A DNAzyme Catalytic Beacon Sensor for Paramagnetic Cu$^{2+}$ Ions in Aqueous Solution with High Sensitivity and Selectivity

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1. Experimental Section:

Sensor preparation: in a typical experiment, 1 µM of Cu_Sub and 2 µM of Cu_Enzyme (see Figure 1A in the paper for DNA sequences and modifications) were prepared in a buffer of 200 µL volume containing 1.5 M NaCl, 50 mM HEPES, pH 7.0. The reason for using excess amount of the enzyme strand is given in Figure S3. The mixture was warmed to 80 ºC for 1 min and allowed to cool naturally to room temperature in 1 hour to anneal the DNAzyme. For each test, the annealed DNAzyme sensor (5 µL) was diluted into 490 µL of buffer (1.5 M NaCl, 50 mM HEPES, pH 7.0) in a quartz cuvette with 0.5 cm path length on each side, and 5 µL of 5 mM ascorbate was also added. The final Cu_Sub concentration was 10 nM, the final Cu_Enzyme concentration was 20 nM, and the final ascorbate concentration was 50 µM.

Detection: the above prepared cuvette was vortexed to mix all the reagents and placed into a fluorometer (SPEX Fluoromax-P, Horiba Jobin Yvon). The cuvette was allowed to incubate in the cuvette holder of the fluorometer for 1 min before taking any measurement. The temperature of the fluorometer was set at 23 ºC (around room temperature). The fluorometer was set at the kinetics
mode by exciting at 490 nm and monitoring emission at 520 nm with 15 sec intervals. After the first 4 readings, the cuvette was quickly taken out and a small volume (0.5 to 3 µL) of concentrated metal stock solutions was added. The cuvette was rapidly vortexed to mix the metal with the sensor, and was then placed back into the fluorometer to continue the kinetics monitoring. The obtained kinetics traces were normalized to the fluorescence intensity before metal addition. The slope of fluorescence increase over background from 1 min to 3 min after Cu²⁺ addition was calculated and plotted against metal concentration (Figure 2B).

**96-well plate based selectivity assays**: 5.4 µL of 100 µM Cu_Sub and 10.8 µL of 100 µM Cu_Enzyme were annealed in 400 µL of buffer (1.5 M NaCl, 50 mM HEPES, pH 7.0). After annealing, the solution was diluted with 5 mL of the same buffer. Ascorbate was added to a final concentration of 50 µM. Therefore, the final Cu_Sub and Cu_Enzyme concentrations were 100 nM and 200 nM, respectively. The sensor solution was spotted into three rows (each row contained 8 wells) of a 96-well-plate with 200 µL in each well. Metal solutions at 2× of designated concentrations were prepared in the same buffer with 50 µL volume and were spotted in the plate. The sensor solution in the wells was taken out with an 8-channel pipet (each channel transferred 50 µL of sensor solution) and added to the metal solutions. At 12 min after mixing, the plate was imaged with a Fuji fluorescence scanner (FLA-3000G, Fuji) by exciting at 473 nm and the filter was set at 520 nm. The metal salts used include: MgCl₂, CaCl₂, SrCl₂, BaCl₂, Mn(OAc)₂, Fe(NH₄)₂(SO₄)₂, FeCl₃, CoCl₂, NiCl₂, Cu(NO₃)₂, ZnCl₂, Cd(ClO₄)₂, Hg(ClO₄)₂, Pb(NO₃)₂, TbCl₃, EuCl₃, and UO₂(OAc)₂. Under the reaction condition (pH 7.0, 1.5 M NaCl), Fe(NH₄)₂(SO₄)₂ was quickly (within 5 min) oxidized into Fe³⁺ species and the color of the well turned from colorless to yellow. The well with Fe(NH₄)₂(SO₄)₂, FeCl₃, Pb(NO₃)₂, and UO₂(OAc)₂ formed visible precipitation.

