Simple Method to Reduce Interference from Excess Magnesium in Cadmium Immunoassays

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In order to develop a rapid inexpensive test for cadmium in rice, we identified an antibody specific for cadmium-EDTA complexes; this antibody binds to cadmium-EDTA with a K_d of $\sim 10^{-8}$ M. Although the antibody's cross reactivity to magnesium was minimal ($K_d \approx 10^{-5}$ M), the high toxicity of cadmium coupled with the high natural occurrence of magnesium in rice resulted in a situation where magnesium interfered with cadmium determination and resulted in falsely elevated estimates of cadmium. Fortunately, the formation constant of EDTA for cadmium is approximately 5×10^7 times higher (at pH 7) than the formation constant of EDTA for magnesium, and we were able to eliminate the magnesium interference by judicious selection of the EDTA concentration used in the assay. The resulting equilibria are complex, but we show that a relatively simple two-step model in which cadmium and magnesium compete for EDTA followed by cadmium-EDTA and magnesium-EDTA competing for antibody provided a good fit to the measured data. These analyses enabled appropriate selection of the optimum EDTA concentration for an immunoassay with improved selectivity.

INTRODUCTION

Cadmium is considered an increasingly important environmental pollutant in many countries. It is a cumulative poison associated with a variety of syndromes and effects, including renal dysfunction, reproductive toxicity, and bone defects (*1*). The main source of cadmium exposure is dietary via contaminated water and crops grown on polluted soil (*2*), resulting in a need for methods of detecting cadmium in both environmental and food samples.

The most frequently used methods in environmental analysis of cadmium today are atomic absorption spectroscopy (AAS), inductively coupled plasma–atomic emission spectrometry (ICP-AES), and inductively coupled plasma–mass spectroscopy (ICP-MS). These methods are sensitive and accurate, but they are time-consuming and require sophisticated equipment, generally in a laboratory setting. In cases where suitable antibodies and extraction protocols are available or can be developed, immunoassays offer a simple, fast, cost-effective alternative (*3*). This is especially true in cases where high-throughput and/or on-site screening analysis is needed.

At present, all antibodies specific for cadmium recognize a chelated form of cadmium (e.g., Cd·EDTA *4–7*) necessitating the addition of EDTA to the sample prior to measurement. Since nearly all metal present in near neutral pH is expected to be coordinated with other compounds, the addition of a high activity chelator such as EDTA also serves as a metal specific extraction step. Since the chelator will extract all metals to some degree (depending on the complexation constant) and since all antibodies identified so far have at least some cross reactivity to other chelated metals, there is at least a possibility of interference. For example, monoclonal antibody 2A81G5 described by Blake et al. (*4*) shows relatively high affinity binding to Cd·EDTA with an equilibrium dissociation constant (K_d) of 2.1×10^{-8} M, but it also binds to Mg·EDTA at a much weaker K_d of 2.2×10^{-4} M (*4*). Magnesium is a harmless metal (in fact, an important nutrient) that is often present at high concentrations (micromolar to millimolar) in samples such as rice (*8*), while cadmium is toxic in nanomolar concentrations. This large difference in concentrations is enough to overcome the relatively good cadmium specificity (cross reactivity to magnesium is 0.01%) for this antibody. A recently described anti-Cd·EDTA monoclonal antibody (clone no. Nx2C3) produced in our laboratory shows very similar binding properties and has proven susceptible to magnesium interference in rice samples (*6*). One approach to solving the problem would be to further reduce the antibody's cross reactivity through genetic modification. However, the process is technically complex, and

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it can require more than 2 months, even in a lucky case, to make an improved antibody using phage-display methods (*9*). We show here a simpler approach, exploiting the difference in complexation constants between Cd·EDTA and Mg·EDTA.

MATERIALS AND METHODS

Materials. The Nx2C3 antibody that recognizes Cd·EDTA complexes was prepared from a mouse hybridoma (*6*). It was deposited at International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan) as deposition no. FERM P-19703. Cd·EDTA−protein conjugates were prepared, as described by Darwish and Blake (*10*). 1-(4-Isothiocyanobenzyl) ethylenediamine-*N,N,N′,N′*-tetraacetic acid (isothiocyanobenzyl−EDTA) was obtained from Dojindo (Kumamoto, Japan). Keyhole limpet hemocyanin, bovine serum albumin (BSA), and ovalbumin (OVA) were obtained from Sigma-Aldrich (H7017, A9647, and A2512; St. Louis, MO). Cy-5 conjugated F(ab′)$_2$ fragment of goat antimouse IgG (no. 286402) was obtained from Jackson ImmunoResearch (West Grove, PA). The myeloma cell line (NS0) was purchased from The Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan).

