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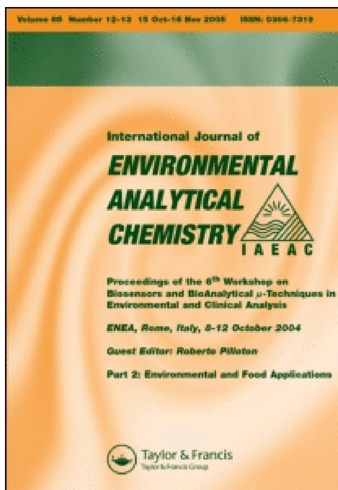

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An immunosensor for autonomous in-line detection of heavy metals: validation for hexavalent uranium

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An immunosensor for autonomous in-line detection of heavy metals: validation for hexavalent uranium

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An automated immunosensor, based on the commercially available KinExA 3000™, has been developed as a collaboration between academia and industry. The sensor had the ability to autonomously run a standard curve from stock reagents and to prepare environmental samples for analysis. Assays for both a model analyte (biotin) and the environmental contaminant hexavalent uranium (UO_2^{2+}) have been constructed. The sensor measured biotin at levels from 20 to 1000 nM and UO_2^{2+} at concentrations from 5.8 to 100 nM (1.4–24 ppb). The coefficients of variation (CV) in the uranium assay ranged from 3.5 to 5.9%, with an average of 4.6%. Spike-and-recovery experiments in the uranium assay yielded a mean % recovery of 99.17 ± 7.05. The sensitivity and specificity of this uranium sensor will support the rapid, inexpensive analysis of hexavalent uranium in both environmental and clinical samples.

Keywords: Immunosensor; Flow fluorimeter; Monoclonal antibodies; Uranium; Heavy metals; Biotin

1. Introduction

The Cold War era processing of uranium has led to environmental problems on an international scale. In the USA, more than 200 million metric tons of mine tailings and other waste have been identified for clean-up and disposal [1], and the European community has also experienced environmental degradation as the result of uranium mining and processing [2, 3]. More recently, the use of depleted uranium for both military and civilian applications has renewed interest in the bioaccumulation and toxicity of uranium [4–8].

The toxicological effects of uranium differ according to its chemical form; the more soluble hexavalent form, UO_2^{2+}, has been shown to be the most potent systemic toxicant [9, 10]. Chronic exposure to uranium in drinking water is weakly associated with altered proximal tubulus function without a clear threshold, which suggests that even low uranium concentrations in drinking water can cause nephrotoxic effects [10]. Uranium has also been shown to be genotoxic and mutagenic *in vivo* [11–13].

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Uranium in environmental and clinical samples is currently speciated and quantified using ICP-MS, alpha spectrometry, or laser spectrofluorimetry [14, 15]. Although these methods accurately measure the UO_2^{2+} in environmental and clinical samples, they all require relatively complicated and expensive equipment that must be housed in a central facility. In this study, we describe a new approach for uranium analysis, based on a new immunosensor and an antibody with specificity for chelated UO_2^{2+}. 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 both the model analyte biotin and UO_2^{2+}, an environmentally relevant ligand.

2. Experimental

2.1 *Materials*

HEPES buffer, biotin, biotin–BSA and bovine serum albumin (fraction V, minimum 98%) were purchased from Sigma/Aldrich (St. Louis, MO). Cy5-labeled anti-biotin and a Cy5-labeled Fab fragment of goat anti-mouse IgG were products of Jackson ImmunoResearch Laboratories (West Grove, PA). 2,9-Dicarboxyl-1,10-phenanthroline (DCP) was obtained from Alfa Aesar (Ward Hill, MA). Poly(methylmethacrylate) and azlactone beads ($98 \pm 8\,\mu m$ diameter) were obtained from Sapidyne Instruments Inc. (Boise, ID). Bovine serum albumin with covalently conjugated 2,9-dicarboxyl-1,10 phenanthroline (BSA–thioureido–DCP) and monoclonal antibody 8A11, which binds specifically to UO_2^{2+}–DCP complexes, were available from previous studies [16, 17]. Standard uranium solutions were obtained from the National Institute of Standards and Technology (Gaithersburg, MD). ACS grade uranyl acetate was a product of Mallinckrodt Chemical Works (St. Louis, MO).

