | 516 | 128 | −18 |
| Electrostatic | −16911 | −13289 | −1146 | −2476 |
| | | | | −2494 |
| 2 O' clock Loop | 2 O'clock Loop Complex | Aptamer only | Ligand only | |
| Van der Waals | 650 | 530 | 131 | −11 |
| Electrostatic | −16688 | −13239 | −1178 | −2271 |
| | | | | −2282 |
| | | | Net 10 O'clock Preference=−212 kcal/mol′ | |

The electrostatic energy is greatly overestimated because the simulation was performed *in vacuo* and the effects of water and counter ions, such as $Mg^{2+}$ are not considered.

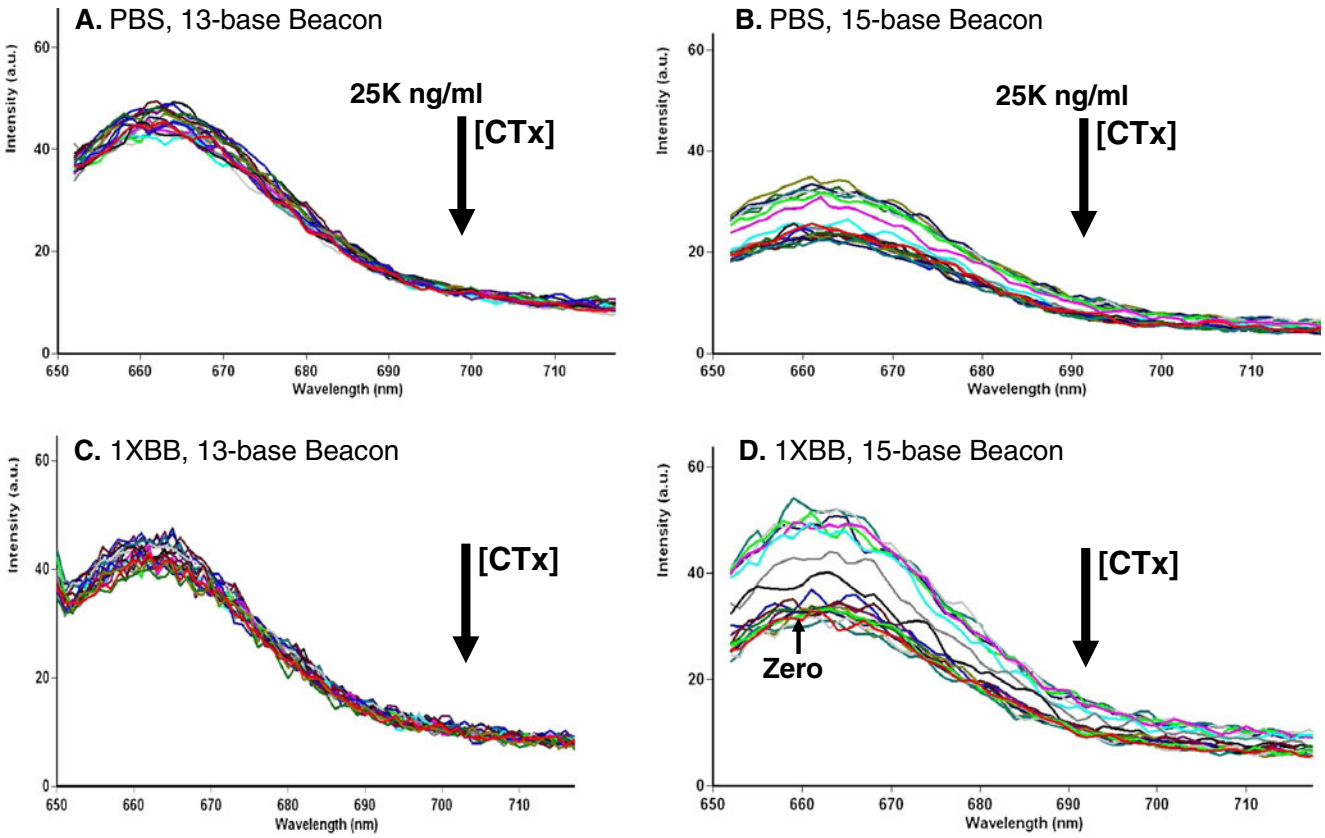


**Fig. 4** Comparison of the 13-base and 15-base versions of the CTx 2R 2h aptamer beacon in PBS having no $Mg^{2+}$ and 1XBB containing 1 mM $Mg^{2+}$as labeled. Serial two-fold dilutions of CTx peptide (26 amino acids) beginning at 25,000 ng/ml and ending at zero (beacon only red traces) were analyzed with 1.25 μg/ml of each beacon for 30 min at RT. PMT voltage was set at 700 V

