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Practical Screening of Mercury Contamination in Fish Tissue

Oliver Brümmer, James J. La Clair and Kim D. Janda*

Department of Chemistry and Molecular Biology and the The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

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Abstract—The increased release of heavy metals over the last century poses an unknown detriment to our ecosystem. Already, poisoning by toxic heavy metals has been documented in a number of species, including man. Estimation of the toxicological context of this release requires screening methods that rapidly process large numbers of samples with minimal cost, effort and ecological impact. We now describe a practical colorimetric kit to quantify mercuric ion in tissue, and demonstrate its application to screen fish. Advantageously, this test can easily be amended for field use and catch-and-release programs.

Introduction

Emissions from automobiles, coal burning, mining, industrial activities, and trash incineration has increased the natural release of heavy metals.[1,2] The potential risks associated with enhanced contact to toxic metals has drawn attention to their vectors of presentation. This concern has been furthered by the finding that aquatic organisms convert elemental mercury to methylmercury, which subsequently concentrates through the food chain in the tissues of fish and marine mammals.[3] Already, a number of cases have linked mercury poisoning in man to the consumption of fish.[4–8]

Mercury contamination in tissue is currently determined using a combination of techniques, including capillary electrophoresis, flame atomic absorption spectroscopy, inductively coupled plasma emission spectroscopy and gas chromatography.[9–14] The labor, chemical hazards, instrumental and technical sophistication associated with these methods requires their conduct in a laboratory setting. While these techniques serve to screen modest sample arrays, the hazard of mercury poisoning lies at the level of providing confidence for a large number of small tissue samples. Screening would be enhanced through the development of field ready kits. This report now describes an important advance through effectively combining a new sample preparation and a reliable colorimetric response.

One means to provide a practical indication arises through the device of an appropriately tuned color change. Dyes based on thiocarbazone moieties, such as dithizone and β-napthylthiocarbazone are known to respond colorimetrically to mercuric ion.[15] While their response has been suggested for the analysis of mercury contamination in tissue,[16,17] the method has not gained acceptance because their interaction with mercuric ion expresses a series of different colors depending on a diverse equilibrium between various states of complexation. We sought to prepare a material whose response presents a defined color change.

Results and Discussion

We recently described the synthesis and metal binding properties of chemosensor **1**.[18] This material contains both a handle to grab mercuric ion, a phosphorodithioate, and an adjacent charge transfer dye to relay this recognition to a visual signal (Fig. 1).[19] When examined at $\geqslant 8\,\mu M$ in **1**, the addition of mercuric ion resulted in a distinct color change from yellow to red. The red complex immediately precipitated, leaving the aqueous solution colorless or bleached. The selectivity was verified by screening 42 different metal salts (Fig. 2). Within a second after addition, only Hg^{2+} produced an immediate red color and precipitate. This observation was further validated by subsequent processing of the solution and precipitate (Fig. 3). After the reaction was complete, the remaining solution was transferred to another plate leaving only the red precipitate (the complex of $Hg^{2+} \cdot$ **1**) in the original plate. Upon addition of acetone to the original plate, wells containing the pre-

*Corresponding author. Fax: +1-858-784-2595; e-mail: kdjanda@scripps.edu

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cipitate turned yellow (Fig. 2B). Inspection of this change was verified by processing the color of the transferred solution (Fig. 2C). Addition of an equiv of bromophenol blue converted trials which contained excessive mercury blue (colorless plus blue), and those, which did not, violet (yellow plus blue). Within 30 min, a similar response also occurred in wells containing Ag^{+}, Cd^{2+}, Cu^{2+}, and Pb^{2+}. The relative visual sensitivity was determined to be $Hg^{2+} \gg Ag^{+} \gg Cu^{2+} > Pb^{2+} > Cd^{2+}$ (Table 1). This analysis provided a confident visual signal when adding 2 μM mercuric ion to 10.5 μM **1** as judged by the immediate observation of colored or bleached dye solutions. Further quantification of this approach was possible using a conventional absorption-based plate reader or spectrophotometer. Changes in absorption that are not distinguishable by the eye were apparent upon inspection at a single wavelength between 415–450 nm, extending the detection limit to 0.5 ± 0.1 μM Hg^{2+}.

