JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Monoclonal Antibodies for Lead−Chelate Complexes: Applications in Antibody-Based Assays

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Monoclonal antibodies against lead were generated by immunizing BALB/c mice with lead conjugated to keyhole limpet hemocyanin (KLH) via a bifunctional chelator, *S*-2-(4-aminobenzyl)diethylenetriamine pentaacetic acid (DTPA). Stable hybridoma cell lines were produced by fusion of murine splenocytes and SP2/0 myeloma cells. One of the hybridomas generated from this fusion (4/7) synthesized and secreted an antibody that bound tightly to Pb^{2+}−DTPA complexes but not to metal-free DTPA. The performance for a competitive inhibition enzyme-linked immunosorbent assay (ELISA) incorporating this antibody was assessed for its sensitivity to changes in pH, ionic strength, and blocking reagents. The cross-reactivities in this ELISA were less than 3% for Fe^{3+}, Cd^{2+}, Hg^{2+}, and Cu^{2+} and less than 0.3% for Cr^{3+}, Mn^{2+}, Mg^{2+}, In^{3+}, Ag^{1+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Cu^{1+}, and Hg^{1+}. The IC_{50} value achieved for lead was 2.72 ± 0.034 μM, showing the detection range of 0.092−87.2 μM and the lowest detection limit of 0.056 ± 0.005 μM. Recoveries from the analyte-fortified tap water and ultrapure water were in the range of 80−114% . These results indicate that the ELISA could be a convenient analytical tool for monitoring lead residues in drinking water.

KEYWORDS: Lead; monoclonal antibody; ELISA; immunoassay

INTRODUCTION

Food safety is a major public concern worldwide. During the last decades, increasing concerns about food safety have stimulated research regarding the risk associated with consumption of foodstuffs contaminated by pesticides, heavy metals, and/or toxins (*1*). Heavy metals are among the major contaminants of the food supply and may be considered one of the most important problems in our environment (*2*). The heavy metal contamination of food supplies is a particular problem in developing countries. Such contamination can occur as a result of irrigation with contaminated water, the application of fertilizers and metal-based pesticides, industrial emissions, transportation, harvesting processes, and storage. In general, heavy metals are not biodegradable, have long biological half-lives, and have the potential for accumulation in different body organs, which leads to unwanted side effects and toxicity (*3*−*5*). In addition, heavy metals are also implicated in causing carcinogenesis, mutagenesis, and teratogenesis (*6*, *7*). Lead is the most abundant heavy metal and shows particular toxicity. The excessive lead in food is associated with the etiology of a number of diseases, including cardiovascular, kidney, nervous, and bone diseases (*8*, *9*).

Some typical analytical methods for highly selective detection of toxic heavy metals are flameless atomic absorption spectrometry (*10*), flame atomic absorption spectrometry (*11*−*13*), inductively coupled plasma mass spectrometry (*14*), voltammetry and ion chromatography (*15*), and electrothermal atomic absorption spectrometry (*16*−*19*). These methods have the drawbacks that are typically encountered when using instrumental analysis. They require very expensive and sophisticated equipment and highly qualified personnel. In addition, sample throughput is limited and these methods are not suitable for on-site analysis. Recently developed immunoassays present an interesting alternative for the detection of certain metal ions (*20*−*26*). These novel techniques involve raising antibodies against metal chelates. Reardan (*27*) first reported the selective recognition of an ethylenediaminetetraacetic acid (EDTA)−indium chelate by monoclonal antibodies (mAbs). In this study, we report the isolation and characterization of lead-specific mAbs.

MATERIALS AND METHODS

All chemicals were ultrapure grade. Lead granules (99.999% purity) were obtained from Aldrich Chemical Co. (Milwaukee, Wl). Other metal ions, including Cr(III), Fe(III), Mn(III), Mg(II), Cu(II), In(III), Cd(II), Hg(II), Ag(I), Ni(II), Co(II), Zn(II), Ca(II), Cu(I), and Hg(I), were supplied by Merck (Darmstadt, Germany). *S*-2-(4-Aminobenzyl)-diethylenetriaminepentaacetic acid (*p*-NH2-Bn-DTPA) was purchased from Macrocyclics Co. (Dallas, TX). Bovine serum albumin (BSA),

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10.1021/jf070787d CCC: $37.00
Published on Web 06/05/2007

keyhole limpet hemocyanin (KLH), Freund's complete and incomplete adjuvants, glutaraldehyde, 3,3′,5,5′-tetramethylbenzidine (TMB), lead atomic absorption standard metals (975 μg/mL in 2% HNO_3), diethylenetriamine pentaacetic acid (DTPA), and the Mouse Monoclonal Antibody Isotyping Kit were purchased from Sigma Chemical Co. (St. Louis, MO). Reagents for mAb production included in the ClonaCell-HY kit were purchased from StemCell Technologies (Vancouver, British Columbia, Canada). Hydrochloric acid and nitric acid were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). Fetal bovine serum (FBS) was the product of Hangzhou "Sijiqing" Co. (Hangzhou, China). Goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (GAM-HRP) was purchased from Sino-American Biotechnology Co. (Luoyang, China). BD Cell serum-free Mab medium and Cell-Line 1000 were purchased from BD Co. (Franklin Lakes, NJ); enzyme-linked immunosorbent assay (ELISA) high-binding microplates and tissue culture plates were obtained from Corning-Costar (Cambridge, MA); ImmunoPure IgM Purification Kit was purchased from Pierce Co. (Rockford, United States); ultrapure water (18.2 MΩ cm) obtained from a Milli-Q water purification system (Millipore, Bedford, MA) was used throughout. All glassware was treated with mixed acids (concentrated HCl:concentrated HNO_3 = 1:1, v/v) for 4 h and liberally rinsed with ultrapure water; all plasticware was kept in 3 M HCl overnight and rinsed liberally with ultrapure water before use (*28*). A Wellwasher Plus microplate washer from Thermo Electron Co. (Marietta, OH) was used to wash ELISA plates. A Multiskan Ascent reader for microtiter plates was from Thermo Electron Co.; this device was controlled by a personal computer containing the standard software package EasySoft ware.

