

# Selection of fluorescent aptamer beacons that light up in the presence of zinc

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**Abstract** In order to generate nucleic acid biosensors that could undergo a reversible conformation change in the presence of the metal zinc, a random sequence pool of single-stranded DNA was immobilized on an oligonucleotide affinity column. In the presence of zinc, those species that underwent a conformational change were released from the column, collected, and amplified. A series of negative and positive selections refined the metal specificity of the selected aptamer beacons. Since the aptamer beacons contained a fluorophore, while the bound oligonucleotide contained a quencher, zinc binding also resulted in an increase in fluorescence. One of the selected beacons, Zn-6m2, bound zinc in the low micromolar range, gave a dose-dependent fluorescence signal, and showed an approximately sixfold increase in fluorescence on zinc binding. While some cross-reactivity with cadmium was observed, it should nonetheless prove possible to use the novel selection method to generate and tune the specificity of a variety of reversible metal biosensors. Such biosensors could potentially be used for continuous monitoring of metals in environmental samples.

**Keywords** Aptamer · In vitro selection · SELEX · Biosensor · Zinc · Conformational switch

## Introduction

Sensors for metal ions are important in a variety of applications ranging from environmental monitoring to toxicology studies. Considerable progress has been made in the development of sensors against biologically important metal ions such as  $\text{Ca}^{2+}$  [1–4], and  $\text{Zn}^{2+}$  [5–10] ions. For example, to analyze the in vivo roles of zinc, fluorescent indicators [7, 8] based on peptides, proteins (for example, carbonic anhydrase [10] and zinc-finger motifs [9, 11]) organic macrocyclic receptors [for example, TSQ(6-methoxy-8-quinolyl-*para*-toluenesulfonamide), Zinquins, ZnAF-2 (6-((2-[bis(2-pyridylmethyl)amino]ethylamino)fluorescein diacetate)) [12–14], and Zinpyrs [5, 6, 15]) have been developed. Nonetheless, the development of sensitive and selective sensors that can report target metal ions even in the presence of interfering metals remains a challenge. Ideally, it would be extremely useful if a generalizable strategy could be employed to develop sensors against a variety of different metal ions.

We have now adapted a novel method that directly selects for nucleic acid conformational changes [16] to the development of aptamer beacons that fluoresce (“light up”) in the presence of  $\text{Zn}^{2+}$ . The use of nucleic acids as biosensors was dictated in part by previous successes in identifying nucleic acids that could specifically interact with metal ions. Most functional nucleic acids require metal ions for proper folding, for maintenance of their tertiary structure, and for function [17]. In addition, RNA aptamers that bound zinc with  $K_d$  values of approximately 100–400  $\mu\text{M}$  have been selected from a random sequence pool [18, 19]. In vitro selection has also been employed to isolate novel nucleic acid enzymes which require a specific metal ion for activity. Pan and Uhlenbeck [20] selected  $\text{Pb}^{2+}$ -

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dependent, RNA-cleaving ribozymes, while Breaker and Joyce [21] selected Pb<sup>2+</sup>-dependent, RNA-cleaving deoxyribozymes. In the same way, Cu<sup>2+</sup>-dependent, DNA-cleaving deoxyribozymes [22–24] and Zn<sup>2+</sup>-dependent, RNA-cleaving deoxyribozymes [25, 26] have been selected. In vitro selection was also used to evolve the group I intron [27] and the RNase P ribozymes [28] to utilize Ca<sup>2+</sup> instead of Mg<sup>2+</sup>.

The nucleic acid selection method we have developed can in principle be adapted to almost any analyte, and has already been used to identify aptamer beacons against oligonucleotides [16] and nucleotides [29]. The method relies on using analytes to trigger the release of bound, fluorescent nucleic acids from hybridized oligonucleotides bearing quenchers, and thus leads not only to specific binding properties but also directly to fluorescent signaling without need for further engineering. Therefore, by first generating zinc biosensors using this method, we provide a first step towards the development of a generalized method for generating reversible, light-up biosensors with tailored specificities for virtually any metal or ion. By employing the method with single-stranded DNA, we hope to generate low-cost and robust sensors.

## Experimental methods

### Synthetic DNA

All oligonucleotides were either made in our laboratory with an Expedite 8909 DNA synthesizer (PE Biosystems, Foster City, CA, USA) using synthesis reagents purchased from Glen Research (Sterling, VA, USA), or ordered from Integrated DNA Technologies (Coralville, IA, USA). A single-stranded DNA pool containing 50 randomized positions (N50; 5'-CATCAGTTAGTCATTACGCTTACG-N50-ATTGTGAAGTCGTGTCCCTATAGTGAGTCGTATAGAA-3') was synthesized using a previously reported method [30], and was used as a starting point for in vitro selection. The pool was amplified using primers 25a.50 (5'-GCATCAGTTAGTCATTACGCTTACG-3'), which included a fluorescein-deoxythymidine residue (Glen Research, Sterling, VA, USA) at the 12th position, and 38.50 (5'-TTCTAATACGACTCACTATAGGGACACGACTTCACAAT-3', the underlined region is a T7 RNA polymerase promoter). The fluorescently labeled single-stranded DNA pool was generated by a combination of chemical synthesis, PCR amplification, in vitro transcription, and reverse transcription, and was purified on a denaturing polyacrylamide gel. The final pool complexity was estimated at 5.5 × 10<sup>14</sup> different sequences. A primer that contained biotin at its 5' end, 18.50 (5'-biotin-GGGACACGACTTCAACAAT-3'), was used instead of 38.50 during later rounds of

selection. 4-(Dimethylaminoazo)benzene-4-carboxylic acid (DABCYL; Glen Research, Sterling, VA, USA) was incorporated into the capture oligonucleotide q12.50 at its 5' end (5'-DABCYL-GAAGACAGTGACT-biotin-3').

