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Immunochemical Technology for Environmental Applications

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Chapter 3

Rapid Mercury Assays

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We have developed rapid assays with the potential of being applied to mercury analysis in environmental samples. Our methods combine the simple ELISA-format with the selective, high affinity complexation of mercuric ions by sulfur-containing ligands. The first assay format is based on a sandwich chelate formed by a ligand conjugated to a reporter enzyme, a mercuric ion of the analyzed sample, and a protein bound ligand immobilized on the wells of a microtiter plate. The second assay format involves competition between mercuric ions and an organomercury-conjugate to bind to a chelating conjugate. The assays detect mercuric ions in ppb/high ppt concentrations with high selectivity.

Toxicity of Mercury

Mercury is one of the most hazardous toxic metals. The amount of mercury emitted to the environment is continuously increasing mainly due to industrialization and urbanization. This trend has raised concerns about the risk of human exposure to mercury and the resulting adverse health effects (*1*).

Most of the biological effects of mercury can be explained in terms of the very high affinity of mercurials towards endogenous thiols (*1*). Toxicologically, the most important mercury-species are elemental (Hg^0) and mercuric (Hg^{2+}) mercury, and methylmercury (CH_3Hg^+). Different forms of mercury exhibit different bioavailabilities, toxic potencies, tissue and organ distributions, and patterns of biological effects (*1*).

Exposure to Hg^0 vapor is mostly an occupational hazard for dental personnel, workers in the chloralkali industry, plants manufacturing mercury thermometers, and mercury mines (*2-4*). The toxic impact of the Hg^0 vapor released from dental amalgam fillings has recently been discussed (*2*, *4*, *5*). The initial toxic effects of Hg^0 are observed in the nervous system and later nephrotoxicity develops (*1*). Hg^0

is rapidly oxidized to Hg^{2+} in erythrocytes or in tissues (*1*, *4*); thus, the physiological effects of exposure to either of these two forms of mercury are somewhat similar.

The kidneys are the primary target organ of deposition and toxicity of Hg^{2+} (*1*, *6*). Exposure to Hg^{2+} induces metallothionein synthesis followed by the accumulation of this heavy metal-binding protein and mercury in the kidney (*7*). Hg^{2+} provokes renal injury by damage to proximal tubule cells (*6*). Hg^{2+} also causes strong immunological effects (*5*, *8*, *9*). Repeated exposure of rats to low doses of mercuric chloride resulted in a number of autoimmune responses (*9*).

Wide public, research, and regulatory interest has been focussed on the toxicity of CH_3Hg^+ since the tragic outbreaks of human poisoning in Japan ("Minamata disease") and Iraq. The highly potent neurotoxicity of CH_3Hg^+ is due, in part, to its lipophilicity, which enables it to pass the blood-brain barrier (*1*). CH_3Hg^+ has been known to produce selective damage to the nervous system in adults and, in the case of prenatal exposure, mental impairment in newborn infants (*6*, *10*). CH_3Hg^+ is able to cross the placental barrier and has been found in higher levels in the fetus than in the mother (*10*). CH_3Hg^+ is formed by microbes from inorganic mercury in natural waters and bioconcentrates in fish and other aquatic animals. Prolonged consumption of mercury-contaminated seafood results in the neurological symptoms of CH_3Hg^+-poisoning (*10*, *11*). CH_3Hg^+ also has nephrotoxic effects (*1*)

Analysis of Mercury

Mercury analysis and chemical speciation have been extensively reviewed (*12-18*). Mercury speciation often means that only the amounts of Hg^{2+} and CH_3Hg^+ are determined because these two forms of mercury are toxicologically very important and usually the most common in environmental and biological samples. Hg^{2+} and CH_3Hg^+ are frequently identified as inorganic and organic mercury, respectively, for the same reasons. Most conventional methods used for the determination of trace and ultratrace mercury levels require costly and sophisticated instruments and are not readily adaptable for inexpensive laboratory screening of large numbers of samples nor for on-site analyses. In addition, most mercury speciation methods are tedious to apply (*15*).

Several immunoassays have recently been reported which can detect Hg^{2+} (*11*, *19*, *20*) and other metal ions (*21-23*) either in chelated or free form. Antibodies used in these assays were elicited against chelates of the target metals (*11*, *19-23*). These immunoassays open new avenues in the environmental monitoring of hazardous metals due to their high sample throughput and because they may be formatted as sensors for *in situ* analysis. These selective and sensitive assays employ highly specific monoclonal antibodies, possessing high affinity for the target analyte, which are expensive to develop.