Application to the screening of fish required a means to provide consistent solutions of Hg^{2+} from tissue. For this study, a confidence level was set at 0.55 ppm in tissue based on guidelines suggested by the EPA for safe human consumption.[20] We examined this approach using a series of fish samples collected from Michigan whose mercury content had previously been determined

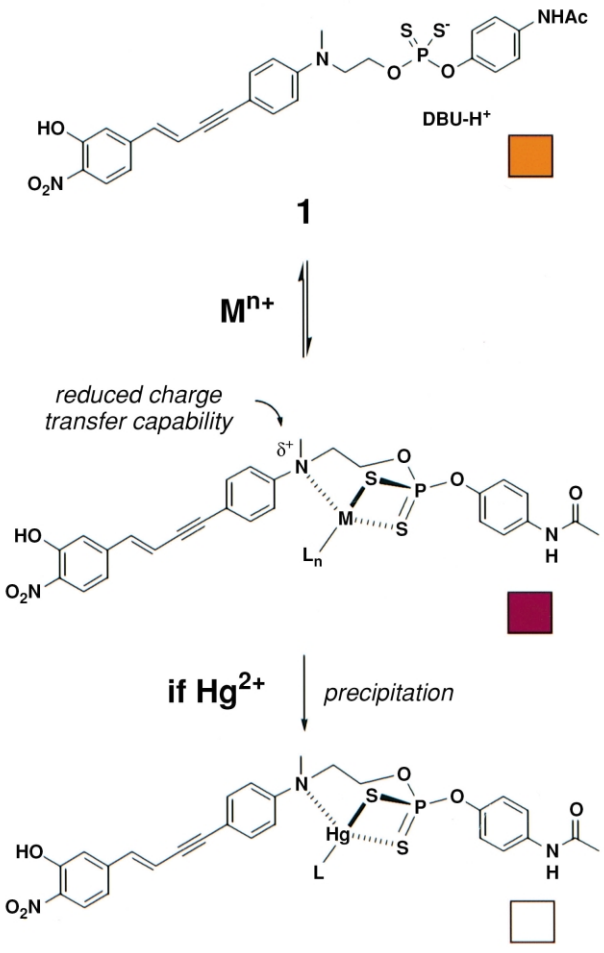

Figure 1. Indicator **1** senses mercury through subsequent regulation of its ability to undergo charge transfer. Most metal ions exist in equilibrium between metal-bound and free states of **1**. Certain metals, such as mercury, form strong complexes that subsequently precipitate, leaving the aqueous phase colorless (bleached). The apparent color of the solution is provided next its chemical equivalent.