SP2/0 cells were obtained from College of Veterinary Medicine in Nanjing Agricultural University, and BALB/c mice were from Shanghai Experimental Animal Center. All animal studies were performed in accordance with institutional guidelines.

Conjugation of Lead Ions with Carrier Protein. Lead ions were conjugated to carrier protein (BSA and KLH) via the bifunctional chelate reagent *p*-NH_2-Bn-DTPA. The amino group of the bifunctional DTPA derivative was linked to the carrier protein using glutaraldehyde (*29*, *30*). All steps of conjugation were performed at room temperature with stirring.

The KLH−DTPA conjugate was prepared by adding 15 mg of KLH dissolved in 2 mL of HBS (HEPES-buffered saline, 10 mM HEPES, 137 mM NaCl, and 3 mM KCl, pH 7.4) to 8 mg of *p*-NH_2-Bn-DTPA in 0.8 mL of H_2O. Glutaraldehyde (2 mL of a 20 mM aqueous solution) was then added with continuous stirring. After overnight incubation, low molecular weight reactants were removed by buffer exchange into HBS using a Centricon-30 concentrator device treated with 100 mM EDTA.

BSA−DTPA was prepared by adding BSA (30 mg in 6 mL of HBS) to 18 mg of *p*-NH_2-Bn-DTPA in 1.8 mL of H_2O. Glutaraldehyde (1.8 mL of a 20 mM aqueous solution) was then added with gentle stirring to couple the *p*-NH_2-Bn-DTPA to BSA. The mixture was purified by buffer exchange as described above. The BSA−DTPA conjugate was divided into two parts: One was used directly as a coating antigen (cAg) for ELISA, and the other part was loaded with lead ions as follows: Lead granules (99.999%, 87.6 mg) were dissolved in 1 mL of 10 M HNO_3, and 14.6 μL of this solution was added directly to each protein−DTPA conjugate (volume, 4.8 mL) to make a final Pb^{2+} concentration of 1.3 mM. After stirring at room temperature for 3 h, low molecular weight reactants were removed by buffer exchange as described above.

Immunization and Monitoring of Mice. Six female BALB/c mice (6−8 weeks old) were used for immunizations. Routinely, the immunogen (Pb^{2+}−DTPA−KLH) in HBS was thoroughly emulsified with an equal volume of Freund's adjuvant and the emulsion was injected intraperitoneally (200 μg of conjugate per animal in a total of 200 μL). Freund's complete adjuvant was used for the first two injections, and Freund's incomplete adjuvant was used for subsequent boost injections. Boosts were given every 2 weeks in the same manner. The antibody response of each mouse was determined by indirect ELISA. These indirect ELISAs were performed by coating 96 well microplates with solutions (5 μg/mL, 50 μL/well) containing either metal-free DTPA−BSA or the DTPA−BSA conjugate loaded with Pb^{2+}. The plates were allowed to incubate overnight at 4 °C. On the following day, the plates were washed five times with PBST (phosphate-buffered saline containing 0.05% Tween 20) and blocked by incubation with 1% gelatin in phosphate-buffered saline (PBS, 100 μL/well). After incubation at 37 °C for 1.5 h, the plates were washed as described above. Mouse sera from immunized animals were serially diluted through the wells using HBS as the diluent, and the plates were incubated for 1 h at 37 °C. After a subsequent wash step, an enzyme-labeled antispecies antibody (goat-anti mouse labeled with horse radish peroxidase, GAM-HRP) was diluted 1:1000 with PBST and added to the plates. The plates were incubated for 1 h at 37 °C. After the plate was washed, 50 μL/well of a substrate solution (3.3 μL of 30% hydrogen peroxide and 0.4 mL of 0.6% TMB in dimethyl sulfoxide added to 25 mL of citrate−acetate buffer, pH 5.5) was added to each well. After 15 min at 37 °C, the reaction was stopped by adding 25 μL/well of 2 M sulfuric acid. The absorbance was measured at 450 nm. The mice showing highest serum reactivity were selected for hybridoma production; these mice were "hyperimmunized" by intravenous (tail vein) injection of 100 μg of Pb^{2+}−DTPA−KLH conjugate in 50 μL of HBS 3 days prior to cell fusion (about 66 days after the initial immunization).

Hybridoma Production and Isolation of Monoclonal Antibodies. SP2/0 murine myeloma cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 20% FBS and 1% penicillin−streptomycin. Splenocytes of two selected mice were harvested aseptically. The splenocytes were added to myeloma cells to give a ratio of five splenocytes per myeloma cell. The hybridoma cells were produced according to the protocol included in the ClonaCell-HY kit. The fused cells were grown in semisolid medium selective for the growth of hybridomas. Each clone was transferred into an individual well of a 96 well tissue culture plate and incubated at 37 °C in 5% CO_2 for 1−4 days. After the hybridoma cells had grown to 30−40% confluence in the well, the culture supernatant was initially screened by indirect ELISA for the ability to bind to immobilized Pb^{2+}−DTPA−BSA or DTPA−BSA. Culture supernatants from unfused SP2/0 cells served as the negative control. Positive hybridoma cells were subcloned by limiting dilution, and stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen. The immunoglobulin subclass and light chain isotype of the antibodies were determined by using a mouse monoconal antibody isotyping kit according to the manufacturer's recommendations.