Materials and Methods

Safety Note. Carbon disulfide, nitric acid, sodium sulfide, as well as numerous heavy and noble metal salts are hazardous. Care has to be exercised while handling and disposing of these chemicals (*24-26*). Safety precautions, compiled in the literature (*24-26*), are to be followed when dealing with very toxic mercury

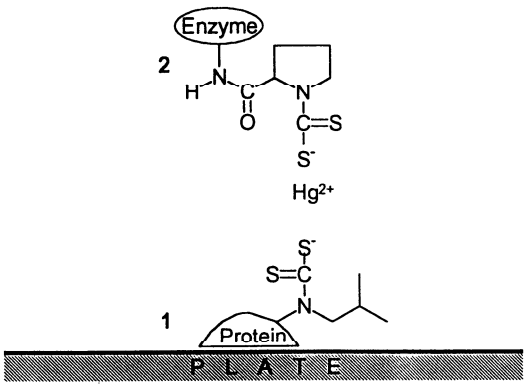

Figure 1. Chemical structures of the reagents, 1-CONA and 2-AP, involved in assay format 1.

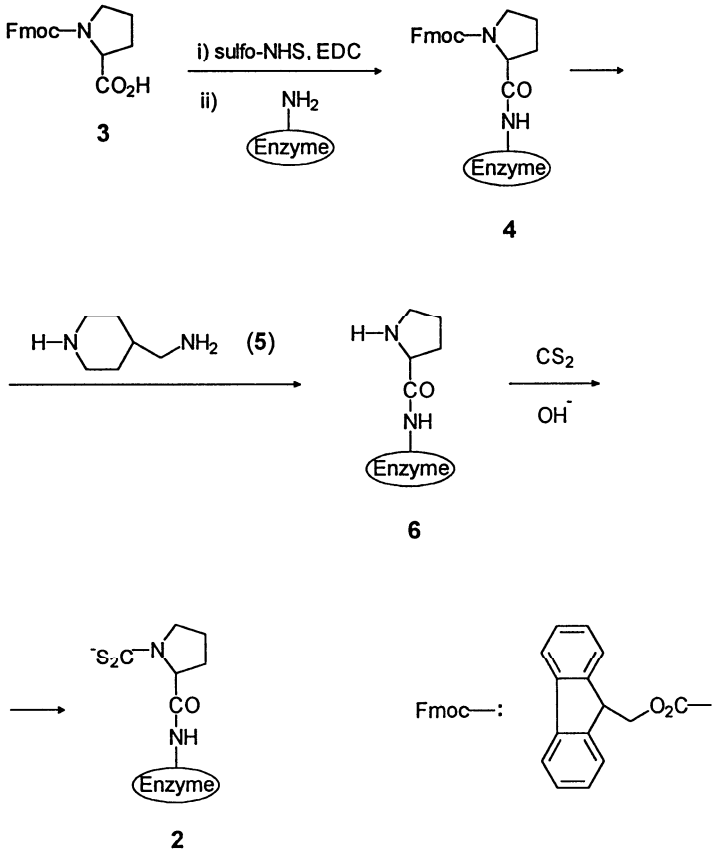

Figure 2. Synthesis of 2-AP, the chelator linked to the reporter enzyme.

derivatives especially organomercurials. Sodium sulfide is an effective reagent for separation of heavy metal salts from wastewaters by sulfide precipitation (*27, 28*). This compound should not be mixed with acidic solutions because highly poisonous hydrogen sulfide may be formed (*24-26*). [Work with this salt and with carbon disulfide, nitric acid, and volatile organomercurials should be done in a laboratory fume hood (*24-26*).] The pH-value of the mixture of sodium sulfide solution and waste should be greater than 8 to achieve optimal cleanup efficiency (*27*). Other methods (e.g., treatment with ion-exchange resins, thioacetamide, thiourea) to eliminate mercury from wastes before disposal are also recommended in the literature (*29-30*).

