

A Highly Selective DNzyme Sensor for Mercuric Ions**

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DNAzymes are DNA molecules that display catalytic activity, which have no precedent in nature and are obtained by *in vitro* selection.^[1] Since the discovery of the first DNAzyme,^[2] intensive research has focused on the identification and elaboration of both catalytic RNAs and DNAs with novel properties. Indeed, deoxyribozymes have been found to catalyze reactions including ligation,^[3,4] DNA phosphorylation,^[5,6] RNA cleavage,^[7] thymine dimer photoreversion,^[8] and the formation of nucleopeptide linkages.^[9] Most of the nucleic acid enzymes require metal cations to function, and many ribozymes/deoxyribozymes have been tailored to extend the variety of metal ions used as well as their specificities, either to gather structural information or to design new metal sensors.^[10] Of the common d-block metals, lead played a primordial part in the advent of DNAzymes because the catalytic activity of the first RNA-hydrolyzing DNAzyme was lead-dependent.^[2] In addition, the first DNAzyme metal sensors were lead-dependent.^[11] DNAzymes sensitive to other metal cations, such as Cu^{2+} ,^[3,6,12] Co^{2+} ,^[13] Zn^{2+} ,^[14] and UO_2^{2+} ,^[15] have been reported.

Mercury is a highly toxic and widespread pollutant that has natural sources, such as oceanic and volcanic emissions, but originates mainly from anthropogenic sources, such as gold mining and combustion of waste and fossil fuels.^[16–18] Mercuric ions (Hg^{2+}), the most stable form of inorganic mercury,^[19] damage the brain, nervous system, kidneys, and endocrine system resulting in often severe effects.^[17,20] Furthermore, microorganisms in streams and oceans are able to biomethylate Hg^{2+} to methylmercury (CH_3Hg^+), which in turn readily bioaccumulates and provides a prime gateway to move high up the food chain and consequently be of concern for public health.^[16,20]

Therefore, there is a high demand for the development of sensitive and selective mercury detectors for both environmental and biological samples.^[21,22] A plethora of methodologies, including sensors based on fluorophores,^[23] chromophores,^[24] foldamers,^[25] genetically engineered bacteria,^[26] conjugated polymers,^[27] gold nanoparticles,^[28] electroanalytical methods such as capillary electrophoresis^[29] and voltam-

metry,^[30] peptides,^[31] proteins,^[32] and enzymes,^[33] have been reported in the past few years. However, these methodologies can be hampered by interference from chemically closely related metals, poor water solubility, delayed response to Hg^{2+} , and/or insufficient sensitivity.

Another emerging methodology for the sensing of mercury involves the use of oligonucleotides. Indeed, DNA has long been known to form strong and stable thymidine- Hg^{2+} -thymidine base pairs,^[34] a fact that had not been unequivocally proven until very recently.^[35] This chelating property of thymidines has successfully been applied in the design of two oligonucleotide-based mercuric ion sensors.^[22,36] Furthermore, the thymidine- Hg^{2+} -thymidine coordination chemistry prompted Liu, Lu, and co-workers to engineer a UO_2^{2+} -dependent DNAzyme^[15] into a very sensitive and selective allosteric DNAzyme that can detect mercuric cations.^[37] They rationalized that because the exact composition of the stem loop had no dramatic effect on the catalytic efficiency of the DNAzyme, the appendage of up to six thymine-thymine mismatches would provide an ideal binding place for Hg^{2+} . Indeed, the generated DNAzyme $\text{E}_{\text{Hg}}5\text{T}$ had a rate constant of 0.61 min^{-1} in the presence of $10 \mu\text{M}$ Hg^{2+} , displayed a high selectivity for mercuric cations, and showed a detection limit as low as 2.4 nM .^[37] Nevertheless, this system required the use of the uranyl ion (UO_2^{2+}) for activity.^[15,38] Finally, the *in vitro* selection of RNA-cleaving DNAzymes requiring mercuric ions for catalysis has recently been reported.^[39] However, these DNAzymes suffer from interference from other metal cations (in particular Cu^{2+} and Zn^{2+}) and a relatively high detection limit.^[39]