Antibody Production and Purification. Large-scale production of mAbs was accomplished by growing selected hybridoma clones as an ascites. Ascites fluid was produced in BALB/c mice primed with 0.5 mL of pristine oil by intraperitoneal (ip) injection of 2×10^6 log-phase hybridoma cells in 0.5 mL of sterile HBS. Ascites fluids were harvested by peritoneal tap with 16-gauge needle on the tenth day after the cells were introduced.

mAb was also produced in vitro by culturing hybridoma cell lines in BD Cell serum-free Mab medium in a membrane-based disposable cell cultivation system (Cell-Line 1000) at 37 °C in a 5% CO_2 atmosphere. The culture supernatant was harvested using the 7/21 day harvest protocol recommended by the manufacturer.

The 4/7 mAb was partially purified by precipitation with 50% ammonium sulfate (*31*). The partially purified mAbs fractions were then pooled and buffer-exchanged into Tris-NaCl buffer (20 mM Tris, pH 7.4, and 1.25 M NaCl) using a Centricon-100. Antibodies in the Tris-NaCl buffer were subsequently purified using the ImmunoPure IgM Purification Kit according to the manufacturer's recommended protocol. The fractions with absorbance greater than 0.02 at 280 nm were pooled, concentrated, and dialyzed against PBS. The concentration of protein in the purified antibody preparations was determined by the Bradford method (*32*).

Competitive Indirect ELISA (CI-ELISA). CI-ELISA was performed to investigate whether the 4/7 mAb would recognize soluble chelated Pb^{2+}. In preliminary experiments, checkerboard assays were employed to determine the concentration of the Pb^{2+}−DTPA−BSA to use for coating the microwells and the concentration of soluble antibody to use in competitive assays. Preliminary experiments were also performed to determine the highest DTPA concentration that would not affect the binding of mAbs to immobilized Pb^{2+}−DTPA−BSA in

subsequent experiments and the blocking proteins that worked best in the assay (data not shown).

Lead standard solutions were prepared in various concentrations (0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 1000 μM) in HBS containing 2 mM DTPA and mixed with equal volumes of diluted hybridoma culture supernatant. After mixing for 30 s and incubation at 37 °C for 45 min, an aliquot of the antibody−lead mixture (50 μL) was added to each antigen-coated microwell and the plates were incubated at 37 °C for 1 h. After the plate was washed, goat anti-mouse IgM conjugated with horseradish peroxidase (diluted 1:1000 with PBST, 50 μL/well) was added to plates and incubated for 1 h at 37 °C. After the plate was washed, 50 μL/well of a substrate solution (3.3 μL of 30% hydrogen peroxide and 0.4 mL of 0.6% TMB in dimethyl sulfoxide added to 25 mL of citrate−acetate buffer, pH 5.5) was added to each well. The subsequent color formation was performed as described for the indirect ELISA. In the CI-ELISA format, analytes that do not react with the antibody will produce absorbances near 100% of the zero analyte control; conversely, analytes that do react with the antibody would decrease the absorbance. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration; these curves were fitted to a four-parameter logistic equation: $y = (A - D)/[1 + (x/C)^B + D]$, where x is the concentration of analyte, y is the absorbance at 450 nm (OD_{450}), A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is IC_{50}, and D is the minimum absorbance at infinite concentration of analyte (*33*).

Assay Optimization. Concentrations of the coating conjugates along with concentrations of 4/7 mAb were optimized to provide the inhibition curve with the lowest IC_{50}, giving adequate maximum absorbance around 1.0 in the absence of analyte. First, saturating conditions were determined by a noncompetitive two-dimensional titration covering a wide range of concentrations. Then, competitive inhibition curves were measured for different antibodies and antigen combinations, and the one with the lowest IC_{50} was selected for further assay development. The effects of pH and ionic strength on the competitive assay were also investigated.

pH Effect. To evaluate the effect of the pH on assay performance, HBS buffers with pH values between 4 and 8 were prepared. All other assay conditions were as described above. The standard curve was run in three well replicates.

Ionic Strength. The effect of ionic strength on the performance of the competitive immunoassay was studied by preparing lead standard solutions and diluting the hybridoma culture supernatant in HEPES-KCl buffer (10 mM HEPES and 3 mM KCl) that had been augmented with 0−0.8 M NaCl. All assays were conducted at pH 7.4 as described above.

Cross-Reactivities (CRs). Data were obtained from standard curves of lead. In addition, other metals such as Cr^{3+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, In^{3+}, Cd^{2+}, Hg^{2+}, Ag^{1+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Cu^{1+}, and Hg^{1+} were also tested for CR by CI-ELISA. Each metal was prepared in HBS and tested at the concentration range of 0.0001−1000 μM. The CR was obtained from the IC_{50} values of lead standard and the other metals from the same plate where %CR = (IC_{50} of lead/IC_{50} of tested metals) × 100.

