Fluorescence Polarization Immunoassays for Metal Ions

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Abstract: Antibodies raised against a given metal ion complex of a polyaminopolycarboxylate chelating agent can display specificity for the immunizing chelate and, when used in conjunction with a fluorophore-labeled analog of that chelate, can form the basis for highly sensitive and specific methods for detecting that metal ion by competitive inhibition fluorescence polarization immunoassay (FPIA). Chelate complexes of ethylenediamine-N,N,N',N'- tetraacetic acid (EDTA) and of a hetrocyclic ring-substituted derivative of diethylenetriamine-N, N', N"-triacetic acid (DTTA) have been used to configure such assays for the heavy metal ions lead(II) and cadmium(II) respectively. Limits of detection for the 1:1 metal chelates under ideal conditions are 20 ppt for lead(II) and below 100 ppt for cadmium(II). Standard curves for 0 - 100 nM cadmium (II) chelate can be constructed in the presence of fixed 250 nM concentrations of the corresponding, potentially cross-reactive chelates of zinc(II), copper(II) and mercury(II). Cross-reactivity of the lead (II) FPIA with 15 non-target metals is below 0.2% in all cases except for mercury(II) (0.37%). These characteristics have allowed the development of FPIA methods for the quantitative analysis of lead in a variety of samples relevant to environmental monitoring, including soil, dust, solid wastes and drinking water. Although applied thus far to heavy metals that are of concern as toxic contaminants in the environment, anti-chelate FPIA methods are also in principle applicable to a wide variety of other metal ions, including precious metals and various transition and main group elements used or monitored in a range of industrial applications. As conventional methods for trace metal analysis based on atomic spectroscopy are relatively slow, expensive and cumbersome, anti-chelate FPIA methods have the potential to supplant many existing techniques and in so doing extend the use of immunoassay technology beyond the biomedical, veterinary and agricultural spheres in which it has historically found use.

1. TRACE METAL ANALYSIS TECHNIQUES

Elemental analysis is a basic tool in analytical chemistry. While non-metallic elements (notably carbon, hydrogen, nitrogen, phosphorus, sulfur and the halogens) represent a significant fraction of such testing, the majority of the known elements (some 70% of stable elements in the Periodic Table) are metallic in character and much of elemental analysis is directed to measuring metal concentrations. Well-established methods are available for measuring metal concentrations down to the sub-millimolar range. Such techniques are generally adequate to meet existing and projected needs for bulk metal analysis and will not be discussed further. Detection of metals at trace concentrations (μmolar and below) poses greater challenges and is the focus of most current efforts directed towards alternative or improved analytical methods. Existing techniques for trace metal analysis are generally based on one of three underlying principles:

Colorimetry: Metals react with small organic molecules (dyes) that undergo a characteristic change in their visible absorption spectrum upon binding to the metal.

Electrochemistry: Metals are, or can be, ionized and the charge transport properties are characterized.

Atomic spectroscopy: At high temperatures metals undergo transitions between the ground state and excited atomic states producing characteristic absorption and emission spectra.

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Colorimetric methods are routinely used for bulk metal analysis and can sometimes also be applied to trace metal determination in cases where a highly colored dye is available and/or where pre-concentration of the sample is feasible (e.g. by ion exchange). Electrochemical methods also are generally more applicable to bulk metal analysis but can sometimes be used to measure trace metal concentrations, again with or without pre-concentration of the sample. These techniques offer the advantages of relative simplicity and low cost but they tend to be subject to a variety of interferences and are not sufficiently sensitive to detect most metals at the lowest concentrations of interest (nanomolar and below). Atomic spectroscopy, although more complex and expensive to perform, is the most sensitive and versatile of the three techniques and constitutes the workhorse of trace metal analysis and the gold standard against which any new technique must be compared.

2. CHARACTERISTICS AND LIMITATIONS OF ATOMIC SPECTROSCOPY METHODS

Atomic spectroscopy techniques have been in use for the better part of a century, beginning with simple qualitative "flame tests" in which metals were identified by the color produced when the sample was placed in the flame of a bunsen burner. Over subsequent decades of development, the combination of a bunsen burner plus the human eye has evolved into sophisticated instrumentation with capital costs ranging well into six figures, but the underlying mechanics of making the measurement remain unchanged. The sample is heated to very high temperature either by introducing it into a flame (FAAS), by placing it in an electrically heated

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graphite tube (GFAAS) or by aspiration into a high temperature plasma (ICP-AES) and selected lines in the absorption or emission spectrum are monitored via dedicated optics. FAAS is generally somewhat less sensitive than is GFAAS but throughput in GFAAS instruments is restricted because the furnace must be allowed to cool between samples. Atomic absorption techniques measure one metal at a time whereas atomic emission methods can quantitate multiple metals in a single determination and are thus preferred when panels of metals are to be evaluated. The most sensitive, most complex and most expensive methods for measuring trace metal concentrations combine ICP analysis with mass spectrometry (ICP-MS).

Atomic spectroscopy instrumentation requires dedicated venting so that the operator does not inhale the gas phase metals produced during the measurement. In the case of ICP instruments, an external source of compressed gas to feed the plasma is also needed. Instruments generally have quite a large footprint and are subject to loss of optical alignment if treated roughly. Because of these characteristics, atomic spectroscopy is generally performed in a fixed base laboratory facility on discrete samples that have been collected in the field, packaged and returned to that facility for testing. Typical commercial testing laboratories will provide a routine result within several days of receiving the sample and a "rush" result within 24 hours. Throughput for a typical FAAS system equipped with an autosampler is on the order of 50 samples per hour. For some forms of atomic spectroscopy minimum sample size requirements can be quite large (10 - 20 mL).

