Mercury has played a rich role in the field of bioanalytical chemistry, as witnessed by the number of diverse mercury-based applications found throughout the literature. For example, mercury's unique electronic properties have proved to be useful in designing electrochemical-based trials assay methods [1,2]. Mercury metal has aided electron microscopy characterization of proteins [3] and has been incorporated into fluorescent peptide and protein tags [4]. More recently, chemical and fluorescent sensors have been fabricated to detect mercury and other metal species [5–8], and numerous nanotechnology methods have emerged [9,10]. There has been interest in developing anti-mercury monoclonal antibodies (mAbs) [3] for their potential use as diagnostic tools capable of providing highly specific, inexpensive, easy-to-use mercury detection assays [11–14]. Mercury metal, mercury salts, and organomercury compounds are ubiquitous and persistent environmental toxins, and the associated health hazards to humans, mammals, and aquatic systems are of keen interest [15–18]. Despite the growing advancements in sophisticated instrumental techniques that are capable of detecting heavy metals at exceedingly low concentrations [19–21], there remains interest in the development of easy-to-use immunoassays that provide efficient, cost-effective, and sensitive mercury detection.

The preparation of metal-containing bioconjugates for the purpose of generating antibodies capable of binding metals and metal ions has been the focus of numerous research efforts for more than 20 years [22–28]. Because metals, like many low-molecular-weight haptenes, are not immunogenic, one common approach taken to generate metal-based immunogenic species has been to covalently attach ethylenediaminetetraacetic acid (EDTA) or other structurally similar metal chelating agents to protein surfaces [22–26]. The chelating moiety facilitates encapsulation of metals in a cage-like structure on the carrier's surface via formation of coordination bonds between the chelate's electron-rich atoms and the electron-deficient metal. The earliest examples of bioconjugates prepared from metal chelation methodologies have resulted in several useful substrates. Although some of these earlier bioconjugates did not yield antibodies with adequate binding specificity for analytical assays, metal chelate bioconjugates have found practical applications as metal-labeled therapeutic antibodies [23,29], as magnetic resonance imaging substrates [30,31], and in radioimaging techniques [32]. One recent extension of the EDTA chelation methodology has been the synthesis of a fluorescent optical sensor designed to detect Hg^{2+} ions [33]. In this

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**Abbreviations used:**
- mAb, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid; KII, keyhole limpet hemocyanin; HgCl\textsubscript{2}, mercuric chloride; BSA, bovine serum albumin; IgG, immunoglobulin G; HRP, horseradish peroxidase; SMCC, succinimidyl 4-[N-maleimidomethyl]-cyclohexan-1-carboxylate; 2-IMT, 2-iminothiolane; BS\textsubscript{2}, bis(sulfosuccinimidyl)suberate; OPD, o-phenylenediamine; BOC, di-tert-butyl dicarbonate; ELISA, enzyme-linked immunosorbent assay; ICP, inductively coupled plasma; NMR, nuclear magnetic resonance; TMS, trimethylsilyl; RT, room temperature; TLC, thin-layer chromatography; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; GaM–HRP, HRP-labeled goat anti-mouse IgG; MALDI–MS, matrix-assisted laser desorption/ionization–mass spectrometry; UV, ultraviolet; TiNBS, trinitrobenzene sulfonate; NaBH\textsubscript{4}, sodium borohydride; Hg(NO\textsubscript{3})\textsubscript{2}, mercuric nitrate; HgCl\textsubscript{2}, mercuric chloride; BSA, bovine serum albumin; IgG, immunoglobulin G; HRP, horseradish peroxidase; SMCC, succinimidyl 4-[N-maleimidomethyl]-cyclohexan-1-carboxylate; 2-IMT, 2-iminothiolane; BS\textsubscript{2}, bis(sulfosuccinimidyl)suberate; OPD, o-phenylenediamine; BOC, di-tert-butyl dicarbonate; ELISA, enzyme-linked immunosorbent assay; ICP, inductively coupled plasma; NMR, nuclear magnetic resonance; TMS, trimethylsilyl; RT, room temperature; TLC, thin-layer chromatography; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; GaM–HRP, HRP-labeled goat anti-mouse IgG; MALDI–MS, matrix-assisted laser desorption/ionization–mass spectrometry; UV, ultraviolet; TiNBS, trinitrobenzene sulfonate; NaBH\textsubscript{4}, sodium borohydride; Hg(NO\textsubscript{3})\textsubscript{2}, mercuric nitrate;
work, Hg^{2+} ions are trapped in a cage-like structure arising from a porphyrin dimer. Preliminary work has shown that the chemical sensor is capable of detecting Hg^{2+} at concentrations of 10^{-5}–10^{-7} M with high degrees of specificity preferentially over other divalent metal ions.