Determination of Affinity Constants (K_{aff}) for Pb^{2+}−DTPA−BSA. Beatty et al. (*34*) described a method for the determination of K_{aff} based upon the law of mass action. Briefly, ELISA plates precoated with three different concentrations of Pb^{2+}−DTPA−BSA (0.45, 0.9, and 1.8 μg/mL diluted in HBS, pH 7.4) were separately incubated with serial concentrations of mAb. Sigmoid curves were constructed using OD values obtained for different concentrations of mAb. Three curves were selected to calculate the affinity constant. The half-maximum OD (OD_{50}) was assigned for the selected curves from which the corresponding antibody concentration ([Ab], [Ab′], and [Ab″]) was extrapolated. Accordingly, [Ab], [Ab′], and [Ab″] are the measurable total Ab concentrations at OD_{50}, $OD_{50'}$, and $OD_{50''}$ for plates coated with [Ag], [Ag′], and [Ag″] respectively.

Fortification of Lead in Water. For recovery studies, a standard dilution series of lead was spiked into tap water and ultrapure water. The samples were conditioned by addition of a 50% volume of a two-fold HBS + DTPA buffer. Recoveries were determined by comparing the results to a lead standard curve generated by competitive inhibition ELISA.

Table 1. Reactivity of Serum from BALB/c Mice Injected with Pb^{2+}–DTPA–KLH[a]

mouse	BSA–DTPA	Pb^{2+}–DTPA–BSA	difference (%)
1	0.773 ± 0.108	0.783 ± 0.120	1.29
2	0.691 ± 0.012	0.756 ± 0.016	9.4
3	0.644 ± 0.033	0.995 ± 0.025	54.5
4	0.442 ± 0.012	0.806 ± 0.036	48.7
5	0.673 ± 0.010	0.762 ± 0.016	13.2
6	0.599 ± 0.0152	1.010 ± 0.025	68.6
normal serum	0.198 ± 0.022	0.202 ± 0.039	2.02

[a] Serum was collected from the tail vein of each mouse, diluted into HBS, and used for ELISA as described. Normal serum was obtained from an unimmunized mouse and served as background reactivity for each antigen. Each value represents the mean of three replicates ± SD.

RESULTS

Design of Immunogen, Animal Response, and Antibody Production. Metal ions are too small to elicit an immune response; thus, to increase the likelihood of inducing lead-specific antibodies, hapten−carrier complexes were designed that would maximize the exposure of lead. Design of the immunogen is very important for development of a sensitive and specific lead immunoassay. It is desirable to immunize with an antigen that exposes the most unique portions of the target analyte for antibody development. Therefore, the bifunctional chelator *p*-NH_2-benzyle-DTPA was covalently coupled to a carrier protein (KLH), loaded with Pb^{2+}, and used as the immunogen. After five injections of Pb^{2+}−DTPA−KLH, blood was collected from the tail vein of each mouse and assayed by indirect ELISA for the presence of Pb^{2+}-specific antibodies. **Table 1** shows the reactivity of each serum sample on microwell plates coated with Pb^{2+}−DTPA−BSA or DTPA−BSA. Sera from three of the six mice (designated as 1, 2, 3, 4, 5, and 6 in **Table 1**) reacted more strongly with Pb^{2+}−DTPA−BSA than with BSA−DTPA, suggesting that these serum samples might contain antibody molecules specific for either Pb^{2+} itself or to an epitope composed of both $Pb^{2+}$and DTPA. Because mouse 3 and mouse 6 demonstrated the highest relative reactivity with Pb^{2+}−EDTA−BSA, they were used for subsequent hybridoma production.

After fusion of lymphocytes from the immunized mice with myeloma cells, a total of 973 clones were screened in three groups; of these clones, 26 hybridoma cell lines reacted strongly with Pb^{2+}−DTPA−BSA but showed only background reactivity with DTPA−BSA. These antibodies were presumed to be binding to Pb^{2+} or the Pb^{2+}−DTPA complex. To confirm that these antibodies would recognize soluble Pb^{2+}−DPTA complexes, CI-ELISA was performed. When Pb^{2+} was serially diluted into 2 mM DTPA and incubated with the immobilized Pb^{2+}−DTPA−BSA conjugate in the presence of hybridoma culture supernatant, the lead ions inhibited binding in a dose-dependent fashion. This result demonstrated that reactivity of antibodies with lead ions was dependent upon the presence of DTPA, although not inhibited by DTPA in the absence of Pb^{2+}.

These positive hybridoma cells were subcloned by limiting dilution, and 6−10 stable clones of each subclone were expanded and frozen. Selected clones were subsequently expanded to produce culture medium and ascites. Three clones designated 4/7, 6H3, and 7/4 were expanded and archived for further study. Unless otherwise specified, all experiments

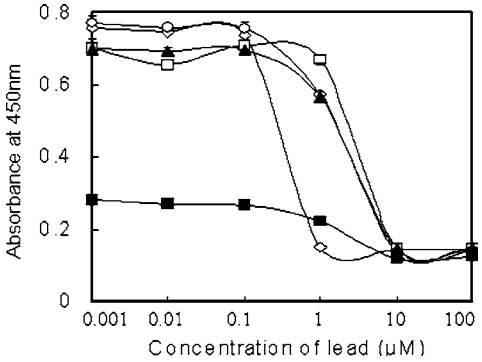

Figure 1. CI-ELISA for Pb^{2+} at varying ionic strengths. Reagent concentrations: cAg (Pb^{2+}–DTPA–BSA), 0.95 μg/mL; culture supernatant, mAb 4/7 1:10 in HBS; and GAM-HRP, 1:1000. Data points are the mean values of triplicates ± SD. In some cases, the error in the analysis was less than the diameter of the plotted points. Key: ◇, 0 M HEPES-KCl; □, 0.15 M HEPES-KCl; ○, 0.2 M HEPES-KCl; ▲, 0.4 M HEPES-KCl; and ■, 0.8M HEPES-KCl.