Solid Phase Preparation. Antigen coated plastic beads were prepared as follows: 0.4 g of polymethylmethacrylate (PMMA) beads (Sapidyne Instruments, Inc., Boise, ID) were suspended in 1 mL of Cd·EDTA−OVA conjugate solution (1 mg protein/mL) and mixed gently overnight. The overlying solution was removed, and the PMMA beads were subsequently blocked against nonspecific binding with BSA solution (1 mg/mL) by gently mixing for >2 h.

Immunoassay Procedure for KinExA. Antibody assays were performed with KinExA 3000 (Sapidyne Instruments, Boise, ID). The KinExA 3000 consists of a single flow cell located at the focal point of a filter fluorometer. Solid phase material (OVA−Cd·EDTA coated PMMA particles in the present case) was suspended and flowed into the flow cell where it was trapped against a screen.

Assay samples consisted of Nx2C3 monoclonal antibody (final concentration of 0.5 nM) mixed with a solution containing metal ions and EDTA in 20 mM Tris and 5 mM sodium citrate (pH 7.0). Samples were supplemented with 2% blocking reagent N101 (NOF Corp., Tokyo, Japan).

An equilibrium measurement began with flow of running buffer, which was 20 mM Tris and 5 mM sodium citrate (pH 7.0) containing 2% blocking reagent N101, at 0.25 mL/min for 30 s to establish a baseline. Next, the sample was flowed through the solid phase for 50 s at a rate of 0.6 mL/min. During this period, free antibody present in the sample (if any) accumulated on the solid phase Cd·EDTA. The solid phase was then washed with running buffer for 30 s, followed by flowing 2 nM Cy-5 labeled antimouse IgG for 96 s, followed by an additional 30 s of the running buffer, all at 0.25 mL/min. Finally, residual bulk fluorescence (i.e., not attached to the solid phase) was removed by a 90 s flow of running buffer at 1.5 mL/min. The signal difference between the fluorescent signal after the final wash and the initial baseline was calculated and used as a measure of free antibody in the sample. K_d values were calculated directly from the measured free antibody using the KinExA Pro software supplied with the instrument in the manner described previously (*11, 12*).

Binding Theory for KinExA. As described previously (*11−13*), the signal level on the KinExA instrument is directly proportional to the free antibody present in the sample at equilibrium. In the present case, the equilibrium is the fairly complex result of two competitive equilibria, one between cadmium and magnesium for EDTA and a second between the Cd·EDTA and Mg·EDTA for the antibody. The calculations can be simplified somewhat by making an assumption that the two equilibria proceed in a serial fashion; that is, the cadmium, magnesium, and EDTA equilibrium occurs first and is not perturbed by the Cd·EDTA or Mg·EDTA antibody equilibrium. This is physically plausible because the measurement protocol envisioned calls for adding EDTA to the sample prior to the antibody, and it is expected that the total antibody added will be well below the cadmium working range of the assay. The second assumption assures that the antibody bound Cd·EDTA will be a small fraction of the total Cd·EDTA, implying that the first equilibrium is unlikely to be significantly shifted by
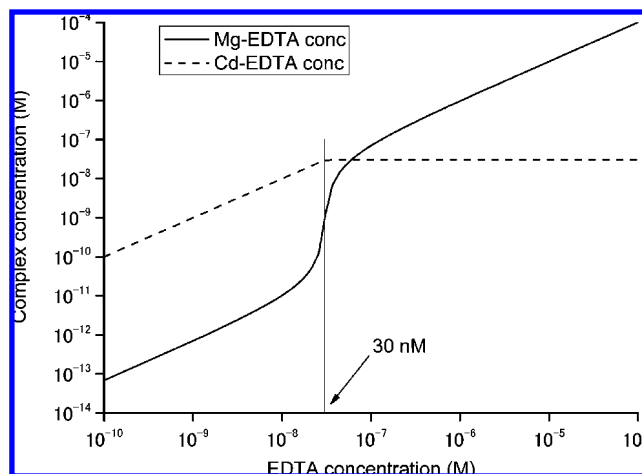

Figure 1. Estimation of formation of EDTA complexes. Concentrations of Cd·EDTA and Mg·EDTA were estimated when EDTA was added to a solution that included 30 nM cadmium and 1 mM magnesium.

antibody binding. Under these assumptions, we can use the competition equations we have previously published (*14*) to write equations for the free antibody as a function of the cadmium, magnesium, and EDTA concentrations.