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2.2 *Preparation of beads with immobilized capture ligands*

For experiments with biotin, poly(methylmethacrylate) microbeads (200 mg) were adsorption-coated for 1 h at 37°C in 1 mL phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 10 mM phosphate, pH 7.4) containing 0.1 mg of the biotin–BSA conjugate. After centrifugation and removal of the supernatant solution, any non-specific protein binding sites were blocked by subsequent incubation of the beads with 1% BSA in PBS. The beads could be stored for several days in this blocking buffer; on the day of use, they were diluted to a final concentration of $6.7\,mg\,mL^{-1}$ and loaded into the bead reservoir of the sensor (see figure 1A).

For experiments with UO_2^{2+}, DCP–BSA was covalently conjugated to the azlactone beads according to Sapidyne's protocol. Briefly, 1 mL of 50 mM sodium carbonate buffer, pH 9.0 containing 50 µg of DCP–BSA was added to 50 mg of dry, pre-weighed beads and the solution was allowed to rock gently overnight at 4°C. The next day, the beads were allowed to settle, the supernatant fluid was removed and the beads were rinsed once with 1 mL of blocking solution ($10\,mg\,mL^{-1}$ BSA in 1 M Tris buffer, pH 8.0). The beads were centrifuged gently and the supernatant fluid was replaced by an additional 1 mL of blocking solution. Beads were blocked for at least 1 h at room temperature before use in the sensor. The conjugated beads were transferred to

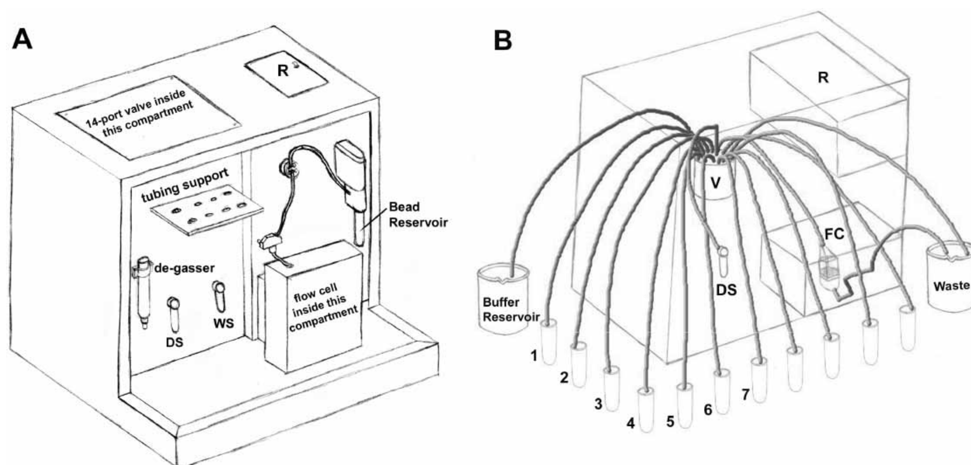

Figure 1. Schematic of in-line immunosensor. (A) Schematic of front panel of the in-line sensor. For clarity of presentation, the tubing and 14-port valve responsible for reagent transfer are not shown in this view. Reagents are stored in a refrigerated compartment (R). The microbeads with immobilized analyte are stored in the bead reservoir; a fresh aliquot of beads is packed into the flow cell for each analysis. A de-gasser prevents bubbles in the flow system. A drive syringe (DS) pulls assays components from individual test tubes; the waste syringe (WS), pumps used components into waste. (B) Fluidics of the in-line system. A 14-port valve (V) pulls fluid from the buffer reservoir or tubes (1–7 in the examples presented herein) to the drive syringe (DS) and subsequently controls and mixing of reagents and transfer to the flow cell (FC).

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30 mL of HEPES buffered saline (HBS, 137 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.4) containing 16 μM uranyl acetate (to load UO_2^{2+} into the DCP chelator) and 0.03% NaN_3 and stored at 4°C.

2.3 *General features of the in-line immunosensor*

The in-line sensor is an arrangement of tubing, connectors, valves, syringes and pumps whose purpose is to accurately mix and deliver soluble reagents to the observation cell of a flow fluorimeter. The operating principals employed by instruments developed by Sapidyne Instruments Inc. have been described previously [18–20]. Rigid beads were coated with an immobilized version of the analyte (biotin or UO_2^{2+}–DCP) and deposited as a mini-column in an observation cell. A fresh bed of beads was used for each determination. Fluorescently-labeled antibody and varying concentrations of the analyte to be measured were autonomously mixed by the sensor; an aliquot of this mixture was then rapidly passed over the bead column. Antibodies with unoccupied binding sites were available to bind to the immobilized ligand on surface of the microbeads; antibodies whose binding sites were already occupied with analyte were not. The amount of fluorescently-labeled antibody bound to the microbeads was measured after a buffer wash to remove soluble components. The quantity of free antibody bound to the microbeads was inversely related to the amount of analyte in the sample, because binding of the antibody to the analyte reduced the free antibody concentration in a dose-dependent fashion.