The primary limitations on existing atomic spectroscopy techniques arise from the difficulties involved in trying to make such measurements rapidly, continuously and/or in the field. In many instances, it would be ideal to have a truly in situ measurement in which a sensor is implanted into the matrix of interest and then remains in place, unattended, making periodic measurements of the concentration of target metal(s) in that matrix. Only in the case of gas phase matrices such as stack gasses has it proven possible to adapt atomic spectroscopy techniques to such continuous monitoring uses. In other applications, it may not be essential to measure metal concentrations in situ but it may still remain highly desirable that the measurement be made close to the sampling event in terms both of elapsed time and geographic location. Such scenarios range from pure field testing with no supporting infrastructure beyond that which can be hand carried to mobile laboratories of varying size, complexity and cost. Pure field testing by atomic spectroscopy remains a goal of ongoing attempts to miniaturize the technology but currently the only practical way to deploy atomic spectroscopy testing in the field is to mount an existing lab-based instrument in a modified trailer or RV with appropriate air handling capabilities. Although this can be done, it is expensive and is only cost effective when a large number of samples are to be analyzed and/or when there is a particularly high premium placed on a timely result.

$$M + CHEL = (M\text{-}CHEL)$$

$$K_a = [(M\text{-}CHEL)] / [M]\,[CHEL]$$

$$Ab + (M\text{-}CHEL) = Ab\text{-}(M\text{-}CHEL)$$

$$K'_a = [Ab\text{-}(M\text{-}CHEL) / [Ab]\,[(M\text{-}CHEL)]$$

For polyaminopolycarboxylate chelators K_a is between 10^{10} and 10^{40} while K'_a is between 10^7 and 10^{11}

M = Metal ion, CHEL = Chelating agent, Ab = Antibody

Fig. (1). Binding characteristics in chelate/anti-chelate systems.

3. IMMUNOASSAY AS AN ALTERNATIVE TO ATOMIC SPECTROSCOPY FOR TRACE METAL ANALYSIS

3.1 Context

Alternative technologies that would be capable of performing rapid, continuous, on-site or in situ trace metal analysis are the focus of much current research. This ranges from attempts to use modified forms of atomic spectroscopy (such as SIBS [1]) through a variety of sensor and biosensor approaches to advanced "lab on a chip" concepts. Many novel trace metal analysis methods currently under development are bioassay techniques based on a biological molecule (such as a metalloenzyme [2,3] or a catalytic DNA strand [4]) that contains a metal-binding site. Discussion here will be confined to those bioassay approaches that use antibodies and immunoassay technology to perform trace metal analysis

3.2 Anti-Chelate Antibodies

Attempts have been made to produce antibodies against metal ions themselves by in vitro stimulation of B lymphocytes with a metal salt and there have been reports that patients with metal implants can develop an immune response to that metal. In general however metal ions themselves do not make attractive targets for antibody recognition as most are highly charged and tend to react strongly and irreversibly with biological molecules (the same properties that are responsible for the toxicity of heavy metals). Sequestering a metal ion in the form of its coordination complex with a chelating agent reduces the reactivity of the resulting molecule and provides an outer shell of organic material that should be processed by the immune system in much the same way as any other organic hapten. This concept remained untested until the early 1980's when the first anti-chelate antibodies were described. The original work was undertaken with no thought of applying anti-chelate antibodies to analytical applications and with no expectations of, or need for, metal ion specificity in the antibodies that were generated.

The original report by Meares and co-workers [5] described two monoclonal antibodies that were produced against the indium(III) complex of EDTA. The intended use of these reagents was in the eventual production of bispecific antibodies that would bind to a tumor associated antigen via one antibody binding site and to the indium-EDTA chelate through the other, enabling an alternative approach to performing indium-111 radioimmunoscintigraphic imaging studies in colorectal cancer patients [5,6]. While the observed ability to produce antibodies against a chelate

complex was not in itself particularly surprising, one antibody that was studied in detail (CHA255) displayed a specificity for the indium complex relative to any other metal-EDTA chelate that had not been anticipated (and had not been necessary for the application envisioned). Single crystal X-ray diffraction studies of CHA255 Fab fragments containing either In-EDTA or Fe-EDTA in the binding site [7], showed a multitude of interactions between the chelate and the polypeptide, with five of the six CDR's of the antibody contributed to the binding site. A total of nine bonding interactions were identified, including a coordinate bond between the metal atom and a histidine residue. This could not be formed in the case of the iron analog. Most bonding interactions involved the residual charge on the chelate carboxylate groups, which formed both direct hydrogen bonds and indirect hydrogen bonds (mediated via a water molecule) with antibody side chains..

The origins of metal ion specificity are not completely understood but are believed to reflect the ability of an antibody to detect subtle changes in the shape of the "outside" of the EDTA chelate as the central metal ion is varied. The coordination number and coordination geometry of an EDTA complex depend on the identity of the metal ion. The CHA255 Fab data are an example of the coordination sphere being expanded by an amino acid side chain functioning as an additional ligand. It is similarly possible to expand the coordination sphere by having one or more solvent molecules or other low MW species form coordinate bonds to the metal. Even in the case of two identically charged metals that form EDTA chelates of identical coordination number, the fine structures of the complexes (i.e. the precise metal-donor atom bond lengths and bond angles) are not identical. The non-bonding carboxylate oxygen atoms are several bonds removed from the metal center and relatively modest changes in bond angles about that center can translate into substantial changes in the resulting location of those oxygen atoms in 3-dimensional space. In a sense, small structural changes "inside" the chelate complex (i.e. at the metal center) are amplified when they make themselves manifest on the "outside" of the structure.

Since the initial report describing antibodies against the indium-EDTA chelate, a variety of other anti-chelate antibodies have been described. These have included antibodies produced against other metal complexes of EDTA [8,9], analogous chelates of other polyaminopolycarboxylate chelates [10 - 12], various porphyrins [13,14] and macrocycles [15]. In all but a few cases, these reagents were developed for use in bispecific antibody mediated tumor targeting or as catalytic antibodies and analytical applications were neither intended nor pursued. The first accounts of the use of an anti-chelate antibody in an immunoassay did not appear until the early 1990's.

3.3 ELISA Methods

The first report of an immunoassay for a metal ion described an ELISA [16] based on a monoclonal antibody [17] recognizing the mercury(II) complex of glutathione, a naturally occurring tripeptide chelator. The first account of an immunoassay for measuring a synthetic chelate [15] used polyclonal antibodies to configure an RIA that detected a macrocyclic gadolinium complex. Blake at al [18] first demonstrated an ELISA for the detection of indium(III) using the CHA255 antibody then subsequently described additional ELISA assays for cadmium [9] and lead [12]. The intended uses of these assays have been in environmental monitoring and the choice of an ELISA configuration was driven partly by the ready availability of field portable microtiter plate ELISA readers and partly by the expectation that such tests could eventually be added to the menu of ELISA kits for organic pollutants (herbicides, pesticides, petroleum hydrocarbons, etc.) that had been commercialized in the late 1980's and early 1990's [19].