New synthesis of tracer 2-AP. (See Figures 1, 2.) This synthetic pathway provided us with a simple tool, UV-spectroscopy, to verify the complete removal of the blocking groups of intermediate 4-AP; thus, the formation of conjugate with secondary amino-groups (6-AP) (*31*). The *N*-(9-fluorenylmethyloxycarbonyl) (Fmoc) protecting group was cleaved by 4-(aminomethyl)piperidine (5) in aqueous solution with only minor loss of enzymatic activity (*31*). Intermediate 4-AP was obtained using the efficient reagent combination of *N*-hydroxysulfosuccinimide (sulfo-NHS) and water soluble carbodiimide (EDC, Figure 2) (*32*).

Assay Procedures. The assays were performed according to the usual solid phase immunoassay methods, the protocols were similar to those routinely used in our laboratory (*33, 34*). Features specific to the mercury assays are indicated below. Spectramax 250 microplate reader (Molecular Devices, Menlo Park, CA) and Softmax Pro (Molecular Devices) software package were used to construct the standard curves, based on Rodbard's four-parameter logistic method (*35*), and for interpolation of unknown sample concentrations. Aqueous solutions were prepared with nanopure water (16.7 megohm/cm) obtained from Sybron/Barnstead Nanopure II system (Barnstead Co., Newton, MA). Trace amounts of heavy metals were removed from buffers used in the assay work by Chelex 100 ion exchange resin (100-200 mesh, sodium form, Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Glassware and non-disposable plasticware (e.g., containers for buffers, troughs) were kept in 20% nitric acid at room temperature overnight, followed by thorough rinsing with nanopure water (*36, 37*). Special care was taken to completely eliminate nitric acid because it might interfere with the assay. Microtiter plates (96-well Nunc-Immuno Plate, Nunc InterMed, Denmark) and metal-free micro-pipet tips were used without soaking in acid. Between incubation steps, plates were washed four times with sodium/acetate buffer (0.1 M acetate, pH 5.5).

Assay Format 1. (See Figures 1, 3.) Conjugate 1-CONA, dissolved in a sodium/carbonate/bicarbonate buffer (50 mM carbonate/bicarbonate, pH 9.6), was immobilized on the wells of a microplate. The wells were washed, incubated with the mixture of the tracer (2-AP) and mercury standard or sample in a sodium/acetate buffer (0.1 M acetate plus 0.05% Tween 20, pH 5.5), and washed again. The wells were treated with the solution of the enzyme substrate (*p*-nitropheny phosphate), and the optical density (OD) values measured following the standard protocol (*34*).

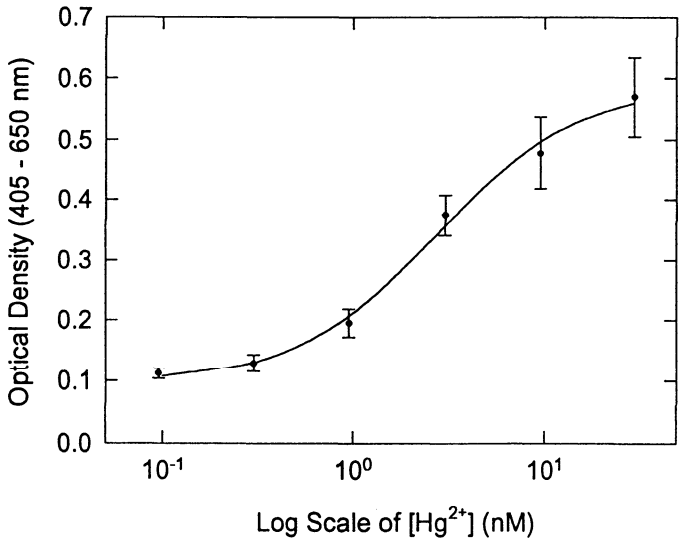

Figure 3. Standard curve of assay format 1 with simultaneous incubation.

Assay Format 2B. (See Figures 4, 5.) The wells of a microtiter plate, coated with conjugate 7-CONA as above, were washed and incubated with the mixture of the tracer (2-AP) and mercury standard or sample in a sodium/acetate buffer (0.25 M acetate plus 0.05% Tween 20, pH 6). After a washing step, the color reaction was performed and the absorbance values were determined as described above.