Our interest in Hg^{2+} -sensing issues stems from the discovery of $9_{25}\text{-}11\text{c}$, the first RNaseA-like self-cleaving DNAzyme that cleaves an embedded ribophosphodiester bond in the absence of divalent metals by utilizing two modified nucleobases for catalysis, namely dA^{im} (8-histaminyl-dA) and dU^{aa} (5-aminoallyl-dU).^[40] Both functionalized nucleosides were incorporated enzymatically from triphosphate (TP) precursors^[41] (Figure 1a) in an *in vitro* selection that led to the discovery of $9_{25}\text{-}11$. The self-cleaving $9_{25}\text{-}11\text{c}$ was subsequently converted to $9_{25}\text{-}11\text{t}$, the *trans*-cleaving species,^[42] which exhibited multiple turnover.^[43] The introduction of two amino acid side chains reminiscent of the active site of RNaseA confers both electrostatic complementarity and acid/base catalysis to this DNAzyme. Notably, these side chains are soft ligands that would bind soft metals. Indeed, as $\log\beta_2(K_a)$ for imidazole- Hg^{2+} is at least 10^{15} , it was not surprising that we observed strong and specific inhibition by mercuric ions with an apparent dissociation constant (K_d^{app}) of 110 nM .^[44] Thus, $9_{25}\text{-}11\text{c}$ is able to act as a mercuric cation sensor, albeit with some interference from Cu^{2+} , which displayed a K_d^{app} of $2.5 \mu\text{M}$.

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Supporting information (including an extensive experimental section) for this article is available on the WWW under <http://www.angewandte.org> or from the author.

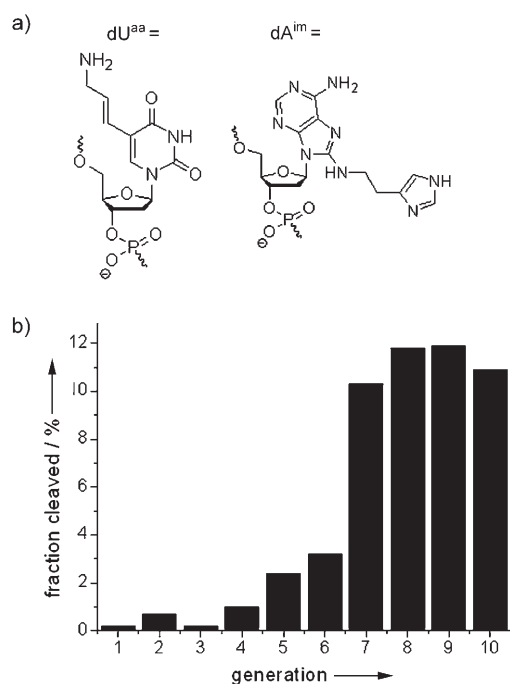


Figure 1. a) Structures of dU^{aa} and dA^{im} . b) Progress of the fraction cleaved after 60 min as a function of the generation (round of selection).

Although inhibition of self-cleavage can be used for sensing, it would be advantageous for a sensor to be induced to react rather than be inhibited, which nevertheless is not always necessary as demonstrated by Dickerson et al.^[45] Towards this goal, we hypothesized that the same modified deoxyribonucleotide triphosphates (dNTPs) could be exploited in a combinatorial selection to afford a self-cleaving species that would be active in the presence of Hg^{2+} rather than inactive. Herein, we present the *in vitro* discovery of a self-cleaving DNAzyme that displays a high sensitivity and selectivity for Hg^{2+} .

To select for a DNAzyme that is activated by mercuric ions, an initial population was created by polymerizing the two modified nucleoside triphosphates $dA^{imr}TP$ and $dU^{aa}TP$ onto a template containing 40 degenerate positions (about 10^{24} possible sequences) in a similar way to the method used previously for the selection of 9₂₅₋₁₁ (see the Supporting Information).^[40] This design was surmised to give ample sequence space to accommodate enough nucleosides bearing imidazole residues to ensure both catalysis and chelation to the mercuric cation. A “negative” selection step was performed to avoid the propagation of sequences that would cleave in the absence of Hg^{2+} .