A heterogeneous immunoassay format is not well suited to many potential environmental testing and process monitoring applications, particularly those involving continuous monitoring or remote operation. Homogeneous assay formats are generally faster, simpler, more resistant to artifacts and easier to automate. The ELISA methods for organic pollutants have not found wide acceptance, in part because the use of enzyme labels can be problematic when testing environmental samples, which tend to be far more heterogeneous than the clinical and biomedical materials to which the ELISA format has historically been applied. Most commercial ELISA assays for analytes of environmental and industrial concern have been semi-quantitative [20], with the notable exception of tests for atrazine in water [21]. The context in which immunoassay technology has been introduced into the environmental analysis field over the past 15 years has resulted in the technique being viewed as providing inherently lower data quality (accuracy, precision, comparability) than do the laboratory based HPLC, GC and GC-MS methods typically used to measure organic pollutants

4. FLUORESCENCE POLARIZATION METHODS

FPIA is an attractive alternative to ELISA formats for environmental testing applications and the ongoing development by Eremin and co-workers of FPIA methods for organic pollutants is described elsewhere in this issue. The application of FPIA methods to trace metal analysis using anti-chelate antibodies involves some modifications that reflect the bimolecular nature of the analyte but in most other respects anti-chelate FPIA assays differ little from those that have been developed over the past two decades to measure various organic haptens [22 -24].

FP methods for detecting metal ions have been described that were not based on an anti-chelate approach. Elbaum et al used an indirect enzyme-based assay to measure zinc(II) by FP [2] and proposed that the method would be useful in field testing. The first report of an immunoassay for lead (II) [3] described a method in which an FPIA for porphobilinogen was used as an indirect measure of the lead content of the sample. Such approaches in effect couple FP detection with an enzymatic activity that either requires or is inhibited by the target metal. Appropriate enzymes are not available for many metals of interest however. A significant attraction of anti-chelate antibody technology is that it is in principle applicable to some seventy metals that are known

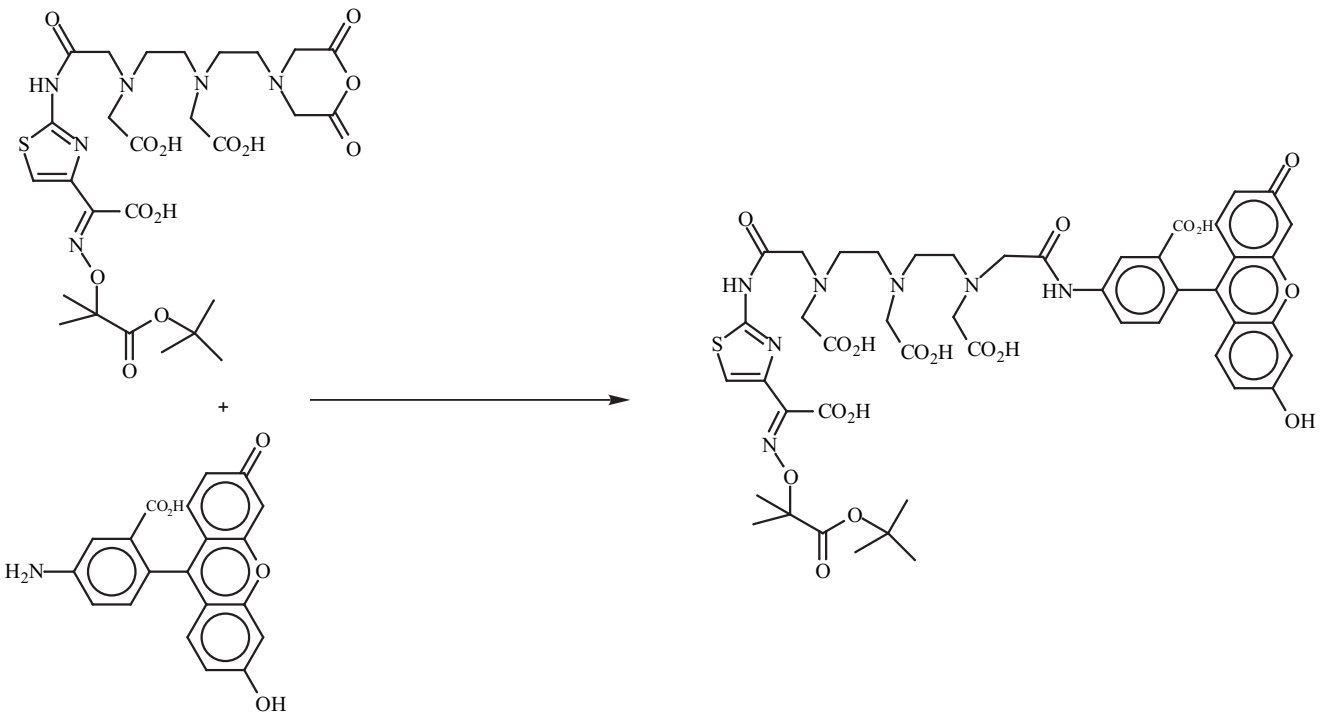

Fig. (2). Preparation of the chelator-fluorescein conjugate used to prepare the cadmium tracer.

to form chelate complexes [25] and is thus of potentially broad applicability.

Anti-chelate FPIA tests for metals in real samples involve pre-treating the sample with excess chelating agent so that all chelatable metal ions present are converted into the corresponding chelates. The immune complex that is formed from the analyte and measured in an anti-chelate FPIA is thus the end product of two successive binding reactions (Figure 1). Specificity in such assays does not reside in the chelating agent, which is assumed to bind indiscriminately to all chelatable metal ions in the sample. The chelating agent serves to produce highly stable species having an idiosyncratic shape unique to each metal. The antibody binds to the chelate with lower thermodynamic affinity than does the chelating agent to the metal but it confers specificity to the overall reaction by recognizing chelate shape.