Currently, there are only a few examples of protocols that have yielded metal-binding antibodies arising from methodologies other than chelate complexation. Benkovic and coworkers chemically modified the binding site of a catalytic antibody to accommodate a tripeptide of glutamic acid, cysteine, and glycine, to keyhole limpet hemocyanin (KLH) via carbodiimide chemistry, mercuric chloride (HgCl2) was attached to the glutathione-modified carrier by sulfur-mediated ligand exchange. The resulting immunogen yielded antibodies capable of detecting low concentrations of mercury(II) salts in water.

Our interest in this area prompted us to design and prepare a synthetic organomercury hapten amenable to protein conjugation methods, with the goal of generating an antibody capable of binding both Hg(II) metal and organometallic forms of mercury in a variety of sample media. Our overall strategy employed the intramolecular oxymercuration reaction to synthesize a structurally simple, but chemically robust, organomercury hapten that was readily covalently linked to carrier proteins through an available amine, a strategy that is quite unique compared with previously described chelation methods. In this study, the highly versatile mouse anti-mercury antibody afforded by applying our strategy is a particularly potent, resulting in a versatile and highly sensitive mercury detection system.

**Materials and methods**

**Reagents and materials**

Bovine serum albumin (BSA), chicken immunoglobulin G (IgG), phosphate salts, and Tween 20 were purchased from Sigma (St. Louis, MO, USA). Goat anti-mouse IgG labeled with horseradish peroxidase (HRP) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Succinimidyl 4-[N-maleimidomethyl]-cyclohexan-1-carboxylate (SMCC), 2-iminothiolane (2-IMT), bis[sulfosuccinimidyl]suberate (BS3), O-phenylidiamine (OPD), and stable peroxide buffer were purchased from Pierce (Rockford, IL, USA). Analytical-grade solvents, CDC13, Hg(NO3)2, allylamine, di-tert-butyl dicarbamate (t-BOC), 1,2-pentanediol, and all other organic reagents were purchased from Aldrich (Milwaukee, WI, USA) and were used without further purification. Enzyme-linked immunosorbent assays (ELISAs) were run on BD Falcon 96-well, flat-bottomed polystyrene plates purchased from Fisher Scientific (Pittsburgh, PA, USA).

The toxicological properties of the organomercury compounds prepared in this study have not been characterized. All mercury-containing compounds were handled using standard safety guidelines that included protective clothing, safety goggles, and double gloves. Prior to spectral characterization, the synthetic organomercury intermediates prepared in this study were reduced with NaBH4 to avoid excessive handling of mercury-containing substrates whenever possible.

**Apparatus**

The plate reader used in this study was a Bio-Tek Instruments model EL800. The inductively coupled plasma (ICP) spectrometer was a PerkinElmer Optima 2000 OES. 1H nuclear magnetic resonance (NMR) and 13C NMR were recorded on a Varian EM360L (60 MHz) using tetramethylsilane (TMS) as an internal standard. Flash column chromatography was carried out on a silica gel column (32–63 mm particle size) at an elution rate of 2 inches/min [35].

**Synthesis of haptenes and bioconjugation procedures**

1. **t-Butyl allyl carbamate (2)**

   This was prepared as described in Ref. [36].

