Dissecting the effect of anions on Hg^{2+} detection using a FRET based DNA probe

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Abstract

Many biosensors have been developed to detect Hg^{2+} using thymine-rich DNA. While sensor response to various cations is often studied to demonstrate selectivity, the effect of anions has been largely overlooked. Anions may compete with DNA for metal binding and thus produce a false negative result. Anions cannot be added alone; the cation part of a salt may cause DNA compaction and other effects, obscuring the role of anions. We find that the sensitivity of a FRET-based Hg^{2+} probe is independent of Na^{+} concentration. Therefore, by using various sodium salts, any change in sensitivity can be attributed solely to the effect of anions. Halide salts, sulfide, and amines are strong inhibitors; anions containing oxo or hydroxyl groups (e.g. nitrate, sulfate, phosphate, carbonate, acetate, and citrate) do not interfere with Hg^{2+} detection even at 100 mM concentration. Mercury hydrolysis and its diffusion into polypropylene containers can also strongly affect the detection results. We conclude that thymine-rich DNA should be useful for Hg^{2+} detection in many environmental water samples.

Introduction

Heavy metal ions can cause severe adverse health effects including damage to organs, developmental retardation and cancer.[1-6] A major source of human metal exposure is drinking water, attracting an immense amount of research effects to develop metal sensors for water analysis. An emerging method is to use DNA for metal recognition.[6-11] For example, DNA-based sensors for Pb^{2+},[12-16] Cu^{2+},[17] Hg^{2+},[18-20] Zn^{2+},[21] K^{+},[22-25] Ag^{+},[26-28] and UO_2^{2+} [29] have been demonstrated. In particular, many sensors have been prepared for Hg^{2+} detection using thymine-rich DNAs,[18, 30-44] where even low nanomolar Hg^{2+} can still tightly bind to these DNAs and be detected.

Most metal sensors have been evaluated only in carefully formulated clean buffers, and certain cations and anions are intentionally avoided. For example, mercury detection is usually carried out in the presence of $NaNO_3$ instead of NaCl. Environmental water samples, however, may contain various interfering cations and anions, which affect with DNA-based sensors in different ways. Cations may compete for the metal binding site on DNA to result in a false positive signal, or induce DNA compaction, change duplex stability or even quench fluorescence. We recently studied the effect Mg^{2+}, Ca^{2+} and Na^{+} on a Hg^{2+} sensor and found that detection was affected mainly through metal induced DNA compaction.[45] To the best of our knowledge, the effect of anions has not been systematically explored. It is conceivable that anions have very little interaction with DNA due to charge repulsion. Instead, their effects may come from chelating target metal ions to reduce their effective concentration. Other effects may include the change of solution properties such as dielectric constant and viscosity or hydrophobic interactions with DNA bases, which should be weak for most small anions. Most metal sensors have been tested in the presence of non-coordinating anions (e.g. nitrate or perchlorate). Coordinating anions such as Cl^{-} may compete with DNA for metal binding. While some DNAs possess nM metal binding affinity, the sensor DNA concentrations are usually just several nM. Therefore, the competition between nM DNA and mM buffer anions poses an interesting problem in analytical and inorganic chemistry.

Anions cannot be added alone and a cation must also be added at the same time. As mentioned previously, cations always interfere with DNA-based detection, especially at high concentration. Therefore, dissecting the anion effect from the overall signal is a challenging analytical problem. We recently reported a fluorescence resonance energy transfer (FRET)-based DNA probe for Hg^{2+} detection, whose sensitivity did not change as the concentration of $NaNO_3$ was varied from 0 to 500 mM.[45] Based on this observation, we reason that by using sensor sensitivity as the analytical index, it might be possible to isolate the effect of anions by using the sodium salts of various anions. We found that while several anions including Cl^-, Br^-, I^-, S^{2-} and amines can strongly inhibit Hg^{2+} detection, others such as phosphate, sulfate, carbonate, acetate and citrate did not affect the sensor performance. This is the first report to link specific metal binding by DNA, non-specific DNA folding and metal chelating by anions, showing that fundamental ion interactions can be studied using DNA probes.

Materials and Methods

Chemicals. The FAM and TAMRA dual labeled DNA was purchased from GeneLink (Hawthorne, NY) and was gel purified by the vendor. $NaNO_3$, NaCl, $NaHCO_3$, Na_2SO_4, sodium citrate, sodium acetate, tris(hydroxymethyl) aminomethane (Tris), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Mandel Scientific (Guelph, Ontario, Canada). $Hg(ClO_4)_2$, NaBr, NaI, Na_2S, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma-Aldrich. The polypropylene microcentrifuge tube was from Axygen. Lake Ontario water was collected from Colonel Samuel Smith Park in Toronto, Ontario, Canada.

