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Research Article

A Review of Heavy Metals Immunoassay Detection

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Abstract: Contamination of heavy metals in soil has been a significant problem, which resulted in food pollution and diseases through bioaccumulation. Traditional methods utilized to determined content of metal ions are time-cost, expensive and laboratorial. Since the introduction of antibody against In-EDTA, immunoassay has been developing for several decades. It filled in the blank of determination in situ with lower price and a short period. In this study, we mainly presented the research process of monoclonal antibody special binding to metal-ligand and the immunoassay utilized in detection of food and environment.

Keywords: Application, binding affinity, heavy metals, immunoassay

INTRODUCTION

Heavy metals which used in many different areas are commonly defined as the excessive deposition of toxic heavy metals in the soil caused by human activities. The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury and arsenic (arsenic is a metalloid, but is usually classified as a heavy metal). Sometimes a few of radioactive metal elements are also involved in the group.

Human can take in the toxic metals by consumption of contaminated food crops, water or inhalation of dust. The intake via ingestion depends upon food habits. It has been estimated that more than 70% of dietary intake of cadmium is contributed via food chain and respiratory tract (mainly smoking). Prolonged consumption of contaminated foodstuff may lead to the unceasing accumulation of toxic metals in the liver and kidney of humans resulting in lesions on varietal organs (Markus and McBratney, 2001). Recently, the content and concentration of heavy metal have been increasing with the activity of population, especially industrial effluent discharge, irrigation of waste water, sewage sludge and e-waste recycling (Jaradat *et al*., 2005; Kumar *et al*., 2007; Tang *et al*., 2010), resulting in degradation of soil health and microbe environment, sometimes irreparably (Hiroki, 1992; Oliveira and Pampulha, 2006).

Existing technologies for measuring metal contamination require expensive instrumentation (atomic absorption spectroscopy, inductively coupled plasma emission spectroscopy, X-ray fluorescence spectroscopy) in a centralized facility. Immunoassay offer significant advantages over more traditional methods of metal ion detection. Immunoassays are quick, inexpensive, simple to perform and reasonably portable; they can also be both highly sensitive and selective. The microscopic information of the bond between metal ions and organic chelate in aqueous solution was firstly proposed by Nakamoto *et al*. (1961). And In 1985, Meares and partners described monoclonal antibodies that were produced against In (III)-EDTA chelate complex (Reardan *et al*., 1985). This was the first report for monoclonal antibodies against metal-ligand. Since then, these antibodies have subsequently been used to develop immunoassays for special metal ions (Wylie *et al*., 1991). In this study, we are intended to describe immunoassay procedure and briefly review the binding properties of some monoclonal antibodies and application of the mAbs in the detection methods for the heavy metals.

METHODOLOGY

Binding theory for monoclonal antibody with metal-ligand:

Description of chelators in metal detection: The first synthetic chelator and still most abundant chelator from the family of poly amino-carboxylic acids is EDTA. It was patented by F. Münz in 1935. Ethylenediamine Tetraacetic Acid (EDTA), which has coordinated atoms containing four acetate groups and nitrogen atoms, forms a very stable chelate between one molecule of EDTA and metal ion. Since the chelating (ch = k) agent proposed by Calvin and Martell (1952), fifty-seven species of metal including some Lanthanide elements and over 11 kinds of metal-complexes have been studied extensively, with the stability constant and structure strength determined by varieties of measurement methods (Table 1). Expect for EDTA, NTA (nitrilotriacetic acid), DTPA

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Table 1: Deferential molecules applying to heavy metal chelation