For the first four rounds of selection, this counter-selection consisted of an incubation with buffer containing solely $MgCl_2$ for 30 min at room temperature. The incubation time was then increased to 4 h for rounds 5 to 7. The DNA population of round 7 showed high and equal background cleavage both in the absence of any divalent metals and in the presence of either Mg^{2+} , Zn^{2+} , Cu^{2+} , or Hg^{2+} (see the Supporting Information). Therefore, the negative selection was upgraded to include incubation for 2 h without divalent

metals followed by a 2-h exposure to Mg^{2+} only and then to a buffer containing a mixture of equal amounts of Zn^{2+} and Cu^{2+} (5 μM). Cleaved (active) species were obtained after the addition of 5 μM Hg^{2+} , 200 mM NaCl, 5 mM $MgCl_2$, and 25 mM cacodylate (pH 7.5) at room temperature. Over the course of the selection experiment, the reaction time was gradually diminished from 60 to 1 min. A clear and marked increase in intramolecular cleavage was observed after round 4, which seemed to plateau after generation 8 (Figure 1 b).

Individual molecules were cloned from the population following the tenth round and the clones were screened for activity. Amongst the 50 clones screened, 15 gave a reasonable activity towards mercuric cations and were thus sequenced (see the Supporting Information). The synthetic oligonucleotides corresponding to these sequences were then used to assess the sensitivity and selectivity of the various clones and the most promising candidate was then further characterized. The sequence and hypothetical 2D structure of the selected DNAzyme 10-13 is shown in Figure 2 a. Note that the degenerate region partitions into two domains, one of which is rather AT-rich and thus concentrated in amino/imidazole modifications (loop II), whereas the other is GC-rich and thus modification-poor (loop III).

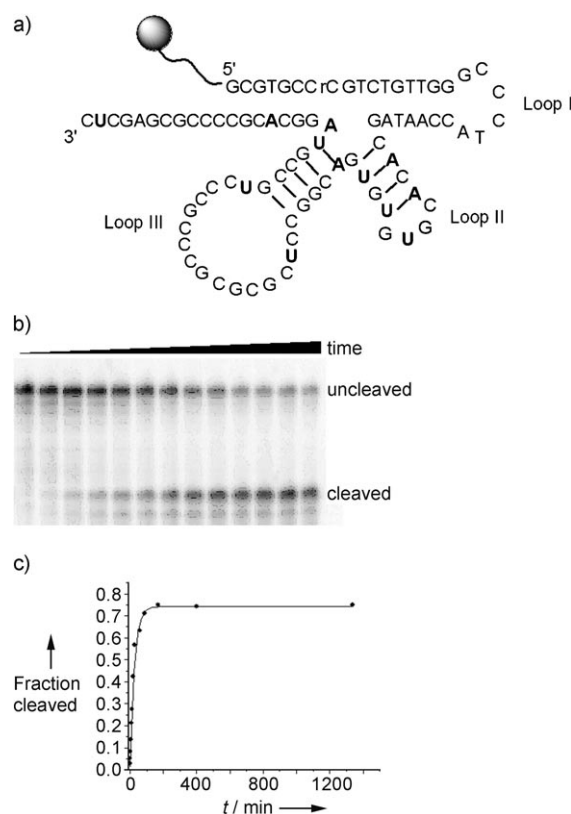


Figure 2. a) Sequence and hypothetical 2D structure of the selected DNAzyme 10-13 (bold-face **A** and **U** indicate the positions of the modified nucleosides). b) Gel image (7% PAGE) showing the fraction cleaved over a period of 1300 min in the presence of 5 μM Hg^{2+} . c) Graphical analysis of this particular kinetic run: $k_{obs} = (0.039 \pm 0.003) \text{ min}^{-1}$; error is standard deviation from the exponential fit.

The selected self-cleaving DNzyme 10-13 has a rate constant k_{obs} of $(0.037 \pm 0.002) \text{ min}^{-1}$ (three independent measurements carried out on different days with differently modified DNA) in the presence of $5 \mu\text{M Hg}^{2+}$ (Figure 2b and c). In the absence of any divalent metal or in the sole presence of MgCl_2 , no appreciable ($< 10\%$) cleavage could be detected over a period of 42 h. Not unexpectedly, a maximum cleavage of approximately 80% is noticed and no further conversion seems to occur after 120 min.^[40,46] Interestingly, the number of bases in the degenerate region diminished from 40 to 39, as noted in the selection of 9₂₅-11.