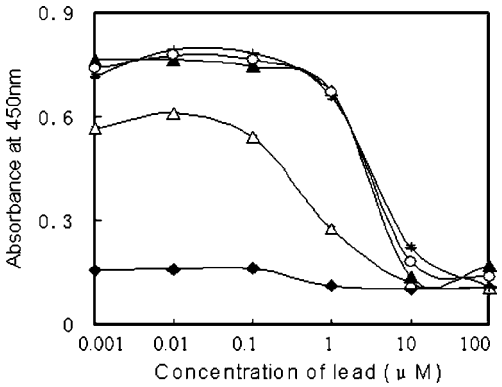

Figure 2. CI-ELISA for Pb^{2+} at varying pH values. Reagent concentrations: cAg (Pb^{2+}–DTPA–BSA), 0.95 μg/mL; culture supernatant, mAb 4/7 1:10 in HBS; and GAM-HRP, 1:1000. The data points are the mean values of triplicates ± SD. In some cases, the error in the analysis was less than the diameter of the plotted points. Key: ♦, pH 4.0; △, pH 5.0; ▲, pH 6.0; ○, pH 7.0; and +, pH 8.0.

reported herein were done with the purified mAb 4/7, which gave higher titer (data not shown). The isotypes of the obtained mAbs were IgM with κ light chains, as determined using a mouse mAb isotyping kit.

Assay Optimization. To determine lead residues in farm produce and environmental samples, it is essential to develop an ELISA with optimum sensitivity. For that purpose, the effects of the assay buffer-related factors such as ionic strength and pH were evaluated. Because the ionic strength of the buffer can affect antibody binding (*34*), the ionic strength of the assay buffer was varied by augmenting HEPES-KCl buffer with additional NaCl (0−0.8 M). Increasing the salinity of the assay buffer resulted in a decrease in sensitivity (IC_{50} increased significantly) but also resulted in a decrease of the control absorbance (absorbance in the absence of added lead) as shown in **Figure 1**. HEPES-KCl buffer with no additional NaCl was chosen as the optimum.

Figure 2 shows the influence of varying the pH of the assay buffer during the competition step in the presence of the analyte. Lead standard curves were obtained at several pH values, and pH effects were evaluated based on the shape and IC_{50} value of each curve. The absorbance values were depressed at pH 4.0, and the curves flattened out. Because the assay was more sensitive and had higher control absorbance under more acidic conditions, a pH of 5.0 was used for subsequent assays.

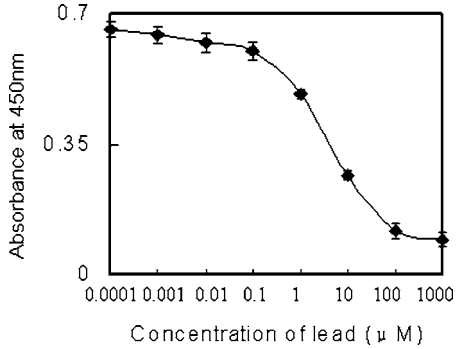

Figure 3. Pb^{2+} standard curve obtained under the optimized condition of the ELISA. The error bars represent the SD calculated from replicate calibration curves, which were obtained with the same set of standards ($n = 6$). In some cases, the error in the analysis was less than the diameter of the plotted points.

Characteristics of the Optimized Immunoassay. The optimized lead ELISA used 0.95 μg/mL of cAg Pb^{2+}−DTPA−BSA, hybridoma culture at a dilution of 1/10, blocking with 3% BSA, and lead standard solution in 2 mM DTPA/HEPES-KCl buffer (3 mM KCl and 10 mM HEPES, pH 5.0). As shown in **Figure 3**, the IC_{50} value of the optimized assay was 2.72 ± 0.034 μM, showing the detection range (IC_{20-80}) of 0.092−87.2 μM, and the lowest detection limit [defined as two standard deviations (SDs) above the lowest detectable level] was estimated as 0.056 ± 0.005 μM.

CR. mAb 4/7 was highly selective for the target analyte Pb^{2+}. Thirteen other metals, including Cr^{3+}, Fe^{3+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, In^{3+}, Cd^{2+}, Hg^{2+}, Ag^{1+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Cu^{1+}, and Hg^{1+}, were tested for CRs (Table 2). The optimized ELISA system had low CRs for Fe^{3+} (1.19%), Cd^{2+}(2.60%), Hg^{2+} (2.49%), and Cu^{2+} (2.59%). The CRs of the other tested metals were negligible (less than 1%). The interference to the assays was negligible in all cases.

Apparent Affinity Constant (K_{aff}) of mAb 4/7 for Immobilized Pb^{2+}−DTPA−BSA. The apparent affinity constant (K_{aff}) was measured by indirect ELISA using serial dilutions of purified antibody and three concentrations of Pb^{2+}−DTPA−BSA for coating of the microwell plates (0.45, 0.9, and 1.8 μg/mL). The general equation used to calculate the affinity constant, adapted from Beatty et al., was $K_{aff} = (n - 1)/2(n\,[Ab'] - [Ab])$, where [Ab] and [Ab′] were the measurable total antibody concentrations in the wells at OD_{50} and $OD_{50'}$ for plates coated with (Ag) and (Ag′), respectively, with (Ag′) = (Ag)/n. Representative curves obtained for 4/7 mAb are illustrated in **Figure 4**, and the calculated average K_{aff} values are presented in **Table 3**.