### In vitro selection

The selection was initiated by annealing the fluoresceinated, single-stranded N50 DNA pool (1.5 pool equivalents) with a twofold molar excess of the biotinylated capture oligonucleotide q12.50 in 50 μl 1× zinc selection buffer [ZSB; 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid pH7.0, 300 mM NaCl, 0.5 mM MgCl<sub>2</sub>]. The oligonucleotides were annealed by heating them to 94 °C for 30 s, and cooling them to 45 °C for 90 s and then to room temperature. The capture oligonucleotide and bound pool were immobilized on streptavidin-agarose (Sigma-Aldrich, St. Louis, MO, USA) and transferred to a column (Poly-Prep column, Bio-Rad, Hercules, CA, USA). The column volume varied from 500 μl in the first round to 130 μl in rounds 2–8, to 40 μl in rounds 9–12. The immobilized pool was washed several times with selection buffer to remove pool members not bound to the column (Table 1). The contents of the column were then eluted by resuspending the column material with selection buffer containing 2 mM Zn<sup>2+</sup> (1× ZSB containing 2 mM ZnCl<sub>2</sub>; 600 μl in round 1, 300 μl in rounds 2–7, and 200 μl in rounds 9–12) and mixing for 25 min at room temperature. The column material was drained and was subsequently washed three times with 400-μl aliquots of the selection buffer. All the eluates were collected and DNA was precipitated with ethanol. Selected aptamer

**Table 1** Selection conditions

Selection round	Input ssDNA (μM)	Input Zn <sup>2+</sup> (mM)	Negative selection <sup>a</sup>	Pre-selxn washes <sup>b</sup>	
1	1.842	2	No	8	t1.3
2	1.098	2	No	12	t1.4
3	1.098	2	No	14	t1.5
4	1.098	2	1	16	t1.6
5	1.098	2	1	20	t1.7
6	1.098	1	1	35	t1.8
7	1.098	1	1	40	t1.9
8	1.098	1	1	50	t1.10
9	0.500	0.5	2	180	t1.11
10	0.500	0.25	2	220	t1.12
12	0.500	0.0002	2	220	t1.13
11	0.500	0.01	2	220	t1.14

ssDNA single-stranded DNA

<sup>a</sup> The two different negative selections are described in the text.

<sup>b</sup> The number of column volumes used for washing prior to elution.

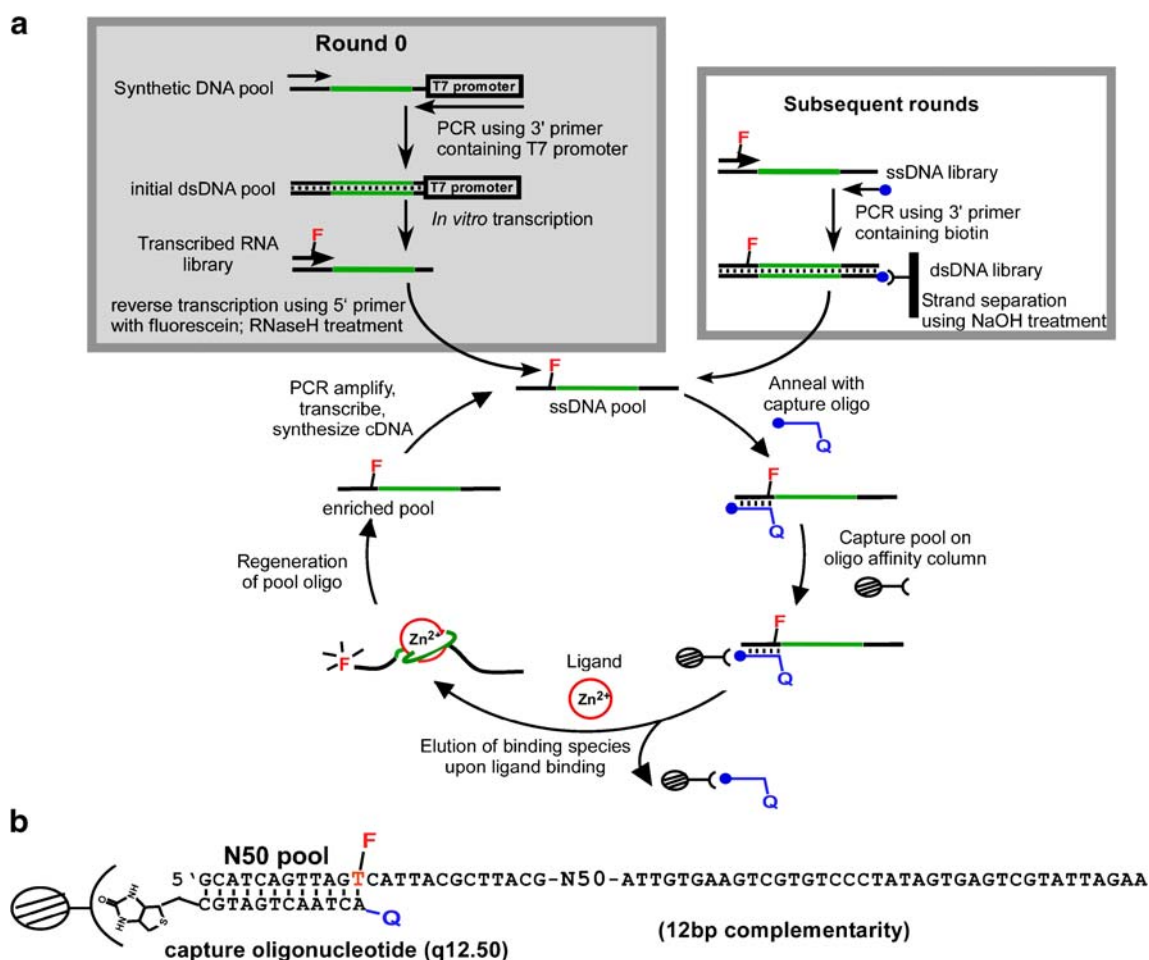
beacons were amplified by the PCR, and regenerated either by transcription followed by reverse transcription (as with the original pool), or by NaOH elution of single-stranded DNA from immobilized, biotinylated, double-stranded DNA bound to a streptavidin column. The amplified, single-stranded DNA was then used for the next round of selection.

