Field Portable and Autonomous Immunosensors for the Detection of Environmental Contaminants

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Abstract. A monoclonal antibody that binds with high affinity to UO22+ complexed with the chelator 2,9-dicarboxyl-1,10-phenanthroline was used to develop a sensor-based assay for UO22+. The assay range for UO22+ was 0.5 to 25 nM (0.12 to 6 ppb). The average coefficients of variation in the assay was 2.2%. The immunoassay results were comparable to those obtained using the Kinetic Phosphorescence Assay. The versatility of this sensor platform was further demonstrated by the development of an immunoassay for caffeine.

Introduction

Antibody-based techniques are alternative approaches for environmental analyses. These approaches are attractive to local and governmental agencies because they have significant advantages over the traditional analytical methods. Immunoassays are remarkably quick, reasonably portable to the analysis site, and simple to perform. Because most of the technology of an immunoassay is "built" into the antibody that is at the core of this detection system, sample pretreatment is usually minimal and the instrumentation designed to transduce the antibody binding event to a measurable signal can be relatively small and inexpensive. The immunoassay and instrument can also be formatted for high throughput analysis. In addition, studies have shown that the use of immunoassays during remediation processes can reduce analysis costs by 50% or more (Szurdoki et al. 1996). Although most environmental immunoassays are directed toward complex organic chemicals, (Giraudi and Baggiani 1994; Van Emon et al. 1998; Abad et al. 1999) this technique is theoretically applicable to any analyte, including a metal ion, if a suitable antibody can be generated.

In this study, we describe a new approach for uranium analysis, based on an immunosensor and an antibody with specificity for chelated UO_2^{2+} (Blake et al 2004). The immunosensor described herein is a flow fluorimeter with broad applications for the assay of low molecular weight analytes. In the present study, we show how this sensor can be employed to measure two different environmentally relevant analytes, UO_2^{2+} and caffeine.

Methods

The uranium-selective chelator 2,9-dicarboxyl-1,10-phenanthroline (DCP) was obtained from Alfa Aesar (Ward Hill, MA). A Cy5-labeled Fab fragment of goat anti-mouse IgG was a product of Jackson ImmunoResearch Laboratories (West Grove, PA). Poly(methylmethacrylate) and azlactone beads (98 $\pm$ 8 μm diameter) were obtained from Sapidyne Instruments Inc (Boise, ID). A UO_2^{2+} standard was obtained from the National Institute of Standards and Technology (Gaithersburg, MD). The 12F6 monoclonal antibody, which binds to UO_2^{2+}- DCP complexes, and UO_2^{2+}-DCP-BSA conjugate were available from a previous study (Blake et al. 2004). Reaction mixtures for UO_2^{2+} were prepared in HBS buffer (137 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.4). The inline immunosensor timing files used for the analysis were described in detail in (Yu et al. 2005).

A sensor assay for caffeine was also developed using commercially available reagents. A caffeine-bovine serum albumin conjugate (caffeine-BSA) and a monoclonal antibody to caffeine were obtained from YJ BioProducts (Rancho Cordova, CA). Additional monoclonal antibodies to caffeine (clones 9410, 8.F.28, and 1.BB.877) were obtained from Biodesign (Saco, ME) and U.S. Biologicals (Swampscott, MA). A standard solution of caffeine (1 mg/ml) was purchased from Sigma-Aldrich (St. Louis, MO). Equilibrium dissociation constants for the interaction of these antibodies with caffeine were determined as described by (Blake and Blake 2003) in PBS (137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4).

Results

Immunosensor-based assay for uranium

The method employed for the analysis UO_2^{2+} is diagrammed in Fig. 1. The sensor consisted of a capillary flow/observation cell containing microbeads coated with an immobilized version of the metal-chelate complex. The fluorescently labeled antibody was mixed with soluble metal-chelate complex derived from the environmental sample, and the assay components were allowed to come to equilibrium. This mixture was then passed rapidly through the observation cell. Only those antibody molecules with unoccupied binding sites were available to bind to

the immobilized ligand on the surface of the beads; antibodies whose binding sites were already occupied with ligand were not. An LED was used to excite the fluorescently labeled antibody bound to the microbeads and a photodiode measured the amount of fluorescence emanating from the observation cell. The amount of antibody bound to the microbeads was inversely proportional to the amount of metal-chelate complex in the sample, because the binding of the antibody to the complex reduced the free antibody concentration in a dose-dependent fashion.