Metal titration. The titration experiments were carried out in a quartz cuvette with a sample volume of 500 μL at room temperature using a Varian Eclipse fluorometer. The excitation wavelength was set at 485 nm and emission was scanned from 500 to 620 nm. All of the titrations were run in triplicates. For each sample, the cuvette contained 498 μL of 10 mM HEPES, pH 7.6 with varying concentrations of

salt and 2 μL of 5 μM dual-labeled DNA to achieve a DNA concentration of 20 nM. After this, 10 μM Hg^{2+} was titrated in increments of 1 μL to achieve a final Hg^{2+} concentration of 20 to 80 nM. Fresh mercury dilutions using 1 mM HNO_3 were made about once every week from a 10 mM Hg^{2+} stock dissolved in 100 mM HNO_3.

Results and Discussion

The probe design. We recently studied the effect of cations on a Hg^{2+} binding DNA.[45] This DNA was first reported by Ono *et al* in 2004.[18] Since then, it has been widely used as the Hg^{2+} recognition motif.[18, 30-44] We designed a FRET probe with its two ends labeled by a FAM and a TAMRA, respectively (Figure 1A). Hg^{2+} induced DNA conformational change from a random coil to a hairpin, resulting in shorter end-to-end distance and enhanced FRET efficiency. To quantify energy transfer, the fluorescence intensity ratio of TAMRA at 580 nm over FAM at 520 nm (called FRET ratio) was calculated. Using 20 nM DNA, FRET ratio increased linearly with Hg^{2+} concentration from 0 to 80 nM. We performed Hg^{2+} titration experiments in buffers containing various concentrations of $NaNO_3$. While the initial FRET ratio increased due to DNA compaction by Na^+, the slope of the titration curves (i.e. sensitivity) was independent of $NaNO_3$ concentration from 0 to 500 mM (Figure 2A, B). Based on this observation, we derived a strategy of dissecting the effect of anions (Figure 1B). In a low salt buffer, the DNA is in an extended coil conformation, giving a low FRET ratio. Adding non-coordinating $NaNO_3$ only results in DNA compaction because of Na^+ (e.g. shorter end-to-end distance) but the compacted DNA can still react with Hg^{2+} with the same sensitivity. By using the sodium salts of coordinating anions, any change in sensitivity can therefore be attributed to sequestering of Hg^{2+} by the anions.