A schematic of the front of the in-line sensor is shown in figure 1A; the liquid handling system unique to the in-line instrument is shown in figure 1B. Reagents (fluorescently labeled antibodies, buffers, soluble analytes required for construction

of standard curves) can be stored as concentrated stocks in a refrigerated compartment built into the side of the instrument (see R in figures 1A and 1B) or maintained at room temperature (see tubes 2–7 of figure 1B for the examples presented herein). These reagents were drawn into the fluid handling system of the instrument via flexible sample lines that were assigned to individual ports in a rotary valve (see V in figure 1B). The reagents were drawn into a drive syringe (see DS in figures 1A and 1B), then subsequently delivered to a mixing tube (see tube 1 in figure 1B). The drive syringe then mixed reagents via sequential transfers from the mixing tube to the chamber of the drive syringe. The final, mixed sample was injected into the flow/observation cell (see FC in figure 1B), where the amount of antibody bound to the microbeads was quantified by measuring the fluorescent signal after a buffer wash to remove soluble components from the beadpack.

3. Results and discussion

3.1 *Assay for biotin*

The in-line sensor was controlled via sequential execution of the commands of a programmable 'Timing Routine'. A timing routine for the packing of a microbead column, washing of instrument components, and construction of a calibration curve for biotin is shown in table 1. Each numbered line in the routine represents a command that instructed the instrument to perform a specific fluid transfer. The lines of command were executed sequentially in numerical order. Lines 1–7 of table 1 comprised the 'bead-handling' routine. A 20-s backflush removed the previous beadpack from the flow/observation cell; this was followed by a 20-s buffer wash to allow the beads in the particle reservoir to be lifted into suspension. The suspension of analyte-coated microbeads was subsequently added to the flow cell from the bead reservoir. After a buffer wash, the beads were gently lifted and allowed to settle for 20 s in the absence of flow. A 6 s final wash helped remove any remaining blocking solution and firmly pack the column. In normal operation, a 450 µL aliquot of analyte-coated beads resulted in a beadpack 4 mm high (a height that permitted optimal observation of the fluorescent signal). Lines 8–11, which directed buffer from the buffer reservoir to the mixing tube and subsequently to waste, were included to wash the mixing tube. This process was repeated two additional times (lines 12–19) in order to ensure that there was no carryover of reagents in the mixing tube and drive syringe.

The next steps in the timing routine (lines 20a–22h) were specific to the particular analyte being measured. In this example, sample line 1 was assigned to the mixing tube, sample line 2 to a tube containing Cy5-labeled anti-biotin antibody, sample lines 3 and 4 to two different stock solutions of biotin, and sample line 5 to buffer. The instrument executed lines 20a–22a, and then continued with line 23 of the routine. Any line that contained a 'Yes' in the Loop column was executed for only one assay. Lines 20–22 instructed the sensor to draw antibody, biotin stock solution, and buffer into drive syringe. Line 23 then directed the transfer of these reagents to the mixing tube, and subsequent commands (lines 24–26) mixed these reagents by sequential transfers from the mixing tube to the drive syringe. A small volume of material (400 µL) was sent to waste to avoid diluting the mixed sample with residual material in the tubing (Line 27), then the remaining 2 mL of the mixed sample was

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Table 1. Timing routine for the construction of a biotin calibration curve using the in-line immunosensor.[a]

Line	Fluid movement	Sample line[b]	Time (s)	Draw		Dispense		Loop	Stir
				Vol (μL)	Rate (μL min^{-1})	Vol (μL)	Rate (μL min^{-1})		
Bead handling									
1	Backflush	–	20	0	0	–	–	No	Off
2	Buffer	B	20	500	1.5	–	–	No	On
3	Particle reservoir	–	18	450	1.5	–	–	No	On
4	Buffer	B	34	850	1.5	–	–	No	Off
5	Waste	–	10	50	0.3	–	–	No	Off
6	Buffer	B	20	0	0	–	–	No	Off
7	Buffer	B	6	150	1.5	–	–	No	Off
Wash of mixing tube									
8	From buffer to DS	B	60	3000	3.0	–	–	No	Off
9	From DS to mixing tube	1	10	–	–	3000	18.0	No	Off
10	From mixing tube to DS	1	60	3000	3.0	–	–	No	Off
11	From DS to waste	W	10	–	–	3000	18.0	No	Off
Lines 8–11 are repeated two more times (lines 12–19)									
20	*Addition of antibody*								
20a	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20b	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20c	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20d	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20e	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20f	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20g	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20h	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
21	*Addition of biotin*								
21a	From tube 5 to DS	5	12	600	3.0	–	–	Yes	No
21b	From tube 4 to DS	4	4	200	3.0	–	–	Yes	No
21c	From tube 4 to DS	4	10	500	3.0	–	–	Yes	No
21d	From tube 4 to DS	4	16	800	3.0	–	–	Yes	No
21e	From tube 4 to DS	4	24	1200	3.0	–	–	Yes	No
21f	From tube 3 to DS	3	5	240	3.0	–	–	Yes	No
21g	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21h	From tube 3 to DS	3	24	1200	3.0	–	–	Yes	No

