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# A Blue Fluorescent Antibody–Cofactor Sensor for Mercury

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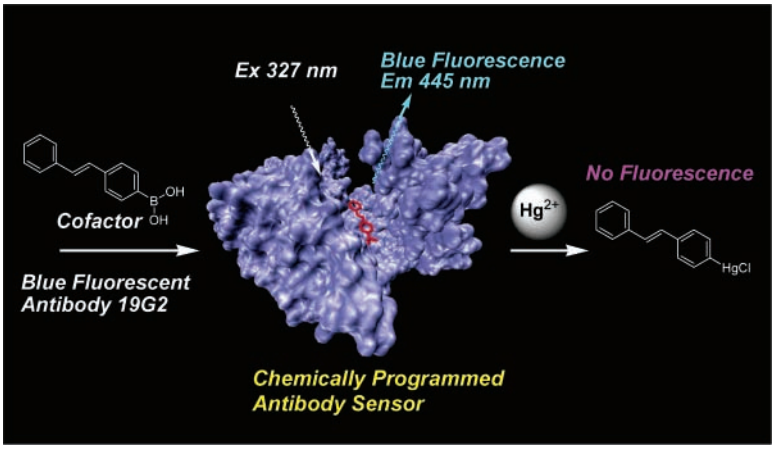
## ABSTRACT




**A chemically programmed antibody sensor, consisting of a stilbenyl boronic acid cofactor and monoclonal antibody EP2–19G2, provides a new method of mercury detection. The fluorescent antibody sensor generates an intense powder blue fluorescence when bound to the stilbenyl boronic acid cofactor; however, it is quenched in the presence of $Hg^{2+}$ ions. The EP2–19G2-cofactor biosensor provides micromolar sensitivity and selectivity toward $Hg^{2+}$ ions over a wide range of metal ions in aqueous solution.**

Contamination with heavy metal ions poses risks for human health. Mercury, in particular, is a highly toxic element and can cause a wide variety of symptoms, including digestive, cardiovascular, and especially neurological diseases.[1] Despite its toxicity, mercury and mercuric salts are still used in a large number of industrial processes and products.[2] Development of new tools for detecting $Hg^{2+}$ has attracted much attention recently.[3] A number of mercury sensors based on azacrown ethers,[4] dithio-aza compounds,[5] calixarene-based ionopheres,[6] DNA,[7] MerR family proteins,[8] fluorescent dye-doped crystalline complexes,[9] and others[10] have been re-

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ported. One major challenge involves the creation of $Hg^{2+}$ sensors that function in water and are highly selective for $Hg^{2+}$ against a background of competing analytes.[5d,9]

Recently, we have reported a series of monoclonal antibodies (mAbs, e.g., mAb EP-2−19G2), prepared against a *trans*-stilbene hapten EP-2 (**1**) (Figure 1), in which the

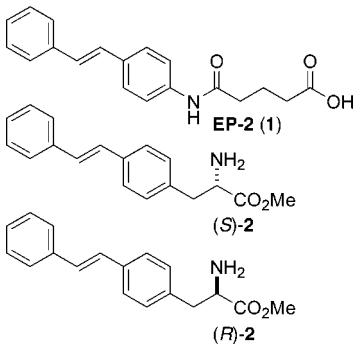


**Figure 1.** Structures of stilbene hapten **1** and ligands (*S*)- and (*R*)-**2**.

19G2−**1** complex emits a powder blue fluorescence with high quantum yield ($\lambda_{exc}$ = 327 nm, $\lambda_{em}$ = 410 nm, $\Phi_f$ = 0.78).[11] The remarkable change in emission occurs over a narrow temperature window, between 240 and 260 K, and temperature-dependent excited-state dynamics have been proposed to explain this unusual fluorescent emission.[12] The novel immunochemistry of 19G2 complexation to stilbene **1** was demonstrated by the introduction of pro-fluorescent tags on nucleosides,[13] DNA,[14] or the cowpea mosaic virus coat protein.[15] We also discovered that each of the chiral *trans*-stilbene amino acid esters (*S*)-**2** and (*R*)-**2** could bind to 19G2 ($K_d$ = 5.2 and 46 $\mu$M, respectively), but only 19G2−(*S*)-**2** afforded a blue fluorescence.[16] Using this fluorescent biosensor, a high throughput screening method of a chiral phase transfer library used for the synthesis of (*S*)-**2** and (*R*)-**2** was demonstrated.[16,17] Hence, it occurred to us that chemical modification of the stilbene moiety in the combining site of 19G2 would result in changes in excited energy surface and fluorescent emission. Our hypothesis is that stilbene derivatives that bind $Hg^{2+}$ via covalent or noncovalent bonds could result in altered fluorescent properties, through changes in antibody paratope conformation, affinity, or perturbation/stabilization of the excited state of the stilbene moiety in the complex. Such a complex between 19G2 and a stilbene derivative could provide a convenient biosensing method for $Hg^{2+}$ ions based on fluorescence; as such, the stilbene derivative would function in a manner analogous to the way a cofactor confers a certain functionality to an enzyme. Here, we present experimental details of this conceptually new chemically programmed antibody sensor for $Hg^{2+}$.[18]