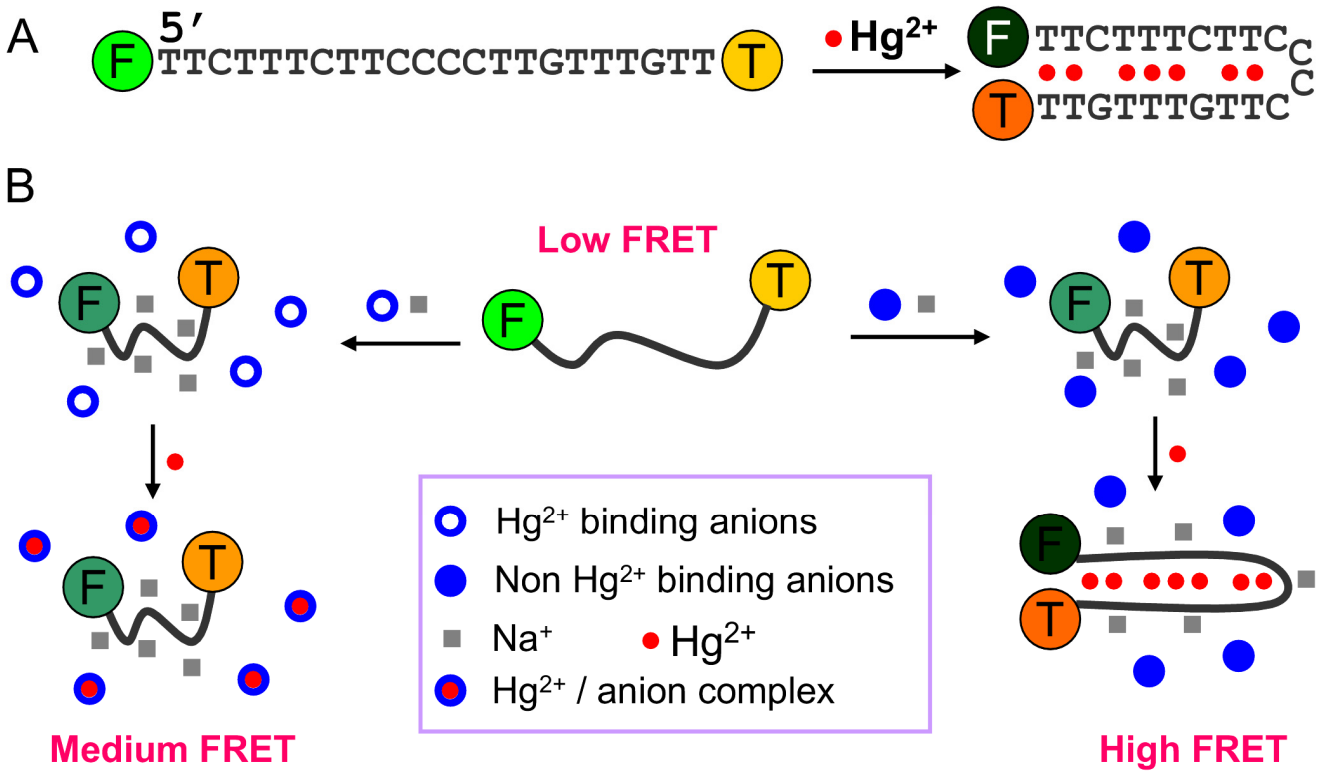

Figure 1. (A) The sequence of the Hg^{2+} binding DNA. F and T denote for FAM and TAMRA fluorophores, respectively. (B) Hg^{2+} titration experiments carried out in the sodium salts. If the salt concentration is high, the DNA forms a more compact structure due to charge screening, producing an increase in FRET efficiency. If the anion can bind Hg^{2+} tightly, no Hg^{2+}-induced DNA folding is observed. Otherwise, the DNA folds into a hairpin to further increase the FRET efficiency.

Effect of Cl^-, Br^- and I^-. Since Cl^- is one of the most common anions, its effect was studied first (Figure 2C). NaCl induced DNA compaction was observed, as reflected by the increased initial FRET ratios in the absence of Hg^{2+}. The highest slope (sensitivity) was achieved in the absence of NaCl. The sensitivity progressively decreased with increasing NaCl. In particular, no response was observed if NaCl concentration was higher than 100 mM (Figure 2D). Since $NaNO_3$ had no effect on the slope (Figure 2A, B),[45] the change in the presence of NaCl can only be attributed to Cl^-. In Figure 2D, an inhibition curve with K_i = 13.7 ± 2.1 mM NaCl was obtained. The solubility of $HgCl_2$ is 74 g/L or 270

mM,[46] and the added Hg^{2+} is far from this solubility limit. It is known that Cl^- and Hg^{2+} can form the $[HgCl_4]^{2-}$ complex with a formation constant of 15.4.[47] With ~20-80 nM Hg^{2+}, the complex formation is expected at ~10 mM NaCl, which is consistent with our experimental data and suggests the inhibition effect of Cl^- can be attributed to the complex formation.