2. **t-Butyl N-[2-(chloromercurio)-3-[(1-hydroxypentane-2-yl)oxy]propyl] carbamate (3)**

   Mercuric nitrate (162 mg, 0.50 mmol) was added to an oven-dried round-bottomed flask under an N2 purge. After dissolving the Hg(NO3)2 in 3.0 ml of dry CH3CN, a solution of (76 mg, 0.50 mmol) dissolved in 1.0 ml of dry CH3CN was added to the flask, resulting in a bright yellow solution. After stirring for 15 min at room temperature (RT), a yellow solid formed and thin-layer chromatography (TLC) indicated that the starting material (2) was consumed. A solution of 1,2-pentanediol (105 μl, 0.98 mmol) dissolved in 2.0 ml of dry CH3CN was added to the flask, and the mixture was stirred overnight under N2 at RT. The reaction was quenched with brine and allowed to stir for 3 h at RT to generate the more stable mercuric chloride. The resulting mixture was extracted with CH2Cl2. The organic fractions were concentrated in vacuo, and the resulting yellow oil was passed through a thin column of silica gel while eluting with CH2Cl2. No attempt was made to separate the regioisomeric products.

3. **t-Butyl N-[3-[(1-hydroxypentane-2-yl)oxy]propyl] pentane-1-ol (5)**

   Crude 3 (0.50 mmol) in 2.0 ml of CH2Cl2 was reduced with 37 mg (1 mmol) of sodium borohydride (NaBH4) dissolved in 1.0 ml of 0.2 M NaOH. Within minutes, while stirring at RT, the reaction mixture turned to a gray suspension, indicating mercury(II) reduction. After stirring for 15 min at RT, the crude product was filtered through Celite to remove the mercury metal formed during the reaction, and the resulting filtrate was extracted with CH2Cl2. The organic fractions were washed with brine, dried over Na2SO4, and concentrated in vacuo to yield a pale yellow oil: 1H NMR (CDCl3) δ 3.8 (overlapping multiplets, 3H, CH2, and CH3, 3.5 (t, 2H, CH2), 2.2 (m, 2H, CH-N), 1.9 (m, 2H, CH2), 1.4 (s, 9H, t-butyl), 1.45–1.30 (complex multiplet, 4H, CH2CH2O), 0.9 (t, 3H, CH3). 2-[3-Amino-2-(chloromercurio)propoxy] pentane-1-ol (5)

   Crude 3 (248 mg, 0.50 mmol) in 2.0 ml of CH2Cl2 was deprotected with 114 mg (1.0 mmol) of trifluoroacetic acid (TFA) dissolved in 2.0 ml of CH2Cl2. The reaction progress was followed by TLC. After stirring for 1 h at RT, the majority of the starting material had been consumed. Unreacted TFA and solvent were removed in vacuo to yield a viscous yellow oil, which was resuspended in CH2Cl2 and extracted with H2O. The resulting aqueous layer was basified (pH 8–9) with NaOH. The mixture was filtered and the filtrate was concentrated in vacuo to yield a yellow oil (53%), which was used in conjugation protocols without further purification.

**Preparation of solid-phase conjugates**

**Preparation of BS3 bioconjugates**

To 1.0 ml of a 10-mg/ml solution of BSA diluted in 20 mM phosphate buffer with 150 mM saline at pH 7.2 (PBS) was added
Preparation of SMCC bioconjugates

Crude hapten 1 (5.0 mg, 0.014 mmol) was placed in a vial, crude hapten 5 (6.0 mg, 0.015 mmol) was placed in a second vial, and both samples were diluted with 200 µl of PBS. To each vial was added 200 µl of 30 mg/ml (0.018 mmol) SMCC. Both mixtures were stirred at RT for 1 h. In a separate vial, 20 mg of BSA (10 mg/ml in PBS) and 1.0 ml of a 4.0-mg/ml (0.030 mmol) stock solution of 2-IMT in PBS were mixed together. After incubating for 1 h at RT, the BSA/2-IMT solution was passed through a Sephadex G-25 column using PBS as eluent. The collected BSA/2-IMT fractions were divided equally by volume between the vial containing hapten 1 and the vial containing hapten 5. Both conjugation mixtures were incubated overnight at 4 °C. Before the resulting conjugates were coated on polystyrene plates, they were passed through a Sephadex G-25 column using PBS as eluent.