Stilbene derivatives with various functionalities (**3**−**9**, Figure 2) and potential degrees of affinity for mercury were

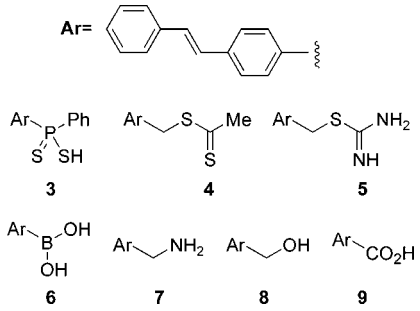


**Figure 2.** Structures of stilbene derivatives.

designed and synthesized. The functional moieties in these ligands, such as the phosphinodithioic acid in **3**,[5a] the thio ester in **4**[9] and the thiourea group in **5**[19] are known to coordinate $Hg^{2+}$ ion. Arylboronic acid **6**[20] was expected to undergo transmetalation with $Hg^{2+}$ to form aryl mercuric chloride.[21] These structures, along with amine **7**,[22] alcohol **8** (Aldrich), and carboxylic acid **9**[23] were each mixed with 19G2 and florescence intensities ($\lambda_{exc}$ = 327 nm, $\lambda_{em}$ = 410 nm) were measured in the presence and absence of $Hg^{2+}$ (0, 1 and 10 equiv, [19G2] = 10 $\mu$M (20 $\mu$M binding sites), [stilbene derivatives] = 20 $\mu$M, in PBS (10 mM sodium phosphate, 10 mM NaCl, pH 7.4) with 5% DMF as cosolvent.

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From all of the structures, only **1**, **6**, and **8** gave rise to blue fluorescence ($\lambda_{exc}$ = 327 nm, $\lambda_{em}$ = 410 nm) in the presence of 19G2 (Figure 3). The 19G2−EP-2 (**1**) complex

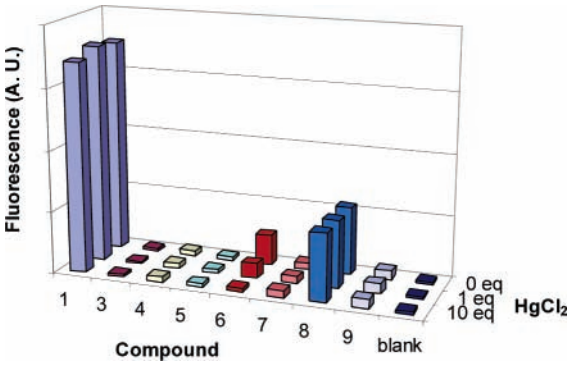



**Figure 3.** Fluorescence emission of 19G2 and stilbene derivatives in the presence of $Hg^{2+}$ ions. Compound numbers correspond to the structures in Figures 1 and 2. [19G2] = 10 $\mu$M, [stilbene derivatives] = 20 $\mu$M, [$Hg^{2+}$] = 0, 10, 100 $\mu$M in PBS (10 mM sodium phosphate, 10 mM NaCl, pH 7.4) with 5% DMF cosolvent. $\lambda_{exc}$ = 327 nm, $\lambda_{em}$ = 410 nm.

gave the most intense fluorescence, and its intensity was not influenced by the addition of $Hg^{2+}$. The 19G2-stilbenyl alcohol **8** complex displayed blue fluorescence, and as with 19G2−**1**, its intensity was not affected by $Hg^{2+}$. The complex between 19G2 and **6**, however, showed moderate fluorescence intensity, and it decreased dramatically with the addition of $Hg^{2+}$. From this we concluded that the 19G2−**6** complex can chemoselectively respond to the addition of $Hg^{2+}$ ions.

Fluorescence spectra of the 19G2−**6** complex ([19G2] = 10 $\mu$M, [**6**] = 20 $\mu$M) in the presence of $Hg^{2+}$ are shown in Figure 4. The excitation maximum was blue-shifted, while the fluorescence emission of 19G2−**6** displayed a red shift ($\lambda_{exc}$ = 300 nm, $\lambda_{em}$ = 445 nm) and also decreased with the addition of $Hg^{2+}$. When the concentration of $Hg^{2+}$ ion was increased up to 80 $\mu$M (4.0 equiv relative to **6**), which include 1.2 $\mu$g of $HgCl_2$, 93% quenching of the initial fluorescence was observed.