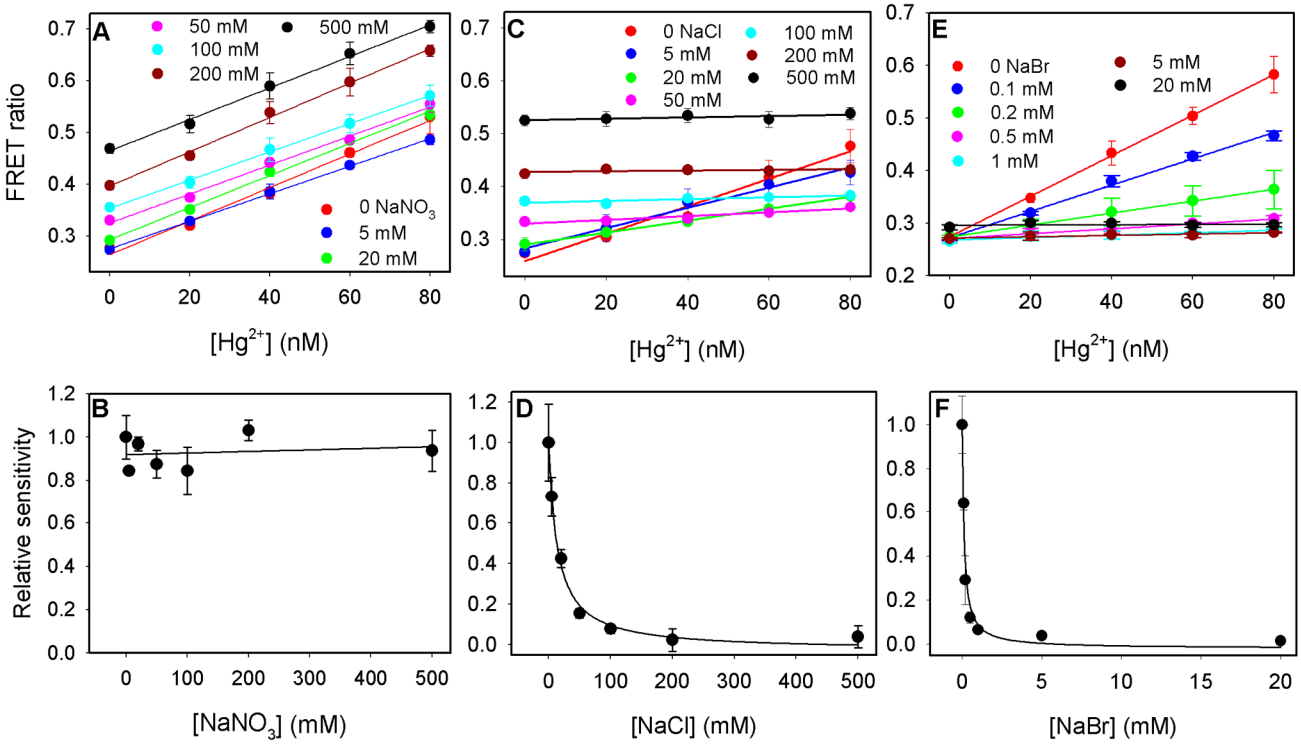

Figure 2. Hg^{2+} titration curves in the presence of varying concentrations of $NaNO_3$ (A), NaCl (C) or NaBr (E). The relative sensitivity (slope) of the titration curves as a function of $NaNO_3$ (B), NaCl (D) or NaBr (F). The buffer contained 10 mM HEPES, pH 7.6. Note that the data in (A, B) have already been published and they are presented here for comparison.[45] Reprinted with permission from ref 45. Copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Along the same lines, the effect of Br^- was studied next (Figure 2E). Br^- showed a much stronger inhibition effect; no signal was observed with >1 mM Br^- and K_i was determined to be 0.12 mM (Figure 2F), two orders of magnitude stronger than that for Cl^-. This is consistent with the

formation constant of $[HgBr_4]^{2-}$ being 21. Since the highest NaBr added was only 20 mM, little increase in the initial FRET ratio was observed and all the calibration curves appear to start from the same point. I^- binds to Hg^{2+} even more tightly with a formation constant of 30.3 and we obtained a K_i of 0.13 μM (see Supporting Information), consistent with the expected trend. It needs to be pointed out that the observed inhibition by Br^- and I^- were not related to the heavy atom effect of fluorescence quenching. In the salt concentration range we tested, no significant change of the fluorescence spectra were observed in the absence of Hg^{2+} (see Supporting Information). These experiments confirm that sensitivity measurement is useful for studying the anion effect.

Effect of Other Anions. In addition to halides and nitrate, other common anions that might be present include sulfate, phosphate, carbonate, and various carboxylates. According to the *CRC Handbook of Chemistry and Physics*, $HgSO_4$ decomposes in water. Figure 3A shows the sensor response in the presence of Na_2SO_4. The sensor sensitivity is scarcely affected by up to 100 mM SO_4^{2-} (Figure 3B). Therefore, binding of Hg^{2+} by sulfate is very weak and even 100 mM sulfate cannot compete with 20 nM DNA. Next the effect of phosphate was studied. NaH_2PO_4 and Na_2HPO_4 were mixed to achieve pH 7.6, which was used as the source of phosphate. According to the *Handbook*, mercury phosphate is insoluble in water but soluble in acid. Therefore, phosphate is expected to interfere with the detection. However, up to 100 mM phosphate had little effect on Hg^{2+} detection (Figure 3C, D). Next the effect of carbonate was measured. There is no data about $HgCO_3$ in the *Handbook*, but Bilinski *et al* reported that this salt is quite soluble and forms colored precipitations at high concentrations.[48] We found that the sensor performance was not affected by carbonate either (Figure 3E, F). Therefore, for the extremely low Hg^{2+} concentrations in this work, conclusions previously drawn from the 1:1 salts may not be directly applied.