The zinc concentration was kept high (2 mM) during the first nine rounds of selection, but was decreased in the last three rounds to increase competition between pool molecules for  $Zn^{2+}$  ions (Table 1). The stringency of the selection was also progressively increased by increasing the number of washes carried out prior to elution. From the fourth round of selection onwards, a negative selection step was introduced in which the immobilized pool was incubated in selection buffer lacking zinc for approximately 20 min prior to incubation in the presence of  $Zn^{2+}$ . During the ninth round of selection an additional negative selection

step was introduced in which the immobilized pool was incubated in the presence of a mixture of other transition metal ions excluding zinc ( $1\times$  ZSB containing 2 mM  $MnCl_2$ , 2 mM  $NiCl_2$ , 2 mM  $CoCl_2$ ). After round 12, the selected pool was cloned (TA cloning kit, Invitrogen, Carlsbad, CA, USA) and sequenced using the dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) and a CEQ 2000 XL DNA sequencer (Beckman Coulter) [31].

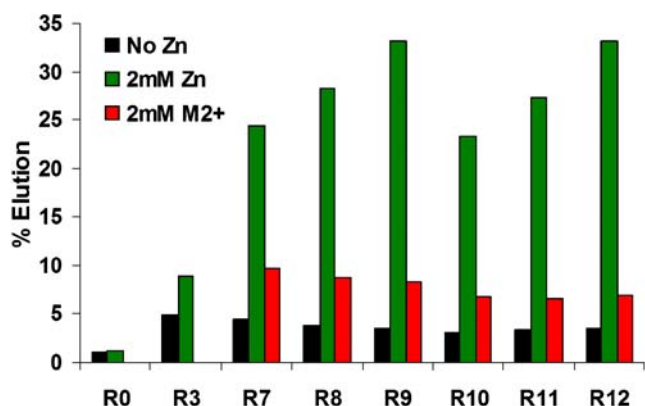
#### Binding assays

Following rounds 3, 7, 8, 9, 10, 11, and 12, the amplified, single-stranded DNA pools were 5' end-labeled using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA, USA) and [ $\gamma$ - $^{32}P$ ]ATP (2.0 mCi, 7,000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA, USA). Binding assays with pools and clones were performed in a manner similar to the selection



**Fig. 1** Selection of  $Zn^{2+}$ -specific aptamer beacons. **a** In vitro selection. Selection is based on target-mediated elution of immobilized nucleic acids from an oligonucleotide affinity column and is described in greater detail in the text. The closed circle at the termini of the capture oligonucleotide represents biotin. The oligonucleotide affinity column is derived from streptavidin-coated agarose beads. **b** The

sequences of the single-stranded DNA library and the capture oligonucleotide used in the selection experiments. *F* an embedded fluorescein, *Q* a quencher, 4-(dimethylaminoazo)benzene-4-carboxylic acid, *ssDNA* single-stranded DNA, *dsDNA* double-stranded DNA, *cDNA* complementary DNA



**Fig. 2** Progress of the selection. Binding assays from different rounds of selection. The vertical axis indicates the percentages of the pools that were specifically eluted with zinc. Black bars show elution in the absence of target (buffer wash alone), green bars show elution in the presence of 2 mM Zn<sup>2+</sup>, and red bars show elution in the presence of a mixture of nontarget transition metal ions (2 mM MnCl<sub>2</sub>, 2 mM NiCl<sub>2</sub>, and 2 mM CoCl<sub>2</sub>)

experiments themselves, except that fractions were collected for scintillation counting. In short, 40 pmol of gel-purified, labeled single-stranded DNA was annealed with 80 pmol of the capture oligonucleotide q12.50 in a 50- $\mu$ l reaction. The radiolabeled DNA was immobilized on 50  $\mu$ l of streptavidin-agarose (Sigma-Aldrich, St. Louis, MO, USA) and the unbound fraction was collected. The column was washed three times with 300  $\mu$ l of 1 $\times$  ZSB and the washes were again collected. A 2 mM solution of Zn<sup>2+</sup> in 1 $\times$  ZSB was added to the immobilized DNA in a total volume of 200  $\mu$ l. As with the selections themselves, the binding reactions were incubated for 25 min prior to washing the column two times with 500  $\mu$ l of selection buffer. Radioactivity in the eluants and in the remaining solid resins were quantitated using a scintillation counter, and the proportion of DNA that was specifically eluted was determined.