5. CHELATOR SELECTION AND DESIGN OF TRACERS FOR ANTI-CHELATE FPIA APPLICATIONS

The fluorescent tracers needed to configure anti-chelate FPIA methods originate from the same bifunctional chelators that are used to produce the immunogens. Bifunctional chelators are chelating agents that contain a reactive side chain through which the chelating moiety may be covalently linked to other molecules without seriously compromising its metal binding properties. Such agents have been widely used as labels, in conjunction with radioisotopic, fluorescent, catalytic or magnetic metal ions [26]. Polyaminopolycarboxylate chelating agents are particularly useful as they form high affinity water-soluble chelates with a very wide range of metal ions [25]. There are two positions in such structures through which covalent linkage to a second molecule is commonly achieved: via a

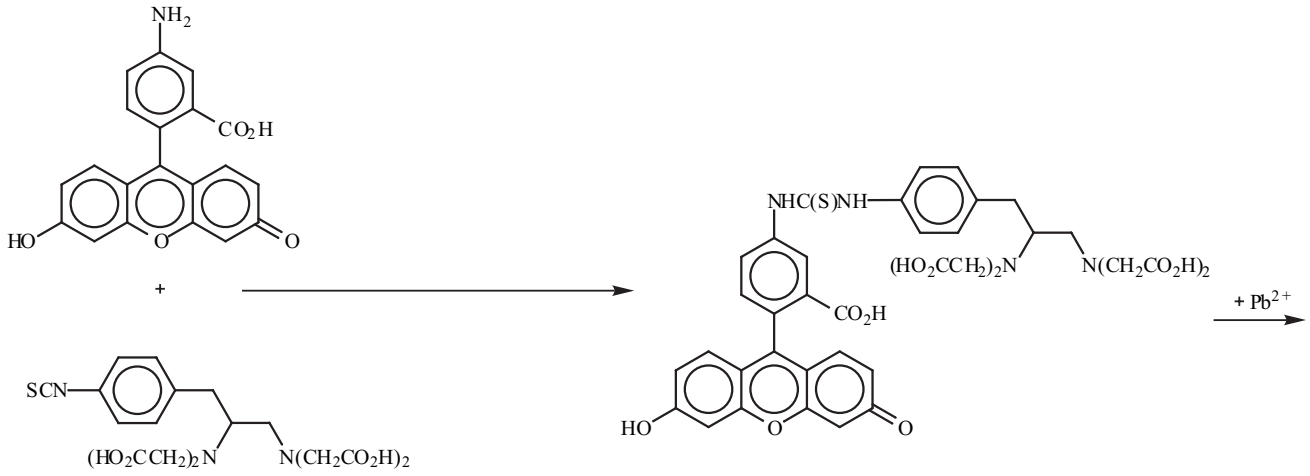

Fig. (3). Preparation of the chelator-fluorescein conjugate used to prepare the lead tracer.

methylene carbon atom in the polyamine backbone [27] or through one of the carboxylic acid functions [28]. Both types of linkage chemistry have been explored. A prototype cadmium (II) assay was developed using carboxylate-linked immunogens and tracers [29] while a prototype FPIA for lead (II) [30] used the same methylene-linked benzylisothiocyanate derivative of EDTA that had originally been developed as a protein label by Meares et al [27, 31] and subsequently used by the same workers to produce the first anti-indium-EDTA antibodies [5] and by Goodwin et al [8] and Blake et al [9,32] respectively to produce antibodies to the cobalt (III) and cadmium(II) complexes.

Reaction schemes for the preparation of the cadmium and lead tracers appear in Figures 2 and 3. Polyclonal antibodies to the corresponding target chelate-BSA immunogens were produced in rabbits by standard methods [29,30]. An immediate measure of the metal ion selectivity of the immune response was available by titering serum samples against both target and non-target tracers (Figure 4) using the same tracer concentration and serum dilutions in all cases. This ability to readily and directly evaluate specificity in solution at equilibrium is one of the unusual aspects of working with a bimolecular hapten.

To date, the prototype cadmium FPIA has been used as a testbed for attempts to develop antibody/tracer combinations based on newly developed and untested chelating structures. In a parallel effort, development of a lead (II) FPIA has taken advantage of the well understood chelating properties and ready availability of EDTA and its derivatives to extend the application of anti-chelate FPIA methods to real samples and real world situations.

6. FP ASSAYS FOR PURE CHELATES IN BUFFER

As an intermediate step before attempting to apply anti-chelate FPIA methods to real samples, the ability of selected antibody/tracer combinations to produce a standard curve for the pure 1:1 target chelate in buffer was evaluated. In some cases, the preliminary cross-reactivity of these assays was also evaluated by attempting to establish such standard curves in the presence of a fixed excess of a non-target chelate. Such assays for pure analyte in buffer also provided a preliminary indication of the sensitivity of the method under ideal conditions.

6.1 Sensitivity

Figure 5 shows the effect of pre-formed 1:1 Pb-EDTA cehelate diluted into PBSA on binding of Pb-EDTA-fluorescein tracer to an anti-Pb-EDTA polyclonal antiserum. The standard curve was obtained by fitting polarization values produced by calibrators containing 0, 30, 50 and 100 ppt of Pb-EDTA to a single exponential curve. The LOD (the blank signal + 3 x the SD of the blank signal transposed into an analyte concentration according to a dose = response curve) and LOQ (blank + 10 SD) calculated from the curve are 20 ppt and 75 ppt respectively. The lowest measured analyte concentration (30 ppt) was 4 SD's above the blank.