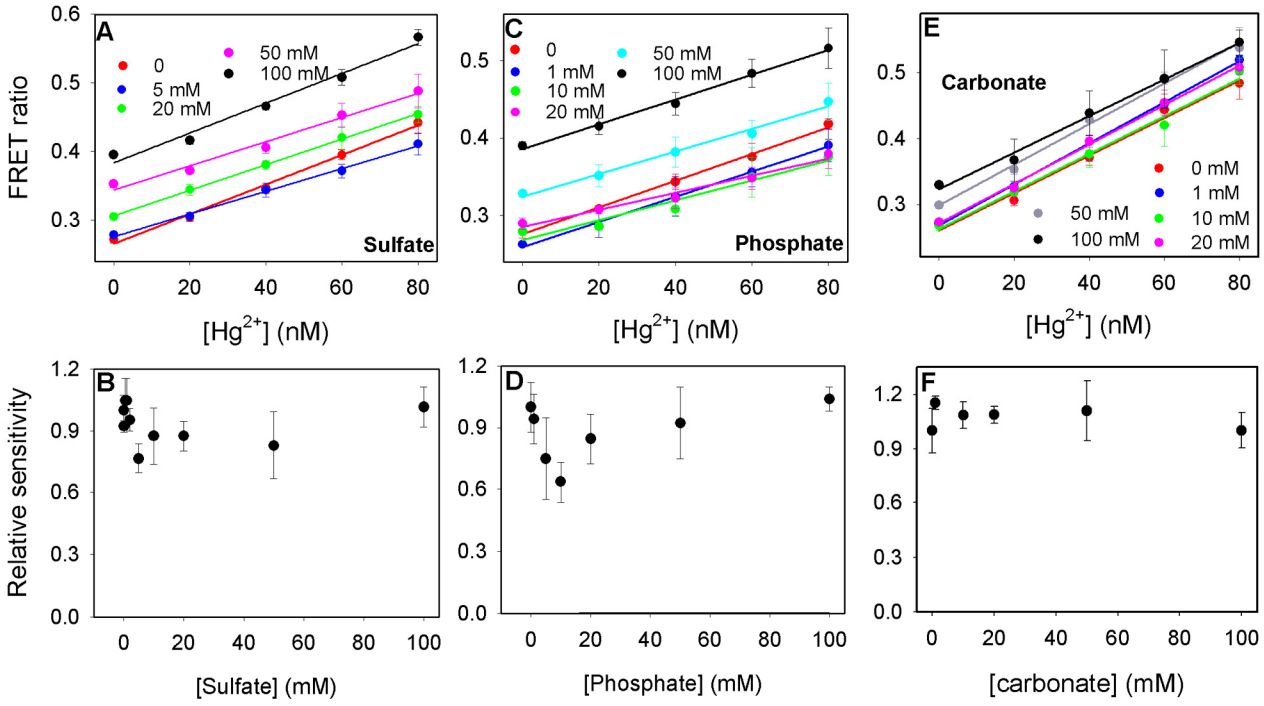

Figure 3. Hg^{2+} titration curves in the presence of varying concentrations of sodium sulfate (A), sodium phosphate (C), and sodium carbonate (E). The relative sensitivity (slope) of the titration curves as a function of sodium sulfate (B), sodium phosphate (D), and sodium carbonate (F).

We next employed sodium acetate and sodium citrate to study the effect of carboxyl anions. Each citrate contains three carboxyl groups and therefore it might interact with Hg^{2+} more strongly compared to the monovalent acetate. There is no record in the *Handbook* about mercury citrate but mercury acetate is known to be soluble in water. As shown in Figure 4A-D, both acetate and citrate had little effect on Hg^{2+} detection. A related anion is oxalate and $[Hg(C_2O_4)_2]^{2-}$ has a formation constant of 9.5×10^6, meaning that 1 M oxalate is required to bind 100 nM Hg^{2+}. Therefore, it is plausible that acetate and citrate do not interfere with mercury detection. Sampled together, the interference brought by anions containing only oxo and hydroxyl groups is minimal. This can be explained by the fact that Hg^{2+} is a soft metal and these oxygen containing anions are hard ligands. Environmental water samples

are unlikely to contain such high concentrations of these anions. Therefore, these anions are not considered to be interfering species.