t2.1 **Table 2** Sequences of individual aptamer beacons

	R12 aptamer beacons	Sequence	% Elution with 2mM Zn	% Elution with 2mM Mg	
	R0		1.00	1.16	
	R12		33.13	6.95	
Family 1	36	gcatacagtttagtcattacgcttacgGCCCGATCCTAACTGCAAAGGTTTCGTCGTCGCAATCTGCGTGGAGGGCG	37.26	2.96	
	14	gcatacagtttagtcattacgcttacgGCCCGATCCTaACCGAaGAAGAAGAGgTTAaTAACGTTCCGGACcTGSTA	31.66	3.48	
	13	gcatacagtttagtcattacgcttacgGCCGGATCCTAACTGGATGTTTCATAGGcTCAGACCCGCAATACATTCGC	37.19	5.09	
	25	gcatacagtttagtcattacgcttacgGCC-GATCCTAACTGCTTTGGATGAGACATTCTGGGACACGTGTCTGTGG	35.76	4.08	
	16	gcatacagtttagtcattacgcttacgGCCGGCCGATCCTAAcTCawGTTGgAaTCCGTTAACACGTCGAGCTAGTGGG	34.19	4.10	
	11	gcatacagtttagtcattacgcttacgGCCGGCCGATCCTAACTGCTGCTGCTGCTCTACgTTCACAAAGGCATCC	31.57	2.75	
	17	gcatacagtttagtcattacgcttacgGCCGGCCGATCCTAACTGCGGCTAACACGTTAGCAAAATGTCATCTGATTGC			
	7	gcatacagtttagtcattacgcttacgGCCGGCTTATCCTA-CTGATATTACTGTCCCTTCCGCCAGTTGTGCCGCG	38.77	2.41	
	6	gcatacagtttagtcattacgcttacgGCCGGCCGATCCTAACTAATAATGGAGCGGAaAACAGCTTCATACGCGTC	31.40		
	35	gcatacagtttagtcattacgcttacgGAGGCAGCATCCTAACTGTAAGTGTTCAGACTTACAAAGCCAGCGTCG			
	21	gcatacagtttagtcattacgcttacgGCCGGCTTATCCTAATTTGAAGkGGTAATGGAAGGaGTTAAGGACGATAG			
	10	gcatacagtttagtcattacgcttacgGCTCGTAGCGGCCATCCTAACGGTACGCCAGGTCGACAGCCAGTGTTC	30.07	2.27	
	2	gcatacagtttagtcattacgcttacgGCCAGCGTCCGCTCCATCCTAACTGCAAGGGCCGCTAGTCACATTGTGAC	31.79	2.33	
	33	gcatacagtttagtcattacgcttacgGTGTGCGTATGCGGGAGATTCCTAACTGGGACaTTCCTCCACTAGAGACTGC	30.78	3.34	
	3	gcatacagtttagtcattacgcttacgGCAGCCTGCCTTCTACGAGCGCCGATCCTAACGATCATAGTCCCGACG	35.03	2.75	
	31	gcatacagtttagtcattacgcttacgGCCCATTACGGTGGCAGGCGGCTCGATCCTAACTGTGAAGGAACGTC			
	1	gcatacagtttagtcattacgcttacgGCCCATTACGACTGGGTAAGAAAGCGGCCGCATACTAGCTGATTTGTTC	30.20	3.50	
	8	gcatacagtttagtcattacgcttacgGCCAGCATGCACGGAGCGGCCGCATc-TTACGGGTGTGTGGCGGTAGCACT			
18	gcatacagtttagtcattacgcttacgGCCGGCCGATTAACCTGTTGGTATyAACAACTGCTGCTCGGAAGCTCAGG	24.97	3.15		
	30	gcatacagtttagtcattacgcttacgGCTAAGCGGCTGTATCCTAACATATGATGAGTTATCCTCACTAGTAGC	27.74	2.51	
	34	gcatacagtttagtcattacgcttacgGCCGGCTGGGTGTAATCCTAACTGCGCCCTAACTGCTGGGGCTGGCGTGCC	27.01	2.38	
Family 2	12	gcatacagtttagtcattacgcttacgTTCGGCGCTAACTTAGGGCATATCAATAAGCCACmATACGGGCGATCCGG	18.29	2.73	
	28	gcatacagtttagtcattacgcttacgCAGTCCGTCGGCGAGTAACTGAAAACCTCAATGGCTCGACGGTCACCCGT	9.60	3.64	
	5	gcatacagtttagtcattacgcttacgGCCAAGCGTTCGGCGGCTATCTGAACGtTgGtTcmCGGATGTCTTCACTG			
	9	gcatacagtttagtcattacgcttacgCCCAGCGAAGAGTTCCGCGAGTAACTGTGTCGATTACAAATTCCGATTGGAC	31.70	3.66	
	4	gcatacagtttagtcattacgcttacgGAGTGCCACGTTGTGAGCGTTTGGCGGCTAGCTGGGGATGTGTGATTTCG			
	20	gcatacagtttagtcattacgcttacgGTGGGCAAAAtcCAGTGsTGTTagAAGTTGcGAGTtACTGCAGATCTGGG			
	15	gcatacagtttagtcattacgcttacgGACGGCAAGTyGTGCAAGCGTTTCCGCGCTTAAcTTAATGAATTAACGCGCC	35.84	6.22	
		26	gcatacagtttagtcattacgcttacgGGTACCGTTAGGCCACCTTGGTTGAGTTAACTGACTAGGCTCAAGACATC	34.82	3.41
		24	gcatacagtttagtcattacgcttacgTTCGCAGATCTAACTGAGAGCATTAAGgAAaTATCACGGGCTCGCTGATC	25.80	4.32
		19	gcatacagtttagtcattacgcttacgGCCCATGGCCGGTAGGTGGTTGCGACTaCGGtGCAGTTCGTCATGGAAC		
	22	gcatacagtttagtcattacgcttacgTAAGGACGCTGTCTcTcGAGTgGTGTTACC GGCGGCTACAAATTCTGTCTGGT			
	23	gcatacagtttagtcattacgcttacgGGTGGCCCTGCACGGTCTCTCAAGTAAGAGGCCGATTCTACTCGGGTAC			
	32	gcatacagtttagtcattacgcttacgCGTGGGTGCGCCAGTTCGGTTCGCGACTACGGAGGCCAGTTACAAATCCC			

t2.2 The 3' constant region is not shown. Yellow indicates motif 1 and light blue indicates motif 2. A minor motif present in only two clones is shown in green