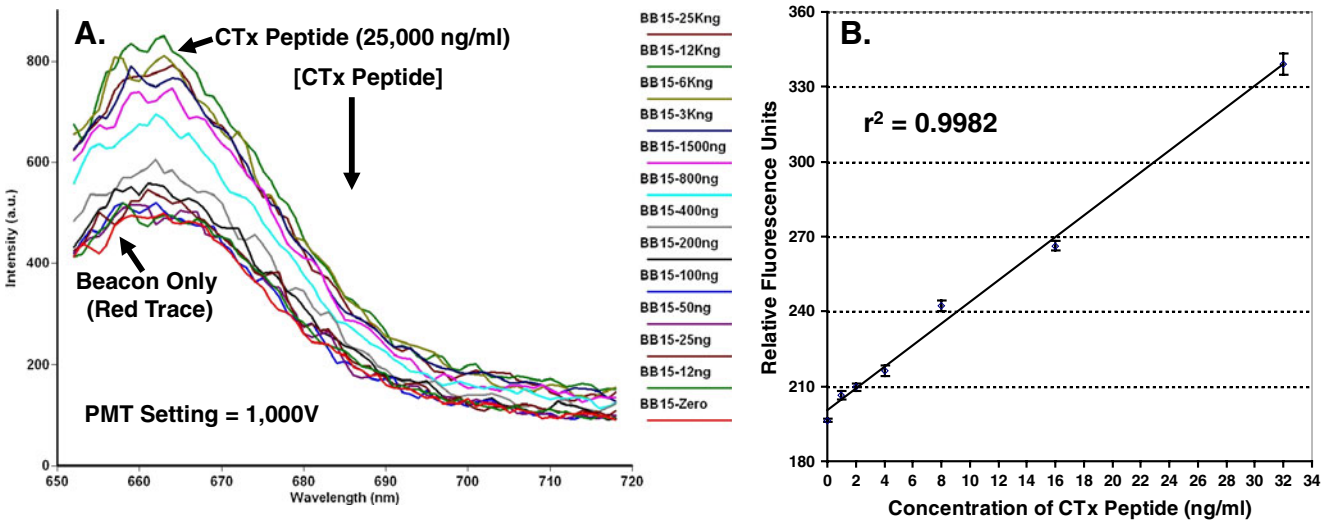


**Fig. 5** **a** Broad concentration range fluorescence spectral behavior of the CTx 2R-2h 15-base aptamer beacon at a final concentration of 1.25 μg/ml versus two-fold serial dilutions of the CTx peptide in 1XBB beginning at 25,000 ng/ml. PMT voltage was set at 1,000 V. **b** Lower limit of detection (LOD) summary for the CTx 2R-2h aptamer beacon versus varying concentrations of 26-amino acid CTx peptide using the modified handheld Quantifluor™-P fluorometer with 650 nm excitation and 660–720 nm bandpass emission filter. The sensitivity level (STD VAL button) was set to 999.0. Data points represent the means ± 3 standard deviations for 5 independent measurements in 1XBB ($r^2$=0.9982). RFU; relative fluorescence units

binding of CTx peptide to the 10 O'clock (10 h) loop as summarized in Table 2, the CTx 2R aptamer demonstrated an overwhelming topological fit and docking preference (96 of the top 100 poses and all of the top 10 poses) for binding to the 2 O'clock (2 h) loop as illustrated in Fig. 3. In essence, these observations support a greater role for geometric fit (Fig. 3 pose preference) versus a slight thermodynamic advantage (Table 2) in determining binding pockets in at least some aptamer systems. We have previously shown that high aptamer affinity and optimal FRET performance are not necessarily well correlated in a competitive displacement FRET scenario [5–7]. Therefore, the presently reported greater FRET capacity from a lower affinity loop is not too surprising for an aptamer beacon system.

Figure 4 illustrates that $Mg^{2+}$ plays a role in 15-base CTx 2R-2h aptamer beacon performance (perhaps as a stabilizer) [9], because the best fluorescence response as a function of CTx peptide concentration occurred in the presence of 1X binding buffer (1XBB) containing 1 mM $Mg^{2+}$ (panel D). In the absence of $Mg^{2+}$, (in PBS), the same 15-base beacon experiment performed poorly (panel B) and neither of the 13-base beacons demonstrated a fluorescence assay (Fig. 4, panels A and C) regardless of the presence or absence of $Mg^{2+}$. This may be because only one G-C pair exists in the stem region of the 13-base beacon and it is easily disrupted [18] or never really closed to quench the TYE 665 dye leading to maximal fluorescence even in the absence of the CTx target.

In order to determine the lower limit of detection (LOD) for CTx peptide using the 15-base CTx 2R-2h aptamer beacon, numerous assay experiments were conducted which culminated in the data presented in Fig. 5. The handheld fluorometer data in Fig. 5b demonstrate a lower LOD of approximately 1 ng/ml making this beacon assay comparable in sensitivity to some of the best commercial CTx ELISA kits [3]. The beacon is also linear over a low to mid ng/ml range of CTx peptide concentrations ($r^2$=0.9982) which more than covers the requisite range of possible physiologic values detectable in urine [19].