The emission of the 19G2−**1** complex ($\lambda_{exc}$ = 327 nm, $\lambda_{em}$ = 410 nm) was not affected when $Hg^{2+}$ ions were added up to 10 equiv (200 $\mu$M). The uncomplexed boronic acid cofactor **6** generates a 4 times lower emission ($\lambda_{exc}$ = 300 nm, $\lambda_{em}$ = 360 nm) compared to that of 19G2−**6**, and the intensity was decreased along with the addition of $Hg^{2+}$ ion. 4-Biphenylboronic acid generates strong fluorescence with a maximum of 300 nm when irradiated at 260 nm, and also, a quenching of the fluorescence intensity was observed upon addition with $Hg^{2+}$. When 2.0 equiv of $Hg^{2+}$ was added, up to 60% of fluorescence decrease was observed.[24] These results suggest that aryl boric acid chromophores chemoselectively respond to $Hg^{2+}$ ions with a decrease in fluorescence emission.

(24) See the Supporting Information.

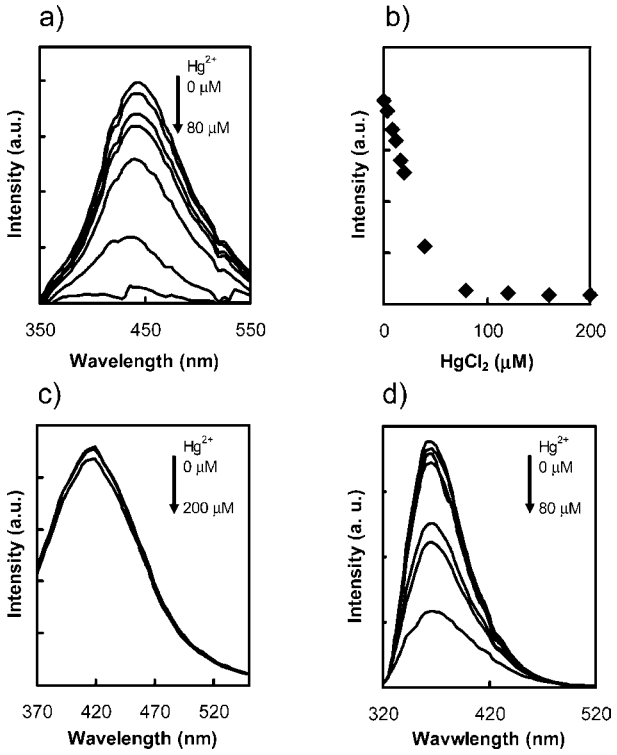
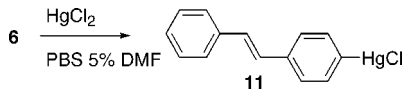



**Figure 4.** Changes in fluorescence intensity of 19G2−**6**, 19G2−**1**, and **6** as a function of $Hg^{2+}$ ions. (a) Fluorescence spectra of 19G2−**6**. 19G2 fluorescence response of 19G2−**6** to addition of $Hg^{2+}$ in 60 $\mu$L of PBS (10 mM sodium phosphate, 10 mM NaCl, pH 7.4) with 5% DMF cosolvent. [19G2] = 10 $\mu$M. [**6**] = 20 $\mu$M. $\lambda_{exc}$ = 300 nm. (b) Fluorescent spectra of 19G2−**6** upon addition of $Hg^{2+}$ ion (0, 4, 8, 12, 20, 40, 80 $\mu$M). $\lambda_{exc}$ = 300 nm, $\lambda_{em}$ = 445 nm. (c) Fluorescent spectra of 19G2−**1** upon addition of $Hg^{2+}$ ion (0, 10, 20, 200 $\mu$M) in 60 $\mu$L of PBS with 5% DMF cosolvent. [19G2] = 10 $\mu$M. [**1**] = 20 $\mu$M. $\lambda_{exc}$ = 327 nm. (d) Fluorescence response of **6** upon addition of $Hg^{2+}$ ion (0, 4, 8, 12, 20, 40, 80 $\mu$M) in 300 $\mu$L of PBS with 5% DMF cosolvent. [**6**] = 20 $\mu$M. $\lambda_{exc}$ = 327 nm.

LC−MS analysis, using a synthetic sample of **11** as standard, unambiguously confirmed that boronic acid **6** quantitatively reacts with $HgCl_2$ to form the nonfluorescent organomercurial species **11** under the fluorescence measurement conditions (Scheme 1), both in the absence and

**Scheme 1**




presence of 19G2. The titration of the 19G2−EP2 (**1**) complex with synthetic **11** suggested that **11** binds to the 19G2 combining site, although its affinity is significntly perturbed.[25] Consequently, we believe that the overall

(25) 5000 equiv of **11** decreased the fluorescence intensity of the 19G2−EP2 (**1**) complex by ~50%. See the Supporting Information.