Primary data for the analysis of UO22+ by a prototype in-line immunosensor that had the ability to autonomously prepare and run a standard curve from reagents stocks is shown in Fig 2A. For clarity, not all instrument traces are shown. The period between 0 and 700 seconds in the trace represents the time of automated reagent mixing, the period between 700-1120 seconds corresponds to the application of the reaction mixture to the microcolumn/observation cell, and the period between 1120 and 1260 seconds represents a buffer wash that removed unbound antibody from the microbead column. Individual instrument traces for assay mixtures containing 0, 1, 5, and 25 nM UO_2^{2+} are shown, as is a control for

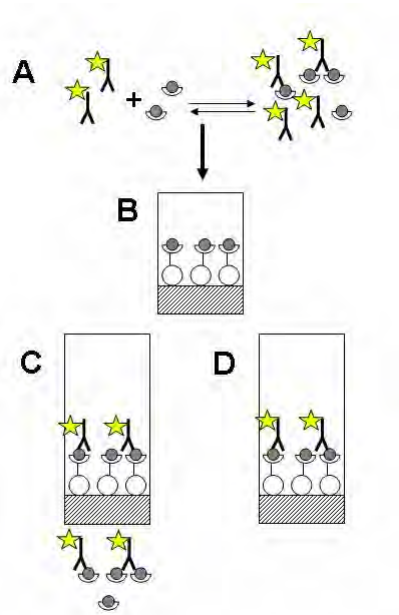

Fig. 1. A. Fluorescently labeled antibody (**Y★**) is mixed with environmental sample containing the analyte (the metal-chelate complex) and the mixture is allowed to come to equilibrium. **B.** The equilibrated sample is passed rapidly through an observation cell that contains an immobilized version of the metal-chelate complex. **C.** Antibodies whose binding sites are already occupied with the metal-chelate complex are not retained by the column. **D**. After a buffer wash, only those antibodies with open binding sites contribute to the final signal, which is inversely proportional to the amount of metal-chelate complex in the environmental sample.

non-specific binding (NSB) in reaction mixture that omitted the uranium-specific 12F6 antibody. The instrument automatically calculated the response to varying concentrations of uranium by subtracting a baseline reading from the reading after the unbound antibody had been washed from the column (the average instrument response from 1250-1255 seconds minus the average instrument response during the first 5-10 seconds of the trace). This instrument response (delta signal) was plotted versus UO_2^{2+} concentration to generate the uranium dose-response curve shown in Fig. 2B. For this demonstration, the instrument responses generated by 3 replicate analyses of each UO_2^{2+} concentration were plotted as individual points. The coefficients of variation in this experiment ranged from 1.4 to 3.9%. This assay accurately measured UO_2^{2+} at concentrations from 0.5 to 25 nM (0.12 to 6 ppb) and the results were comparable to KPA assays run at the same time (data not shown). The drinking water limit for uranium set by the United States Environmental Protection Agency is 30 ppb (US EPA 2000). Thus, this sensor-based assay has sufficient sensitivity to monitor UO_2^{2+} at environmentally relevant concentrations.