**Gel-based assays**: 1 µM of Cu_Sub and 2 µM of Cu_Enzyme were annealed in 750 mM NaCl, 25 mM HEPES, pH 7.0. After annealing, 50 µM of ascorbate was also added. Before addition of Cu²⁺, a 10 µL aliquot was taken out as the zero time point (lane 2 in the inset of Figure S2). After adding Cu²⁺, 10 µL aliquots were taken out at designated time points and were quenched in a stop buffer containing 8 M urea, 50 mM EDTA, 0.05% xylene cyanol, and 50 mM Tris acetate, pH 8.2. A sample with only 1 µM Cu_Sub (no Cu_Enzyme), 20 µM Cu²⁺, and 50 µM ascorbate was also
prepared (lane 1 in the inset of Figure 1C, incubated at room temperature for 1 hour before adding the stop buffer). The samples were loaded into 20% polyacrylamide gel electrophoresis to separate cleaved and uncleaved substrate. The gel was imaged with a fluorescence scanner (FLA-3000G, Fuji) by exciting at 473 nm and setting the emission filter at 520 nm.

**Test of Cu$^{2+}$, Pb$^{2+}$, and UO$_2^{2+}$ DNAzymes:** the design of the Pb$^{2+}$ and UO$_2^{2+}$ sensors have been described elsewhere and are also presented here in Figure S1.\(^1\)\(^-\)\(^3\) The Cu$^{2+}$ sensor was prepared with a final Cu_Sub concentration of 12 nM and Cu_Enzyme of 24 nM in 1.5 M NaCl, 50 mM HEPES, pH 7.0, 50 µM ascorbate. The Pb$^{2+}$ sensor was prepared with a final substrate concentration of 6 nM and enzyme concentration of 6 nM in 100 mM NaCl, 25 mM Tris acetate, pH 8.2. The UO$_2^{2+}$ sensor was prepared with a final substrate concentration of 200 nM and enzyme concentration of 400 nM in 300 mM NaCl, 50 mM MES, pH 5.5. The three sensors were spotted into three rows of a 96-well plate, respectively, with each row containing 8 wells. Eight metal mixtures were prepared with all the eight possible combinations among Cu$^{2+}$, Pb$^{2+}$ and UO$_2^{2+}$. The metal concentrations in each mixture were either 0 or 20 µM. 5 µL of each metal mixture was spotted into three wells along the same column but in different rows. 95 µL of sensor was transferred into the wells containing the metal mixture and the reaction was allowed for 10 min at room temperature. The final metal concentration for each metal after mixing with the sensor was either 0 or 1 µM. The plate was imaged with the Fuji fluorescence scanner.

![Figure S1](image-url). Sequences and modifications of the DNAzyme-based UO$_2^{2+}$ and Pb$^{2+}$ sensors. In both sensors, the 5'-F denotes a FAM fluorophore. The 3'-Q on the substrate strands denotes a Black Hole Quencher. The 3'-Q on the enzyme strands denotes for a Black Hole Quencher and a Dabcyl for the UO$_2^{2+}$ sensor and the Pb$^{2+}$ sensor, respectively.
Results of gel-based assays

Gel-based assays were carried out to confirm the cleavage reaction (Figure S2). Lane 1 contained the substrate alone and Cu$^{2+}$. Lane 2 had the DNAzyme complex but no Cu$^{2+}$. Cleavage was observed only after addition of Cu$^{2+}$ to the DNAzyme complex (lanes 3-5).

![Gel-based assay](image)

**Figure S2.** Gel-based assay of the sensor DNAzyme. Lanes 2 to 5 are 0, 5, 10, and 25 min after addition of Cu$^{2+}$, respectively.

**Figure S3.** The effect of enzyme to substrate ratio on signal enhancement. The Cu$^{2+}$ concentration was 4 μM and the experiments were performed in parallel to have a fair comparison. Other conditions are the same as previously described.