The sensitivity of the cadmium assay has not been formally determined but the mean polarization of a solution containing 100 ppt of the cadmium chelate differed from that of a blank by seven SD's, (Table 1) suggesting that the LOD and LOQ would be on the order of twice those for the analogous lead FPIA. It is possible that this disparity reflects differences in the intrinsic immunogenicity of the two different chelate structures. It is also possible that the stage of the immune response at which the polyclonal antibodies were harvested may have played a role. The cadmium polyclonal antibody pool was obtained within the first 6 months after an initial immunization plus two 0.1mg booster doses. The antibody pool used in the lead LOD study was obtained some 3 years after an analogous initial

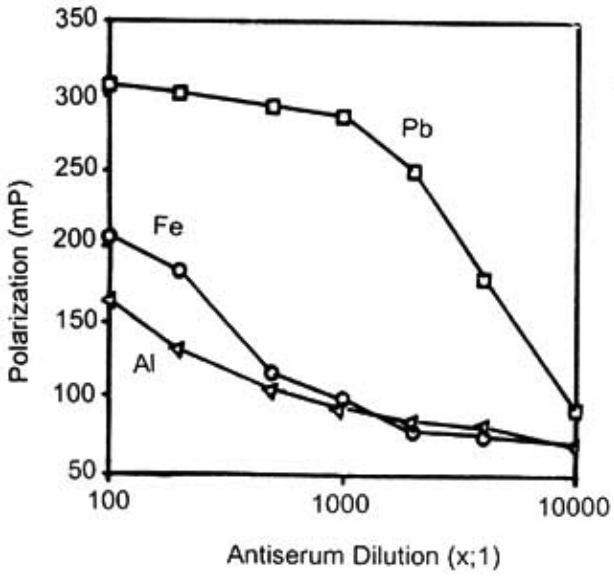

Fig. (4). Titration of rabbit antiserum against target and non-target tracers. Data taken from ref [30].

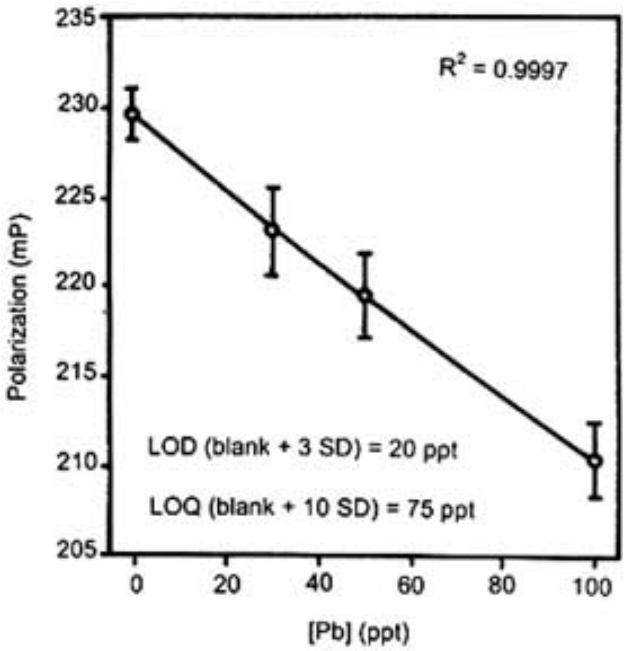

Fig. (5). FPIA standard curve for 1:1 Pb-EDTA chelate in PBSA .A Pb-EDTA stock was formed by mixing a 1.0 mM solution of Pb(II) AA standard with a 1.0 mM solution of EDTA in 0.2 mM HEPES., pH 7.4. Pb-EDTA calibrators were prepared by serial dilution of a 100 ppm stock solution into PBSA to produce solutions containing final concentrations of 10, 5 and 3 ppb Pb. Pb-EDTA-fluorescein tracer was prepared as described elsewhere [30] and diluted into distilled water on the day of the study. Anti-Pb-EDTA antiserum was obtained from a rabbit that had received a primary immunization comprising 1 mg of BSA-EDTA-Pb imunogen, prepared and administered as described in ref [30], followed by 0.1 mg booster doses at 6 weeks and 12 weeks and a 0.02 mg booster dose at approx. 2 years post-primary injection. Assays were performed in 5 mL disposable test tubes to which were added sequentially: a) 2.0 mL PBSA, b) 0.06 mL dilute antiserum in PBSA (1:50), c) 0.02 mL PBSA or calibrator, and d) 0.04 mL of 0.125 nM tracer. Each tube was incubated at room temperature for 10 min. then the polarization was measured (FPM-1 analyzer, Jolley Research & Consulting, Inc., Grayslake, IL). Blanks and calibrators were run in replicates of 10, and the mean polarization values were fitted to a single exponential curve.

immunization regimen followed by very low booster doses of immunogen (20 micrograms) administered after lengthy resting periods (a process termed "hyperimmunization"). Early phase polyclonal antibody responses produce predominantly IgM antibodies, which are generally lower in affinity than are the IgG antibodies that appear in later phases of the immune response. Hyperimmunization protocols were specifically developed to maximize antibody affinity. The observed trends in sensitivity are thus consistent with explanations based on the phase of the immune response but more data will be required to confirm this. As a practical matter, both chelate systems could be used to configure highly sensitive assays, reinforcing the general observation that heavy metal chelates tend to be extremely immunogenic and can produce a high affinity antibody response

Table 1. FP of Cadmium Chelate Standards in PBSA(1)

Sample	Polarization (mP) 0 nM Cd-chelate	Polarization (mP) 1.0 nM Cd-chelate
replicate 1	191.0	182.1
replicate 2	193.5	183.4
replicate 3	194.0	185.7
replicate 4	193.3	185.3
replicate 5	191.4	184.8
Mean	192.6	184.3
SD	1.2	1.3

(1) Data taken from ref [29]

6.2 Specificity

One way to evaluate potential interferences due to cross reactivity with non-target metals is to evaluate the effect that the presence of fixed concentrations of individual non-target chelate has on the ability to establish a standard curve for the target chelate in buffer. Studies of this type have been performed for the cadmium FPIA [29] and showed the feasibility of establishing a standard curve for 0 - 100 nM Cd chelate in the face of fixed 250 nM concentrations of the corresponding Cu (II), Zn(II) and Hg(II) chelates. Span and sensitivity were both reduced somewhat, but the resulting assays were still able to detect cadmium at concentrations well below regulatory limits. This may not hold true for

assays on real samples in the presence of free chelator (see below).