(*Continued*)

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Table 1. Continued.

Line	Fluid movement	Sample line[b]	Time (s)	Draw		Dispense		Loop	Stir
				Vol (μL)	Rate (μL min^{-1})	Vol (μL)	Rate (μL min^{-1})		
22	*Addition of buffer*								
22a	From tube 5 to DS	5	24	1200	3.0	–	–	Yes	No
22b	From tube 5 to DS	5	32	1600	3.0	–	–	Yes	No
22c	From tube 5 to DS	5	26	1300	3.0	–	–	Yes	No
22d	From tube 5 to DS	5	20	1000	3.0	–	–	Yes	No
22e	From tube 5 to DS	5	12	600	3.0	–	–	Yes	No
22f	From tube 5 to DS	5	31	1560	3.0	–	–	Yes	No
22g	From tube 5 to DS	5	24	1200	3.0	–	–	Yes	No
22h	From tube 5 to DS	5	12	600	3.0	–	–	Yes	No
Sample mixing									
23	From DS to mixing tube	1	24	2400	3.0	–	6.0	No	Off
24	From mixing tube to DS	1	48			2400	3.0	No	Off
Lines 23–24 are repeated once (lines 25–26)									
Flushing lines to observation cell									
27	From DS to waste	W	4	–	–	400	6.0	No	Off
Reading sample in observation cell									
28	From DS to flow cell	FC	240			2000	0.5	No	Off
Washing syringe pump									
29	From buffer to DS	B	60	3000	3.0	–	–	No	Off
30	From DS to waste	W	10	–	–	3000	18.0	No	Off
Lines 29–30 are repeated two more times (lines 31–34)									
Washing to remove unbound reagents from observation cell									
35	From buffer to DS	B	30	1500	3.0	–	–	No	Off
36	From DS to flow cell	FC	90	–	–	1500	1.0	No	Off

[a] Execution of specific command lines in table 1 resulted in calibrators containing the following final biotin concentrations: 20a–22a, 0 nM; 20b–22b, 20 nM, 20c–22c, 50 nM; 20d–22d, 80 nM; 20e–22e, 120 nM; 20f–22f, 200 nM; 20–22g, 500 nM; 20–22h, 1000 nm. Each solution (a–h) also contained the Cy5-labeled antibiotin antibody at a concentration of 25 ng mL^{-1} (0.083 nM) and BSA at 25 μg mL^{-1}.

[b] The solutions supplied to sample lines 2–5 were as follows: 2, Cy5-labeled anti-biotin antibody in PBS, 50 ng mL^{-1} in PBS containing 0.1 mg mL^{-1} BSA; 3, 2000 nM biotin in PBS; 4, 240 nM biotin in PBS; 5, PBS.

sent through the flow/observation cell (Line 28), where the fluorescently-labeled antibody bound to the analyte-coated beads. After a buffer wash of the drive syringe and tubing (Lines 29–34), the unbound antibody was washed from the flow cell (Lines 35–36) and antibody that remained bound to the beads was quantified from the fluorescent signal (in volts) emitted from the beadpack.

Once the sensor had completed the entire timing routine for the first point in the standard curve (Lines 20a–22a) it repeated the entire sequence, completing Lines 20b–22b in the next round of samples, then 20c–22c and so forth, until all the commands in table 1 had been executed. The instrument traces obtained upon execution of these timing routines are shown in figure 2. The instrument responses from 0 to 700 s corresponded to the background signal generated during reagent mixing. From 701 to 930 s, reagent mixtures containing varying concentrations of biotin were applied to the flow cell. From 931 to 1170 s, the mixed sample remained in the flow cell while the instrument was washing the syringe pump, and the signal from 1170 to 1255 s represents the signal during a buffer wash of unbound reagents from the flow cell. The instrument automatically calculated the response to varying concentrations of biotin by subtracting a baseline reading (the average instrument response during the first 5–10 s of the trace) from the instrument response after the unbound antibody had been washed from the column (the average instrument response from 1250 to 1255 s in this example).