We next investigated specificity of the CTx 2R-2h aptamer beacon versus a number of other bone markers

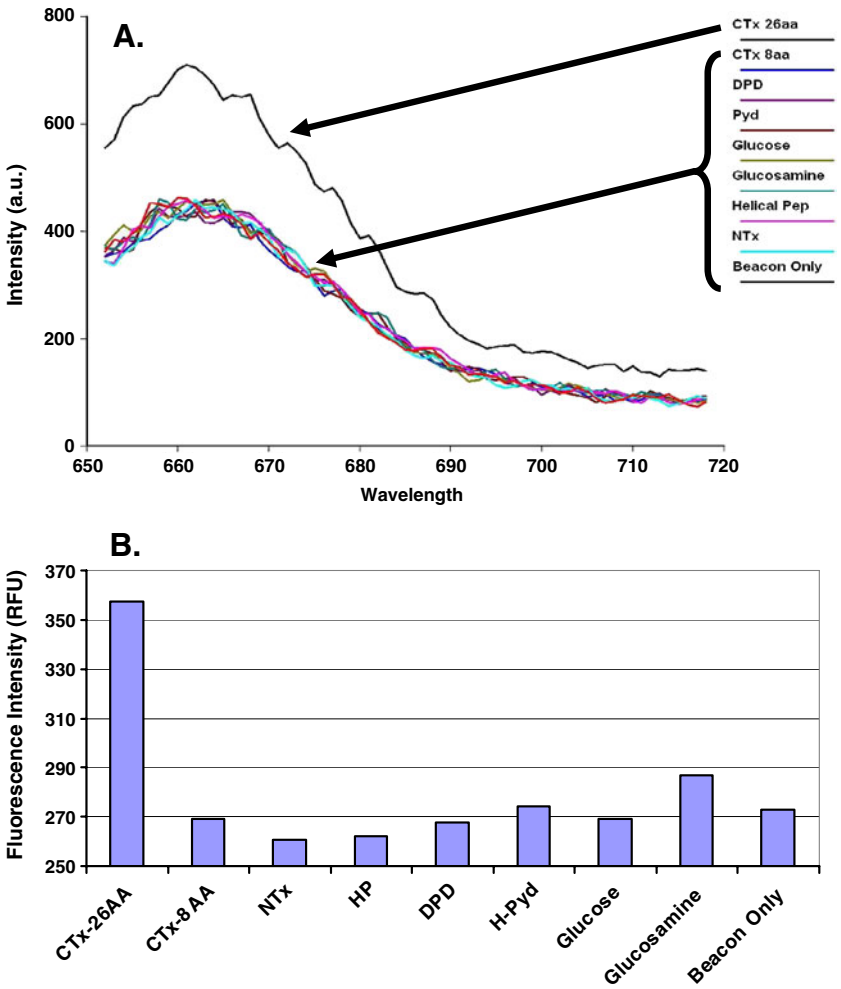


**Fig. 6** Results of a cross-reactivity study for the CTx 2R-2h aptamer beacon including the full-length 26-amino acid CTx and the truncated 8-amino acid CTx peptides. All analytes were assessed at 500 ng/ml except for D-glucose which was analyzed at 200 mg/dl to emulate a high blood glucose level. Spectrofluorometry results from the top panel (**a**) with PMT setting at 1,000 V were corroborated by Quantifluor™-P results in the bottom panel (**b**). RFU; relative fluorescence units. Other abbreviations are listed in the Experimental section

and general analytes that might be expected in human serum or urine. Figure 6 shows that the CTx 2R-2h aptamer beacon demonstrated a significant preference for the full-length 26-amino acid CTx peptide and did not bind bone collagen crosslinkers such as DPD and H-Pyd or other common small molecules found in human body fluids. Fluorescence spectra and handheld fluorescence intensity readings were in relative agreement as seen by comparison of data in Fig. 6a and b. This general lack of cross-reactivity included an 8-amino acid segment (EKAHDGGR) of the full-length 26-amino acid CTx peptide which some commercial ELISA kits recognize [3, 20], but which can be excluded as a binding site for the CTx 2R-2h aptamer beacon based on data in Fig. 6.

A key problem to overcome for implementation of the aptamer beacon assay was to detect CTx in urine. As Figs. 7, 8 and 9 illustrate, many aptamer beacons simply yield fluorescent light in proportion to the level of urine or denaturing amine compounds such as urea and creatinine in urine. We had developed other aptamer sequences against immobilized creatinine (not shown but designated as "Cr" aptamers in Fig. 7) and these as well as other unrelated aptamer beacons simply emit fluorescence in proportion to the creatinine or urea concentration. This was presumably due to nonspecific opening of the closed and quenched beacons by cationic destabilization from urea and creatinine which compete with nucleotides for hydrogen bonding opportunities. Addition of urease failed to ameliorate this problem. Therefore, we were forced to extract CTx peptide from urine by size-exclusion chromatography prior to our fluorescence analysis.