Cadmium assays have thus far been pursued to the point of proving principle using the first of a series of structurally related chelate systems. Other members of this series are being developed with view to basing the eventual selection of a chelate/anti-chelate system for cadmium assays in real samples on a preliminary structure-function study. In the meantime, attempts to extend anti-chelate FPIA methods beyond the detection of pure chelate in water at neutral pH have focused on lead.

7. FPIA METHODS FOR LEAD IN ENVIRONMENTAL SAMPLES

7.1 General Considerations

It is possible that an inert, water soluble complex such as Pb-EDTA that can be detected at ppt concentrations by inexpensive fluorescence methods might find use as a tag in aqueous flow labeling applications. There may also be other special circumstances in which a metal-EDTA complex could be an analyte of interest in a matrix that contains no free EDTA or other EDTA complexes. However, the overwhelming majority of scenarios in which anti-Pb-EDTA FPIA methods might be useful involve testing for the presence of ionic lead (II) in samples containing unknown quantities of one or more non-target metals. This in turn requires pre-treating the sample to convert ionic Pb (II) into the Pb-EDTA chelate and the only way to ensure complete conversion of lead into its chelate is to add more than enough chelating agent to the sample to complex all metal ions present, target and non-target.

7.2 Effects of Excess Free EDTA on Assay Performance

Anti-Pb-EDTA FPIA assays performed in the presence of 25 micromolar EDTA have a maximum sensitivity of approximately 1 ppb [30]. The origins of the significant reduction in assay sensitivity that is consistently seen in the presence of excess free EDTA are not fully understood and may involve multiple mechanisms. As these are polyclonal antisera and free EDTA must be assumed to share some antigenic determinants with its chelates, cross-reactivity with free EDTA could be a factor. Lead is still widely used as a stabilizer in polymers, mold release agents and finishing products used in fabricating plastic consumables (such as disposable test tubes and pipet tips) while buffer salts and assay reagents all contain some residual level of heavy metal contaminants including lead. By definition, the assay system is sensitive to 20 ppt of lead and a significant factor contributing to loss of sensitivity is likely related to adventitious lead chelate formed from solid surfaces and solutions with which the EDTA-containing reagent comes into contact. Even in a blank prepared in the presence of free EDTA, the highest affinity antibodies in the polyclonal antiserum are neutralized by adventitious Pb-EDTA leaving only lower affinity antibodies to participate in the assay. While it should be possible to minimize this effect by using high quality water, ultrapure reagents, metal-free pipet tips and treated assay tubes, such precautions can greatly increase the cost and complexity of an inherently simple and inexpensive technique. An LOD of 1 ppb represents a sensitivity that is routinely achievable using bottled distilled water and ACS grade reagents but no other special precautions. The requirements of individual analytical applications will dictate the level of precaution that is cost effective. For example, the U.S, regulatory limit for lead in drinking water is 15 ppb so there would seem to be little incentive to use elaborate precautions against metal contamination in drinking water FPIA methods. Permits for discharge of lead to groundwater from some facilities stipulate regulatory limits in the range of 1 ppb and greater efforts to control adventitious lead contamination in the assay would be not only justified but probably essential for these purposes. If saturation of the highest affinity IgG molecules in the assay by adventitious Pb-EDTA is confirmed as a mechanism limiting sensitivity, then switching to a monoclonal antibody should make it possible to avoid this effect.

7.3 Soils and Solid Wastes

The initial development of FPIA methods for lead in solid phase samples was done using splits of extracts that had been prepared for atomic spectroscopy, so that initial correlation studies between FPIA and atomic spectroscopy were not complicated by issues of incomplete sample extraction.

Lead can be naturally present in soil at concentrations of several hundred ppm and the range of greatest regulatory interest is approximately 20 - 2000 ppm. These are not strictly speaking trace concentrations and soil methods do not represent a significant challenge in terms of sensitivity requirements. Soil was chosen as an initial focus because it contains a wide range of non-target metals present in highly variable concentrations depending on the origin of the sample. Early testing of soils provided one way to try to identify any pronounced cross reactivities of the lead FPIA with non-target metals beyond those key elements tested during initial screening. The patterns of cross-reactivity of known anti-chelate antibodies with non-target chelates are not sufficiently well understood to permit prediction of which non-target metals are most likely to cross-react, so narrowing the field on theoretical grounds is problematic. An empirical approach has considerable attraction.

FPIA results correlated well with FAAS data [30] for 1 M nitric acid extracts derived from soil samples (FPIA = 1.61 x FAAS - 50 and $r^2 = 0.96$ for n = 138). Such extracts were diluted extensively to bring lead concentrations into the dynamic range of the FPIA, which was 1 - 50 ppb. FPIA data for a second form of extract obtained by leaching solid wastes with acetate buffer showed good agreement with ICP-AES values (ICP-AES = 0.74 x FPIA = 1.6 and $r^2 = 0.93$ for n = 40). As there were no notably discrepant samples in either the soil extracts or the solid waste leachates, these studies provided no clues as to the cross-reactivity in the FPIA of other metals present in these matrices. Subsequent cross reactivity determinations conducted using 15 individual non-target metals indicated less than 0.5% cross reactivity in all cases (Table 2) which is consistent with the soil results. Although results to date suggest that the assay

is highly specific for lead, it is possible that one or more of the remaining 55 metals that have not been directly tested might be strongly cross reactive but not present in significant concentrations in samples analyzed thus far.

Table 2. Cross Reactivity of Non-Target Metal Ions in the Soil Lead FPIA(1)

Metal Ion	Cross-reactivity (%)
Mercury(II)	0.37
Silver(I)	0.19
Copper(II)	0.15
Cadmium(II)	0.15
Bismuth(II)	0.13
Zinc(II)	0.11
Calcium(II)	0.11
Cobalt(II)	0.10
Chromium(III)	0.10
Gold(III)	0.08
Nickel(II)	0.08
Manganese(II)	< 0.05
Magnesium(II)	< 0.05
Aluminum(III)	< 0.05
Iron(III)	< 0.05

(1) Data taken from ref [30]

7.4 Drinking Water

Pilot studies of a prototype method for lead in drinking water [30] provided a counterpart to the studies with solid samples. Whereas soil testing entails a specificity challenge but no significant sensitivity demands, drinking water analysis requires 3-4 logs greater sensitivity but does not for the most part involve high concentrations of non-target metals. An FPIA assay configured to minimize sample dilution showed good recovery of lead spiked into two representative sources of drinking water at concentrations bracketing the regulatory limit. With no need to either extract or dilute the sample, this prototype FPIA procedure is the simplest of those developed thus far for environmental samples and is readily amenable to automation.