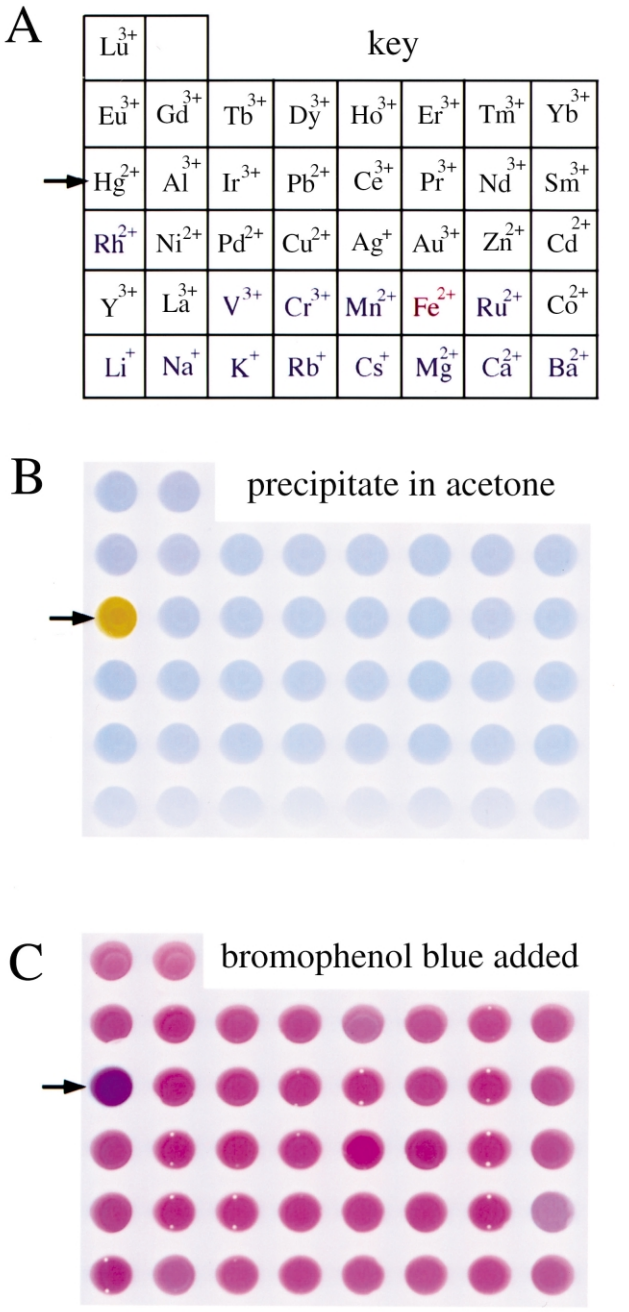

Figure 2. Colorimetric response arising from the addition of various metal salts to probe **1**. (A) The counter ions of each metal are keyed by the color: nitrates in black, chlorides in blue, and sulfates in red. Within seconds of addition, only the well containing mercury turned clear, at which point the remaining solution was transferred to empty wells and the color processed as given in Figure 3. (B) Yellow color appears upon redissolving the precipitate left in the original well containing Hg^{2+} with acetone. (C) Verification of this result can be enhanced by the addition of 1 equivalent of bromophenol blue (see experimental section) to the transferred solution. Blue results from the mixing of bromophenol blue with a clear solution (resulting from complete precipitation of **1**). Violet appears from mixing yellow (from unreacted **1**) and blue (from bromophenol blue).

by atomic absorption spectroscopy (AA). Currently, the digestion protocols for this analysis require over 24 h and conditions are too harsh for field use (i.e., boiling in concentrated mineral acids).[9–14] We devised a mild digestion procedure using detergent solubilization, enzymatic proteolysis, followed by oxidation of methylmercury to mercuric nitrate with 1.0 M nitric acid (Fig. 4A).[21] Using this sequence, fish samples were completely digested in less than 6 h. After buffering to pH 4, metals were sequestered from these digests using Chelex 100 resin affixed to a stirring rod. This additional extraction not only removed potential interference from remaining organic materials but also enhanced the selectivity, as Chelex 100 exerts its greatest affinity to Hg^{2+}.[22] Mercuric ions were subsequently washed from the dipstick by stirring in a second vial containing dilute HCl at pH 1.25. A sample of this solution was diluted in 50 mM HEPES to a level that correlated an expected mercuric ion concentration of 0.12 μM Hg^{2+} in solution with 0.55 ppm in tissue.[20] The assay was calibrated by examining a series of mercury standards ranging from 0.01–1 μM. Two relevant trials are shown in Figure 4B and define a colorimetric distinction at 0.12 μM in stock solutions, as given by yellow at <0.11 μM and violet at >0.15 μM. As indicated by prior AA analysis, samples **A**–**C** contain greater than 0.55 ppm mercury in tissue and presented a color change, and **D** did not. As confirmed experimentally in Figure 4B, the presence of a blue color in **A**–**C** (as given by the addition of bromophenol blue to clear solutions) arose from complete precipitation of Hg^{2+}•**1** and yellow in **D** from adding a trace of blue to yellow from **1**.

The preceding observations extend a visual kit to determine a confidence level for fish tissue.[19] The cutoff of this test can easily be tuned from 0.1–5 ppm by regulating the

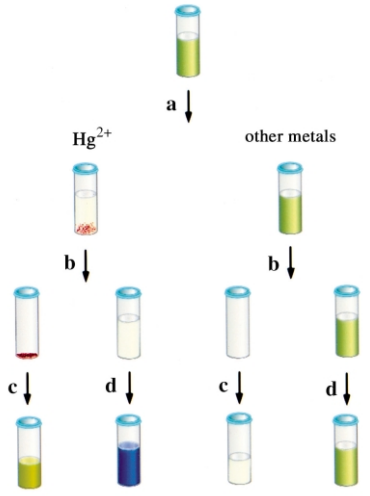

Figure 3. Color processing. (**a**) A stock solution of **1** is treated with a selection of metals. Within seconds only mercuric ion causes the precipitation (left second row) and other metals result in only diluting the color (right second row). (**b**) The mother liquor or remaining solution is transferred to a second vial or well. (**c**) Acetone is added to the original vial or well. A yellow color appears only if precipitation occurred (i.e., from the presence of a significant amount of mercuric ion). (**d**) The color of the transferred solution is enhanced by addition of bromophenol blue. When adding 0.2 equiv, this solution turns blue (blue plus colorless) if significant mercury was present or remains yellow if not (yellow dominates a trace of blue).