We validated extraction of the 2.9 kD full-length CTx peptide from a urine sample using a polyacrylamide D-Salt$^{TM}$ column with 1.8 kD MWCO by Coomassie blue-stained 8% SDS-HEPES-PAGE gel electrophoresis (Fig. 10a). The D-Salt$^{TM}$ columns have a reported 95% recovery efficiency. When fractions 3 and 4 were pooled (2 ml total volume) and the aptamer beacon titration experiment was repeated (Fig. 10b), the lower LOD was slightly elevated to 3 ng/ml. It is not known how much CTx peptide may have already been in the urine sample prior to CTx peptide spiking. However, examination of the "urine only" lane (Fig. 10a) suggests some protein and low molecular weight peptides may have been present and may have influenced the detection limit by elevating background fluorescence or making some of the aptamer beacons unavailable for binding to CTx peptides which were added exogenously.

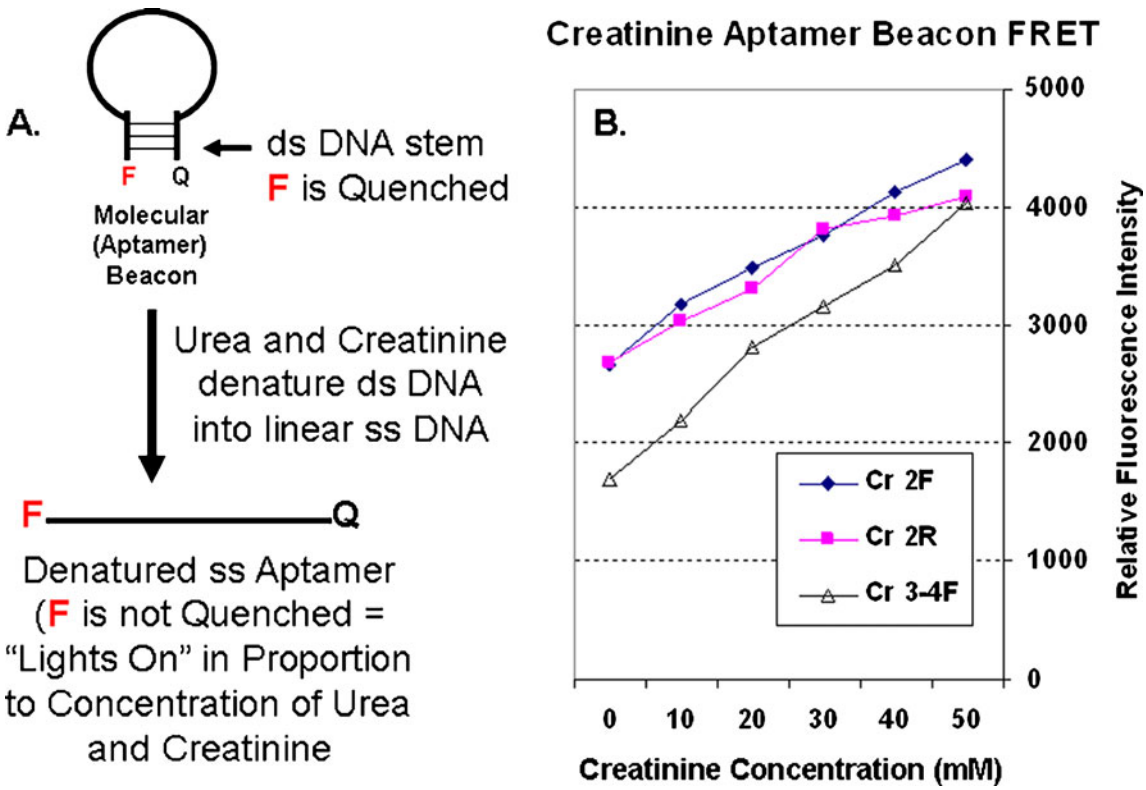


**Fig. 7** **a** Diagram illustrating the hypothesized denaturing effect of urea and creatinine from urine on aptamer beacons. **b** Aptamer denaturation leads to linear fluorescent light output as a function of creatinine concentration in the physiologic range for the creatinine (Cr) aptamer beacons as shown and could be used as a divisor for facile normalization of bone marker levels in urine