Spike and Recovery Studies. To investigate the accuracy of the CI-ELISA, tap water and ultrapure water samples from our laboratory were spiked with different levels of lead (0.00005, 0.0001, 0.001, 0.01, and 0.1 mM) and recoveries of lead from the water were determined by CI-ELISA using three replicates at each spike level. **Table 4** summarized the accuracy of the ELISA in these water samples. The recoveries of lead from water samples were in the range of 80−114%, and the variable coefficients (CV) were from 7 to 19%, whereas those obtained from the same samples by flame atomic absorption spectrometry were 87−102% (*35*). The accurate recovery of the spiked water samples suggests that the residues analysis for lead with the ELISA is suitable for a rapid and simple checking method.

DISCUSSION

Rapid, inexpensive, sensitive, and selective ELISAs have been utilized in environmental science for 20 years or more. Our goal

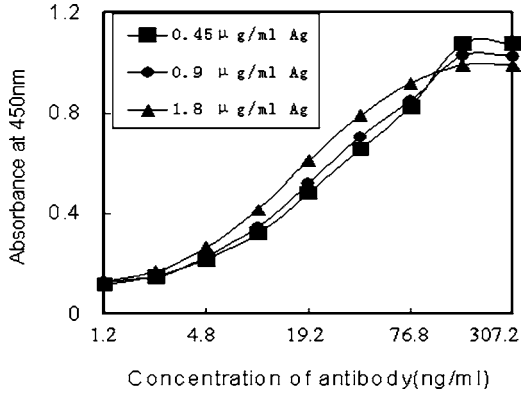

Figure 4. Primary data for affinity measurement. The apparent affinity constant was measured as described in the Materials and Methods. The value for mAb 4/7 was 2.19×10^{10} L/mol. Each point represented the mean of triplicate determinations ± SD. In some cases, the error in the analysis was less than the diameter of the plotted points.

Table 2. CRs of Other Metals to mAb (4/7)

metal ions	IC_{50} (μM)	CR[a] (%)
Pb^{2+}	2.72 ± 0.034	100
Fe^{3+}	225 ± 12.92	1.19
Cd^{2+}	103 ± 8.93	2.60
Hg^{2+}	108 ± 15.61	2.49
Cu^{2+}	104 ± 5.43	2.59
In^{3+}	>1000	<0.269
Ag^{+}	>1000	<0.269
Ni^{2+}	>1000	<0.269
Co^{2+}	>1000	<0.269
Zn^{2+}	>1000	<0.269
Ca^{2+}	>1000	<0.269
Cu^{+}	>1000	<0.269
Hg^{+}	>1000	<0.269
Mn^{3}	>1000	<0.269
Mg^{2+}	>1000	<0.269
Cr^{3+}	>1000	<0.269

[a] % CR was calculated as (IC_{50} of lead/IC_{50} of tested metals) × 100.

Table 3. Affinity Constant of mAb 4/7 Determined by ELISA

Ag (μg/mL)	OD_{50}[a]	[Ab] at OD_{50} (ng/mL)	K_{aff} (L/mol)	average K_{aff} (L/mol)
1.8	0.498	15.6	2.46×10^{10}	2.19×10^{10}
0.9	0.514	17.0	2.32×10^{10}	
0.45	0.54	22.75	1.79×10^{10}	

[a] OD_{50} represents the half-maximum optical density obtained for a given concentration of antigen and the corresponding mAb ([Ab]). The affinity constant (K_{aff}) for each selected concentration of Ag and Ab was determined using the formula described in the Materials and Methods.

was to develop simple analytical methods based on the formation of chelates of the metal ion to be detected. We have utilized the high affinity bifunctional chelator *p*-NH_2-Bn-DTPA, which was coupled to KLH via a primary amine and linked with Pb^{2+} by binding to the carboxyl and nitrogen groups of the chelator. The Pb^{2+} ions are too small to possess an antigenic epitope, and they appear to react with the antibody only when complexed to the chelator, DTPA. Therefore, it is important to choose a suitable concentration of chelator in designing the optimal immunoassay for lead.

In contrast to the previously reported 2C12 antibody (*36*), mAb 4/7 did not show significant CR with other metal−DPTA complexes, indicating that this new mAb is highly specific for lead. Careful development and optimization of an ELISA to

Table 4. Analytical Recovery of Pb(II) Spiked into Water[a]

sample	theoretical (mM)	detected (mM)	mean recovery (%, $n = 3$)	CV (%)
tap water	0.1	0.087	87 ± 2.94	10
	0.01	0.008	80 ± 2.94	13
	0.001	0.0009	90 ± 5.10	9
	0.0001	0.000089	89 ± 7.93	19
	0.00005	<LOD		
ultrapure water	0.1	>0.0872		
	0.01	0.0163	106.3 ± 4.11	7
	0.001	0.00109	109 ± 4.55	17
	0.0001	0.000112	112.5 ± 9.12	13
	0.00005	<LOD		

[a] Ultrapure water was from a Milli-Q water purification system; tap water was obtained from the Nanjing municipal water system. Varying amounts of lead were added to the samples to give final concentrations in water of 0.00005, 0.0001, 0.001, 0.01, and 0.1 mM. After thorough mixing, the samples were mixed 1:1 in 2× HEPES-KCl buffer (20 mM HEPES and 6 mM KCl, pH 5.0) containing 4 mM DTPA prior to immunoassay.