Table 1. Requirements for visually-apparent precipitation within 30 min[a]

Metal	Metal concentration (μM)	Equivalents of metal per dye[b]
Ag^{+}	20	1.9
Cd^{2+}	>100	>10
Cu^{2+}	60	5.7
Hg^{2+}	2	0.19
Pb^{2+}	90	8.7
Zn^{2+}	∞	∞
K^{+}	∞	∞

[a]As determined by adding 10 μl of a series of metal nitrate stock solutions in water to wells containing 60 μl of a 35 μM acetonitrile solution of **1** and 130 μl of 50 mM HEPES (pH 7.0). The final dye concentration was 10.5 μM. Weak binding metals salts showed no effect at greater than 150 equivalents of metal and are designated by ∞. The values presented were established by an average of three repetitions and were chosen at the level where no color could be seen (within 10 % deviation).
[b]Note only mercury can effectively precipitate **1** at less than an equivalent of metal.

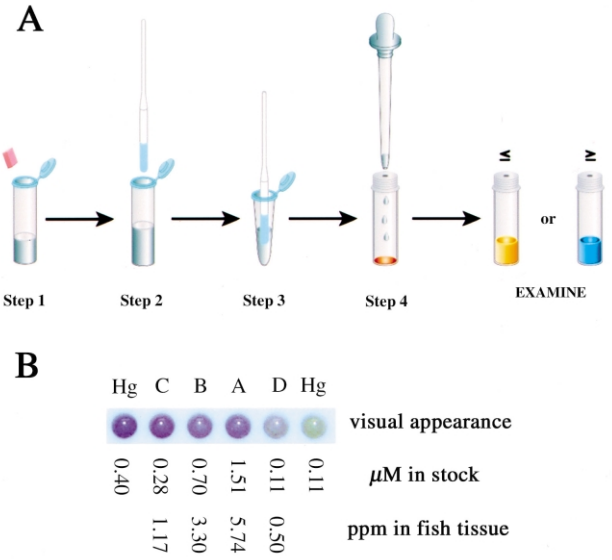

Figure 4. Inspection of fish. (A) Schematic representation of the mercury kit. Step 1: addition and subsequent digestion of fish samples. Step 2: the metal ions are abstracted from a buffered solution using a dipstick coated with Chelex 100 resin. Step 3: metals are eluted from the stick by stirring in an acidic solution. Step 4: this solution is buffered and subsequently added to a sample of chemosensor **1**. Inspection of color prior to and after development with bromophenol blue provides a distinct response. (B) Letters **A**–**D** refer to samples of fish as denoted by letter: **A** northern pike from Deer Lake in Marquette County containing 5.74 ppm mercury, **B** northern pike from Deer Lake in Marquette County containing 3.30 ppm mercury, **C** northern pike from Deer Lake in Marquette County containing 1.17 ppm mercury and **D** walleye from Croton Pond in Newago County containing 0.5 ppm mercury. The mercury content in this fish was independently determined by atomic absorption spectroscopy (AA). **Hg** represents standard solutions of $Hg(NO_3)_2$ that were processed using the aforementioned extraction. The values presented under each well indicate the expected level of mercury given by μM in the stock solution or ppm in tissue, as based on standard or that found by AA. Yellow indicates a lack of complete reaction <0.55 ppm of mercury, and blue to violet a positive reaction >0.55 ppm mercury, as described in the color processing section of the experimental section and Figure 3.

dilution factor used in Step 3 (Fig. 4A). Most importantly, the technique can detect both mercuric ion and methylmercury by virtue of the oxidation step.[21] The use of pre-loaded vials and aliquots simplifies the technique to a level that requires minimal training and laboratory instrumentation. There are no hazardous steps, indeed, the most dangerous reagent used was 1 M HNO_3. In addition, this test requires only 100 μL (equivalent to a 0.5 cm cube) of tissue. This sample size can easily be employed in catch-and-release programs, therein minimizing impact resulting from specimen removal.

Conclusion

We herein demonstrate how practical technology directly monitors mercury contamination in fish. We describe a visual mercuric ion sensor that can be coupled with an efficient tissue processing. This new technique reduced the time and scale of sample preparation, and allows tissue to be analyzed within the same day with minimal laboratory requirements. The material required for over a million assays can be prepared in approximately 1 week from inexpensive materials ($<$\$500), as a single analysis requires less than 150 ng of **1** and minimal reagents and vessels. If desired, a microtiter plate reader or image processing using a CCD camera can be used to quantify the results of this assay within a 2% deviation with high-throughput. This response now delivers a convincing tool to screen and quantify mercury contamination. While demonstrated in fish, the method described within extends a screen for a wide variety of tissue and environmental samples.