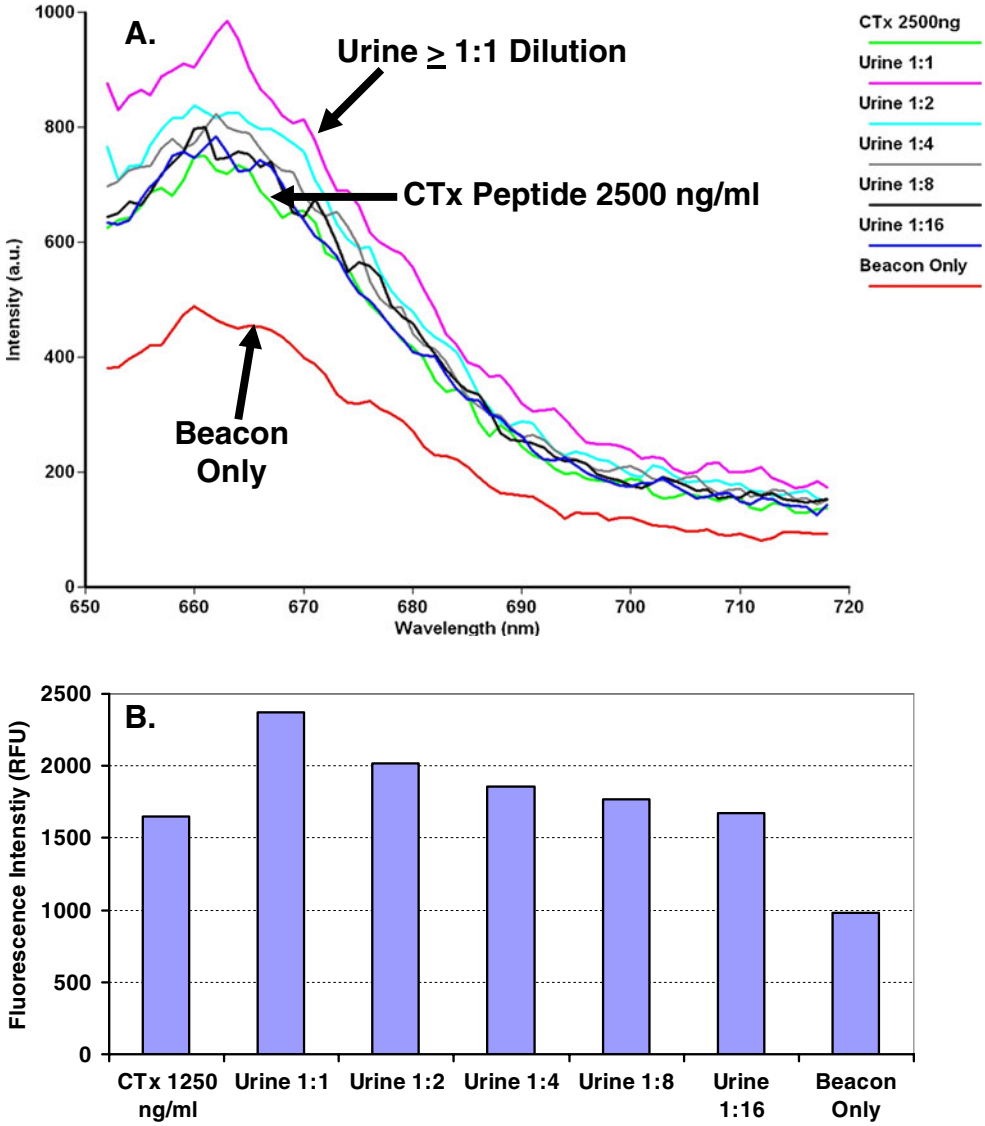


**Fig. 8** **a** Fluorescence spectra illustrating the denaturing effects of as little as a 1:16 dilution of human urine on the CTx 2R-2h aptamer beacon. PMT setting was 1,000 V. **b** Corresponding Quantifluor™-P values. The fluorescence levels achieved with 1,250 and 2,500 ng/ml of CTx peptide were included for comparison. Aptamer beacon concentration was 2.5 μg/ml in 1XBB

## Discussion

To the expert observer, it is remarkable that a useful beacon arose from the 2 h loop since this loop was primarily composed of the fixed PCR primer region. Of the original 15 bases in the CTx 2R-2h aptamer beacon, 13 consecutive bases were part of the fixed-sequence primer region (primer underlined: (5′-GGTGGTGTTGGCTCC-3′). The 6 h loops from CTx 2F and CTx 2R essentially demonstrated no fluorescence changes at all in our screening experiments (Fig. 2c and d), showed no topological fit for the CTx peptide, and yet derived from the randomized or degenerate 36-base region of the template library and went through five rounds of SELEX. The value of the fixed primer ends of aptamers as potential target binding sites has been controversial with most scientists acknowledging that these constant sequence segments can contribute to binding [21] while other researchers are devising methods to eliminate the fixed primer regions [22, 23].