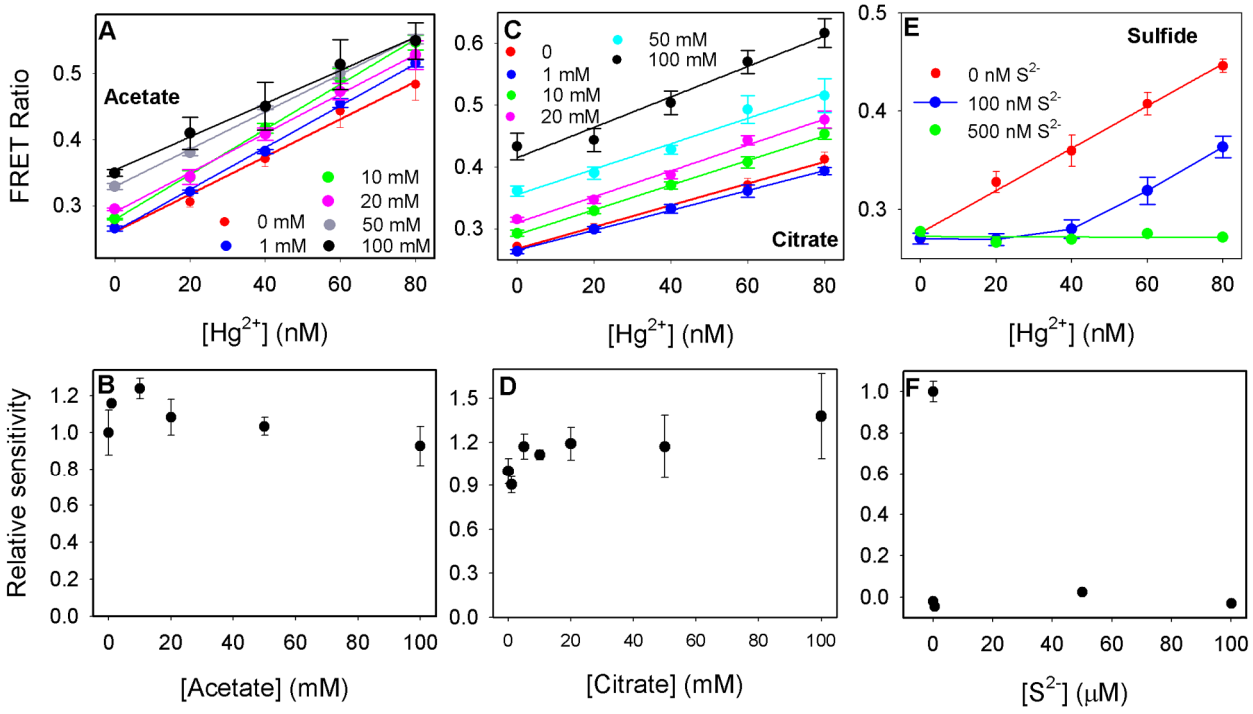

Figure 4. Hg^{2+} titration curves in the presence of varying concentrations of sodium acetate (A), sodium citrate (C), and sodium sulfide (E). The relative sensitivity (slope) of the titration curves as a function of sodium acetate (B), sodium citrate (D), and sodium sulfide (F). In (E) the blue dots were connected by lines instead of fitted. In (F) the 100 nM data point was obtained by fitting only the 0 and 20 nM signals in (E).

Effect of amine, sulfide and buffer capacity. While oxygen containing species do not interfere with detection, nitrogen groups are known to be good ligands for Hg^{2+}. For example, our DNA probe uses its thymine nitrogen to bind Hg^{2+}.[49] We used Tris as the nitrogen source, which is also a commonly used buffer. The sensitivity was significantly reduced by Tris (Figure 5A). In particular, complete inhibition was observed with ~50 mM Tris (Figure 5B). After reading the literature for DNA-based

Hg^{2+} detection, we found that the majority of the published papers used low buffer concentrations (≤ 10 mM), and a diverse range of buffers have been tested such as Tris, HEPES, MOPS, and MES. Using dilute buffers is certainly important for Tris. However, no work on the effect of buffer capacity was carried out. HEPES contains sulfonate and its binding to Hg^{2+} should be weak. Indeed, little sensitivity change was observed in the presence of up to 200 mM HEPES (Figure 5C, D). Therefore, buffer concentration is not a major factor compared to its chemical nature.