Results and Discussion

We have utilized the high avidity of sulfur-containing ligands to mercurials and the high sample throughput of the ELISA methodology in the development of sensitive, selective, and rapid assays for Hg^{2+} ion. We have used dithiocarbamate ligands instead of antibodies for the recognition of mercury. Dithiocarbamates show high affinity to a number of heavy metal ions, but Hg^{2+}-bis(dithiocarbamate) complexes have exceptionally high thermodynamic stability (*38*); Hg^{2+} substitutes most other metal ions from their dithiocarbamate chelates (*39*). Only a few noble metal ions (e.g., Au^{3+}) display affinities for dithiocarbamates similar or greater than that of Hg^{2+} (*40*). Organomercurials with the general structure of RHg^{+} (R: alkyl or aryl) also form stable mono(dithiocarbamate)-chelates (*41*). Dithiocarbamates obtained from secondary amines are known to be fairly chemically stable; thus, we employed these chelators in our assays.

First Assay Format (Sandwich Complex Assay). In our first assay format (format 1, Figure 1) the target ion forms a sandwich complex or chelate with two complexing agents. Chelator 1-CONA (Figure 1) is a protein conjugate with dithiocarbamate groups. This chelator (1-CONA) is coated on the wells of a microtiter plate. In the structure of reagent 2-AP (Figure 1), the chelator groups are covalently bound to an enzyme. In this work, we used conalbumin (CONA) as carrier protein and alkaline phosphatase (AP) as reporter enzyme (*31*). The activity of AP is only marginally diminished by trace amounts of Hg^{2+} (*42*). To furnish the protein- and enzyme-linked dithiocarbamates, secondary amino groups were formed on the surface of these macromolecules, the intermediates (e.g., 6-AP) were then reacted with carbon disulfide (Figure 2). The preparation of the immobilized chelator (1-CONA) and enzyme-conjugate 2-AP was previously reported (*16*). An improved synthesis of tracer 2-AP (Figure 2) and a modified assay protocol with simultaneous incubation resulted in remarkably better assay performance (*31*). The ionic strength, pH, and detergent concentration of the assay buffer were optimized. Conditions promoting formation of insoluble or weakly solvated material with Hg^{2+}, such as high pH-values, may substantially reduce the signal. Inappropriate Tween 20 concentrations also adversely influence the assay (*16*). The chelator would decompose under strongly acidic conditions. Our results demonstrate that Hg^{2+} ion can be selectively detected at high ppt/low ppb concentrations by this assay (format 1, Figures 1 and 3) (*31*). Figure 3 shows the low-concentration part of the standard curve with an OD_{50} value of 2.7 ± 0.5 nM (0.54 ± 0.1 ppb) Hg^{2+} concentration. (OD_{50} is the analyte concentration at the point of the standard curve where the optical density is the half of the maximum OD minus OD at zero concentration.) The limit of detection (LOD) was 0.375 nM (75 ppt). (LOD is the analyte concentration at the

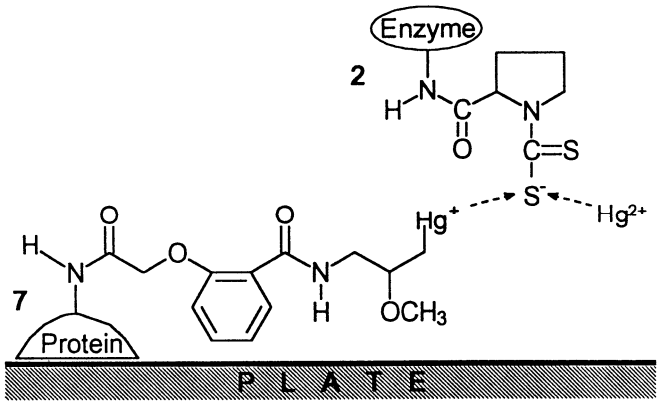

Figure 4. Chemical structures of the reagents, **2**-AP and **7**-CONA, involved in assay format 2B.