7.5 Lead Based Paint Hazards

Lead contamination in the environment can take many forms. In the U.S. the oversight responsibility for identifying and remediating such contamination is often spread among multiple regulatory agencies. Lead hazards in industrial, commercial and military facilities generally reflect past large scale use of metallic lead and lead compounds (such as tetraethyllead) and are regulated by various combinations of the EPA, DOE and DoD. Lead hazards in ongoing manufacturing and mining operations are controlled by the EPA and OSHA. Residential lead hazards derive primarily from the presence of lead-based paint and abatement activities are regulated in the U.S. primarily by HUD.

In seeking to extend the use of anti-chelate FPIA methods to real world situations, one focus is on the development of field testing and mobile laboratory procedures that could be integrated into ongoing residential lead abatement projects. A pilot study of soil samples contaminated with lead based paint residues (Table 3) was consistent with results previously obtained for lead in soil [30]. FPIA values were in good agreement with ICP-AES data even though these were intact soil samples (not extracts) and even though these samples were more heavily contaminated with lead (presumably mostly as lead oxides) than most soils tested previously. Precision of the FPIA soil method was good (RSD of 8%). Soil samples may or may not be collected and analyzed during the identification of residential lead hazards depending on the circumstances. The criterion for acceptable abatement of such hazards is the residual concentration of lead in household dust and such measurements are mandatory.

Table 3. FPIA *vs*. ICP-AES for Lead Contaminated Soil Samples (1)

Sample	Soil [Pb] (ppm) by FPIA	Soil [Pb] (ppm) by ICP-AES
1	1100	980
2	1700	1770
3	2450	2310
4	2300	2470
5	2800	2930
Precision study Sample 5		
Replicate 1	2800	
Replicate 2	2800	
Replicate 3	3000	
Replicate 4	3250	
Replicate 5	3250	
Mean (SD)	3020 (225)	
RSD (%)	8	

(1): Soil samples and ICP-AES data for their lead content were kindly provided by Dr. Lori Allen and Dr. Chris Evans, University of Wisconsin - Parkside, Kenosha, WI. Soil Pb values by FPIA were obtained exactly as described in ref [30] i.e by room temperature extraction of 5 g of dried, ground sample with 50 mL 1 M nitric acid followed by a 2,000-fold dilution of the resulting extract into the immunoassay. Soil Pb values by ICP-AES were obtained after microwave digestion of the sample with conc. nitric acid

Dust samples that are routinely analyzed for lead are of two types. Airborne dust is a concern primarily in facilities where lead is either manufactured or used and workplace safety regulations under OSHA require monitoring of airborne lead. This is done by drawing a known volume of air through a fiberglass filter then eluting the trapped lead with mineral acid and measuring the dissolved lead content by atomic spectroscopy. A small number of such extracts were successfully tested during initial studies of FPIA

methods [30]. Dust samples of relevance to residential lead abatement are settled household dust collected by wiping a known area of the surface to be tested; then digesting the wipe sample in mineral acid. Limits for residual lead content have been established by HUD for dust collected from window sills (250 microgram/sq.ft.) window troughs (400 micrograms/sq.ft.) and floors (40 micrograms/sq.ft.). It is of interest to develop an FPIA method for lead in dust wipe digests and as HUD requires that any method that is to be used for such purposes be accredited under ELLAP [33] it is also of interest to configure the immunoassay to conform to the current ELLAP requirements. These include running blanks and calibrators before analyzing samples and at specified intervals thereafter during the course of each analytical run. One general attribute of FPIA techniques is good standard curve stability and there was little reason to expect problems with standard curve drift given the relatively modest batch sizes involved in this type of testing and hence the short analysis runs (a total of just 8 wipe samples is typically taken per housing unit). However if EDTA-containing reagents were to progressively leach adventitious lead from container surfaces over time this would constitute an additional potential mechanism that could result in standard curve drift and was therefore of potential concern. Figure 6 shows standard curves generated in the course of a blind study that compared FPIA values with the corresponding FAAS data for household dust wipe digests generated during routine lead abatement testing by an ELLAP accredited operation [34]. The standard curve showed adequate stability over the course of a 3 day run and correlation between the two methods was good. Efforts to refine such FPIA dust wipe methods for use in the field are ongoing.