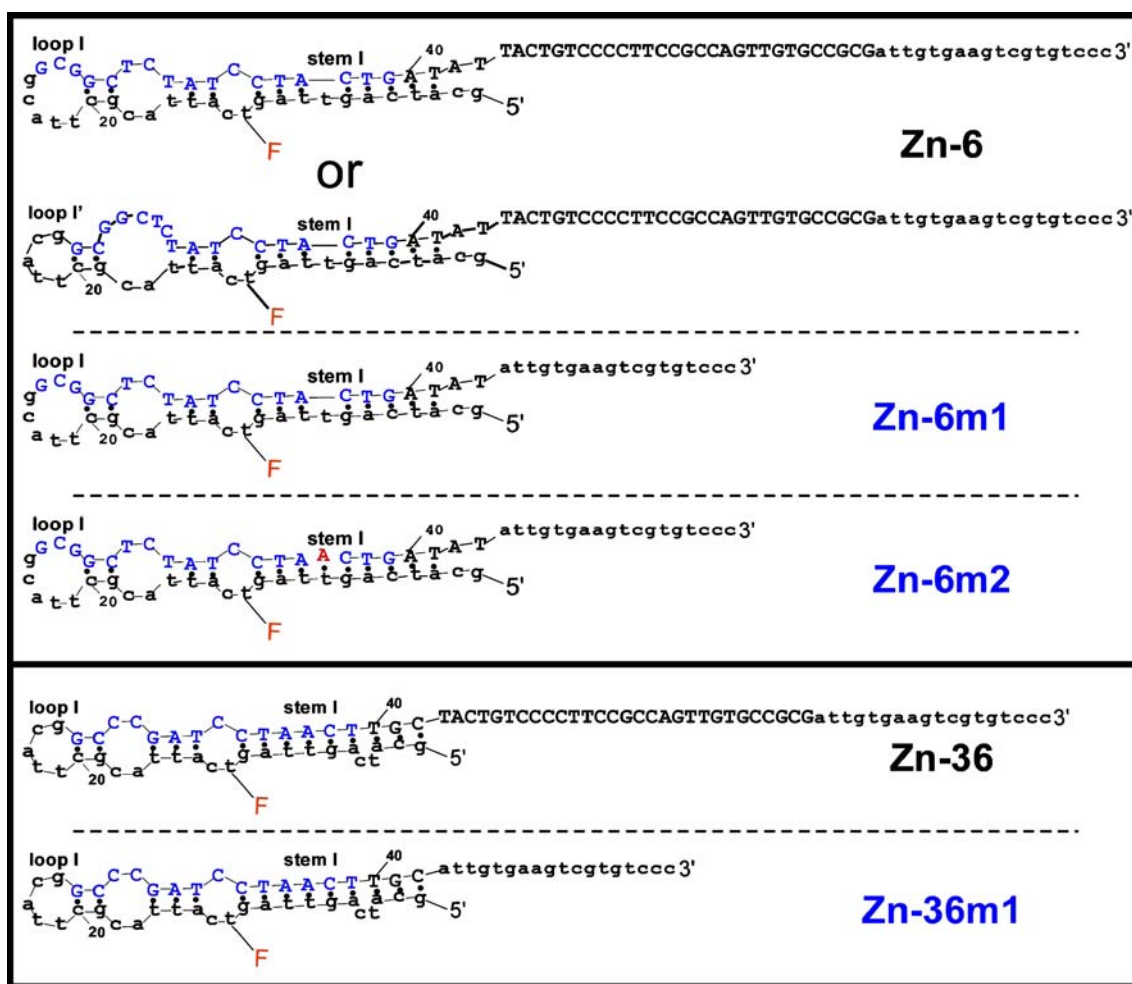
## Fluorescence measurements

Aptamer beacons for fluorescence measurements were generated by NaOH-mediated strand separation of fluorescently labeled DNA from biotinylated, immobilized complementary strands. All fluorescence measurements were made using a PTI Quantmaster QM-4/2003SE spectrofluorimeter (Photon Technology International, London, ON, Canada). The beacons (50 nM final concentrations) were annealed with capture oligonucleotide (100 nM) in ZSB by heating them to 94 °C for 30 s, and cooling them to 45 °C for 90 s and then to room temperature over 10 min. Background fluorescence was first measured by adding 480  $\mu$ l of selection buffer to a fluorimeter cell. The beacon-capture oligonucleotide complexes (10  $\mu$ l) were then added and fluorescence was monitored over time. Once a steady fluorescence signal had been achieved, 10  $\mu$ l of selection buffer containing  $Zn^{2+}$  at various concentrations was added.

The fluorescence response was monitored over 15 min by exciting the samples at 494 nm (the  $\lambda_{ex}$  for fluorescein) and measuring the fluorescence intensity at 518 nm (the  $\lambda_{em}$  for fluorescein). The signal-to-background ratio was calculated as

$$I = (F_{open} - F_{buffer}) / (F_{closed} - F_{buffer}),$$

where  $F_{open}$  is the fluorescence of the aptamer beacon-capture oligonucleotide complex (i.e., Zn-6m2 hybridized to q12.50) in the presence of the target,  $F_{closed}$  is the fluorescence of the aptamer beacon-capture oligonucleotide complex in the absence of the target, and  $F_{buffer}$  is the background fluorescence of the buffer solution alone. The  $K_d$  value of the aptamer beacon was estimated by curve fitting using the program Kaleidagraph (Synergy Software, Reading, PA, USA). For specificity measurements, the fluorescence response was represented as the relative change in fluorescence (i.e., increase in fluorescence versus



**Fig. 3** Sequences and predicted structures of minimized aptamer beacons. Two different predicted conformations are shown for Zn-6. Zn-6m2 contains an additional A residue (red) predicted to pair with the 5' constant region

quenching) in the presence of metal ions. The relative change in fluorescence,  $\Delta\text{RFU}$ , was calculated as

$$\Delta\text{RFU} = (F_x - F_0)/F_0,$$

where  $F_x$  is the fluorescence of the beacon-capture oligonucleotide complex in the presence of 2 mM target metal ion 4 min after addition, and  $F_0$  is the fluorescence of the beacon-capture oligonucleotide complex in the absence of the target.