Despite the unlikely natural selection of the CTx 2R-2h aptamer beacon mostly from a fixed primer region, we adopted the 2 h loop as a beacon because the parent CTx 2R sequence dominated the final selected pool of sequences (31.6%) and the 15-base version of the 2 h loop demonstrated the best fluorescence response as a function of full-length CTx peptide concentration (Fig. 2b). It was of interest to note that the 15-base version of the 2 h beacon did not produce a strong fluorescence response in $Mg^{2+}$ and $Ca^{2+}$-free Dulbecco's PBS (Fig. 4b) and that both 13-base beacons failed to produce a fluorescence response to varied levels of the full-length CTx peptide (Fig. 4a and c). However, the 15-base 2 h beacon worked quite well in 1XBB containing 1 mM $Mg^{2+}$ (Figs. 4d and 5), supporting the generally known and accepted role of divalent $Mg^{2+}$

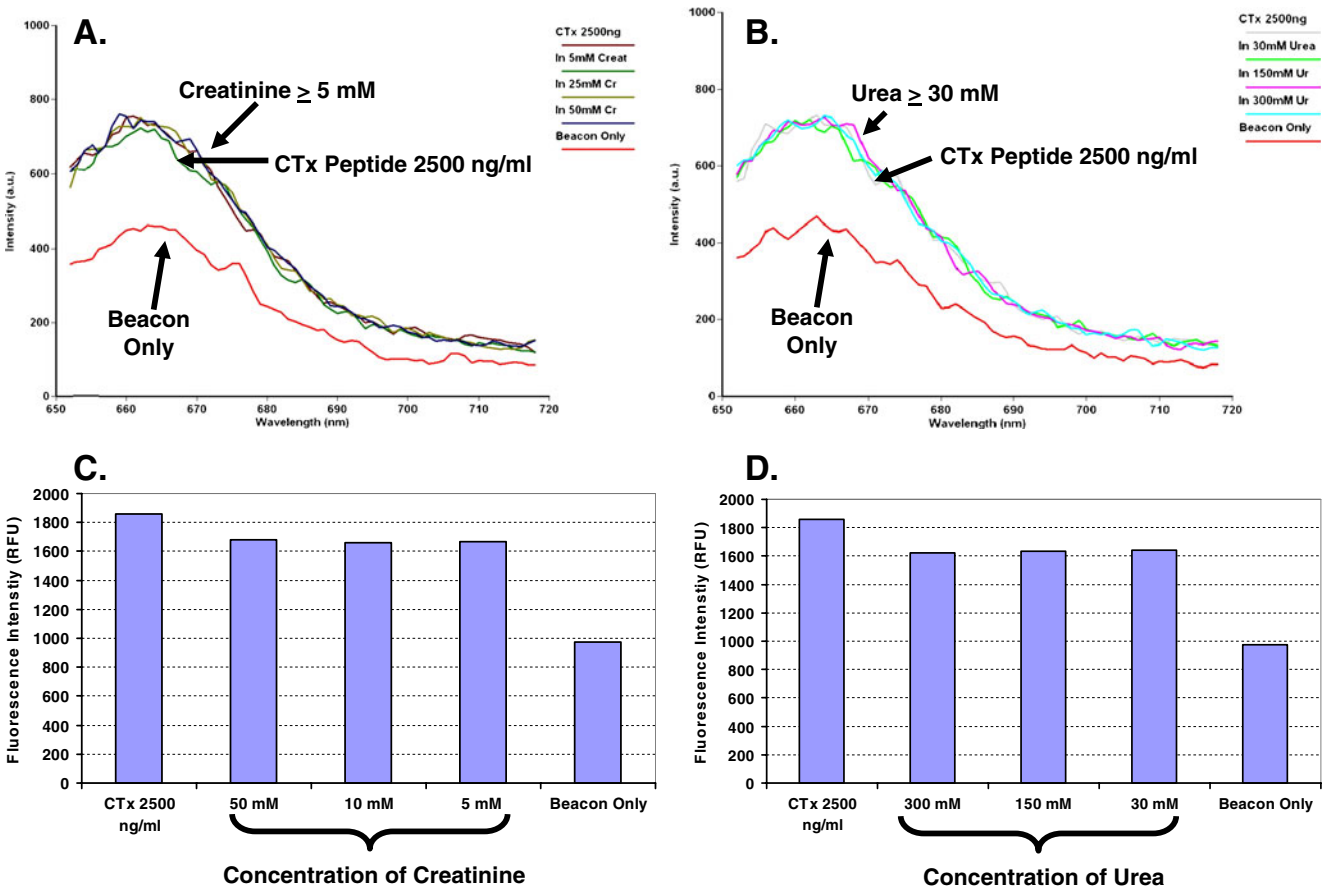


**Fig. 9** **a** Fluorescence spectra illustrating the denaturing effects of ≥ 5 mM creatinine on the CTx 2R-2h aptamer beacon. **b** Fluorescence spectra illustrating the denaturing effects of ≥ 30 mM urea on the CTx 2R-2h aptamer beacon. PMT setting was at 1,000 V. Panels (**c**) and (**d**) corresponding Quantifluor™-P values. The fluorescence levels achieved with 2,500 ng/ml of CTx peptide were included for comparison. Aptamer beacon concentration was 2.5 μg/ml in 1XBB

cations in stabilizing higher-order guanine structures [9] that appear to exist in the 2 h beacon loop. Although the 2 h loop does not appear to be a classic G-quartet or quadraplex, it does have a dual loop nature (Fig. 1), but did not appear to stack in 3-D renderings. The failure of the 13-base beacon suggests that at least two G-C pairs are needed in the stem region to hold the loop closed and effectively quench the fluorophore [9, 18].