To assess the applicability of 10-13 as a mercury sensor, we determined the effect of various concentrations of Hg^{2+} ions on the rate constant (Figure 3). Initially, and as expected,

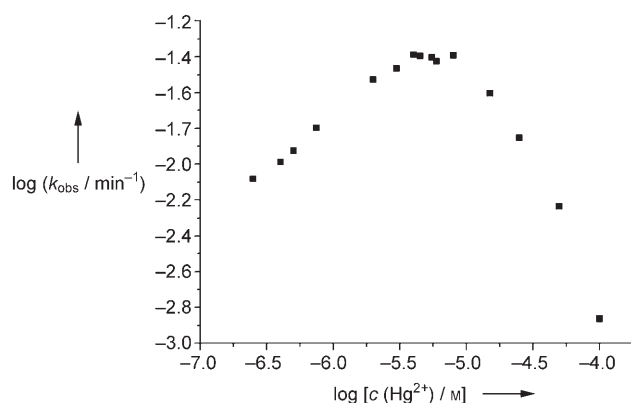


Figure 3. Dependence of the logarithm of the rate constant k_{obs} for cleavage of deoxyribozyme 10-13 on the logarithm of the Hg^{2+} concentration c .

increasing concentrations of Hg^{2+} lead to higher rate constants until a maximum is reached at $\approx 5 \mu\text{M}$, which was the concentration used during the selection experiment. The rate constant then diminishes in a similar fashion upon increasing the Hg^{2+} concentration, which results in a bell-shaped profile. The mercuric cations seem to have a dual role, since they are vital for catalysis but at the same time apparently poison the catalyst at higher concentrations, as observed in the case of 9₂₅-11. Nevertheless, the DNzyme 10-13 expresses a dynamic range over more than two orders of magnitude of Hg^{2+} concentration; cleavage rates can be observed at concentrations as low as 100 nM and as high as $100 \mu\text{M}$. Based on this plot, the value of K_d can be estimated at about $1 \mu\text{M Hg}^{2+}$. Beyond $10 \mu\text{M}$, Hg^{2+} appears to inhibit the system rather than saturating it. Both the rise and diminution of the rate, as a result of increasing mercuric cation concentration, seem to be linear with a slope of ≈ 1 and -1 , respectively, which suggests the effect of only one Hg^{2+} cation despite the four different histaminyl residues that possibly could interact.

To test the selectivity of DNzyme 10-13, 16 competing metal cations were analyzed at three different concentrations (1 , 20 , and $100 \mu\text{M}$) and the reactions were quenched after 60 min (Figure 4). None of the metal ions tested appeared to give any significant cleavage ($< 5\%$), in stark contrast to the effect of Hg^{2+} (about 65% cleavage in 60 min). To demon-

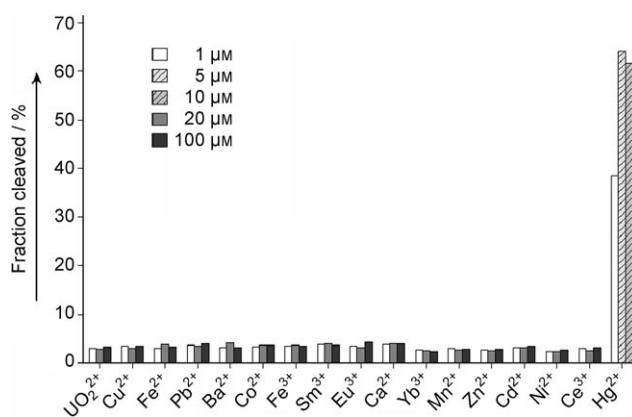


Figure 4. Survey of the selectivity of the DNzyme. All metals were tested at three different concentrations (1 , 20 , and $100 \mu\text{M}$). For comparison, the response of the DNzyme to 1 , 5 , and $10 \mu\text{M Hg}^{2+}$ is also shown. For all reactions: $25 \text{ mM cacodylate (pH 7.5)}$, 5 mM Mg^{2+} , 200 mM NaCl , and transition-metal ion (M^{2+}) at the indicated concentrations.

strate the reversibility of this interaction, we initiated cleavage with $5 \mu\text{M Hg}^{2+}$ and then added ethylenediaminetetraacetate (EDTA; 0.5 M) after 12 min to chelate all the mercuric ions (see the Supporting Information). After the addition of EDTA, the fraction cleaved remains at about 20% and no additional cleavage is observed.