2. The enzyme to substrate ratio in the Cu$^{2+}$ sensor.

The structure of the Cu$^{2+}$-specific DNAzyme is different from the UO$_2^{2+}$ and Pb$^{2+}$ DNAzymes shown above. One of the substrate binding arms of the Cu$^{2+}$ DNAzyme contained a DNA triplex. Therefore, the stability of the DNAzyme complex is not as high as that with normal Watson-Crick
base pairs. To form stable DNAzyme complex at room temperature, high ionic strength conditions (1.5 M NaCl) were used. With the 1:1 enzyme to substrate ratio, the fluorescence enhancement was only ~7-fold in 4 min (Figure S3, red curve). With a 2:1 enzyme to substrate ratio, the enhancement was ~10-fold in 4 min, which was attributed to more complete substrate hybridization. Therefore, we chose a ratio of 2:1 for the Cu$^{2+}$ sensing experiment. Considering the consumption of materials, further increase of the enzyme was not tested.

3. Gel-based assay of the Cu$^{2+}$ sensor in the presence of Fe$^{2+}$ and UO$_2^{2+}$

Since fluorescence enhancement was observed in the presence of 1 mM of Fe$^{2+}$ or UO$_2^{2+}$, gel-based assays were carried out to test whether the fluorescence increase was due to cleavage or due to DNA denature in the presence of high level of metal ions (Figure S4). All the lanes contained the sensor DNAzyme complex shown in Figure 1A with 1 µM Cu_Sub and 2 µM Cu_Enzyme. Lane 1 did not have any added metal ion. Lane 2 contained 1 µM Cu$^{2+}$, lane 3 contained 10 µM Cu$^{2+}$. Lane 4-6 contained 10, 100, and 1000 µM Fe$^{2+}$, respectively. Lane 7-9 contained 10, 100, and 1000 µM UO$_2^{2+}$, respectively. 1 mM Fe$^{2+}$ produced clear cleavage products; while no well-defined cleavage bands were observed with 1 mM UO$_2^{2+}$. Ascorbate (50µM) was included in all the lanes.

![Figure S4](image)

**Figure S4.** Gel-based assay on Fe$^{2+}$ and UO$_2^{2+}$ cleavage of the Cu$^{2+}$ sensor DNAzyme.

4. Quantitative measurement of sensor response to Fe$^{2+}$.

The Cu$^{2+}$ sensor was titrated with varying concentrations of Fe$^{2+}$ and the results are presented in Figure S5. The assay conditions were the same as that used for Cu$^{2+}$ titration described previously. Only 1 mM Fe$^{2+}$ showed enhanced fluorescence, which is consistent with the results in 96-well plate based measurements. The sensor response to 1 µM of Cu$^{2+}$ is also measured for comparison. Based on this data, the selectivity for Cu$^{2+}$ over Fe$^{2+}$ is ~3000-fold.
**Figure S5.** Kinetics of the Cu$^{2+}$ sensor fluorescence enhancement in the presence of 1 µM Cu$^{2+}$ or varying concentrations of Fe$^{2+}$.

**5. Reaction of the Cu$^{2+}$ sensor in the presence of Fe$^{3+}$**

Fe$^{3+}$ with ascorbate is commonly used to generate reduced iron species. Therefore, it is surprising that there was no response from this combination in the selectivity experiment (Figure 2C). To understand the reason behind it, a gel-based assay was carried out as shown in Figure S6. Lane 1 and 2 contained 50 µM ascorbate. Lane 1 did not contain Cu$^{2+}$ while lane 2 contained 10 µM Cu$^{2+}$. These two lanes served as negative and positive controls, respectively. Lanes 3-5 contained 1 mM FeCl$_3$ and 0, 50 µM, and 1 mM ascorbate, respectively. Lanes 6-8 contained 1 mM Fe(NO$_3$)$_3$ and 0, 50 µM, and 1 mM ascorbate, respectively. Cleavage products were observed in lanes 4, 5, 7, and 8. Therefore, Fe$^{3+}$ with ascorbate can indeed induce cleavage. The reason for the failure to generate fluorescence increase in the selectivity experiment was attributed to the following two reasons. First, the cleavage even with 1 mM Fe$^{3+}$ in the presence of 50 µM ascorbate (lanes 4 and 7) was very slow. Because only 50 µM ascorbate was used in the sensing experiment, such slow cleavage may give limited cleavage products. Second, Fe$^{3+}$ is a strong quencher and even fluorescence did increase, the overall fluorescence intensity may still below background. To further confirm the effect of Fe$^{3+}$, solution based fluorescence kinetics experiments were performed and the results are shown in Figure S7. At all the three tested Fe$^{3+}$ concentrations, quenched fluorescence was observed.
There was slight recovery of fluorescence for the 100 μM Fe³⁺ sample. Even based on this enhancement, the selectivity for Cu²⁺ was still ~4200-fold higher for Cu²⁺ than for Fe³⁺.