The 2 h aptamer beacon repeatedly performed well for detection of full-length 26-amino acid CTx peptide with little cross-reactivity (Fig. 6), but it did not bind the 8-amino acid version of CTx which is detected by some commercial ELISA kits [19, 20]. There are a number of peptide fragments of type I collagen that originate from the C-terminus and we chose the longest (26-amino acid) peptide described in the literature [12] as our initial target for aptamer and assay development to ensure that we covered the available epitopes found in urine. Our results, therefore mirror those of Srivastava et al. [19] who developed an ELISA for an 18-amino acid version of CTx peptide from urine which also failed to bind the same 8-amino acid segment of CTx (i.e., EKAHDGGR). The observation that our CTx 2R aptamer binds a different "epitope" than some commercial ELISAs may be advantageous for future sandwich assays, if we screen our aptamer library and identify or develop aptamers that bind the EKAHDGGR segment of CTx or wish to develop a hybrid antibody-aptamer sandwich assay.

At present we have not given consideration to aptamers that might distinguish various stereoisomeric (α and β) forms of CTx peptide [3], but that is a future goal. A problem of more immediate importance was the effect of urea and creatinine from urine (the preferred non-invasive body fluid for assessment of bone loss markers) on the beacon. As Figs. 7–9 show, as little as a 1:16 dilution of human urine and relatively low (mM) levels of urea and creatinine can denature and linearize an aptamer beacon, probably by competing for hydrogen bonds in the stem region [18, 24]. Human urine can contain 0.6–2.4 mg/ml of creatinine (5.3–21.2 mM creatinine) and ≥ 3.5 mg/ml of

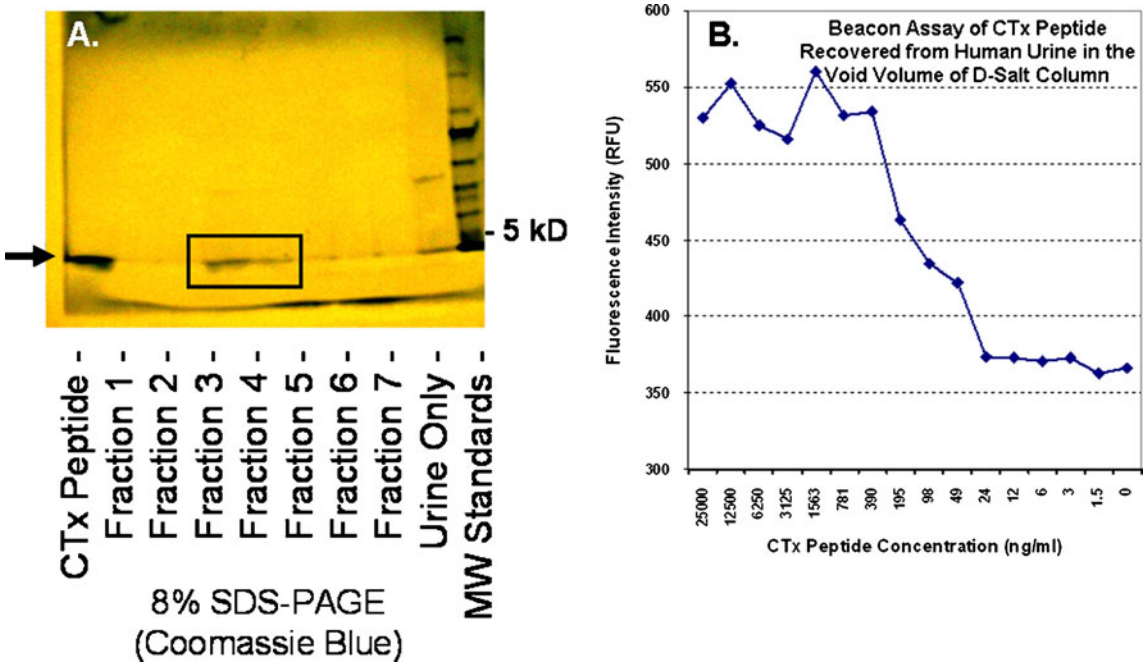


**Fig. 10** Coomassie blue-stained 8% SDS-HEPES-polyacrylamide electrophoresis gel verifying that the 250 μg of full-length CTx peptide spiked into 1 ml of human urine can be extracted by a 1.8 kD MWCO D-Salt™ column and recovered in fractions 3 and 4 (1 ml each). Twenty μl of each fraction were run per lane. Panel B—Fluorescence titration results using 1.25 μg/ml of the 2 h beacon against serial two-fold dilutions of the recovered CTx peptide in 1XBB after extraction from urine in the void volume (2 ml) of a polyacrylamide D-Salt™ column with an exclusion limit of 1.8 kD. Readings were taken with the red channel (650 nm peak excitation) of a handheld Quantifluor™-P fluorometer with STD VAL set to 999.0