Preparation of immunogen and antisera

Because we intended to use BSA as the carrier for the assay solid phase, chicken IgG was chosen as the immunogen carrier to minimize nonspecific binding. In addition, we had obtained good results immunizing mice with IgG in other work. To 500 ml of PBS, 30 mg of BSA was added. The resulting solution was stirred at RT for 2 h. Solid-phase bioconjugates were purified by size exclusion chromatography (Sephadex G-25) using PBS as eluent. Preparations were divided equally by volume between the vial containing hapten 1 and the vial containing hapten 5. Both conjugation mixtures were incubated overnight at 4 °C. Before the resulting conjugates were coated on polystyrene plates, they were passed through a Sephadex G-25 column using PBS as eluent.

Results and discussion

Synthetic organomercury hapten 1 (Fig. 1) was prepared in six steps following procedures previously established in this laboratory [36]. The hapten is stable to strong acid, mild base, and for more than 2 years in the freezer. Cross-linking reaction parameters were studied to determine the optimal coupling conditions prior to preparing bioconjugates by covalently linking hapten 1 to protein carriers. In a model study, benzylamine was coupled to BSA using BSA-B5 as a cross-linker under pH conditions ranging from 6.0 to 9.5 at varying incubation times. Between pH 7.0 and 7.5, the extent of benzylamine coupling to BSA was consistent regardless of the incubation times that were studied (30–120 min). Initially, to evaluate the feasibility of covalently coupling an organomercury substrate under the conditions evaluated in the model study, hapten 1 was cross-linked to a commercially available peptide nine amino acids in length and containing a terminal lysine. The resulting complex was studied extensively via matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS), which clearly demon-
strated that hapten 1 survived the coupling procedure with the organomercury-based hapten intact [36]. MALDI–MS techniques have been shown to be very powerful and accurate tools for analyzing complex bioconjugate structures [37–42]. For example, in a comparative study, Adamczyk et al. demonstrated that MALDI–MS can be a more accurate analytical method than either ultraviolet (UV) spectroscopy or trinitrobenzene sulfonate (TNBS) colorimetric assays [43] for determining the extent of coupling of low-molecular-weight haptons to protein carriers [39]. MALDI–MS is a sensitive ionization technique (detection limits in the low attomole range have been demonstrated) that generates primarily singly charged [M+H]+ ions for peptides and proteins [37]. MS is a sensitive ionization technique (detection limits in the low-molecular-weight haptens to protein carriers [39]. MALDI–MS techniques and Technology (NIST) mercury standards (mercuric mercury standard curve was generated using National Institute of hapten/protein ratio cannot be ascertained from ICP data. A mer-
substrates [41]. When tryptic fragments of the BSA–BS3–hapten 1 bioconjugates were characterized by MALDI–MS, it was found that 8 mol of hapten was covalently attached to 1 mol of protein. Mer-
cury was readily apparent by its characteristic isotopic MS pattern. Subsequent conjugates, including the chicken IgG–hapten 1 immunogen, were analyzed using ICP spectrometry. ICP as an ana-
lytical method was faster and easier than MALDI–MS, but it does not distinguish between free mercury and covalently bound mercury because the sample is hydrolyzed in nitric acid, thereby liber-
ating all forms of mercury as Hg2++, prior to analysis. Thus, the hapten/protein ratio cannot be ascertained from ICP data. A mer-
cury standard curve was generated using National Institute of Standards and Technology (NIST) mercury standards (mercuric ion, Hg2++ in 2% HNO3). Two different preparations of IgG–hapten 1 were analyzed, as were samples of unconjugated BSA and chicken IgG as negative controls. The ICP probe was washed between each sample reading with 2% HNO3. The mercury concentration found in the two IgG conjugates was greater than 250 ppm, and both the unconjugated BSA and chicken IgG controls had negative mercury concentration values when compared with the standard curve.