## Results and discussion

### Selection of aptamer beacons that are eluted with zinc

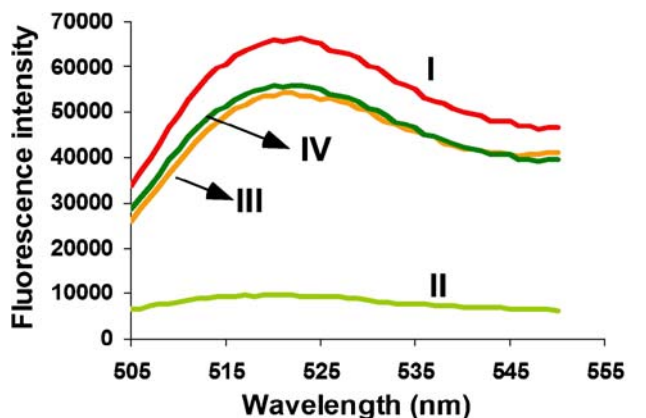
We previously developed an in vitro selection method that directly coupled analyte binding to signaling (Fig. 1a) [16], and now wished to apply this scheme to the selection of metal biosensors. In short, a single-stranded DNA pool that spanned 50 random sequence positions (N50,  $5.5 \times 10^{14}$  different sequences) was amplified with a 5' primer containing a fluorescent thymidine residue at position 11 (T11) and was then annealed via the 5' constant region to an oligonucleotide affinity column containing a 12-residue capture oligonucleotide (q12.50) having a fluorescence quencher (DABCYL) at its 5' end (Fig. 1b). This hybridization event poised the fluorescent reporter on the pool across from the quencher on the capture oligonucleotide. On addition of the target analyte,  $\text{Zn}^{2+}$ , any species that underwent metal-dependent conformational changes should have been concomitantly released from the oligonucleotide affinity column. Eluted species were collected, amplified, and carried into additional rounds of selection. The conformational change and release from column also resulted in the fluorophore and quencher being separated from one another, and therefore in a zinc-dependent increase in fluorescence.

In each round of selection, the immobilized pool was washed several times with buffer to remove any unbound or poorly bound species. The pool was then eluted with selection buffer containing 2 mM  $\text{Zn}^{2+}$ , since the binding affinities of aptamers and small-molecule targets have frequently been found to be in the micromolar to millimolar range [32, 33]. Eluted species were collected, amplified, and carried into the next round of selection. After nine rounds of selection and amplification the pool showed a significant improvement in zinc-dependent elution, and the zinc concentration was decreased to select for higher-affinity species (Table 1). To avoid the accumulation of species that were eluted via a zinc-independent mechanism, a negative selection step was introduced from round 4 onwards in which the pool was first eluted in selection buffer without  $\text{Zn}^{2+}$ , prior to elution in the presence of  $\text{Zn}^{2+}$ . In addition, to

improve the metal specificity of the selected aptamers, an additional negative selection step was introduced at round 9 in which the immobilized pool was eluted with a mixture of three other transition metal ions ( $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Mn}^{2+}$ ) before eluting the pool with  $\text{Zn}^{2+}$ .

After 12 total rounds of selection, zinc-dependent elution had peaked (Fig. 2). The pool was cloned and 34 individual aptamers were sequenced (Table 2). Most (21 of 34) of the selected aptamer beacons were found to have a common motif (motif 1) that spanned from eight to 14 residues (family 1). Interestingly, apart from these 14 residues, the remaining 36 bases in the random region differed amongst the members of this family. A second, smaller family of aptamer beacons (family 2) was also observed that both contained five residues in common with family 1 and also an additional five to eight residue sequence motif (motif 2). A few other sequences had the five-residue motif common to both families 1 and 2, but contained no other similarities.

The majority of the selected aptamer beacons showed  $\text{Zn}^{2+}$ -dependent elution from the oligonucleotide affinity column, and were also specific for  $\text{Zn}^{2+}$  relative to  $\text{Mg}^{2+}$  (Table 2). This is somewhat not surprising, since magnesium was present in the wash buffer, and any magnesium-