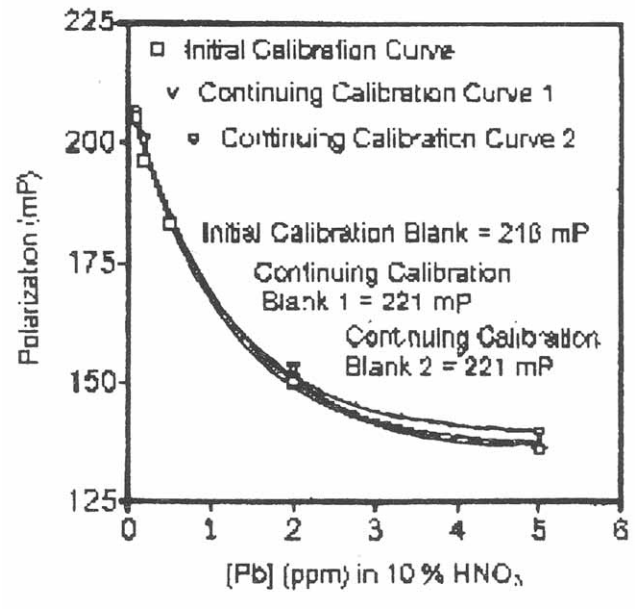

Fig. (6). Stability of standard curves for FPIA of lead in household dust wipe digests. The standard curve conditions were chosen for optimum precision around the regulatory limit for floors of 40 micrograms/sq.ft., which corresponds to 0.40 ppm Pb in the 10% nitric acid extract. This was achieved by incorporating a 50-fold dilution factor into the assay so that a sample at the regulatory limit produced a final concentration of 8 ppb in the assay at the point of measurement. As had been the case for drinking water [30] (which is acidified to 0.5% with nitric acid prior to analysis and is not significantly diluted before measurement) it proved necessary to increase the buffer capacity in the assay to 200 mM from the 20 mM concentration used when testing soil extracts. Calibrators containing 0.1, 0.2, 0.5, 2.0 and 5.0 ppm Pb were obtained by dilution of a 1000 ppm Pb AA standard into 10% nitric acid. The assay was performed in 5 mL disposable test tubes by adding 0.02 mL of calibrator or blank (10 % nitric acid) to 1.0 mL of assay reagent 1 (25 micromolar EDTA + 2.5 nM Pb-EDTA-fluorescein in 0.20 M HEPES, pH 8.0) followed by 0.04 mL of assay reagent 2 (anti-Pb-EDTA antiserum in PBSA) (the tracer and antiserum were obtained as described in ref [30]). After standing at room temperature for 30 min the polarization of the resulting solution was measured (Sentry FP analyzer, Diachemix Corp., Grayslake, IL).

8. ANTI-CHELATE FPIA CHARACTERISTICS AND FUTURE DEVELOPMENT

To date antibody/tracer combinations that produce extremely sensitive, highly specific FPIA tests for metals have been demonstrated using two different chelating structures and two different conjugation chemistries. Testing the metal ion specificity of an antibody by titering against non-target tracers and by examining the effect of non-target chelates on a model FPIA in buffer can be used to screen for critical cross reactivities. Early testing of real samples is also helpful because all possible cross reactivities cannot be predicted. It is probable that the same types of chelating structures and antibody selection strategies can be used to develop analogous FPIA methods for many other metal ions.

FPIA tests for lead have been shown to be usable over a dynamic range of 8 logs overall (3,000 ppm - 20 ppt) and 6 logs in real samples (3000 ppm - 1 ppb). FPIA methods for real samples that would have sensitivities in the sub-ppb domain (which generally requires ICP-MS at present) may be possible if the effects of free chelating agent in the assay can be controlled and/or if suitable high affinity monoclonal antibodies can be developed.

Blind correlation studies of lead in 3 types of solid samples (soil, solid waste, dust) comparing values with 4 independent laboratories (2 running FAAS, 2 running ICP-AES) have produced correlation coefficients (r^2) of 0.93 - 0.99. Grossly discrepant samples that would indicate a severe interference of some kind (including cross-reaction with either non-target metals or organic material present in these types of samples) have not been encountered thus far. Broader testing will be required to confirm the high degree of specificity with which lead can be detected, given the extremely heterogeneous nature of these matrices. Based on experience to date it appears likely that anti-chelate assays can be reduced to practical analytical methods for routine use. The methods are quantitative and can produce data quality comparable to that of atomic spectroscopy. Results with blind samples indicate that anti-chelate FPIA methods are capable of meeting proficiency testing goals under ELPAT [33] and can be configured to meet other ELLAP

requirements regarding calibration, blanks and check samples. Finalization of field-friendly FPIA methods for solid matrices awaits integration of the detection technique with modified sample extraction protocols more suited to such purposes than are the high temperature extractions with mineral acid used in fixed base testing.

Extracts from highly complex solid matrices have been the focus of most initial testing of the anti-chelate FPIA technology because seeking out possible cross-reactivities has been the highest priority. The apparently minimal level of such cross reactivity in materials as heterogeneous as solid wastes suggests that future FPIA methods could be developed for a wide variety of more predictable and homogeneous matrices, particularly liquid phase effluent and process streams. Potential industrial process monitoring applications (monitoring feedstocks and work in process during manufacturing operations that either use metals or are sensitive to metal contaminants) may ultimately outnumber environmental monitoring uses. The residual heavy metal content is a QA/QC testing criterion for innumerable intermediates and products and in many cases is regulated in the finished product (particularly in cases where the latter is for human use or consumption). Many of the strengths of FP technology derive from the fact that it is a solution phase measurement and, with liquid samples, the full versatility of the technique can be brought to bear. Self contained automated systems capable of making anti-chelate FPIA measurements in a flow cell could fulfill many remote metal sensing and continuous monitoring needs. Typical levels of demand and batch sizes in most current trace metal analysis do not require high throughput methods, but user expectations reflect the traditional limitations of atomic spectroscopy. Anti-chelate FPIA chemistries could be combined with a microtiter plate FP system or other format as described elsewhere in this issue to offer high throughput trace metal analysis at comparatively low cost. Such capabilities have the potential to change the ways in which trace metal analysis is used in industrial process control in addition to monitoring the wastes and fugitive emissions produced by such processes.

ABBREVIATIONS

BSA = Bovine serum albumin

CDR = Complentarity determining region

DoD = U.S. Department of Defense

DOE = U.S. Department of Energy

EDTA = Ethylenediamine-N,N,N'N'-tetraacetic acid

ELISA = Enzyme linked immunosorbent assay

ELLAP = Environmental Lead Laboratory Accreditation Program

ELPAT = Environmental Lead Analysis Proficiency Testing

EPA = U.S. Environmental Protection Agency

FAAS = Flame atomic absorption spectroscopy

FP = Fluorescence polarization

FPIA = Fluorescence polarization immunoassay

GFAAS = Graphite furnace atomic absorption spectroscopy

HUD = U.S. Department of Housing & Urban Development

IgG = Immunoglobulin G

IgM = Immunoglobulin M

ICP-AES = Inductively coupled plasma - atomic emission spectroscopy

ICP-MS = Inductively coupled plasma - mass spectrometry

LOD = Limit of detection

LOQ = Limit of quantitation

OSHA = U.S. Occupational Safety & Health Administration

PBSA = 0.1 M Phosphate buffered normal saline + 0.1% sodium azide

QA = Quality assurance

QC = Quality control

ppb = parts per billion

ppm = parts per million

ppt = parts per trillion

RIA = Radioimmunoassay

RSD = Relative standard deviation

RV = Recreational vehicle

SD = Standard deviation

SIBS = Spark Induced breakdown spectroscopy

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