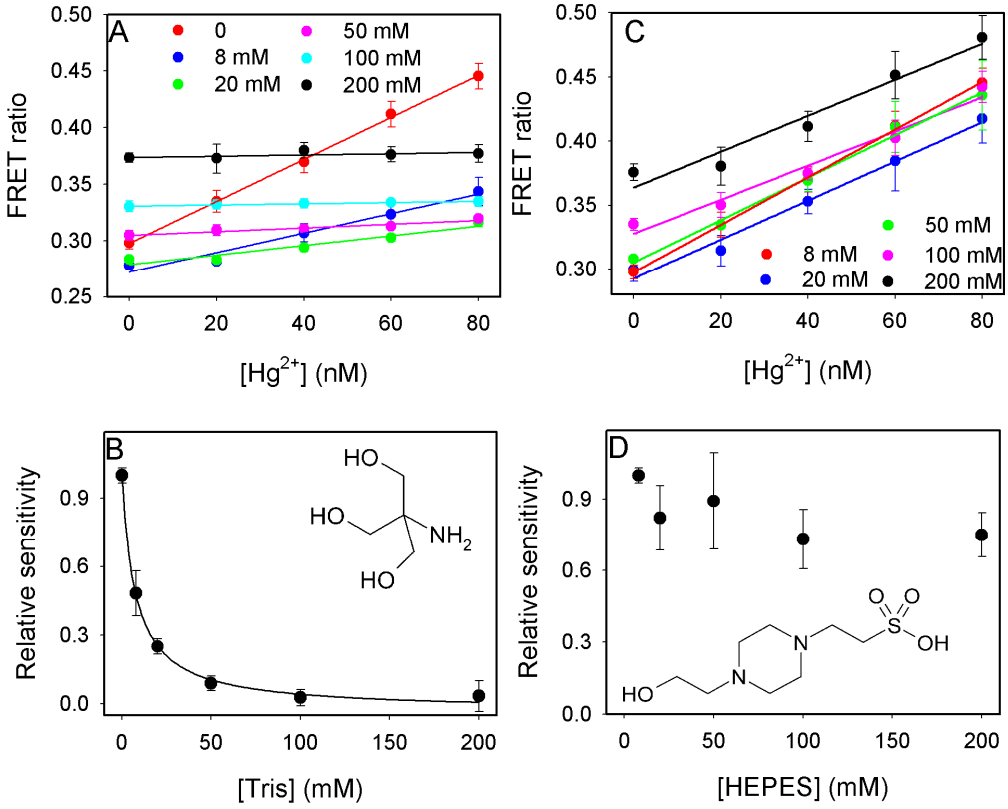

Figure 5. Hg^{2+} titration curves in the presence of various concentrations of Tris (A) and HEPES (C). The relative sensitivity (slope) of the titration curves as a function of Tris (B) and HEPES (D).

Hg^{2+} has a high affinity for thiol-containing compounds and this property has been utilized for thiol detection. For example, Mirkin and co-workers reported a competitive colorimetric assay for

detecting cysteine, where even 2 μM of cysteine was able to completely inhibit the effect of Hg^{2+}.[50] In this work, we tested the effect of S^{2-}, which is known to precipitate Hg^{2+} with a solubility product (K_{sp}) value of 2×10^{-53} for HgS. This means that even a single Hg^{2+} and a single S^{2-} can react. Interestingly, as shown in Figure 4C, 100 nM S^{2-} completely inhibited only ~40 nM Hg^{2+}. After that, Hg^{2+} showed a slope similar to that in the absence of S^{2-} (blue dots). We propose that only ~40 nM S^{2-} was present and the rest was oxidized. Even though the sulfide solution was freshly prepared, a significant level of oxidation could still occur at such a low sulfide concentration. In the presence of 500 nM or higher S^{2-}, the signal was completely inhibited (Figure 4F).

Effect of pH and container. Previous spectroscopic work has shown that mercury binding to thymine occurs over a wide pH range from 3.5 to 10.[51] Since pH can be conveniently controlled by using buffers, the effect of pH on detection was not studied. In this work, we are interested in testing the effect of pH on the storage of Hg^{2+} to mimic the natural water environment. For most biosensor work, mercury salts are dissolved in an acid to assist in dissolution and prevent hydrolysis. However, natural water samples are not buffered and the pH is usually slightly acidic. Therefore, the long-term stability of Hg^{2+} in water needs to be studied. Hg^{2+} is unlikely to present as free ions according to its speciation around neutral pH, where $Hg(OH)_2$ is assigned to be the major species.[47] To have a systematic understanding on this problem, we incubated 5 μM Hg^{2+} in three solutions: water (pH ~6.2), 50 mM HEPES (pH 7.6), and 50 mM HNO_3 (pH ~2). The response of the sensor was measured over a period of ~25 days. Two different types of containers were tested including polypropylene microcentrifuge tubes and glass vials.