Pb			
Edta	1.000 ug/L	Airborne dust, drinking water	Johnson *et al*. (2002)
p-NH2-Bn-DTPA	0.056 um	Tap water	Zuh *et al*. (2007)
p-SCN-Bn-DTPA	0.100 ng/mL	Pearl river, Liuhua lake and Lu lake	Xiang *et al*. (2010)
Cr			
Isothiocyanobenzyl-EDTA	5.000 ng/mL	Pearl river, human serum samples	Liu *et al*. (2012)
EDTA	0.100 ng/mL	Local surface water	Zou *et al*. (2013)
Hg			
EDTA	26.000 nM	Water	Blake *et al*. (1996)
Cd			
EDTA	7.000 ppb	Water sample in bayou trepagnier	Khosraviani *et al*. (1998)
EDTA	0.300 ppb	Water samples form a louisiana bayou	Darwish and Blake (2002)
EDTA	0.240 ug/L	Human serum	Darwish and Blake (2001)
1- (4-isothiocyanobenzyl) ethylenediamine-N, N, N', N'-tetraacetic acid (ITCBE)	1.953 μg/L	Electroplating waste water, bush branches and leaves, apple juice, rice flour, wheat flour, tea and spinach	Liu *et al*. (2009)
1- (4-aminobenzyl) ethylenediamine N, N, N', N' -tertraacetic acid (aminobenzyl-EDTA)	0.051 μg/L	None	Kong *et al*. (2013)
Cu			
p-SCN-Bn-DOTA	0.032 μg/mL	Water samples and blood samples	Liu *et al*. (2013)
U			
1, 10-phenanthroline-2, 9-d icarboxylic acid (DCP)	1.000 nM	Water	Blake *et al*. (2001a)

(diethylentriaminepenta acetic acid) and some other chelate agent are deployed for titration, isolation and separation of metal ions. As we know, bond in noncovalent complexes is indicative of ion-dipole, ion-quadrupole and ion-induced dipole interactions (Amunugama and Rodgers, 2002). Normally, The EDTA complexes of the divalent or trivalent metals have either a sexidentate structure, where two N and four O are coordinated to the metal or a quinquedentate structure, where one carboxyl group is uncomplexed and one water is coordinated to the metal center. However, with the ionic radius of the metals increasing, EDTA cannot encircle the ion completely, for instance, lanthanum in the La-EDTA complex has a coordination number of 10, where four additional water molecules are coordinated (Nowack and Sigg, 1996). And in the NTA complex, the adsorbent acid is a quadridentate chelate former and especially suitable for metal ions with coordination numbers of six (Hochuli *et al*., 1987). As to $M \cdot dtp^2$ or $M \cdot dtp^3$, six to eight orbitals are likely to occur , which give a rise to form hexadentate (e.g., Pd, Ir, Rh), hetadetate (e.g., lanthanides) and octadentate (e.g., In) ligand bind through amino groups and deprotonated carboxylate groups to the metal ion (Jørgensen, 1962; Choppin *et al*., 1979; Maecke *et al*., 1989).

Stability constant of metal-ligand In order to describe the bond strength of metal-ligand and the reversibility of reaction, the stability constant of co-ordination compounds is introduced into the formation of metal-ligand complex in solution. Basically, the stability constant is consisted of a series of equilibrium constant of a reaction obtained through the measure of the heat released in the reaction and entropy change during reaction. Therefore, the stability of reaction products increases with the amount of heat evolved in the reaction and the rising in entropy during the process. Two statistical aspect of the term stability in complex, one deals with the bond energy, stability and redoxpotential that can be called thermodynamic stability, the other kinetic stability gets involved in the rate of the reaction, mechanism of reaction, formation of intermediate complexes, are combined to evaluate the reaction.

Among the techniques for the computation of stability constants, pH-metric method and spectrophotometric method are the initial ones to explain the term. For the majority of stability constants reported, the relevant complex formations are those between M and protonated L and for convenience the successive stability constants treated in this survey are defined as follows:

$$M + L \underset{}{\overset{K_1}{\rightleftharpoons}} ML$$

$$ML + L \underset{}{\overset{K_2}{\rightleftharpoons}} ML_2$$

……

$$ML_{n-1} + L \underset{}{\overset{K_n}{\rightleftharpoons}} ML_n$$

The successive stability constants are related to the overall stability constants β as follows:

$$\beta = K_1 \cdot K_2 \cdots K_n$$

Stability constants K_n are related to the standard free energy change ΔG° by $\Delta G^\circ = -RT\ln K_n$ at a constant pressure. Hence, the enthalpy change ΔH° can be determined not only by calorimetry but also from the temperature dependence of K_n values determined (e.g., by potentiometry) according to the van't Hoff equation:

$$\frac{d(\ln K)}{dT} = \frac{\Delta H^O}{RT^2}$$

Due to the experimental difficulty of determining the stability constants accurately over a wide range of temperature and the slight incensement of temperature, the thermodynamic parameters are generally less accurate. On the basis of these two methods, some new techniques have been used to determine the stability constant. Gel chromatographic method had been increasingly applied to the characterization of Mg (II)-Ligand (Yoza, 1977), Ni (II) -NTA and Ni (II) -IDA (Hochuli *et al*., 1987) and other complexes from 1970s to 1980s. Subsequently, CD spectra and electronic absorption were accepted for determination (Casas and Jones, 1980; Tabata and Tanaka, 1985). In Hynes (1993) had published a paper of NMR spectrometry to detect the variation in the chemical shift of a suitable nucleus on formation of the complex species. This method is particularly useful in non-aqueous solution where potentiometric methods may not be readily applicable. And since then, the fluorescence spectrum which was introduced by Grimm *et al*. (1991) and the electrospary mass spectrometry have been brought into the study of metal-ligand solution equilibria. Recently, Wacker and Seubert (2014) utilized anion/cation exchange chromatography and atomic spectrometry detection for the determination of stability constants of very strong polyaminocarboxylic acid complexes that were hardly detectable with any known methods. Species of the metal-ligand is still extending by the application of new methods or techniques, which can bring more theoretical foundation to the immunoassay of heavy metals.

Structural binding between artificial antigen and antibody: The stability constants of metal-ligand have provided basic condition of the reaction for chelating process. However, the coordination number and coordination geometry of EDTA complex depend on not only the characteristic of the chelate process, also the identity of the metal ion. For example, the ionic radii of Cr (III), Fe (III), Ni (II) are 0.76, 0.79 and 0.83 Å, respectively. In terms of these metals, the affinity order was the inverse of the size of the radii of these metal ions. But there are slight differences between conjugations with various ion radiuses. It decreased the affinity for Fe (III)· EDTA by over 2000-fold compared with Cr (III)· EDTA, although the difference in the ion radius between trivalent chromium and ferric ion is 0.03 Å (Sasaki *et al*., 2009). In the case of two identically charged metals that form EDTA chelates of identical coordination number, the fine structures of the complexes 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. As we can see, slight structural differences can lead to amplifying effects in binding geometry of peptides of Fab fragments.

Several studies had exploited the crystal structure of the antigen-binding fragment (Fab') of CHA255 complexed with its hapten. Single crystal X-ray diffraction studies of CHA255 Fab fragments containing In/Fe-EDTA (Love *et al*., 1993), showed that antibody combined with chelate through nine bonding interactions, especially a bond between the metal atom and a histidine residue. In antibody 5B2, another homology modeling and mutagenesis gave a explanation that three residues(Trp52, His96 in the heavy chain and Arg96 in the light chain) separately mediate hydrophobic stacking with the benzyl moiety, ligation to the Cd (II) ion and hydrogen bond to the chelate portion of the complex (Blake *et al*., 2003). The conformational changes of the chelate arms as they shift to accommodate various metal ions appear to be an important factor in antibody recognition. For example, the mAbs for Cd (II) usually have a cross-reactivity with Hg (II) because there is only a difference of 0.7 Å in the ionic radius between Cd (II) and Hg (II) (Liu *et al*., 2012, 2013, 2009). Most bonding interactions involved the residual charge on the chelate carboxylate groups, which formed both direct hydrogen bonds and indirect hydrogen bonds with antibody side chains. But in any case, elbow angle in Fab structure had no influence with antigen binding (Davies *et al*., 1990; Stanfield *et al*., 1990; Rini *et al*., 1992).