Once we were confident that the organomercury hapten was covalently linked to the carrier, four mice were immunized with chicken IgG–hapten 1 mixed with Imlject Alum adjuvant. Mouse antisera were screened without purification every 2 weeks starting 12 weeks after initial immunization. An indirect ELISA was assem-
bled by initially coating polystyrene plates with BSA–BS3–hapten 1 at a concentration of 16 μg/ml. Once the antisera titer was sufficient, the preliminary assay conditions were optimized and ultimately screening plates were coated with 10 μg/ml BSA–BS3–hapten 1 and the HRP-labeled detection antibody was used at 10 ng/ml. Between the second and fourth bleeds, sera diluted 1:20,000 went from levels of 20% to 30% above background to more than nine times greater than background absorbance. Antisera diluted 1:40,000 were at least seven times greater than background absorbance by the fifth bleed (22 weeks after initial immuniza-
tion). BSA diluted at 10 mg/ml in PBS and BSA–BS3 capped with allylamine was reacted as negative controls in these experiments. We propose that the hydrophilic nonpeptide character of the hapten, coupled with the rigid shape imparted by the cyclopentane back-
bone, yields antibodies demonstrating high degrees of sensitivity and specificity. We perceived hapten 1 as a logical choice for immunization because the availability of mercury seemed to be more favorable for eliciting an immune response than haptns with less rigid structures. To our knowledge, this is the first exam-
ple of an anti-mercury antibody derived from an immunogen pre-
pared from covalently attaching an organomercury-based hapten to protein carriers.

Several solid-phase synthetic modifications were explored to evaluate the antibody specificity toward organic and inorganic forms of mercury and to determine correlations between solid-
phase conjugate structure and assay sensitivity. A second solid-
phase conjugate was prepared by coupling hapten 1 to BSA via SMCC. The coupling conditions were not optimized as they were for the preliminary work with BS3; rather, the manufacturer’s sug-
gested coupling protocol was followed (Fig. 1). The BSA–SMCC–hapten 1 conjugate was coated onto polystyrene plates under the same coating conditions (10 μg/ml in this case nonoptimized) used for coating the BS3 conjugate. The two solid-phase conjugates (BS3 vs. SMCC) were run side-by-side using the above indirect immunoassay format to explore linker arm impacts on the assay sensitivity. Six antiserum dilutions were added to plates in triplicate, and two HRP-labeled antibody concentrations (80 and 40 ng/ml) were used to evaluate antibody binding to each solid phase (Fig. 2). At the higher detection antibody concentration (80 ng/ml), the two curves are quite similar. However, the SMCC conjugate demonstrated improved dynamic range (25%) compared with the BS3 conjugate when the HRP-labeled detection antibody concentration was reduced to 40 ng/ml. These results clearly dem-
strate that the antibody is binding to functionality specifically associated with the hapten structure rather than the linker arm or some portion of the hapten and the linker arm. It seems feasible to predict that the assay sensitivity will likely improve significantly on optimization of the cross-coupling chemistries and/or judicious choice of cross-linking agents. SMCC may afford a better opportu-
ity for antibody binding due to the longer linker arm distance relative to BS3 (1.97 vs. 1.14 nm) and/or due to SMCC’s greater conformational rigidity relative to BS3, ultimately leading to a more sensitive assay. In addition, immunoassay performance is generally improved when the linker arm in the immunogen is structurally different from the linker arm used to prepare the assay compo-
nents. Because the synthetic hapten’s structure includes an amine, it can be readily coupled to a wide selection of commercially avail-
able cross-linking reagents to yield a stable amide linkage between the hapten and the carrier, affording a more durable bond than the sulfur–mercury dative bond used in previously prepared Hg2++

 based conjugates.

An additional organomercury hapten was synthesized (hapten 5 in Fig. 3) to further evaluate the anti-mercury antibody selectivity. Following a similar synthetic strategy employed to prepare hapten 1, allyl amine was protected with t-BOC to yield 2. Under intermo-
olecular oxymercuration reaction conditions [44,45], t-BOC–allyl amine was reacted with mercuric nitrate, followed by in situ addi-
tion of commercially available 1,2-pentanediol to the resulting mercurium intermediate. In preparing hapten 1, it was found that a milder reagent, mercuric acetate, facilitated the oxymercura-

Fig. 2. Mouse anti-mercury antibody binding response to solid-phase conjugates prepared by coupling hapten 1 to BSA via two different cross-linking reagents (BS3 vs. SMCC). Both of the solid-phase conjugates were coated at 10 μg/ml. Antibody response to both solid-phase conjugates was measured using 80 and 40 ng/ml peroxidase-labeled goat anti-mouse IgG.
tion reaction, whereas $t$-BOC–allyl amine (2) required mercuric nitrate, a more potent electrophile, to expedite the addition reaction. TLC analysis of the reaction mixture revealed that substrate 2 did not react completely with Hg(OAc)$_2$; however, in the presence of Hg(NO$_3$)$_2$, the mercury addition reaction was complete in 15 min.