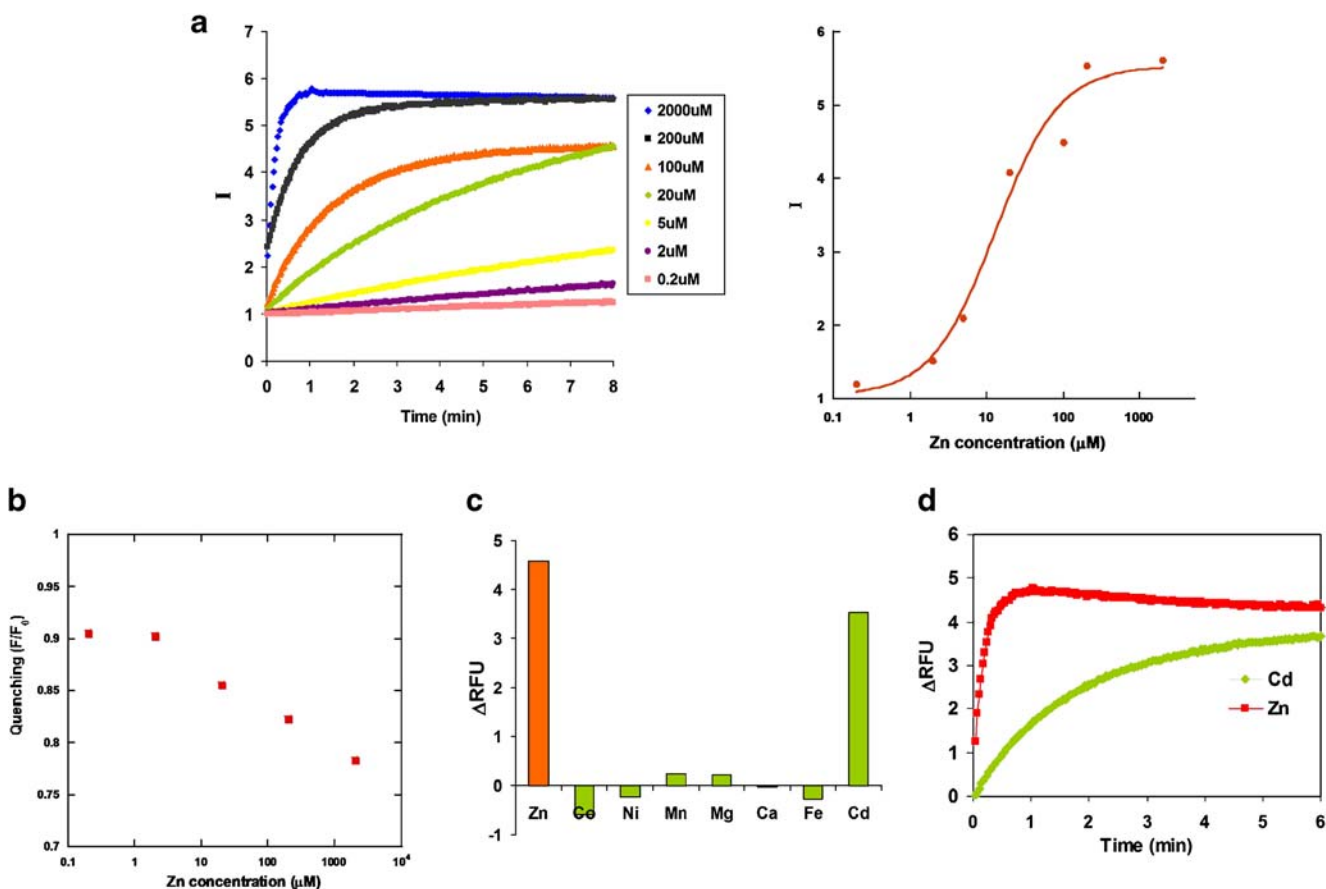
**Fig. 4** Fluorescence activation of Zn-6m2 in the presence of  $\text{Zn}^{2+}$ . Change in fluorescence of the Zn-6m2 aptamer alone (*I* → *III*) and the aptamer-capture oligonucleotide complex (*II* → *IV*) in the presence of  $\text{Zn}^{2+}$ . Here *I* represents Zn-6m2 aptamer alone, *II* the aptamer-quencher oligonucleotide complex, *III* the aptamer alone in the presence of  $\text{Zn}^{2+}$ , and *IV* the aptamer-quencher oligonucleotide complex in the presence of  $\text{Zn}^{2+}$ . The capture oligonucleotide q12.50 was present in a 2:1 (100 nM:50 nM) molar excess to the aptamer. The target  $\text{Zn}^{2+}$  was added at 2 mM concentration and the signal was monitored until it was stable

dependent species would presumably have been washed away prior to the application of zinc.

The two beacons, Zn-6, and Zn-36, that showed the highest elution with  $Zn^{2+}$  and that were most specific for  $Zn^{2+}$  were chosen for further analysis. Because of the similarities between the 5' constant region and motif 1, we hypothesized that the 5' end of the aptamer folded back on motif 1 in the presence of zinc. To test this hypothesis, minimal beacon variants based on the aptamer beacons Zn-6, and Zn-36 were designed in which the sequence between the common sequence motif and the 3' constant region was removed. The elution characteristics of these minimized beacons (Zn-6 m1 and Zn-36 m1; Fig. 3) were almost unchanged from those of the parent beacons. An additional variant of the minimized aptamer beacon was also constructed (Zn-6m2) that contained an insertion and was hypothesized to form a better pairing with the 5' end (Fig. 3).

Selected beacons show  $Zn^{2+}$ -dependent increases in fluorescence

To determine if Zn-6m2 could indeed function as a beacon, we measured changes in fluorescence upon addition of  $Zn^{2+}$ . While immobilization with the capture oligonucleotide was necessary to partition aptamers during selection, a free oligonucleotide containing a quencher was used in fluorescence assays. As expected, hybridization of the beacon with the quencher oligonucleotide led to quenching of the fluorescence signal in the beacon-quencher oligonucleotide complex (curve II in Fig. 4) to 15% of its original value (curve I in Fig. 4) owing to the close proximity between fluorescein and DABCYL in the hybridized state. Upon addition of 2 mM  $Zn^{2+}$ , the fluorescence of the beacon Zn-6m2-quencher oligonucleotide complex (curve II in Fig. 4) was restored to 85% of the maximum possible fluorescence response (i.e., to 85% of the fluorescence of the beacon