urea (≥ 57.8 mM urea) averaged over a 24-h period for a normal adult male [25]. Khan et al. [24] reported that ≥ 50 mM urea from urine was inhibitory for PCR assays and hypothesized that this level of urea prevented primer hybridization to the target DNA (i.e., inhibited duplex formation as in the stems of aptamer beacons). Some other researchers have reported successful detection using aptamers in urine, but these were not beacon systems [26, 27]. Merino and Weeks [28] used a combination of urine dilution (1:3), microfiltration and chemical treatment with sulfosuccinimidyl acetate at elevated temperatures for a total of 44 min to process urine samples prior to analysis with an ATP fluorogenic aptamer assay.

The urinary amine problem for some aptamer beacons can be solved more simply by size-exclusion chromatography as illustrated in the present work (Fig. 10). While chromatography is still somewhat time-consuming with present instrumentation, a number of groups are working on or have already developed micro- and nano-chromatographic sample processing devices for lab-on-a-chip POC diagnostics [29, 30]. Hence, the use of size-exclusion chromatography as a pre-processing step, should not be viewed as prohibitive. Despite reports of 90–100% recovery efficiency for proteins from polyacrylamide size-exclusion columns [31], we were only able to recover 78.6% of the 26-amino acid CTx spiked into 1XBB at 100 μg/ml by Bradford protein assay of samples taken before and after gel filtration (data not shown). Clearly, recovery efficiency is an area where improvement can be implemented.

We also attempted urease-mediated breakdown to solve the nonspecific fluorescence background problem, but of course degradation of urea does not remove the denaturing amines or creatinine and the urease treatment failed to enable a fluorescence response to varied levels of CTx.

While urine represented a hurdle in our CTx assay, the relative linear proportionality of fluorescence light output from some aptamer beacons (Figs. 7–9) as a function of urine or creatinine levels may actually be viewed as a positive or useful aspect, since quantitative bone marker assays require normalization by the creatinine content of urine. Creatinine levels are routinely used as divisors in urinary analyte ratios to normalize other analytes against the overall concentration of urine since creatinine is produced at a relatively constant rate by the body [25]. The linear FRET response as a function of creatinine levels seen in Fig. 7 or simple proportional differences in urine autofluorescence as a function of urine concentration may, therefore, provide a facile means to normalize aptamer beacon-based detection of urinary bone loss markers by fluorescence measurement prior to the size-exclusion chromatography extraction step for bone peptides. Such simple fluorescence measurement methods for estimating

creatinine and urea levels or overall urine concentration appear simpler and easier than the classic multi-step Jaffe colorimetric method for creatinine determination.

Close examination of both the spectra and integrated fluorescence assay values from the Quantifluor™-P reveals nonlinear behavior of the CTx 2R-2h beacon over broad concentration ranges (Figs. 2b, 4d, 5a and 10b). The fluorescence response over the range for zero to 25,000 ng/ml of CTx peptide is actually best modeled by a polynomial function. However, since such high CTx levels are not physiologically relevant, one can focus on much lower levels and the polynomial can be viewed as having two biologically relevant linear regions in the ranges of <50 ng/ml and between 50 and ~780 ng/ml as seen in Figs. 5 and 10b with the latter being more germane for diagnosis of osteoporosis or detection of gross bone resorption.

While commercial ELISA kits for CTx peptides have comparably low limits of detection [3] of ≤ 1 ng/ml versus our prototype CTx aptamer beacon assay, ELISAs are relatively slow and generally confined to a laboratory. Similarly, radioimmunoassay (RIA) [32] and electrochemiluminescence (ECL) [33] assays such as the Elecsys® assays popularized by Roche Diagnostics are more sensitive (sub-ng/ml limits of detection) than our current aptamer beacon assay. However, these systems require specialized instrumentation that most laboratories do not possess, and in the case of RIA, produce hazardous radioactive waste which requires special handling. Overall, the results presented herein are encouraging and suggest further development and validation of the CTx and other bone marker aptamer beacon assays where space and time are limited, such as the interior of a spacecraft or a physician's office. Such rapid and portable assays could aid in bone loss quantification and management of osteoporosis and other bone diseases.

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