RESULTS AND DISCUSSION

The formation constants (log K_f) for Cd·EDTA and Mg·EDTA are 16.46 and 8.79 corresponding to conditional formation constants (designated $K_f′$ and equivalent to affinity) of 1.4 × 10^{13} and 3.1 × 10^5 M^{-1} at pH 7.0, respectively (*15*). The large difference in the affinities of EDTA for these two metals suggests that, when the concentration of EDTA is limiting, a greater relative fraction of the cadmium will be bound than the magnesium. This is shown in **Figure 1** where the calculated concentrations of chelated cadmium and magnesium are plotted as a function of total EDTA. The total concentrations of cadmium and magnesium are 30 nM and 1 mM, respectively, and as long as the total EDTA is less than 30 nM, there is over 1000 times more chelated cadmium than magnesium, despite the large excess of magnesium. Only when the EDTA concentration exceeds the cadmium concentration does the Mg·EDTA concentration grow greater than the Cd·EDTA concentration.

The binding strength (K_d) of Nx2C3 for both cadmium and magnesium was measured with 5 mM EDTA. In the case of cadmium, the K_d was also measured with 5 μM EDTA. The K_d measurement for magnesium was not possible at the lower EDTA concentration because of the high concentrations of Mg·EDTA needed to effect binding. Curves are shown in **Figure 2A**, and the measured K_d values are shown in **Table 1**. The mechanism by which the EDTA affects the K_d for cadmium is not known, but the effect is clear in both **Table 1** and **Figure 2A**.