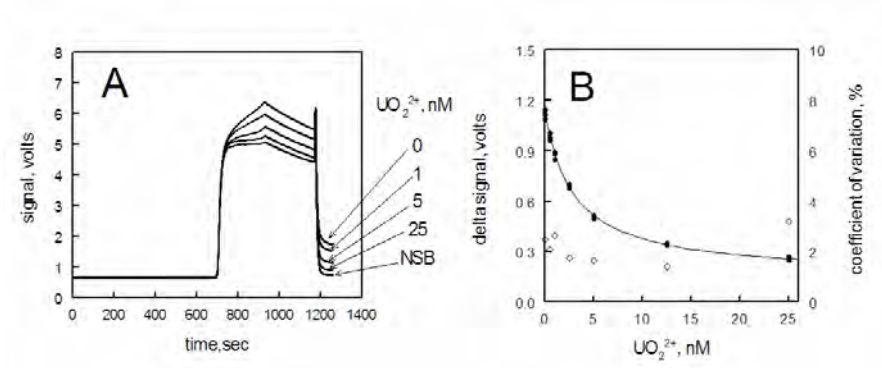

Fig. 2. A, Individual instrumental traces when 12F6 monoclonal antibody (0.33 nM), Cy5-Fab fragment of goat anti-mouse IgG (6.5 nM), DCP (100 nM), bovine serum albumin (12.5 µg/ml) and varying concentrations of UO_2^{2+} (as indicated) were mixed by the immunosensor and applied to a microcolumn of beads containing immobilized UO_2^{2+}-DCP. NSB reaction mixture contained Cy5-Fab fragment (6.5 nM), DCP (100 nM), and bovine serum albumin (12.5 µg/ml). **B**, (●) Instrument response (delta signal) plotted versus UO_2^{2+}concentration. Triplicates were run at each UO_2^{2+} concentration; the data are plotted as individual points. (◇), Coefficient of variation (% CV) at various UO_2^{2+} concentrations.

Table 1. Binding affinities of commercially available antibodies for caffeine.

Vendor	Clone Number	K_d, nM[a]
YJ BioProducts	not applicable	1.21
Biodesign International	9401	0.89
United States Biological	1.BB.877	1.20
United States Biological	8.F.28	1.47

[a] Equilibrium dissociation constants were determined using KinExA 3000™ as described in (Blake and Blake, 2003; Blake et al. 1999) using poly(methylmethacrylate) microbeads adsorption-coated with a caffeine-bovine serum albumin conjugate.

Immunosensor-based assay for caffeine

The handheld and autonomous immunosensors under development in our laboratory have sufficient flexibility to accommodate the analysis of a wide variety of analytes. An example of this functionality is an immunosensor-based assay for caffeine that utilized commercially available reagents. Assays for caffeine are relevant in environmental monitoring and for quality control in the food industry.

The method of analysis employed by the handheld and in-line sensors under development in our laboratories (as outlined in Fig. 1) makes it relatively straightforward to develop new applications. Antibodies must be identified that bind to the analyte at a relevant concentration range, and a microbead column must be developed that contains an immobilized version of the analyte. Once these reagents have been identified, the software associated with these sensors can be easily modified to accommodate the requirements of specific antibodies and/or analytes. Four different commercially available antibodies to caffeine were evaluated for use in this assay, as shown in Table 1.

In our experience, the immunosensors under development in our laboratories have the ability to detect analyte concentrations that are $\geq$ 10% of the K_d value, so any of the four antibodies surveyed in Table 1 could theoretically provide sufficient sensitivity for analysis of environmental water samples.

Another important variable in the development of immunoassays for environmental analyses is the effect of sample matrix on the immunoassay. In this model study for caffeine, we evaluated the ability of the four commercially available monoclonal antibodies to correctly measure caffeine levels in Diet Coke™. Spike and recovery data for two of these antibodies are given in Table 2, below. It is clear that the 2 antibodies tested are influenced to different extents by other components in the Diet Coke™ sample. For this particular application, antibody 1.BB.877 appears most suitable for subsequent assay development.

Discussion

A practical focus of our laboratories is the development of both autonomous, in-line and portable, hand-held immunosensors that operate on the same principle as that of a commercial flow fluorimeter designed to study antibody-antigen binding interactions (Blake and Blake, 2003). The experiments summarized above represent preliminary data collected to illustrate the versatility of the immunosensor devices currently under development in our laboratories. Perhaps the most challenging analyte for any immunoassay is a heavy metal ion such as UO_2^{2+}. Since the uranyl ion is not sufficiently large to elicit an immune response by itself, it must be tightly complexed with a larger molecule, in this case a uranium chelator covalently conjugated to a protein, in order to be rendered as a target for the im-

Table 2. Spike and recovery data for caffeine in Diet Coke™ using 2 different monoclonal antibodies[a]

1.BB.877			YJ BioProducts		
Added (nM)	Found (nM)	% Recovery	Added (nM)	Found (nM)	% Recovery
0.1	.0788	79.9	0.2	0.29	145.3
0.3	0.326	108.7	0.6	0.096	160.8
0.6	0.750	121.8	1.0	1.43	143.5
1	1.073	107.3	4	5.34	133.4
5	5.825	116.5	8	9.63	120.4
10	12.628	126.3	50	71.61	143.2
Average		110.1	Average		141.1

[a]Standard curves for caffeine were constructed as described for the uranium immunoassay, using a commercially available caffeine standard. The caffeine in Diet Coke™ samples (www2.coca-cola.com/mail/goodanswer/soft_drink_nutrition.pdf) was diluted into PBS to the indicated concentrations, and the delta signal was used to determine caffeine concentration from the standard curve.

mune response. Thus any immunoassay for chelated UO_2^{2+} must include at least one additional equilibrium between the metal ion and the chelator in addition to the necessary antibody-antigen recognition event. The highly precise and reproducible antigen inhibition data shown for UO_2^{2+} in Fig. 2B indicates that the autonomous in-line sensor is quite capable of identifying and quantifying UO_2^{2+} in a mixture of antibody and antigen where the 'antigen' is itself the product of an independent metal-chelator binding equilibrium.