**Fig. 5** Fluorescence responsivity of Zn-6m2. **a** Target-concentration-dependent increase in fluorescence of the beacon Zn-6m2-capture oligonucleotide complex. Complexes with the capture oligonucleotide were formed as in Fig. 4, the target  $Zn^{2+}$  was added at various concentrations, and the time-dependent development of the signal was monitored.  $I$  is the signal-to-background ratio, as defined in “Fluorescence measurements.” The data are also shown as a function of concentration, rather than time, for the  $t=6$  min time point. **b** Target-concentration-dependent decrease in fluorescence of the beacon

Zn-6m2 alone.  $F_0$  is the fluorescence of the beacon in the absence of the target, and  $F$  is the fluorescence in the presence of the target, 2 min after addition of the target. **c** Specificity of fluorescence response. The change in fluorescence of the beacon capture oligonucleotide complex in the presence of 2 mM target metal ion 5 min after addition.  $\Delta RFU$  is the relative change in fluorescence, as defined in “Fluorescence measurements.” **d** Discrimination in the kinetics of fluorescence response. The changes in fluorescence were measured as a function of time in the presence of either 2 mM zinc or 2 mM cadmium

alone; curve IV in Fig. 4). It should be noted that there was a slight quenching due to the addition of zinc alone (curve III in Fig. 4; the signal was quenched by approximately 20%).

Beacon Zn-6m2 in complex with quencher oligonucleotide exhibited a target concentration-dependent increase in fluorescence (Fig. 5a). In the presence of 2 mM  $Zn^{2+}$ , beacon Zn-6m2 showed a 5.5-fold increase in fluorescence. Also, unlike molecular beacons, the fluorescence response of the  $Zn^{2+}$ -aptamer beacon was much faster and reached a stable level in less than 5min. The  $K_d$  of the aptamer was estimated to be approximately 15  $\mu$ M. In addition, the fluorescent aptamer alone showed a target-concentration-dependent decrease in fluorescence on addition of  $Zn^{2+}$  (Fig. 5b). The fluorescence of the aptamer was quenched to 78% of its initial value in the presence of 2 mM  $Zn^{2+}$ . Overall, on the basis of the fluorescence increase of the aptamer-quencher oligonucleotide complex in the presence of zinc, the aptamer exhibits a limit of detection of 5  $\mu$ M (signal-to-background ratio of 2) and a dynamic range of approximately 50 fold.

#### Specific fluorescence response of the selected aptamer beacons

To determine the specificity of beacon Zn-6m2, its fluorescence response was monitored in the presence of seven different metals (Fig. 5c). The fluorescence response in the presence of  $Zn^{2+}$  was 4.6-fold higher than the response in the presence of  $Mg^{2+}$  and  $Ca^{2+}$ , the metal ion interferents typically present in abundance in biological media. The beacon was also selective for  $Zn^{2+}$  when compared with other transition metal ions such as  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , and  $Fe^{2+}$ . In fact, rather than lighting up, the beacon showed fluorescence quenching in the presence of some of these metal ions. This has been observed before with other macrocyclic  $Zn^{2+}$  sensors, such as Zinpyrs and ZnAFs: fluorescence increases in the presence of zinc, but quenching occurs with other first-row transition metal ions [6, 13]. However,  $Cd^{2+}$ , which has the same  $d^{10}$  configuration as  $Zn^{2+}$  and occurs right below it in the periodic table, elicited a strong fluorescence response from the aptamer. Only modest selectivity (approximately 1.3-fold at steady state) was observed for zinc relative to cadmium. However, the kinetics of the response for zinc were much more robust than for cadmium (Fig. 5d). This may indicate that the kinetic pathways for association of metals with aptamers and biosensors are complex, and that kinetic measurements can potentially also be used for metal discrimination.

Similar cross-reactivities have been observed before with other zinc sensors [14, 15]. Since cadmium was not originally included in the negative selection regime that led to the generation of Zn-6m2, it is possible that further

negative selections could lead to the identification of metal ion biosensors that are completely specific for zinc. Interestingly, a deoxyribozyme that was originally selected to be activated by  $Zn^{2+}$  [22] ultimately showed much better reactivity with  $Pb^{2+}$  ions, and some activity with  $Mn^{2+}$  and  $Co^{2+}$  [34], in all likelihood because no negative selection regime was included. A selection for a cobalt-dependent deoxyribozyme that did include negative selections for metal interferents yielded catalysts with much better specificities [35].

## Conclusions

We have developed a selection method that leads directly to the generation of fluorescent aptamer beacons that can be used for the detection of metals. In comparison with organic metal ion sensors, such as the macrocyclic Zinpyr-1, Zinpyr-2, and ZP4 sensors, the selected  $Zn^{2+}$ -specific aptamer beacon is much less sensitive but shows similar fluorescence enhancement upon binding  $Zn^{2+}$  [6, 15]. In general, these organic macrocyclic receptors are able to bind zinc with affinities comparable to that of natural zinc receptors such as carbonic anhydrase, by coordinating the zinc ion via multiple nitrogen and oxygen ligands, a feat that may be difficult to achieve with small, structured nucleic acid binding pockets. However, the metal specificities of organic and protein biosensors are generally fixed, while the metal specificities of nucleic acid biosensors can be finely crafted through negative and positive selections. The sensitivities and fluorescence signals achieved with the selected aptamer beacon were similar to those previously seen with a deoxyribozyme biosensor for lead [36–38]. However, the deoxyribozyme biosensors cleave a substrate containing a fluorophore and a quencher in an irreversible reaction, while the aptamer beacon biosensors undergo a reversible conformational change that separates fluorophore and quencher. Therefore, the aptamer beacons can potentially be engineered for reversible and continuous environmental monitoring.

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