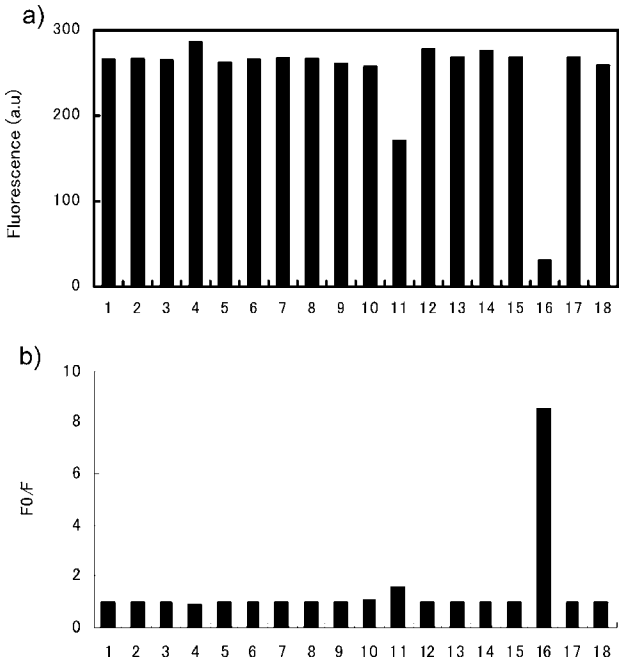



**Figure 5.** Fluorescence response of 19G2−**6** to various cations (10 equiv) in PBS with 5% DMF cosolvent. [19G2] = 10 $\mu$M. [**6**] = 20 $\mu$M. $\lambda_{exc}$ = 300 nm, $\lambda_{em}$ = 445 nm. (a) Fluorescence intensities in the presence of 200 $\mu$M of cation of interest: 1, $Li^+$; 2, $Na^+$; 3, $K^+$; 4, $Mg^{2+}$; 5, $Ca^{2+}$; 6, $Cs^+$; 7, $Cr^{2+}$; 8, $Fe^{2+}$; 9, $Co^{2+}$; 10, $Ni^{2+}$; 11, $Pb^{2+}$; 12, $Pt^{2+}$; 13, $Cu^{2+}$; 14, $Zn^{2+}$; 15, $Cd^{2+}$; 16, $Hg^{2+}$; 17, $Pb^{2+}$; 18, blank. (b) Quenching ratio of fluorescence intensities of 19G2−**6** upon the addition of 10 equiv of metal ions. The response is normalized with respect to the free 19G2−**6** complex.

fluorescence decrease seen occurs through the formation of **11**, and thus most likely through a decrease in affinity of **11** to 19G2, as well as electron transfer from the excited stilbene−19G2 complex to the proximate mercury atom, which thus quenches the fluorescence emission seen.

To investigate the metal selectivity of the antibody−cofactor complex, the fluorescence response of 19G2−**6** to various metal ions ($Li^+$, $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $Cs^+$, $Cr^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Pd^{2+}$, $Pt^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Pd^{2+}$) was investigated. A panel of metal chlorides (66.7 $\mu$M, 10 equiv) was mixed with 19G2−**6** (6.67 $\mu$M) and irradiated ($\lambda_{exc}$ = 327 nm, $\lambda_{em}$ = 410 nm). The results (Figure 5) implied that the 19G2−**6** complex showed high selectivity toward $Hg^{2+}$ over other metal ions tested in aqueous solution. The group 2 metals $Zn^{2+}$ and $Cd^{2+}$, in addition to $Cr^{3+}$ and $Pb^{2+}$ do not quench the fluorescent response of 19G2-**6**. Interestingly, of the transition metals considered, only $Pd^{2+}$ decreased the fluorescence (65%).

In conclusion, we have presented a cofactor approach, employing **6** in combination with blue fluorescent antibody 19G2, to detect $Hg^{2+}$ ions. This unique chemically programmed biosensor 19G2−**6** consists of stilbenyl boronic acid cofactor **6** as a $Hg^{2+}$ ligand and blue fluorescent antibody 19G2 as a fluorescent signal tuner and amplifier. The method is of general interest for the following reasons. (1) It is relatively sensitive with a detection limit lower than 1.2 $\mu$g, which is four times less than the estimated average daily intake of inorganic mercury (4.3 $\mu$g).[2] (2) It is selective toward $Hg^{2+}$ ion over a wide range of other metal ions in aqueous solution. (3) It is convenient, as the blue fluorescence change can be readily recognized by the naked eye. Finally, boronic acids are known to complex small molecules such as sugars, diols, and amino alcohols;[26] hence, further applications of the 19G2−**6** complex for the detection of such molecules are underway and will be reported in due course.

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**Supporting Information Available:** Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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