The effect of added Mg^{2+} may be more obscure, as increasing the concentration to 25 mM or completely removing Mg^{2+} from the cleavage buffer had little effect on the observed constant ($k_{\text{obs}}(\text{rel}) \geq 0.4$). Although it would seem that the presence of Mg^{2+} would have little effect, one cannot exclude opposite yet equal interactions on both conformation and catalysis to give the appearance of indolence. In contrast to Mg^{2+} , cleavage was apparent (albeit markedly reduced) when using Mn^{2+} or the exchange-inert Co^{III} -hexamine instead of magnesium, which suggests that Mg^{2+} is probably involved with folding rather than activity.^[43]

Interestingly, replacing Mg^{2+} by Zn^{2+} , which is notably more aminophilic, led to a total suppression of catalysis (see the Supporting Information). This finding suggests either that Zn^{2+} competes for Hg^{2+} , thus resulting in a catalytically incompetent conformation, or that Hg^{2+} induces a conformational change that leaves at least one imidazole or amine residue free for catalysis, which is consequently chelated by Zn^{2+} .

As with most modified DNzymes, the observed activity necessarily results from the presence of both modified nucleosides. Indeed, when isosequential strands were polymerized under conditions in which one or both modified nucleoside triphosphates were replaced with their natural counterparts, the resulting DNAs had no catalytic activity (Figure 5). Finally, to show that catalysis depended on the specific histaminyl linkage, we replaced dA^{imTP} with dA^{homTP} (see the Supporting Information), which is a close chemical analogue of dA^{imTP} in which the ethylamino linker connecting the histaminyl moiety to the adenine is slightly elongated by one methylene unit borne on the propylamino side chain.^[47] Such a minor change in the chemical environ-

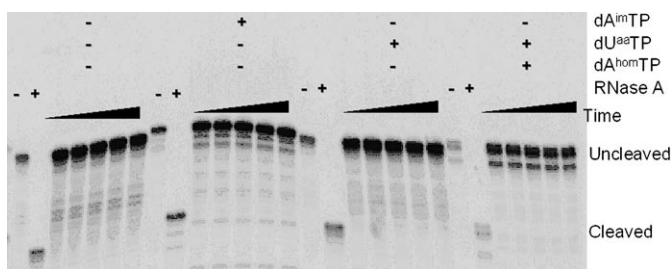


Figure 5. Gel image (7% PAGE) demonstrating the necessity of the presence of both modifications for catalysis.

ment of the dA^{im}TP led to a dramatic suppression in catalysis, which suggests not only that modified dNTPs expand new chemical space to afford new activities, but also that the resulting species represent finely tuned catalytic systems that are intolerant of even the most conservative of modifications.

In summary, we have reported the discovery of a self-cleaving DNAzyme that is highly sensitive and selective for mercuric cations. This novel deoxyribozyme is not stimulated by the presence of any other metal cation, including Cu²⁺, and is active in a wide concentration range. Given the affinity of imidazole for Hg²⁺, thermodynamics would suggest that DNAzyme 10-13 utilizes the imidazole residues^[44,48] to chelate Hg²⁺ cations, and not the usual T-Hg²⁺-T dimeric interaction seen in other systems. Nevertheless, one cannot exclude the possibility that chelation of Hg²⁺ to thymidine residues disrupts the Watson-Crick base pairing in loop II to give a reorganization of this modification-rich region that would position the amines and imidazoles in a new and catalytically competent Mg²⁺-independent conformation. This hypothesis would then account for the further poisoning by higher mercury concentrations and by Zn²⁺.

Although 10-13 displays a significantly lower k_{obs} for self-cleavage compared to the k_{cat} for E_{Hg}5T, these findings highlight the utility of modified nucleotides for expanding the realm of catalytic functions and suggest that modified dNTPs should complement antecedent approaches to metal-sensing DNAzymes. Work is in progress towards the conversion of 10-13 to a *trans*-cleaving catalyst and understanding of the structural basis for Hg²⁺ chelation and the underlying chemical mechanisms. The relatively small size of this catalyst and its ability to bind Hg²⁺ should facilitate both X-ray crystallography and ¹⁹⁹Hg NMR studies aimed at structural elucidation.^[49]

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