The preliminary data developed with caffeine and antibodies directed against caffeine illustrate that the same device and assay principles can just as readily be applied to identify and quantify a simple organic contaminant. A prototype immunoassay for caffeine is more than a simple model chosen to contrast with the more complex immunoanalysis of UO_2^{2+}. Caffeine is one of the more abundant contaminants in surface waters in the United States and Europe (Kolpin et al, 2002; Weigel et al, 2002). The only source of caffeine in the environment is man, and caffeine has been proposed as a chemical surrogate for fecal coliform bacteria of human origin (Standley et al, 2000; Glassmeyer et al, 2003). In the United States, caffeine concentrations in surface waters ranged from non-detect to 31 nM (6 ppb) with a median value of approximately 0.5 nM (0.1 ppb) (Kolpin et al, 2002).

Regardless of the identity of the analyte or the existence of multiple equilibria in the formation of the actual antigen recognized by the antibody, it is important to realize and acknowledge that the performance characteristics of any immunoassay depends on the binding properties of the antibody. Given an acceptable level of reproducibility and uniformity in reagent preparation and handling, it is evident that the sensitivity and precision of an antibody-based assay is critically dependent on the thermodynamics and kinetics of the antibody-antigen binding interaction. The sensitivity and limit of detection of any immunoassay, regardless of format, depends primarily upon the affinity of the antibody for the environmental contaminant; the tighter the binding, the more sensitive the final assay. Similarly, the precision of any immunoassay that is designed to provide a rapid response depends upon the rate of binding between the antibody and the antigen. Most immunosensor formats include an initial incubation step, where a limiting quantity of the antibody is exposed to a molar excess of the analyte, prior to signal generation. If the binding reaction between the antibody and the analyte is not very close to or at equilibrium before the next step in the assay is executed, then the duration of the incubation step must be precisely and reproducibly controlled or the overall precision of the assay will suffer.

When the concentration of the analyte is in a 4-fold or greater molar excess to that of the antibody, the bimolecular association of the limiting antibody with the excess antigen is approximately a first order process (pseudo-first order kinetics), and the approach to the binding equilibrium can be described as a single exponential function of time. The apparent rate constant, k, for this exponential function of time is approximately equal to $k_{on}[\text{analyte}]+k_{off}$, where k_{on} and k_{off} are the association and dissociation rate constants, respectively, for the reversible binding reaction (Moore and Pearson, 1981). The rate equation can be manipulated to determine the half-life of the binding reaction: $t_{1/2}= \ln 2/k$ (Moore and Pearson,

1981) and this $t_{1/2}$ value can then be used to estimate whether a binding reaction is close to equilibrium. As a practical example, the k_{on} and k_{off} for the association and dissociation of the 12Fb antibody and UO_2^{2+}-DCP complex used in this study are 2.0 x 10^7 M^{-1} s^{-1}, and 1.8 x 10^{-2} s^{-1}, respectively (Blake et al, 2004). The $t_{1/2}$ at the lowest analyte concentration in the uranium standard curve (0.5 x 10^{-9} M) can be calculated to be 24.75 s by substitution into the equations above. After 5 half-lives, the binding reaction has achieved 96.875% of its equilibrium value. Therefore, this particular antibody-analyte binding reaction has essentially achieved equilibrium in 5 x 24.75 s, or 2 minutes.

For highly sensitive assays where the k_{on}[analyte] term is very small because [analyte] is a vanishingly small number, the term that defines both k and the $t_{1/2}$ is the dissociation rate constant. Any highly sensitive immunoassay (measurements at the picomolar level or below) will require a very high affinity antibody, and this antibody must necessarily have a slow dissociation rate constant. Consequently, there will always be a practical trade-off between sensitivity and rapidity in any immunoassay; the greater the desired sensitivity, the longer one must wait to achieve effective equilibrium in the initial binding reaction.

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