Recently, a recombinant single-chain variable fragment antibodies (scFv) which recognized UO^2 (II) complexed to 2, 9-Di-Carboxyl-1, 10-Phenanthroline-acid (DCP) were produced (Zhu *et al*., 2011, 2007). Because of the depletion experiments during the panning process, this scFv bound much less tightly to metal-free DCP, which means that it showed best bound tightly to the UO^2 (II) -DCP complex (Kd, 19.6 nM). This procedure provides immunoassay of metal ion an alternative to engineer low-cost antibodies with the desired specificity and affinity.

Measuring equilibrium of artificial antigen and antibody in the KinExA: KinExA™ is a flow fluorimeter used to measure the free molecule concentration in a mixture of both free and bound, composed of a capillary flow and observation cell connected to a microporous screen through which various solutions are drawn under negative pressure. The ability of the KinExA to quantify metal-ligand with unoccupied binding sites was assessed in an experimental system comprised of an anti-metal monoclonal antibody and conjugated antigen coated onto the solid phase, for instance, uniform particles larger than the pore size of the screen or ELISA plate.

When measuring binding interactions in the KinExA, the principal conditions that must be met are as follows:

- Binding of the immobilized antigen and the corresponding soluble antigen must be mutually exclusive.
- Binding to the immobilized antigen must be sufficiently tight to permit efficient protein capture, leading to an instrument response with acceptable signal-to-noise characteristics (Blake II and Blake, 2003).

A novel procedure is that the complete antigens bind to the surface of immobilized phase as a beginning of competitive equilibrium in a period of time. Next, Quantification of metal ions is indirectly determined through complete antigen captured on the immobilized ligand can subsequently be achieved by the brief exposure of the solid phase to a mixture of antibody and metal-ligand, followed by labeled secondary antibody measurement of the resulting fluorescence from the particles after removal of excess unbound reagents.

The ability of KinExA to quantify low-affinity and high-affinity binding interactions in solution are equally broad. Quantification of the protein captured on the immobilized ligand can subsequently be achieved by the brief exposure of the metal-ligand to a fluorescently labeled antibody, since the soluble ligand can effectively compete with the immobilized ligand by rapidly rebinding the newly dissociated. KinExA is a remarkably sensitive tool for characterization of antibody-antigen interactions so that a lot of studies took advantage of the method to investigate the equilibrium and rate constant (Blake *et al*., 1996; Khosraviani *et al*., 2000, 1998; Blake *et al*., 2001b; Blake II and Blake, 2003; Sasaki *et al*., 2009; Xiang *et al*., 2010; Zou *et al*., 2013). Most of the studies determined the equilibrium dissociation constants through the instruments in a short time, while a specialized introduction for the KinExA in protein binding interactions showed an assay time of less than 30 min each (Blake II *et al*., 1999).

APPLICATION OF HEAVY METAL IMMUNE DETERMINATION

Pretreatment procedure of variant metals: Compared with the ICP or AAS for determination, simple pretreatment of samples is needed for immunoassay in liquid. However, for solid or dust, pretreatment is generally required to extract the analyte or to destroy the organic matrix. The choice of the procedure depends to some extent on the target element, food matrix and speed of analysis.

The cadmium in rice foliage and soil were successfully extracted by 0.1 mol/L HCl solution at a rice foliage: HCl ratio of 1: 20 and coexisting metals were removed sufficiently by the column treatment. The 0.1 mol/L HCl-extractable Cd concentration in soil was also determined successfully (Abe *et al*., 2009). The same group extracted Cd from wheat grain and fresh eggplant with 0.1 mol/L HCl ((at solution ratios of 1:10 and 1:4, respectively) and purified the eluate sufficiently using ion exchange column with buffer diluted at appropriate rates (Abe *et al*., 2011).

Besides above pretreatments, some nanomaterials may be used to utilize as solid-phase extractant for trace metal ions. A diethyldithio-carbamate-modified nanometre TiO_2 was developed as preconcentration material prior to measurement by ICP-AES (Zheng *et al*., 2006). Zou *et al*. (2013) used nanometer TiO_2 separate Cr (III) and Cr (VI) after the reduction of Cr (VI) through a series of reactions, while the adsorption percentage for the Cr (III) ions was more than 98%. A column packed with strong base anion exchange resin can also isolate potassium dichromate (Sasaki *et al*., 2009).