Pb^{2+} using the new mAb 4/7 have resulted in an immunoassay range approximately 100-fold lower than previously CI-ELISA reported for 2C12. This new ELISA can detect Pb^{2+} at concentrations as low as 0.056 ± 0. 005 μM and has an IC_{50} of only 2.72 ± 0.034 μM for lead. The chelator *p*-NH2-Bn-DTPA, which has shown higher affinity for Pb^{2+}, may contribute to the increased sensitivity, as compared to previously used chelator CHXDTPA. This optimization has made it possible to detect lead at levels below the threshold promulgated by National Chinese for lead in drinking water (0.05 mg/L or 242.5 nM) (*37*). These mAbs should be very promising analytical tools for rapid and sensitive determination of metal ions in food and in the environment.

ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; cAg, coating antigen; CI-ELISA, competitive inhibition enzyme-linked immunosorbent assay; CR, cross-reactivity; DMEM, Dulbecco's minimal essential medium; DTPA, diethylenetriamine pentaacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAM-HRP, goat anti-mouse immunoglobulin conjugated to horseradish peroxidase; HBS, HEPES-buffered saline (137 mM NaCl, 3 mM KCl, and 10 mM Hepes, pH 7.4); HEPES-KCl buffer (3 mM KCl and 10 mM Hepes, pH 7.4); IC_{50}, concentration of analyte giving 50% inhibition; K_{aff}, equilibrium association constant; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; PBS, phosphate-buffered saline (137 mM NaCl, 3 mM KCl, and 10 mM phosphate, pH 7.4); PBST, phosphate-buffered saline containing 0.05% Tween 20; *p*-NH_2-Bn-DTPA, *S*-2-(4-aminobenzyl)-diethylenetriaminepentaacetic acid; SD, standard deviation; TMB, 3,3′,5,5′-tetramethylbenzidine.

LITERATURE CITED

(1) D'Mello, J. P. F. *Food Safety: Contaminants and Toxins*; CABI Publishing: Wallingford, Oxon, United Kingdom, and Cambridge, MA, 2003; p 480.

(2) Zaidi, M. I.; Asrar, A.; Mansoor, A.; Farooqui, M. A. The heavy metal concentration along roadside trees of Quetta and its effects on public health. *J. Appl. Sci.* **2005**, *5* (4), 708−711.

(3) Jarup, L. Hazards of heavy metal contamination. *Br. Med. Bull.* **2003**, *68*, 167−182.

(4) Sathawara, N. G.; Parikh, D. J.; Agarwal, Y. K. Essential heavy metals in environmental samples from western India. *Bull. Environ. Contam. Toxicol.* **2004**, *73*, 756−761.

(5) Kriegel, A. M.; Soliman, A. M.; Zhang, Q.; El-Ghawalby, N.; Ezzat, F.; Soultan, A.; Abdel-Wahab, M.; Fathy, O.; Ebidi, G.; Bassiouni, N.; Hamilton, S. R.; Abbruzzese, J. A.; Lacey, M.; Blake, D. A. Serum cadmium levels in pancreatic cancer patients from the East Nile Delta Region of Egypt. *Environ. Health Perspect.* **2006**, *114* (1), 113−119.

(6) IARC, Cadmium and cadmium compounds. In *Beryllium, Cadmium, Mercury and Exposure in the Glass Manufacturing Industry*; IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; International Agency for Research on Cancer: Lyon, 1993; Vol. 58, pp 119−237.

(7) Pitot, C. H.; Dragan, P. Y. Chemical carcinogenesis. In *Toxicology International Edition*, 5th ed.; Asarett, Doulls, Eds.; McGraw Hill: New York, 1996; pp 201−260.

(8) WHO. *Lead: Environmental Health Criteria*; WHO: Geneva, 1995; Vol. 165.

(9) Steenland, K.; Boffetta, P. Lead and cancer in humans: where are we now? *Am. J. Ind. Med.* **2000**, *38*, 295−299.

(10) Kloft, C.; Appelius, H.; Schunack, W.; Jaehde, U. Determination of platinum complexes in clinical samples by a rapid flameless atomic absorption spectrometry assay. *Drug Monit.* **1999**, *21* (6), 631.

(11) Bakircioglu, Y.; Segade, S. R.; Yourd, E. R.; Tyson, J. R. Evaluation of Pb-Spec for flow-injection solid-phase extraction preconcentration for the determination of trace lead in water and wine by flame atomic absorption spectrometry. *Anal. Chim. Acta* **2003**, *485* (10), 9−18.

(12) Yaman, M. The improvement of sensitivity in lead and cadmium determination using flame atomic absorption spectrometry. *Anal. Biochem.* **2005**, *339*, 1−8.

(13) Chen, J.; Teo, K. C. Characterizing the risk assessment of heavy metals and sampling. Determination of cadmium, copper, lead and zinc in water samples by flame atomic absorption spectrometry after cloud point extraction. *Anal. Chim. Acta* **2001**, *450* (12), 215−222.

(14) Almeida, C. M. R.; Vasconcelos, M. T. S. D. Lead contamination in Portuguese red wines from the douro region: From the vineyard to the final product. *J. Agric. Food Chem.* **2003**, *51*, 3012−3023.

(15) Buldini, P. L.; Cavalli, S.; Sharma, J. L. Determination of transition metals in wine by ICP, DPASV-DPCSV and ZGFAAS coupled with UV photolysis. *J. Agric. Food Chem.* **1999**, *47*, 1993−1998.

(16) Freschi, G. P. G.; Dakuzaku, C. S.; Moraes, M.; Nobrega, J. A.; Gomes Neto, J. A. Simultaneous determination of cadmium and lead in wine by electrothermal atomic absorption spectrometry. *Spectrochim. Acta, Part B* **2001**, *56*, 1987−1993.

(17) Viitak, A.; Volynsky, A. B. Simple procedure for the determination of Cd, Pb, As and Se in biological samples by electrothermal atomic absorption spectrometry using colloidal Pd modifier. *Talanta* **2006**, *70* (4), 890−895.

(18) Vilar, M.; Barciela, J.; Garcia-Martin, S.; Pena, R. M.; Herrero, C. Comparison of different permanent chemical modifiers for lead determination in *Orujo* spirits by electrothermal atomic absorption spectrometry. *Talanta* **2007**, *71* (4), 1629−1636.

(19) Jurado, J. M.; Martin, M. J.; Pablos, F.; Moreda-Piñeiro, A.; Bermejo-Barrera, P. Direct determination of copper, lead and cadmium in aniseed spirits by electrothermal atomic absorption spectrometry. *Food Chem.* **2007**, *101* (3), 1296−1304.

(20) Khosraviani, M.; Pavlov, A. R.; Flowers, G. C.; Blake, D. A. Detection of heavy metals by immunoassay: Optimization and validation of a rapid, portable assay for ionic cadmium. *Environ. Sci. Technol.* **1998**, *32*, 137−142.

(21) Blake, D. A.; Blake, R. C., II; Khosraviani, M.; Pavlov, A. R. Metal ion immunoassays. *Anal. Chim. Acta.* **1998**, *376*, 13−19.

(22) Darwish, I. A.; Blake, D. A. One-step competitive immunoassay for cadmium ions: development and validation for environmental water samples. *Anal. Chem.* **2001**, *73*, 1889−1895.

(23) Blake, D. A.; Jones, R. M.; Blake, R. C., II; Pavlov, A. R.; Darwish, I. A.; Yu, H. Antibody-based sensors for heavy metal ions. *Biosens. Bioelectron.* **2001**, *16*, 799−809.

(24) Darwish, I. A.; Blake, D. A. Development and validation of a sensitive one-step immunoassay for determination of cadmium in human serum. *Anal. Chem.* **2002**, *74*, 52−58.

(25) Yu, H.; Jones, R. M.; Blake, D. A. An immunosensor for autonomous in-line detection of heavy metals: Validation for hexavalent uranium. *Int. J. Environ. Anal. Chem.* **2005**, *85*, 817−830.

(26) Blake, D. A.; Blake, R. C., II; Abboud, E. R.; Li, X.; Yu, H.; Kriegel, A. M.; Khosraviani, M.; Darwish, I. A. Antibodies to heavy metals: Isolation, characterization and incorporation into microplate-based assays and immunosensors. In *Immunoassay and Other Bioanalytical Techniques*; Van Emon, J. M., Ed.; Taylor and Francis: Boca Raton, FL, 2007; pp 93−111.

(27) Reardan, D. T.; Meares, C. F.; Goodwin, D. A.; McTigue, M.; David, G. S.; Stone, M. R.; Leung, J. P.; Bartholomew, R. M.; Frincke, J. M. Antibody against metal chelatesh. *Nature* **1985**, *316*, 265−268.

(28) Theirs, R. C. Contamination in trace element analysis and its control. *Methods Biochem. Anal.* **1957**, *5*, 273−335.

(29) Parisi, L. T. Monoclonal antibodies to a soluble metallic radioisotope chelator: Development and characterization. *Hybridoma* **1991**, *10*, 696−705.

(30) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1998; p 90.

(31) Reik, L. M.; Maines, S. L.; Ryan, D. E.; Levin, W.; Bandiera, S.; Thomas, R. E. A simple, non-chromatographic purification procedure for monoclonal antibodies. Isolation of monoclonal antibodies against cytochrome P450 isozymes. *J. Immunol. Methods* **1987**, *100*, 123−129.

(32) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(33) Jones, G.; Wortberg, M.; Kreissig, S. B.; Bunch, S. D.; Gee, S. J.; Hammock, B. D.; Rock, D. M. Extension of the four-parameter logistic model for ELISA to multianalyte analysis. *J. Immunol. Methods* **1994**, *177*, 1−7.

(34) Beatty, J. D.; Beatty, B. G.; Vlahos, W. G. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J. Immunol. Methods* **1987**, *100*, 173−179.

(35) Chen, J. R.; Teo, K. C. Determination of cadmium, copper, lead and zinc in water samples by flame atomic absorption spectrometry after cloud point extraction. *Anal. Chim.* **2001**, *450*, 215−222.

(36) Khosraviani, M.; Blake, R. C., II; Pavlov, A. R.; Lorbach, S. C.; Yu, H. N.; Blake, D. A. Binding properties of monoclonal antibody directed toward lead-chelate complexes. *Bioconjugate Chem.* **2000**, *11*, 267−277.

(37) Sillanpaa, M.; Hulkkonen, R. M.; Manderscheid, A. Drinking water quality in the alpine pastures of the eastern Tibetan plateau. *Rangifer* **2004**, *15*, 47−52.

Received for review March 16, 2007. Revised manuscript received April 30, 2007. Accepted May 1, 2007. Financial support was from the National "863" Project (2005AA246031) of China. Partial support was also provided by the Office of Science (BER), U.S. Department of Energy, Grant DE-FG02-98ER62704 (to D.A.B.).

JF070787D