In polypropylene containers, the slope remained the same for Hg^{2+} stored in acid but progressively decreased if stored in water or in pH 7.6 HEPES buffer (Figure 6A). After 20 days, the activity of samples in water was reduced by ~50% and in HEPES by ~80%. In glass containers, however, decayed activity was observed only for the sample stored in HEPES. Even for this sample,

the activity drop did not occur until after day 10; while in the polypropylene tube, the activity dropped immediately. This suggested that adsorption of Hg^{2+} into the polypropylene wall is one of the mechanisms for reduced activity. This process is inhibited at low pH. Given that Hg^{2+} is the species to be associated between DNA thymine base pairs, the conversion of $Hg(OH)_2$ to Hg^{2+} must be very quick since signal increase occurred instantaneously.

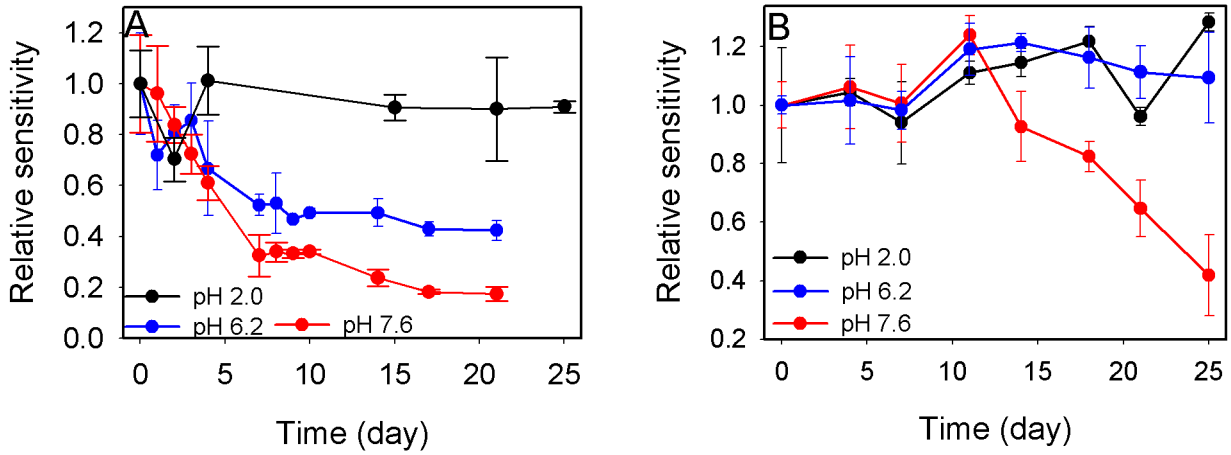

Figure 6. Effect of mercury sample storage in polypropylene tubes (A) and in glass vials (B). The same Hg^{2+} samples were tested over 25 days. The decay of activity indicated the loss of Hg^{2+} due to either container adsorption or precipitation.

Natural water samples. With the above studies, we now have a complete understanding of the behavior of this sensor in the presence of various ions. We next tested the sensor performance in Lake Ontario water, which is known to contain just ~0.6 mM Cl^- while all other major anions should not bind Hg^{2+}. Therefore, this sensor should work very well in it.[52] Indeed, the sensor sensitivity was slightly better in Lake Ontario water than in buffer (see Supporting Information), which was attributed to the presence of Mg^{2+} (0.36 mM) and Ca^{2+} (0.85 mM).[45]

Summary and conclusions. In this study, a FRET pair labeled DNA was used to study the effect of anions on Hg^{2+} detection. It appears that complex formation with Hg^{2+} is the major mechanism of anion interference. While inorganic chemists usually study cation and anion interactions at 1:1 ratio, for analytical chemists, the interaction between low nM Hg^{2+} and high μM or mM anions is more relevant. We found that for many "insoluble" mercury salts, their anions did not bind to low nM Hg^{2+}, supporting the use of such sensors in many environmental water samples. Very strong inhibitors such as sulfide can completely and quantitatively inhibit the sensor signal while most other inhibiting anions (e.g. Cl^{-1}, Br^{-1}) only showed reduced sensitivity. Since it is difficult to design DNA probes insensitive to buffer cations and anions, the standard addition method could be valuable for analyzing environmental water samples. In this particular example, we could simply plot the slope value because of the fortunate observation that the slope of Hg^{2+} calibration curves is independent of $NaNO_3$ concentration. This is unlikely to be a general rule. Therefore, to study the anion effect on other metal sensors, the calibration curve at each salt concentration may need to be compared.

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