Detection methods:

Biosensor kit: An immunochromatographic assay kit for detecting Cd in brown rice was manufactured by Kansai Electric Power Co. of Japan (Tawarada *et al*., 2003; Sasaki *et al*., 2007). This sensor kit uses the antigen-antibody complex reaction between the Cd-EDTA complex and an anti-Cd-EDTA antibody that reacts specifically with this complex to detect Cd at concentrations of 0.01 mg/L or higher.

Fluorescence Polarization Immunoassay (FPIA) for detection: FPIA is a testing application for detecting metal ions based on anti-chelate approach and ELISA formats (Johnson, 2003). The first report about FPIA utilized in metal detection described a method for lead (II) in which the lead content of the sample was measured indirectly through an FPIA for Porphobilinogen (PBG) (Adamczyk *et al*., 1998). The activity of 5-Aminolevulinic Acid Dehydratase (ALAD), the enzyme which converts 5-Aminolevulinic Acid (5-ALA) to PBG, is opposite to the lead concentration. The concentration of lead is then indirectly estimated by measuring the amount of PBG through a competitive FPIA in which the tracer conjugated to hapten would compete with immunogen for attaching the same combining site of antibody. Such approaches with an enzymatic activity that either requires or is inhibited by the target metal. Studies of method for lead (II) had been used for drinking water and solid samples (Johnson *et al*., 2002). To make sure reaction reaching to equilibrium state, the responding signals are taken over 10 min after addition of the Pb (II) -EDTA complex solution (Lin and Chung, 2008).

Lateral Flow Immunosensor Device (LFID) applied for detection: LFID are currently used for qualitative, semi quantitative and to some extent quantitative monitoring in resource-poor or non-laboratory environments. Applications include tests on pathogens,

drugs, hormones and metabolites in biomedical, phytosanitary, veterinary, feed/food and environmental settings (Posthuma-Trumpie *et al*., 2009). A new integrated paper based cadmium immunosensing system in lateral flow format, which integrates the sample treatment process with the analyte detection process was presented (López *et al*., 2013). The device presented here is based on a competitive format by setting Cd-EDTA-BSA-AuNP on the conjugation pad strip and the 2A81G5 mAb specific to Cd-EDTA onto the test line at the optimized concentrations. With a working range from 0.4 to 10 ppb, detection and quantification levels are much lower than the previously reported for metal biosensors based on paper.

The LFIP method has a shorter detection time and much lower cost for the procedure, which shows an excellent choice for the immunoassay with metal ions in the future.

Some other detection methods combined different application in various fields. Fiber-based biosensor with extraordinary optic properties of gold nanoparticles had been introduced into determination of lead ions, which was combined with monoclonal antibody against Pb (II) -EDTA ligand (Lin and Chung, 2008). The combination of functionalized Magnetic Beads (MBs) with electrochemical detection based on immunoassays between metal-ligand and antibodys is another design that can be taken into account. Many chemical agents have been analyzed by the electrochemical magnetoimmuno-sensors (Pedrero *et al*., 2012). This kind of recombination have shortened the time for immune-reaction and decreased coating, competition and blocking, which had a good future in determination of heavy metals *in situ*.

CONCLUSION

The ligand which is exploited for heavy metal coordination and the equilibrium binding properties of the complexes has been investigated for many years. Research in the area of design and fabrication of sensors and biosensors for heavy-metal detection has become of great interest to a variety of scientific communities ranging from biological and chemical sciences to engineering communities. New opportunities for new heavy-metal-sensing technologies with advantages such as high sensitivity and selectivity, rapidness and cost efficiency due also to the possible integration with existing simple platforms and technologies beside the development of new ones are offered. In addition, due to the synergy of nano-materials' properties with nanotechnologies, the development of highly integrated detection systems that can even ensure online or even implanted heavy-metal detectors applicable not only to environmental studies but also to other fields like clinical analysis or safety and security can be previewed.

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