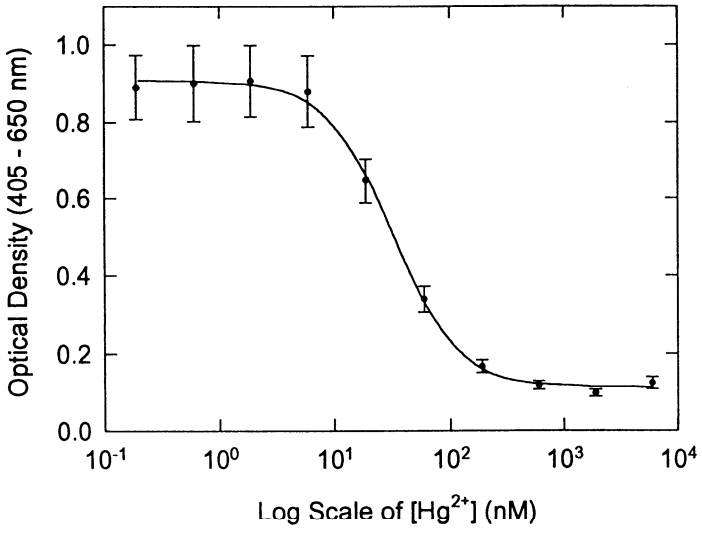

Figure 5. Standard curve of assay format 2B.

point of the standard curve where the OD is the sum of the signal at zero concentration and three times the standard deviation of the signal at zero concentration.) At very high concentrations of Hg^{2+}, a "hook" effect occurs, the signal decreases with increasing concentration, as it is usual with two site immunoassays (*16*). Standard curves were also constructed with a number of foreign ions up to 3000 nM concentrations. Most cations (e.g., ions of alkaline metals, Al^{3+}, Ca^{2+}, Cd^{2+}, Cr^{3+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}) and hexavalent chromium ($Cr_2O_7^{2-}$) demonstrated minimal interference. Ag^+ (7%), Au^{3+} (0.2%), Cu^{2+} (2%), Pd^{2+} (29%), and Zn^{2+} (0.3%) ions displayed some cross-reactivity (CR). The standard curve of CH_3Hg^+ slightly fell below background level at high concentrations which may be due to the competition of CH_3Hg^+ with minute amounts of interfering metal ions contaminating the reagents and buffers used in the assay. An application of this assay, optimized for high-sensitivity detection of Hg^{2+}, could be the determination of the total mercury content of environmental water samples after oxidation of all mercury species to Hg^{2+}. However, CH_3Hg^+ gave essentially background-signal when different assay protocols were employed (*16*, *31*), which makes mercury speciation possible by an indirect method (CH_3Hg^+ = total Hg - Hg^{2+}).

Second Assay Format (Competitive Assay). Our second assay format is based upon competition between mercuric ions and an organomercury-conjugate to bind to a chelating conjugate (format 2, Figure 4). One arrangement of this assay format with a mercury-linked enzyme tracer and an immobilized chelator (format 2A) was extensively characterized (*16*, *31*) and is currently under development for application to the analysis of environmental samples. The opposite arrangement using the same enzyme labeled chelator (2-AP) as seen in format 1 and an organomercury compound bound to the plate coating protein (7-CONA, format 2B), has also been investigated. The immobilized mercury reagent (7-CONA) was obtained from the corresponding acid by the sulfo-NHS/EDC-reagent (*32*). The Hg^{2+}-standard curve is presented in Figure 5; the IC_{50} value is about 30 ± 2 nM (6 ± 0.4 ppb). There was no significant interference from a panel of metals, similar to one studied with format 1, including Ni^{2+} and Zn^{2+} up to at least 300 nM using this assay; however, Au^{3+} (CR: 11%) and CH_3Hg^+ (CR: 14%) cross-reacted.

Conclusions

We have shown that our new, chelation-based rapid assays can be useful analytical tools for sensitive and selective determination of mercury and mercury speciation. Low-ppt levels of mercury in lakes and oceans and less than 1 ppb of mercury in rivers are commonly found. The regulatory maximum of the total mercury concentration in environmental water samples is 1 ppb in numerous countries (*16*), but the amount of mercury in river water sometimes exceeds this limit due to industrial and urban waste water and mine runoff. Thus, an anticipated use of our assay systems is the screening of mercury in water. Promising studies with environmental water samples are in progress. Analysis of mercury content in biological samples is another possible application. The action level of the U.S. Food and Drug Administration for mercury in fish is 1 ppm (*43*). The sensitivity of some of our assays seems adequate for the detection of low-ppm amounts of mercury in

fish after acidic-oxidative digestion of the sample matrix and appropriate buffering and dilution of the resulting corrosive solution (*11*). Application of our assays for real samples and development of further assay systems and formats (e.g., sensors) are the directions of our present and future work.

Acknowledgements

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