**Figure S6.** Gel-based assay on Fe³⁺ cleavage of the Cu²⁺ sensor DNAzyme.

**Figure S7.** Kinetics of the Cu²⁺ sensor fluorescence enhancement in the presence of varying concentrations of Fe³⁺.

### 6. Cu²⁺-induced FAM quenching

FAM was chosen as the signaling fluorophore to detect Cu²⁺ in this work. Although the Cu²⁺ recognition part of the sensor was spatially separated from FAM, Cu²⁺ could still induce quenching due to non-specific fluorophore/metal interactions. A FAM-labeled double stranded DNA was employed to quantitative measure the quenching effect of Cu²⁺ to FAM, and a Stern-Volmer plot was made as shown in Figure S8 (red curve), the remaining fluorescence was less than 10% when Cu²⁺ concentration was higher than 100 μM (quenching efficiency >90%). In the current system, 50
μM of ascorbate was included, which significantly suppressed the quenching effect (blue curve). When Cu$^{2+}$ concentration was lower than 50 μM, the quenching was less than 15%. This experiment was carried out under the identical conditions as the Cu$^{2+}$ detection: 1.5 M NaCl, 50 mM HEPES, pH 7.0 at 23 ºC.

![Stern-Volmer plot](image)

**Figure S8.** Stern-Volmer plot of a FAM-labeled double stranded DNA quenched by Cu$^{2+}$. The DNA concentration was 10 nM for the FAM-labeled DNA and 20 nM for its complementary DNA strand (annealed in the same buffer as Cu$^{2+}$ detection). The sequence of the 5'-FAM labeled 20-mer DNA was: FAM-5'-ACTCACTATAGGAAGAGATG-3'.

7. Test of Cu$^+$ and the effect of ascorbate on the rate of cleavage

To test the effect of ascorbate on the rate of signal enhancement of the sensor, the sensor response in the absence of ascorbate was also carried out. As shown in Figure S9 (pink curve), even though fluorescence did increase, the rate was much slower compared to that with 50 μM ascorbate (red curve). This result is consistent with literature reports that this DNAzyme can operate independent of ascorbate.$^4,5$ The reaction of Cu$^+$ with this DNAzyme has never been reported before and we also studied this effect. Cu$^+$ was tested using [Cu(MeCN)$_4$](PF$_6$) in acetonitrile as the metal source (blue curve). With ascorbate, the rate of fluorescence increase was as fast as that with Cu$^{2+}$. In the absence of ascorbate, Cu$^+$ also can induce fast fluorescence increase (green curve), which was faster than that of Cu$^{2+}$ in the absence of ascorbate (pink curve). Therefore, it is likely that Cu$^{2+}$ was reduced by ascorbate to Cu$^+$, which subsequently reacted with oxygen to oxidatively cleave DNA.
Figure S9. Comparing Cu\textsuperscript{2+} and Cu\textsuperscript{+} and the effect of ascorbate on the rate of cleavage of the DNAzyme.

8. The effect of anions

Figure S10. The effect of anions in the sensor system. The dash curves are the kinetics with varying concentration of NaCl (Cu\textsuperscript{2+} added at the time indicated by the arrow) and the solid curve is the background fluorescence with NaN\textsubscript{3} (no Cu\textsuperscript{2+} added). All the samples contained 50 μM ascorbate. For all the samples with NaCl, the background levels were stable. For the sample with NaN\textsubscript{3}, however, the background was not stabilized (no Cu\textsuperscript{2+} added for the pink curve). Therefore, NaCl was used in the sensor system.
Reference:


