

Antibody-based sensors for heavy metal ions[☆]

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Abstract

Competitive immunoassays for Cd(II), Co(II), Pb(II) and U(VI) were developed using identical reagents in two different assay formats, a competitive microwell format and an immunosensor format with the KinExA™ 3000. Four different monoclonal antibodies specific for complexes of EDTA–Cd(II), DTPA–Co(II), 2,9-dicarboxyl-1,10-phenanthroline–U(VI), or cyclohexyl–DTPA–Pb(II) were incubated with the appropriate soluble metal–chelate complex. In the microwell assay format, the immobilized version of the metal–chelate complex was present simultaneously in the assay mixture. In the KinExA format, the antibody was allowed to pre-equilibrate with the soluble metal–chelate complex, then the incubation mixture was rapidly passed through a microcolumn containing the immobilized metal–chelate complex. In all four assays, the KinExA format yielded an assay with 10–1000-fold greater sensitivity. The enhanced sensitivity of the KinExA format is most likely due to the differences in the affinity of the monoclonal antibodies for the soluble versus the immobilized metal–chelate complex. The KinExA 3000 instrument and the Cd(II)-specific antibody were used to construct a prototype assay that could correctly assess the concentration of cadmium spiked into a groundwater sample. Mean analytical recovery of added Cd(II) was $114.25 \pm 11.37\%$. The precision of the assay was satisfactory; coefficients of variation were 0.81–7.77% and 3.62–14.16% for within run and between run precision, respectively. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Heavy metals are toxic and persistent environmental contaminants. Unlike carbon-based contaminants that can be completely degraded to relatively harmless products, metal ions can be transformed in only a limited number of ways by biological or chemical remediation processes. While such transformations are intended to limit the toxicity or solubility of a given metal species, there are usually competing processes in nature that will eventually recycle at least some of the metal ions back into their original highly toxic state. Metals often persist in the environment for long periods of time bound to soils or sediments; such bound

metals are relatively non-toxic except to bottom-feeding animals (Devi and Fingerman, 1995). Unfortunately, changes in weather, in the pH of the soil or water, or in other combinations of environmental factors can mobilize bound metals and greatly increase their availability and effective toxicity. For this reason, sites contaminated with heavy metals must be monitored on a regular basis. In addition, sites undergoing remediation must be monitored frequently to assess progress in heavy metal removal.

Inductively coupled plasma atomic emission spectroscopy (ICPAES) is the analytical technique most frequently used to measure trace metals in natural materials (Meyer, 1987; Komaromy-Hiller, 1999). ICPAES is based on the principle that the intensity of light emitted from metal atoms undergoing electron transitions at high temperatures is directly proportional to the concentration of these atoms present in an argon plasma. Samples introduced into the plasma

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are atomized and excited to various states, depending upon the amount of energy absorbed in the plasma. The resulting emission, at distinct wavelengths for each given element, can be used for qualitative and quantitative analyses. Sample preparation for this technique usually involves acid digestion at elevated temperatures and pressures, treatment procedures not easily adaptable to field-portable analysis. The technique routinely affords quantification limits as low as 10 ppm for soil and sediments, depending upon the sample matrix and method sensitivity for a given element. Utilization of an ultrasonic nebulizer extends the limit of detection to the low ppb level for dissolved metals in natural waters. ICPAES instruments measure the total amount of a specific metal in an environmental sample, but provide no information about metal oxidation state or speciation. Because these laboratory methods for metal ion analysis are labor-intensive, time-consuming, and expensive, fewer than the optimal numbers of samples are analyzed, leading to undesirable ambiguity in estimates of contaminant extent and risk.

Antibody-based assays offer an alternate approach for metal ion detection. These assays have significant advantages over the more instrument-intensive methods described above. Immunoassays are quick, inexpensive, simple to perform, and reasonably portable; they can also be highly sensitive and selective. Sample analysis is one of the major costs in the remediation of a contaminated site, and studies have shown that the use of antibody-based assays can reduce analysis costs by 50% or more (Szurdoki et al., 1996). Although most of the presently available environmental immunosensors have been designed for the analysis of toxins, explosives, and pesticides (Guilbault et al., 1992; Narang et al., 1997; Schipper et al., 1998; Charles and Kusterbeck, 1999), the availability of antibodies to heavy metals would permit the construction of immunosensors for this important class of contaminants as well. Very few antibodies have been reported with the ability to bind heavy metals. Monoclonal antibodies directed toward mercuric ions have been generated by immunization of animals with a glutathione–Hg derivative (Wylie et al., 1992). Lerner and coworkers have reported the isolation of recombinant antibody fragments that preferentially recognized certain metals in complex with iminodiacetic acid; these recombinant antibodies were obtained by screening a library with randomized amino acids in the third complementarity determining region of the heavy chain (Barbas et al., 1993). In the present study, we briefly review the binding properties of four different metal-specific monoclonal antibodies developed in our laboratory and delineate two different competitive immunoassay formats for the immunological detection of heavy metals in water samples.

2. Experimental

2.1. Reagents

Purified mouse monoclonal antibodies with specificities for chelated cadmium, lead, cobalt, and uranium were available from previous studies. The metal ion binding properties and metal-ion specificities of these antibodies have been described (Blake et al., 1996, 1998a, 2001; Khosraviani et al., 2000). The antibody to cadmium (2A81G5) has been previously used in a microplate assay that accurately measured cadmium at low ppb levels in environmental water samples (Blake et al., 1998b). Atomic absorption grade Cd, Pb, and Co were obtained from Perkin-Elmer (Norwalk, CT). Uranyl acetate (ACS grade) was a product of Mallinckrodt Chemical Works (St Louis, MO). *Trans*-cyclohexyldiethylenetriamine pentaacetic acid (CHXDTPA) and 1,10-phenanthroline-2,9-dicarboxylic acid (DCP) were synthesized for previous studies (Khosraviani et al., 2000; Blake et al., 2001). Ethylenediamine tetraacetic acid (EDTA), diethylenetriamine pentaacetic acid (DTPA), Hepes buffer, and goat anti-mouse IgG coupled to horseradish peroxidase were purchased from Sigma Chemical Co. (St Louis, MO). The fluorescein and Cy3 conjugates of affinity purified goat anti-mouse IgG were products of Jackson ImmunoResearch Laboratories (West Grove, PA). Bovine serum albumin (fatty acid ultrafree) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Microwell plates for ELISA (high-binding, flat-bottom) were products of Corning/Costar (Cambridge, MA). 3,3',5,5'-Tetramethylbenzidine peroxidase substrate (TMB microwell substrate) was a product of Kirkegaard-Perry Laboratories (Gaithersburg, MD). Poly(methyl methacrylate) and polystyrene beads (98 ± 8 μ m diameter) were obtained from Sapidyn Instruments (Boise, ID). Metal-loaded chelators covalently bound to bovine serum albumin (BSA-thioureido-L-benzyl-EDTA–Cd(II), BSA-thioureido-L-benzyl-cyclohexyl-DTPA–Pb(II), BSA-thioureido-L-benzyl-DTPA–Co(II), and BSA-thioureido–DCP) were available from previous studies (Blake et al., 1996, 1998a, 2001; Khosraviani et al., 1998, 2000).

2.2. Competitive immunoassays for heavy metals on microwell plates

The general procedures for metal ion immunoassays on microwell plates have been described elsewhere (Blake et al., 1998a,b; Khosraviani et al., 1998). For the cadmium immunoassays, microwell plates were coated (50 μ l/well) with 0.5 μ g/ml of BSA-thioureido-L-benzyl-EDTA–Cd(II) in Hepes-buffered saline (HBS, 137 mM NaCl, 3 mM KCl, 10 mM Hepes, pH 7.4); for the

lead and cobalt immunoassay, BSA-thioureido-L-benzyl-DTPA-Pb(II) or -Co(II) diluted to 1.0 $\mu\text{g/ml}$ in HBS was used. Microwell plates for hexavalent uranium analysis were coated with 2.0 $\mu\text{g/ml}$ BSA-thioureido-DCP in HBS containing 5 μM UO_2^{2+} . For coating, plates were incubated for at least 2 h at 37 °C. Coated plates were stable at least 4 weeks when stored at 4 °C. Before use, plates were washed three times with phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) containing 0.05% Tween 20 (PBS-Tween), blocked for at least 15 min with 3% BSA in HBS, and washed again three times with PBS-Tween.

Purified antibody (1–4 $\mu\text{g/ml}$) was incubated in the presence of a fixed concentration of chelator and varying concentrations of metal ion in a total volume of 50 μl of HBS in the coated, blocked microwell plates (shown in Fig. 1). After 1 h at 25 °C, the plates were washed with PBS-Tween and the amount of bound primary antibody was quantified using a goat anti-mouse IgG-horseradish peroxidase conjugate and TMB microwell substrate.

2.3. Competitive immunoassays for heavy metals on the KinExA automated immunoassay instrument

Details of the KinExA 3000 instrument and assays procedures have been described elsewhere (Blake et al.,

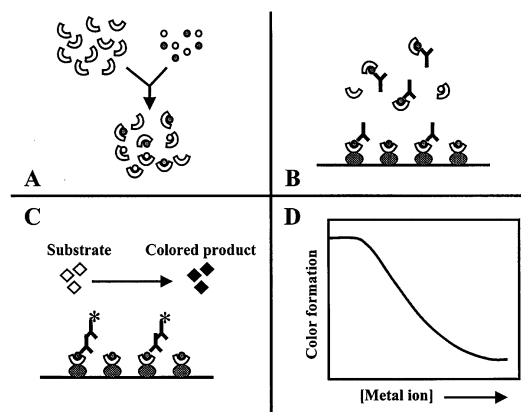


Fig. 1. Format for microwell-based assay of metal ions. (A) Sample containing metal ions (circles) is diluted into buffer containing a molar excess of metal-free chelate (crescents). (B) The solution containing the chelate and metal-chelate complexes is mixed with antibody (Y) in microwell plates containing immobilized protein-chelate-metal conjugates. The lower affinity, soluble metal-chelate complex and the higher affinity, immobilized metal-chelate-BSA conjugate compete for antibody binding sites. (C) After a wash step to remove any antibody bound to the soluble metal-chelate complexes, an enzyme labeled anti-species antibody (Y*) is added. A second wash step removes unbound anti-species antibody, and a signal is generated by the addition of colorimetric substrate. (D) Signal versus metal ion concentration follows the classic pattern observed for competitive ELISAs. Standard curves for specific metal ions are shown in Figs. 3A–6A.

1996, 1999; Khosraviani et al., 2000). Rigid polymer beads (200 mg) were adsorption-coated for 1 h at 37 °C in 1 ml of metal-chelate-BSA conjugate (100 $\mu\text{g/ml}$ in HBS). After centrifugation and removal of the supernatant solution, any non-specific protein binding sites were blocked by subsequent incubation of the beads with 1% BSA. The beads could be stored for up to 4 weeks at 4 °C in the blocking buffer. HBS was added to the blocked beads to achieve a concentration of 6.7 mg beads/ml before use in the KinExA instrument.

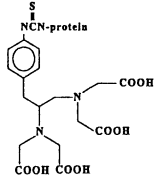
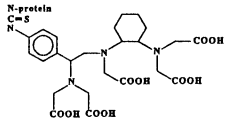
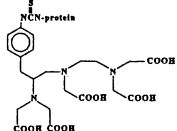
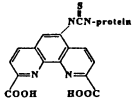
Purified monoclonal antibody (0.1–1 $\mu\text{g/ml}$) was mixed with a fixed concentration of chelator and varying concentrations of metal ion in a total volume of 1.5 ml. These reaction mixtures were allowed to come to equilibrium (10–20 min at 25 °C), then 0.5 ml of each reaction mixture was passed rapidly over beads coated with the appropriate trapping reagent. After a wash with HBS, the amount of bound primary antibody on the beads was quantified using Cy3- or fluorescein-labeled goat anti-mouse IgG. Data acquisition and instrument control were as previously described (Blake et al., 1996, 1999; Khosraviani et al., 2000).

2.4. Analysis of environmental samples using the KinExA 3000

Groundwater samples were collected at a well located in a background area of the Field Research Center established at Oak Ridge National Laboratories, Oak Ridge, TN, by the Natural and Accelerated Bioremediation Research Program (NABIR), United States Department of Energy. This well is approx. 25 feet deep, its waters are filtered through shale saprolite, and it serves as relatively uncontaminated, background control for other groundwater at the Field Research Center. Water samples from the reference well were collected in precleaned glass bottles with plastic tops and transported to the laboratory on ice. Analyses for inorganics were performed at the Oak Ridge site according to established protocols (US EPA, 1983, 1992). The water used in making spiked samples was filtered through a 0.45 μm filter and stored in precleaned 50 ml plastic centrifuge tubes. A series of cadmium-spiked samples was prepared in the concentration range 10–50 ppb by diluting a cadmium standard (1000 ppm Cd(II) in 5% nitric acid) with groundwater. The pH of the environmental water samples was subsequently adjusted to 7.4 by the addition of a 10% volume of a concentrated buffer solution (1.37 M NaCl, 30 mM KCl, 100 mM HEPES, 50 mM EDTA, pH 7.4). The samples were further diluted into HBS containing 5 mM EDTA to bring them into the linear range of the assay. For analysis, the diluted samples were mixed with an equal volume of purified 2A81G5 antibody (0.5 $\mu\text{g/ml}$ in HBS containing 2 mg/ml BSA). The samples were incubated for 10 min at 25 °C, then run on the KinExA 3000 as described above.

Table 1

Interactions of monoclonal antibodies with metal–chelate complexes

| Antibody Designation | Chelate Conjugate used as Immunogen | Soluble Metal–Chelate Complex | Equilibrium Dissociation Constant (M)* |
|----------------------|---|--|--|
| 2A81G5 |  | EDTA–Cd(II) <i>p</i> -nitrobenzyl–EDTA–Cd(II) BSA–thioureido–L–benzyl–EDTA–Cd(II) | 2.1×10^{-8} 5.6×10^{-10} 7.2×10^{-11} |
| 2C12 |  | DTPA–Pb(II) BSA–thioureido–L–benzyl–DTPA–Pb(II) CHXDTPA–Pb(II) BSA–thioureido–L–benzyl–CHXDTPA–Pb(II) | 1.0×10^{-5} 1.8×10^{-9} 8.4×10^{-9} 1.7×10^{-9} |
| 15B4 |  | DTPA–Co(II) BSA–thioureido–L–benzyl–DTPA | 5.2×10^{-8} not determined |
| 8A11 |  | DCP–U(VI) BSA–thioureido–DCP | 5.5×10^{-9} 1.2×10^{-9} |

*Constants for antibodies 2A81G5 and 2C12 have been published previously. (Blake et al., 1996; Khosraviani et al., 2000). Constants for antibodies 15B4 and 8A11 were determined as described in (Blake et al., 1999) in HBS amended with 5 mM DTPA (15B4) or 12.5 μ M DCP (8A11).

A standard curve for Cd(II) was generated in HBS solutions containing 0.25 μ g/ml purified 2A81G5 antibody, 5 mM EDTA, and 1 mg/ml BSA. Replicates ($n = 3$) were analyzed for each Cd(II) concentration and a calibration curve was generated by fitting the data to the following equation:

$$IF_{\text{exp}} = IF_0 - \frac{IF_{\infty} [Cd(II)]}{[Cd(II)] + Cd(II)_{50}} \quad (1)$$

where IF_0 is the integrated fluorescence obtained in solutions containing antibody but no Cd(II), IF_{exp} is the integrated fluorescence at each individual Cd(II) concentration $[Cd(II)]$ used to generate the curve, IF_{∞} is the difference in integrated fluorescence between cadmium concentrations of zero and infinity, and $Cd(II)_{50}$ is the cadmium concentration that produced a 50% inhibition in the signal. The concentrations of cadmium in the environmental samples were then obtained by interpolation on the standard curve.

3. Results and discussion

3.1. Binding characteristics of metal-specific monoclonal antibodies

Monoclonal antibodies with specificities for different metal–chelate complexes were prepared by immunizing animals with metal-loaded chelators covalently coupled to keyhole limpet hemocyanin. Binding properties and metal ion specificities of the antibodies used in this study have been described previously. The 2A81G5 monoclonal antibody has a primary specificity for EDTA complexes of Cd(II) and Hg(II). All other metal–EDTA complexes tested, including Mn(II), In(III), Ni(II), Zn(II), Co(II), Cu(II), Ag(I), Fe(III), Pb(II), Au(III), Tb(III), Ga(III), Mg(II), and Al(III), bound with affinities from 20 to 40,000 less than that determined for the Cd(II)–EDTA complex (Blake et al., 1996). The 2C12 antibody bound to CHXDTPA complexes of Pb(II) with nanomolar affinity. A survey of 15 different di-, tri-, and hexavalent metals demon-

strated that the only Pb(II)–CHXDTPA complex bound with higher affinity to the 2C12 antibody than did metal-free CHXDTPA (Khosraviani et al., 2000). The 15B4 antibody had a primary specificity for DTPA complexes of cobalt (K_d , 5.2×10^{-8} M); it bound to DTPA complexes of Ni(II) and Zn(II) with approx. 5-fold lesser affinity and showed little or no recognition of other metal–DTPA complexes or metal-free DTPA (Blake et al., 1998a). The 8A11 antibody recognized uranyl complexes of 1,10-phenanthroline-2,9-dicarboxylic acid (DCP) with nanomolar affinity (K_d , 5.5×10^{-9} M). Only Cu, Er, and Ti (of 21 additional metals tested) showed any detectable binding to the 8A11 antibody, and the DCP complexes of these three metals bound with affinities approx. 300-fold less than that determined for DCP- UO_2^{2+} (Blake et al., 2001).

Table 1 shows the structure of the original immunogen used to generate each of the four antibodies used in this study and summarizes the binding affinities of these antibodies for selected soluble metal–chelate complexes and metal–chelate–protein conjugates that closely resemble the original immunogen. Three of the antibodies used in this study (2A81G5, 15B4, and 2C12) appear to have an extended binding site that recognizes features of the original immunogen (a benzyl group that linked the chelator to the protein via a thioureido bond) not present in the soluble metal chelate complex derived from the environmental sample. For example, the 2A81G5 antibody bound to EDTA–Cd(II) complexes with an apparent dissociation constant of 21 nM. The addition of a *p*-nitrobenzyl group to the EDTA, to create a soluble ligand that more closely resembled the epitope on the original immunogen, lowered the K_d to 5.6×10^{-10} M. Covalently coupling the Cd(II)–EDTA–benzyl moiety to the ϵ -amino group of a lysine on the BSA molecule, to create a thioureido linkage identical to that in the original immunogen, decreased the K_d to 7.2×10^{-11} M (Blake et al., 1996). Thus, this antibody bound to the metal–chelate–BSA conjugate with an affinity approx. 3000 fold greater than that determined for the soluble metal–chelate complex.

The 2C12 antibody also exhibited preferential binding to the metal–chelate–BSA conjugate over the soluble metal–chelate complex, as shown in Table 1. This antibody has the ability to recognize Pb(II) in complex with two structurally related chelators, DTPA and CHXDTPA (Khosraviani et al., 2000). The Pb(II)–DTPA ligand bound the 2C12 antibody with relatively low affinity (K_d , 1.0×10^{-5} M). Like the 2A81G5 antibody, addition of a thioureido-L-benzyl group increased the affinity for the Pb(II)-loaded protein conjugate by approx. 300-fold. Substitution of CHXDTPA for DTPA in the Pb(II)–chelate complex increased the affinity of the antibody more than 1000-fold. However, the 2C12 antibody also recognizes metal-free CHXDTPA with relatively high affinity (K_d , 2.3×10^{-7}

M) and care must be taken when formatting immunoassays with CHXDTPA, since high concentrations of this chelator will inhibit signal in the assay.

The binding specificities of the 15B4 and 8A11 antibodies are not as well characterized as those of the 2A81G5 and 2C12 antibodies. However, the original immunogen used to generate 15B4 had a linker region (a thioureido-L-benzyl group) identical to that used to generate the 2A81G5 and 2C12 antibodies, and preliminary experiments have demonstrated that it binds with greater affinity to soluble complexes containing these structures (data not shown). It should be noted that a monoclonal antibody directed against a similar epitope [thioureido-L-benzyl–EDTA–In(III)] was also reported to bind with greater affinity to a nitrobenzyl derivative of EDTA–In(III) than to the soluble EDTA–In(III) (Reardan et al., 1985; Meyer et al., 1990). Perhaps participation of the benzyl portion of such immunogens in the binding interactions with the respective high affinity antibodies will be found to be a general feature of the properties of this class of metal–chelate specific antibodies.

In contrast to other immunogens used in these studies, where a hydrophobic and apparently highly immunogenic benzyl group was used as linker to attach the metal–chelate complex to the carrier protein, the immunogen exploited to generate antibody 8A11 employed a metal chelator that already possessed a fused aromatic ring system to which a reactive functional group could be directly attached. By eliminating the need to covalently add a separate, bulky benzyl moiety in the linker region between the chelator and the protein, the thioureido–DCP–U(VI) represented an immunogen and protein conjugate that more closely resembled the corresponding soluble analogue, the DCP–U(VI) complex. Thus the differences in the affinity for the binding of 8A11 to soluble DCP–U(VI) and the protein–thioureido–DCP–U(VI) conjugate are much smaller than those observed for the antibodies directed against the immunogens containing the thioureido-L-benzyl linker group discussed above.

3.2. Comparison of microwell and KinExA formats

The ability of these four antibodies to detect heavy metal ions in aqueous samples was assessed using two different immunoassay formats. The format for the microwell-based assay is shown in Fig. 1. The sample that contains metal ions is diluted into a buffer containing a molar excess of chelator (Panel A). Because all of the chelators used in these studies bind very tightly to metal ions (Margerum et al., 1978), the presence of a molar excess of chelator insures that all of the metal ions in the sample will be converted to metal–chelate complexes. These metal–chelate complexes are subsequently mixed with monoclonal antibody and a

protein-chelate-metal conjugate immobilized on the surface of the microwell plate (Panel B), where the lower affinity, soluble metal-chelate complex derived from the environmental sample and the higher affinity immobilized conjugate compete for antibody binding sites. After a wash step to remove soluble antibody-antigen complexes, an enzyme-labeled antispecies antibody is added and excess antispecies antibody is removed in a second wash step (Panel C). Finally, an appropriate colorimetric enzyme substrate is added to quantify the enzyme-labeled antibody captured and retained on the surface of the microwell. The resulting instrument response developed in the assay conforms to the log-linear concentration dependence characteristic of such competitive immunoassays (Panel D).

The format for the KinExA-based assay is illustrated schematically in Fig. 2. The KinExA is a computer-controlled flow fluorimeter designed to achieve the rapid separation and quantification of free, unbound protein in reaction mixtures of free antibody, free antigen, and antibody-antigen complexes (Blake et al., 1999). Briefly,

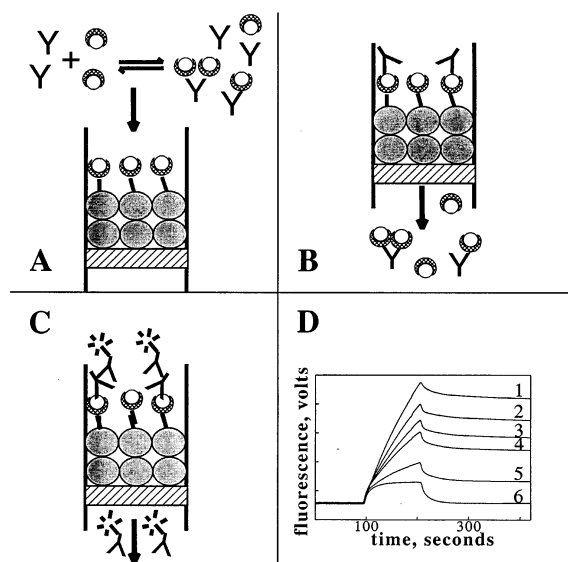


Fig. 2. Format for KinExA-based assay of metal ions. (A) Protein-chelate-metal conjugate is immobilized on rigid polymer beads and held in the observation cell by a microporous screen. The equilibrium mixture of antibody, soluble metal-chelate complex, and antibody-antigen complex is passed rapidly over the beads (~ 240 – 400 ms interaction time). (B) A portion of those antibody molecules with free binding sites is captured by the column, while antibody bound to soluble metal-chelate complex, and free metal-chelate complex are washed through the beads. (C) Fluorescently labeled anti-species antibody is used for detection of antibody bound to the beads. Fluorescence is continuously monitored and recorded via a PC interface. (D) Raw data curves from the KinExA instrument. Curve 1 corresponds to zero ligand concentration; curve 6 corresponds to a saturating ligand concentration. Curves 2–5 are concentrations of ligand between zero and saturation. The later, linear portions of these instrument response curves are integrated over time and plotted against metal ion concentration to yield the standard curves shown in Figs. 3B–6B.

the KinExA consists of a capillary flow/observation cell (inner diameter, 1.6 mm) fitted with a microporous screen through which various solutions are drawn under negative pressure. Uniform particles larger than the average pore size of the screen (98 and 53 μm , respectively) are adsorption-coated with the protein-chelate-metal conjugate and deposited above the screen in a packed bed. A fresh bed of beads is used for each determination. As in the microwell format, all the metal ions in the sample are first converted to metal-chelate complexes by the addition of a molar excess of chelator (not shown). The antibody and the soluble metal chelate complexes are then mixed together and the binding reaction between the two soluble components is permitted to approach equilibrium (Panel A). An aliquot of this equilibrium mixture is then rapidly (250–400 ms) passed over the beads in the capillary/observation flow cell, followed by a buffer wash step to remove excess soluble metal-chelate complexes and antibodies with occupied binding sites (Panel B). A fluorescently labeled antispecies antibody is then passed through the observation cell and excess unbound fluorescent antibody is removed with another wash step (Panel C). Data acquisition is initiated immediately following the establishment of the microcolumn, and representative examples of instrument responses as a function of time are shown in Panel D for various concentrations of soluble metal-chelate complexes. The instrumental response from 0 to 95 s corresponds to the background signal generated while the unlabeled equilibrium mixture is exposed to and washed out of the packed microbead column. The beads were then exposed to a solution of fluorescently-labeled goat anti-mouse antibody (95–215 s), and excess unbound labeled secondary antibody was removed from the beads with a buffer wash (215–420 s). When the equilibrium mixture contained a saturating concentration of free ligand (curve 6), the instrument response approximated a square wave corresponding to the fluorescence of the secondary antibody during its transient passage past the beads in the observation cell. The signal failed to return to background, indicating a 0.5% nonspecific binding of the fluorescently-labeled antibody to the beads. When soluble ligand was omitted from the equilibrium mixture (curve 1), the instrument response from 95 to 215 s reflected the sum of two contributions: the fluorescence of unbound secondary antibody in the interstitial regions among the beads and that of the labeled secondary antibody that had bound to the primary antibody captured by the antigen immobilized on the beads. Binding of the secondary antibody was an ongoing process that produced a positive slope in this portion of the curve. When the excess unbound antibody was washed from the beads, the signal that remained was the sum of that from the nonspecifically bound antibody plus that of the labeled antispecies antibody specifically bound to the primary antibody captured on the beads. Equilibrium mixtures comprised

of soluble ligand present at concentrations intermediate between those of zero and saturation (curves 2–5) thus provided intermediate instrument responses from which calibration curves could be generated.

In principal, information could be derived from the slopes of the curves in the 95–215 s interval, from the average value of a portion of the plateau in the 215–420 interval, or from the corresponding integrals of the area under selected portions of the curve. The fluorescence signal was integrated from 300 to 349 s for the studies presented herein.

Both of the formats described above represent antigen-inhibition assays where a soluble and immobilized version of the antigen compete for a limited number of binding sites on the antibody. If the soluble and immobilized forms of the antigen bind with differing affinities to the same antibody, then the format of the assay may be expected to influence the performance characteristics of the assay (sensitivity, range of detection, etc.), even when exactly the same reagents are employed in each.

In the ELISA described herein, the antibody, soluble metal–chelate complex, and immobilized protein-conjugated metal–chelate complex were incubated together until no further changes in the binding of the soluble antibody to the immobilized conjugate could be detected (50–60 min, data not shown). The purpose of this long incubation was to allow sufficient time for the limited number of antibody molecules in solution to overcome the mass transport limitations attendant with the binding and equilibration of the soluble reagents with a surface-bound capture reagent. That is, a sufficient number of antibody molecules must be bound to the microwell surface to create a quantifiable and reproducible signal when the enzyme-labeled secondary is added in the subsequent step of the ELISA. One consequence of this long incubation time is that operational equilibrium is achieved among the three principal components in the assay. If the antibody binds with significantly higher affinity to the immobilized metal–chelate conjugate than to the soluble metal–chelate complex, then excess soluble antigen is required to compete with the immobilized conjugate. As a consequence, this ELISA format should create a less sensitive assay.

In the KinExA assays described herein, the antibody and soluble metal–chelate complex were incubated and allowed to approach binding equilibrium in solution before subsequent exposure of the mixture to the immobilized, protein conjugated metal–chelate complex on the surface of the beads. The time of exposure of each equilibrium mixture to the immobilized capture reagent was kept sufficiently short to insure that negligible dissociation of the soluble antigen–antibody complexes occurred during the swift passage of the mixture through the beads in the observation cell. Consequently, the immobilized antigen served merely as a tool to separate and quantify only those antibodies in the equilibrium

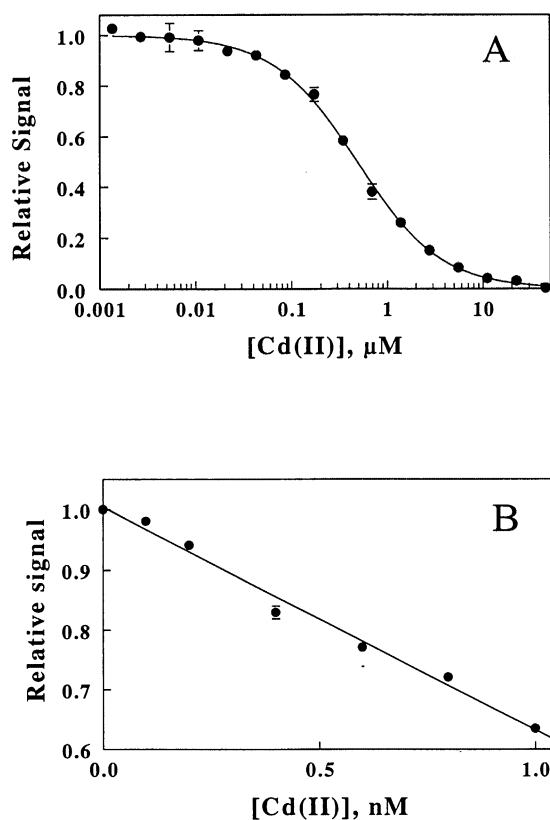


Fig. 3. Immunoassays for cadmium. Assays were performed in HBS containing 5 mM EDTA and the indicated concentrations of atomic absorption-grade cadmium. (A) Microwell-based immunoassay for Cd(II). Error in the determination is expressed as SD. (B) KinExA-based assay for Cd(II). Points represent integrated fluorescence from duplicate determinations.

mixture that bore unoccupied binding sites. Since the immobilized antigen has limited time to compete for antibody binding sites, the KinExA format should create a more sensitive assay with a lower limit of detection in those cases where the antibody binds with higher affinity to the immobilized than the soluble antigen. Quantifiable and reproducible instrument responses were achieved in the KinExA format by using beads (approx. 10,000/column) with a higher surface to maximize the opportunities for the capture of free antibody area (surface area in KinExA is approx. 260 mm² (Blake et al., 1999), compared to the 64 mm² calculated for each microwell in the ELISA format). In addition, the high flow rate of the reagent through the beads minimizes mass transport limitations at the reaction surface.

3.3. Comparison of assays using the ELISA and KinExA formats

Immunoassays were assembled for ionic cadmium in the ELISA and KinExA formats using 5 mM soluble EDTA, immobilized BSA-thioureido-L-benzyl–EDTA–Cd(II), and antibody 2A81G5; the results are shown in

Fig. 3. Fig. 3A shows the results obtained using the ELISA format where the relative colorimetric signal is plotted as a function of the logarithm of the concentration of ionic cadmium in the sample. Fig. 3B shows the corresponding results obtained using the KinExA format where the relative integrated fluorescence signal is plotted as a linear function of the concentration of Cd(II) in the sample. In both cases, a conservative estimate of the limit of detection for each assay was obtained by noting the concentration of Cd(II) necessary to decrease the relative signal by 10%; those values corresponded to 50 and 0.25 nM for assays conducted using the ELISA and KinExA formats, respectively. The large difference in apparent assay sensitivity is readily rationalized in view of the different affinities determined for the binding of 2A81G5 to soluble and protein-conjugated EDTA–Cd(II) (Table 1).

In the ELISA format, the 3000-fold greater affinity of the antibody for the protein conjugate over the soluble EDTA–Cd(II) dictated that the antibody would bind preferentially to the immobilized form of the metal-chelate complex in the three-component binding reaction present in the microwell. That meant that a relatively

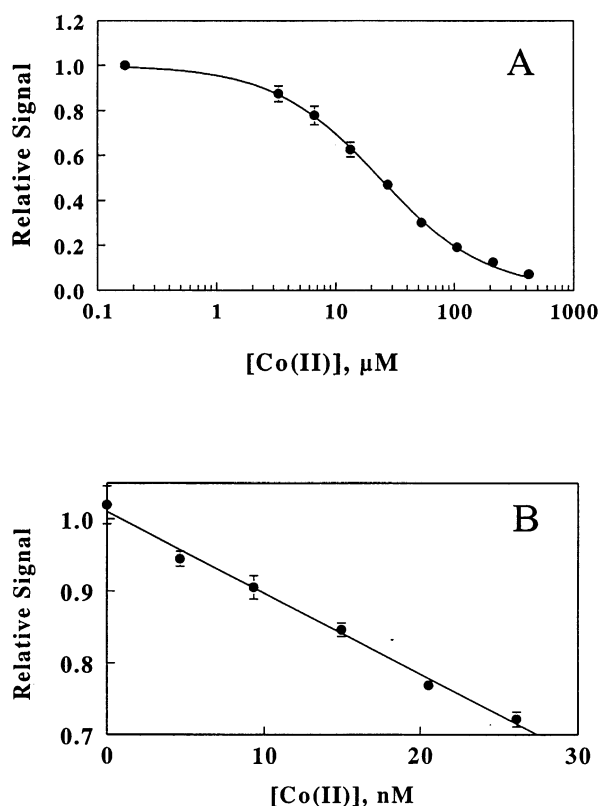


Fig. 4. Immunoassays for cobalt. Assays were performed in HBS containing 5 mM DTPA and the indicated concentrations of atomic absorption-grade cobalt. (A) Microwell-based assay for Co(II). Error in the determination is expressed as SD. (B) KinExA-based assay for Co(II). Points represent integrated fluorescence from triplicate determinations.

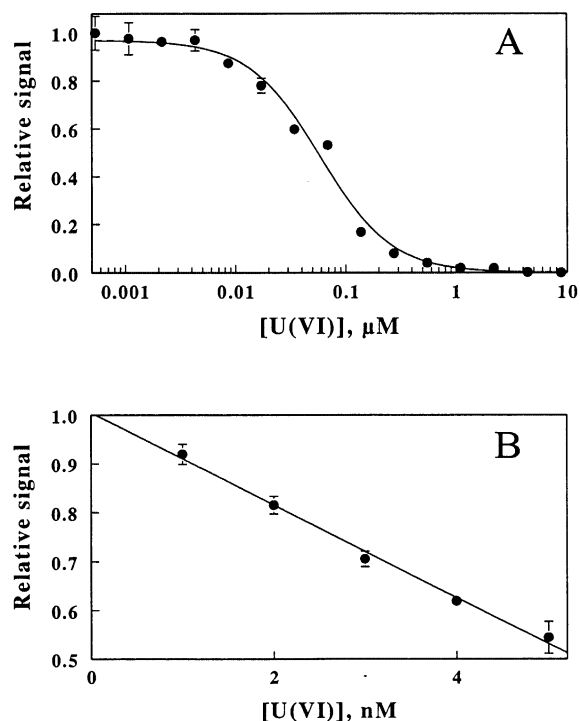


Fig. 5. Immunoassays for uranium. Assays were performed in HBS containing 12.5 μM DCP and the indicated concentrations of uranyl acetate. (A) Microwell-based assay for U(VI). Error in the determination is expressed as SD. (B) KinExA-based assay for U(VI). Points represent integrated fluorescence from triplicate determinations.

high concentration of soluble EDTA–Cd(II) was required to effectively compete with the immobilized antigen for the limited antibody in solution. Consequently, the assay showed a relatively high limit of detection (50 nM) and effective range.

In the KinExA format, the same 3000-fold difference in affinity only insured that the immobilized protein conjugate would be an effective trapping reagent to secure a quantifiable signal in the instrument; the binding equilibrium between the antibody and the soluble metal-chelate complex was undisturbed for all practical purposes. Therefore, a relatively low concentration of soluble EDTA–Cd(II) was sufficient to affect a detectable change in the concentration of free antibody captured in the flow cell, and the KinExA assay was approx. 200-fold more sensitive than the corresponding ELISA.

Similar influences of assay format on the effective limits of detection of soluble Co(II) were obtained when assays were conducted for ionic cobalt using 5 mM soluble DTPA, immobilized BSA-thioureido-L-benzyl-DTPA–Co(II), and antibody 15B4. Fig. 4A and Fig. 4B show the results obtained in the ELISA and KinExA formats, respectively. Conservative estimates for the limits of detection for Co(II) using the ELISA and KinExA were greater than 1.0 μM and 10 nM, respectively. Although a precise value for the equilibrium

dissociation constant for the binding of BSA-thioureido-L-benzyl-DTPA-Co(II) to antibody 15B4 is unavailable (Table 1), estimations from preliminary data indicate that the protein conjugate binds with at least 10-fold greater affinity to 15B4 than does the soluble DTPA-Co(II) complex. Thus the rationale offered above for the difference in sensitivities for the ELISA and KinExA for Cd(II) apply equally well to the differences in sensitivities apparent in the results represented in Fig. 4.

The influence of assay format on the effective limits of detection of soluble U(VI) were much smaller than those reported above when assays were conducted for ionic uranium using soluble DCP, immobilized BSA-thioureido-DCP-U(VI), and antibody 8A11. Fig. 5A and Fig. 5B show the results obtained in the ELISA and KinExA formats, respectively. Estimates for the limits of detection for U(VI) using the ELISA and KinExA were 10 and 1.0 nM, respectively. The difference in affinities for the binding of 8A11 to its soluble and its protein-conjugated antigens was significantly less than the corresponding differences observed for antibodies 2A81G5 and 15B4, because the DCP-protein conjugate used as the original immunogen did not contain the highly immunogenic benzyl linker group present in the other three protein conjugates (see Table 1 for a comparison

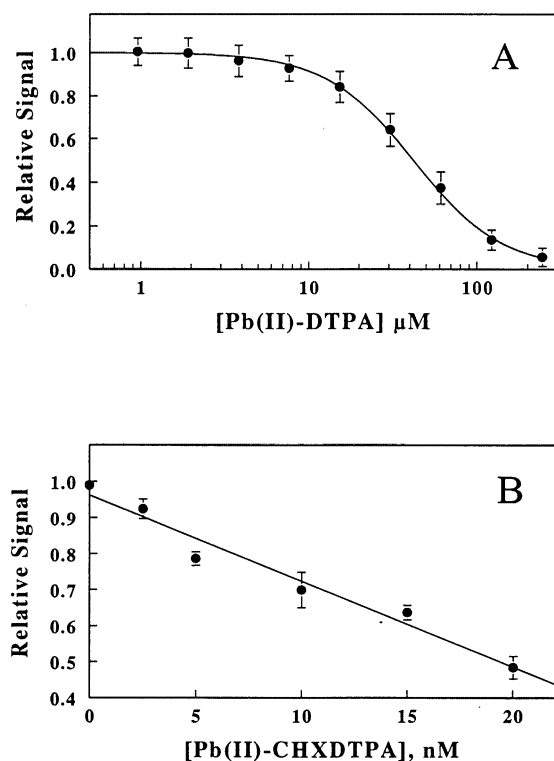


Fig. 6. Immunoassays for lead. (A) Microwell-based assay for Pb(II) was performed in HBS containing 5 mM DTPA and the indicated concentrations of atomic absorption-grade Pb(II). (B) KinExA-based assay for Pb(II) was performed in HBS containing 125 nM CHXDTPA and the indicated concentrations of Pb(II). Points represent integrated fluorescence from triplicate determinations.

Table 2
Precision of KinExA 3000 assay for Cd(II)

| Cd(II) (ppb) | Within run, $n = 2$ | | Between runs, $n = 7$ | |
|--------------|---------------------|---------------------|-----------------------|--------|
| | SD (ppb) | CV (%) ^a | SD (ppb) | CV (%) |
| 0.25 | 0.01 | 1.09 | 0.03 | 3.62 |
| 1.00 | 0.00 | 0.81 | 0.02 | 4.29 |
| 2.50 | 0.03 | 7.77 | 0.02 | 6.85 |
| 6.00 | 0.01 | 4.30 | 0.03 | 14.18 |
| 15.00 | 0.01 | 7.28 | 0.01 | 12.55 |

^a CV, coefficient of variation.

of structures). The three-part equilibrium binding reaction in the ELISA format was not expected to as greatly favor the immobilized form of the antigen, and as a consequence, the limits of detection obtained using the ELISA and KinExA formats would be more comparable. The accuracy of that prediction is evident from the data in Fig. 5.

Finally, the influence of assay format on the effective limits of detection of soluble Pb(II) are illustrated by the data in Fig. 6. Fig. 6A represents the ELISA conducted using soluble DTPA, immobilized BSA-thioureido-L-benzyl-CHXDTPA-Pb(II), and antibody 2C12. Since soluble DTPA-Pb(II) binds with very low affinity to the 2C12 antibody (Table 1), the resulting ELISA is relatively insensitive, with an estimated limit of detection of greater than 10 μM. The estimated limit of detection for the corresponding KinExA assay using the same reagents was only about 2-fold lower (data not shown).

As part of an ongoing effort to enhance the sensitivity of immunoassays for this environmentally important toxic metal ion, the consequences of substituting CHXDTPA for DTPA as the soluble chelator in the initial reaction mixture were also investigated. Since 2C12 binds soluble CHXDTPA-Pb(II) with 10,000-fold greater affinity than DTPA-Pb(II), it was anticipated that the greater affinity of the antibody for the former ligand would enhance the performance characteristics of the immunoassay. Unfortunately, we were unable to construct a useful ELISA when the cyclohexyl derivative was the soluble chelator; even sub-micromolar concentrations of the metal-free CHXDTPA appeared to prevent acceptable binding of the antibody to the immobilized antigen in the microwells (data not shown). In contrast, an immunoassay for Pb(II) was successfully performed using the corresponding KinExA format. Fig. 6B represents the KinExA assay conducted using soluble CHXDTPA, immobilized BSA-thioureido-L-benzyl-CHXDTPA-Pb(II), and antibody 2C12. The estimated limit of detection was 6 nM, more than three orders of magnitude lower than that obtained with the soluble DTPA. These experiments comprised an example where the unique binding properties of the antibody exerted a direct influence on the most effective format for conducting the immunoassay.

Table 3

Heavy metal analyses of a representative groundwater sample from Oak Ridge National Laboratories

| Metal ^a | Concentration (ppb) | Analysis method |
|--------------------|---------------------|-------------------|
| Calcium | 64,900 | ICP, EPA 6010 |
| Magnesium | 2580 | ICP, EPA 6010 |
| Strontium | 98.4 | ICP, EPA 6010 |
| Manganese | 38.9 | ICP, EPA 6010 |
| Uranium | 0.717 | ICP/MS, EPA 200.8 |

^a All other di- and trivalent cations tested (Cd, Al, Fe, Pb, Mb, Ni, Ag, Th, Tl, Cr, Co, Cu, and Zn) were below the detection limits of the instruments.

3.4. The KinExA 3000 as a metal ion immunosensor

In preliminary experiments to demonstrate the ability of the KinExA 3000 instrument to function as an immunosensor for environmental samples, a prototype assay for cadmium was developed using the 2A81G5 antibody. The within-run and between-runs precision of the assay were determined at different levels of cadmium (0.25, 1.00, 2.50, 6.00, and 15.00 ppb). The within-run precision was assessed by analyzing duplicate samples in a single run and between-runs precision was assessed by analyzing the same sample in seven separate runs. The assay gave acceptable results over all tested concentrations; the coefficients of variation were 0.81–7.28% and 3.62–14.18% for within-run and between-runs precision, respectively (Table 2). The accuracy of the method was tested by spike and recovery tests. Various known amounts of atomic absorption grade cadmium were added to purified water and a well-characterized groundwater sample from Oak Ridge National Laboratories (Table 3). Each sample was subsequently analyzed in duplicate (purified water) or triplicate (groundwater) for cadmium content. The mean analytical recovery was calculated as the ratio, expressed as a percentage, of the cadmium concentration found to the cadmium concentration added. As shown in Table 4, a quantitative

Table 4

Analytical recovery of Cd(II) added to spiked water samples

| Purified water | | Environmental water | |
|----------------|---------------------------|---------------------|----------------------------|
| Cd(II) (ppb) | Recovery (%) | Cd(II) (ppb) | Recovery (%) |
| 0.25 | 84.76 ± 1.01 ^a | 20 | 129.70 ± 2.87 ^b |
| 1.00 | 103.13 ± 4.14 | 30 | 104.27 ± 5.70 |
| 2.50 | 96.82 ± 18.68 | 40 | 107.35 ± 2.44 |
| 6.00 | 123.98 ± 3.88 | 50 | 115.66 ± 3.25 |
| Average | 102.17 ± 16.42 | | 114.25 ± 11.37 |

Recovery values were calculated as the ratio, expressed as percentage, between the Cd(II) found to the Cd(II) added.

^a Values are the mean of duplicate determinations ± SD.

^b Values are the mean of triplicate determinations ± SD.

recovery (102.17 and 114.25% for purified water and groundwater, respectively) was obtained. The differences in the sensitivities of the two assays arose from interfering substances in the groundwater sample matrix that required a 20-fold dilution of the spiked sample before analysis. Further studies are in progress to identify the nature of these interfering agents. Nevertheless, this prototype immunosensor was able to accurately measure cadmium in a moderately complex sample matrix at levels comparable to those achieved by ICPAES.

4. Conclusion

These data demonstrate that the format of the assay may influence its performance characteristics (sensitivity, range of detection, etc.), even when exactly the same reagents are employed. The superior performance of the KinExA format is most likely due to (1) the high surface area of beads containing the immobilized capture reagent (protein-thioureido-L-benzyl-chelate-metal) in the flow cell of the instrument, (2) the high flow rate of the reagent through the beads, which minimizes the diffusion limitations at the reaction surface, and (3) the limited time (250–400 ms) that the antibody is in contact with the capture reagent. The KinExA is currently available only as a research grade, bench top instrument; however, experiments are in progress to miniaturize this instrument for field use. The availability of a portable instrument for field analysis that could detect heavy metals in near real-time would significantly decrease the cost of site monitoring and remediation activities, and greatly improve risk assessment efforts.

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