Experimental

Materials

Sensor **1** was prepared as described previously.[18] All reactions were conducted in microcentrifuge tubes using freshly prepared media and reagents. All buffers used in this work were stirred with Chelex 100 resin (Bio-Rad, Hercules, CA) overnight at room temperature to remove any adventitious metal ions (1 g of resin per 100 mL of buffer). The resin was then removed by filtration through a 0.2 μM membrane (Corning Costar). The dipstick was fabricated by glueing Chelex 100 resin onto the bottom 2.5 cm of a 10 cm long, 3 mm diameter, polyethylene rod with commercial contact cement (3M). Comparable extraction was also obtained using porous bags containing $\sim$50 mg of Chelex 100. Samples of Northern Pike and Walleye were collected from Michigan.

Determination of metal selectivity

The metal selectivity of **1** was determined using the following procedure and the outcome of this study is displayed in Figure 2: Ten μl of a 200 μM stock of each metal salt (Fig. 2A) were added to a distinct well in a 96-welled white Teflon plate (Berghof America, Coral Springs, FL) containing 60 μl of a 35 μM acetonitrile solution of **1** and 130 μL of 50 mM HEPES (pH 7.0) per well. Within seconds of addition, only mercuric ion changed the solution's color from yellow-orange to clear. The results of this outcome were further verified by color processing (following section). Use of a Teflon plate was not required but rather its opaque white appearance enhanced our ability to visualize and photograph.

Color processing

Precipitation by mercuric ion occurred within a second. Usually, this result was readily distinguished from unreacted trials (yellow-orange color) by the formation of a colorless solution with red precipitate (Fig. 3). As this distinction may be difficult for certain users to distinguish, we developed a practical processing to further verify this result. This procedure began by transferring the solution remaining in each well to another plate and then examining both the remaining precipitate and mother liquor, as illustrated in Figure 3. The result was verified twofold. First, addition of acetone to the original well resulted in either a clear solution (lack of reaction) or a brilliant yellow color (resulting from redissolving the mercury complex of **1**). This result was further verified by adding between 0.2–1 equiv of bromophenol blue per equiv of **1** to the transferred solution. At 0.2 equiv, wells containing insufficient or non-reactive metals remained yellow (resulting from mixing only a trace of blue with a strong yellow color) and those that did react or contained sufficient mercuric ion appeared blue (as indicated by adding a trace of blue to a colorless solution). At 1 equiv, blue was again attributed to wells that underwent complete precipitation but now violet was obtained from mixing an equivalent amount of yellow (from unprecipitated **1**) with blue (from bromophenol blue). One equiv (10 μM) bromophenol blue was added in Figure 2C and 0.2 equiv (2 μM) were added for the fish analysis (Fig. 4B).

Screening of fish tissue

Three tissue samples were prepared from each fish. A total of four fish were examined that range from low to high mercury content (Fig. 4B). The mercury content in each processed sample was determined in triplicate. Each trial remained within 3% deviation and 5% of the concentration determined by AA. This analysis was conducted using a four-step procedure as depicted in Figure 4A. The procedure for each step is given below:

Step 1: digestion and oxidation. A sample of each fish (115 ± 8 mg), prepared by filling a 100 μL volume with tissue, was added to a microcentrifuge tube containing 880 μL of lysis buffer (50 mM TRIS-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1% SDS and 20 μL of 10 mg/mL proteinase K (Sigma)) sealed and shaken at 55 °C for 3 h. The resulting homogenous solution was converted to pH 1.4 by addition of pre-measured 0.5 mL aliquot of 1.0 M HNO_3 and then heated to 70 °C. After 3 h of incubation, the reaction was adjusted to pH 4.0 with a 0.5 mL aliquot of 0.6 M NaOH.

Step 2: extraction. The preceding solution was stirred for 10–15 min with a polyethylene stick (Hellma Mülheim,

Germany) whose tip was coated with ~50 mg Chelex 100 (BioRad).

Step 3: isolation. The metal ions were eluted from the resin by stirring the rod in a second microcentrifuge tube loaded with 400 µl of aq HCl (pH 1.25) for 15 min. This solution was then buffered to a final pH of 7.5 by the addition of a 1.8 mL aliquot of 50 mM HEPES (pH 12.2).

Step 4: analysis. A 140 µL sample of this solution was mixed with 60 µL of a 35 µM acetonitrile solution of **1** examined after 1.5 h at 23 °C by transfer of the remaining layer to another vial or well. The outcome was verified by further color processing as described in the previous section and Figure 3.

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