A small sample of compound 3 was reduced to 4 with sodium borohydride (NaBH$_4$) to minimize exposure to mercury during characterization. NMR analysis of 4 revealed that both possible pentanediol regioisomers were present in the product mixture (only one regioisomer is shown in Fig. 3). In addition, $t$-BOC deprotection conditions were explored using compound 4 as a model. Repeating the reaction conditions that were used in the model study, substrate 3 was deprotected with TFA. After extraction and pH adjustments, organomercury hapten 5 was coupled directly to BSA with the cross-linking reagent SMCC without further purification to separate regioisomers. The design of hapten 1 precluded formation of regioisomers, affording a substrate that was easier to isolate, purify, and characterize compared with hapten 5.

Plates were coated with BSA–SMCC–hapten 5 and BSA–SMCC–hapten 1 to compare the antibody binding with two structurally different organomercury substrates. The coating concentration of both solid-phase conjugates was 10 µg/ml in coating buffer, and the anti-mercury antibody response was evaluated at 40 and 20 ng/ml HRP-labeled detection antibody. The anti-mercury antibody demonstrated a high degree of binding to both solid-phase conjugates (Fig. 4). Overall, antibody binding to hapten 5 was higher than the binding displayed toward hapten 1. The dynamic range displayed by both solid-phase conjugates increased when using the lower HRP-labeled detection antibody concentration (20 ng/ml) relative to 40 ng/ml. The antibodies’ elevated sensitivity toward hapten 5 relative to hapten 1 seems a bit surprising; however, the BSA–SMCC bioconjugates were not analyzed with MALDI, which reveals hapten/protein ratios. Perhaps hapten 5 coupled more efficiently to BSA because it is a more polar hapten than hapten 1, affording a hapten/protein ratio higher than 8:1 and a solid-phase surface more densely packed with hapten. Both linker arm length [46] and epitope density [42,47] have been shown to impact antibody sensitivity. These results are encouraging because the anti-mercury antibody generated in this work displays unique binding capability to a variety of solid-phase configurations, offering a high degree of flexibility in assay format design.

A preliminary competitive inhibition study was conducted using BSA–BS3–hapten 1-coated plates. Mouse antisera were diluted 1:10,000, 1:15,000, 1:20,000, and 1:30,000 in PBS. Each dilution was preincubated with mercuric acetate (Hg(OAc)$_2$) at 20 ppm, mercuric nitrate (Hg(NO$_3$)$_2$) at 20 ppm, and 10.0 ppm of unconjugated hapten 1 for 1 h at room temperature prior to adding 50 µl of each sample in duplicate to the plate. Each serum dilution was also incubated under the same conditions in the absence of any inhibitor. When the anti-mercury antibody solid-phase bind-
ing was inhibited with Hg(OAc)₂ and Hg(NO₃)₂, the average signal for all of the inhibited samples was equivalent to negative control levels (Fig. 5). Hapten 1 did not block the solid-phase binding capability of the antibody as efficiently as the mercury salts. Perhaps unconjugated hapten 1 is less available for antibody binding because there is greater conformational flexibility and less steric impeding when freely soluble compared with the rigid conformation that results when the hapten–BSA conjugate is adsorbed to the plate surface.

Immunoassays often constitute powerful, but easy-to-use, analytical tools for detecting environmental contaminants present in low concentrations [42,48,49]. This work validates our synthetic strategy for preparing a unique organomercury-based hapten and holds promise for developing a highly sensitive immunoassay. In addition, strategies aimed at preparing other organometallic haptens are currently under investigation.

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