With the individual K_ds from **Table 1** and the competition equation derived previously, we could predict the equilibrium system response for arbitrary mixtures of antigen and antibody. For the case of Nx2C3 and the mixture of Cd·EDTA and Mg·EDTA, theoretical signals were calculated and compared to the results of the experiments for both 5 mM EDTA (**Figure 2B**) and for 5 μM EDTA (**Figure 2C**). Correlation coefficients (R^2) between calculated signals and experimental signals are greater than 0.99, confirming the agreement between theory and experiment apparent in **Figure 2B** and **C**. As shown in **Figure 2B**, the percent inhibition observed when the cadmium assay is performed in 5 mM EDTA is a function of both the cadmium
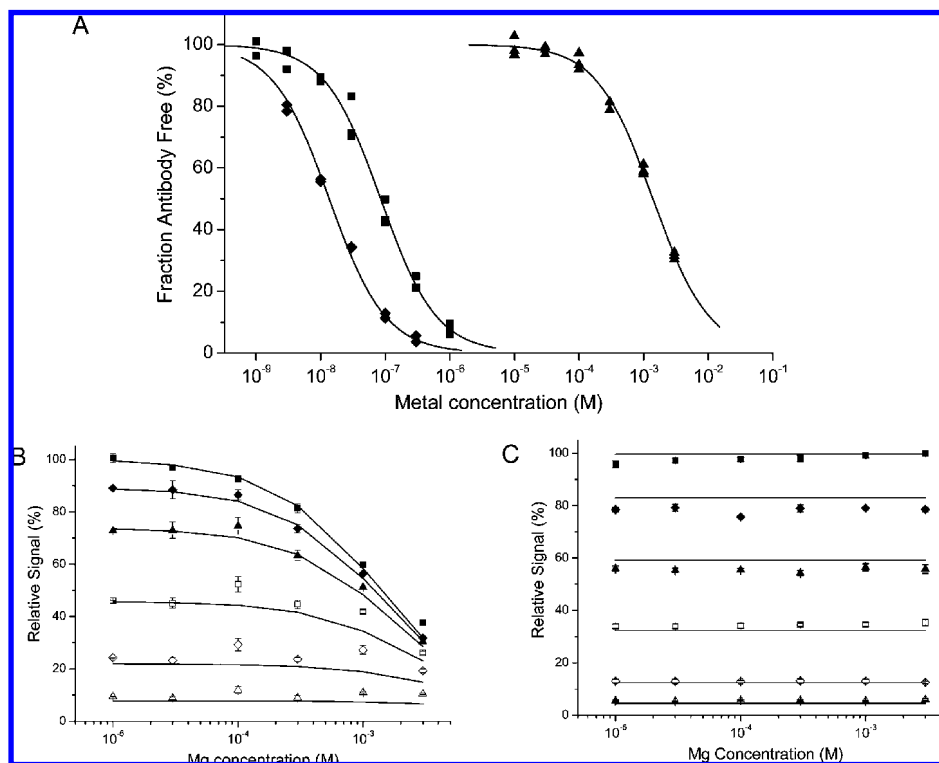
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Figure 2. Binding assays for antibody Nx2C3 and EDTA complexes at different EDTA concentrations. (**A**) Response curves for Nx2C3 reacting with Cd·EDTA and Mg·EDTA: (■) Cd·EDTA with 5 mM EDTA; (▲) Mg·EDTA with 5 mM EDTA; (◆) Cd·EDTA with 5 μM EDTA. Solid lines are calculated from the best fit K_d for each data set. K_d values for each curve are 84×10^{-9}, 1.4×10^{-3}, and 14×10^{-9} M for Cd·EDTA (5 mM), Mg·EDTA (5 mM), and Cd·EDTA (5 μM), respectively. (**B**) Response signals for Nx2C3 reacting with EDTA complex formed from mixture of cadmium and magnesium with 5 mM EDTA. Cadmium concentrations: (■) 0 μM; (◆) 0.01μM; (▲) 0.03 μM; (□) 0.1 μM; (◇) 0.3 μM; (△) 1.0 μM. Solid lines drawn through the data points are calculated from the model of equilibrium binding for antibody reacting with mixed antigens and EDTA using the K_d (Cd·EDTA, 5 mM) and the K_d (Mg·EDTA, 5 mM) in the fashion described in the text. (**C**) Response signals for Nx2C3 reacting with EDTA complex formed from mixture of cadmium and magnesium with 5 μM EDTA. Cadmium concentrations: (■) 0 μM; (◆) 0.003 μM; (▲) 0.01 μM; (□) 0.03 μM; (◇) 0.1 μM; (△) 0.3 μM. Solid lines are calculated in the same way but with the lower EDTA concentration.

Table 1. K_d Values for Nx2C3 with Mg and Cd

EDTA	metal	K_d
5 mM	Mg	1.4 mM
5 mM	Cd	84 nM
5 μM	Cd	14 nM

and magnesium concentrations; under these experimental conditions, the assay would give false positive results. In **Figure 2C**, the system response depended only on the cadmium concentration, even when the magnesium concentration was as high as 3 mM. Even in the case of zero cadmium, the maximum concentration of Mg·EDTA was limited to 5 μM by the EDTA concentration, and this concentration of Mg·EDTA is too low to bind to Nx2C3 (**Figure 2A**). Therefore, the system response in **Figure 2C** can be regarded as resulting solely from the binding of Cd·EDTA and Nx2C3.

An appropriate choice of EDTA concentration allowed us to take advantage of the large difference of formation constants between Cd·EDTA and Mg·EDTA. The result was a sensitive and specific immunoassay for Cd·EDTA that could be performed regardless of the contaminating magnesium concentration.

Magnesium is a very common element in environmental soil and water. The method described above exploits both the specificity of antibodies and the relatively large difference in formation constants between magnesium and cadmium, to enable a sensitive and selective immunoassay for cadmium in the presence of a large excess of magnesium. This method would also be effective for Ca contamination because the formation constant of Ca·EDTA is

10.96, which is still much lower than the formation constant of Cd·EDTA. The method described will not be effective against potentially interfering metals like Zn and Cu whose formation constants (16.5 and 18.8, respectively (*15*)) are comparable to that of the target metals. Some crop extracts might have these elements present in concentrations high enough to affect measurement. These problems would have to be solved by using other approaches such as affinity chromatography.

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