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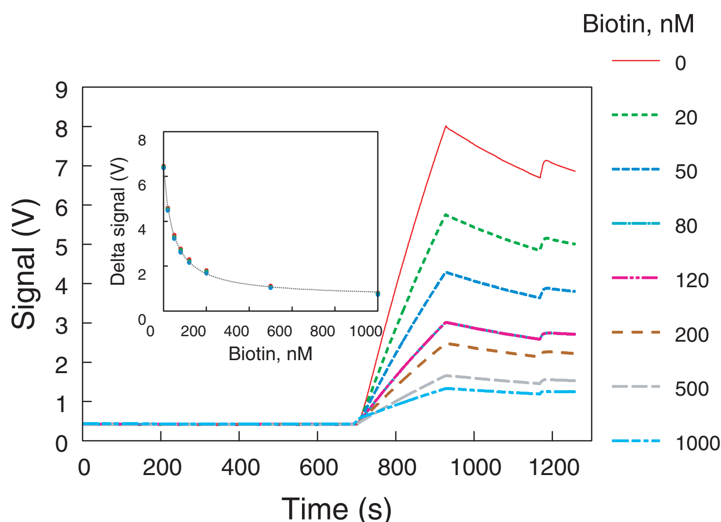

Figure 2. Dose–response curve for biotin. The Cy5-labeled anti-biotin antibody (final concentration, 0.083 nM) and varying concentrations of biotin were mixed by the instrument according to the timing routine described in table 1. These mixtures were applied sequentially to the sensor and the time course of the instrument response obtained at different biotin concentrations was: 0–700 s, background signal during reagent mixing; 701–930 s, reagent mixtures applied to the flow cell; 931–1170 s, sample in the flow cell during wash of the syringe pump; 1170–1255 s, signal after wash of unbound reagents from the flow cell. For clarity of presentation, the individual time courses for only a single data set are shown in this figure. The anti-biotin antibody stock solution (50 ng mL^{-1} PBS containing 0.1 mg mL^{-1} BSA), and the two biotin stock solutions (2000 nM and 240 nM in PBS) were made on the day of use. Inset, the delta signal (average instrument response from 1245–1250 s minus average instrument response from 5 to 10 s) was plotted *vs.* biotin concentration. The entire experiment was performed in triplicate and individual datum for each biotin concentration is plotted.

This instrument response (delta signal) was plotted *versus* biotin concentration to generate the biotin dose–response curve shown in the *inset* of figure 2. For this demonstration, the instrument responses generated by three replicate analyses of each biotin concentration are plotted as individual points. The coefficients of variation in this experiment ranged from 0.69 to 5.3%. This assay measured biotin from 20 to 1000 nM and the sensitivity of the assay was limited by the affinity of the anti-biotin antibody for soluble biotin [20]. The intent of these experiments, however, was not to generate an assay with high sensitivity for biotin but rather to demonstrate the proper function of the immunosensor using commercially available reagents.

3.2 *Assay for UO_2^{2+}*

3.2.1 General considerations. Because phosphate is known to bind UO_2^{2+}, HEPES buffered saline (HBS, 137 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.4) was used in place of PBS in all buffers for uranium analysis [21]. The monoclonal antibody used in this study, 8A11, recognizes UO_2^{2+} in a complex with 2,9-dicarboxyl-1,10 phenanthroline (DCP) [17] and DCP was added to generate the UO_2^{2+}–DCP complex. DCP binds to UO_2^{2+} with a K_d of 3.5 nM at the pH and ionic strength employed in these experiments [17]; the addition of DCP to a final concentration of 200 nM in all assays was therefore sufficient to insure that $>98\%$ of the UO_2^{2+} added to the sample was complexed with DCP. The addition of bovine serum albumin (BSA) in these assays stabilized the 8A11 antibody and improved reproducibility in the assay; however, high concentrations of BSA have been shown to compete with the 8A11 antibody for the UO_2^{2+}–DCP complex [17]. BSA added to achieve a final assay concentration of $25\,\mu g\,mL^{-1}$ stabilized the 8A11 antibody without significantly affecting the sensitivity of the assay for UO_2^{2+}.

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3.2.2 Addition of a fluorescent label to the 8A11 antibody. Covalent conjugation of 8A11 with the Cy5 label significantly changed the antibody's binding properties [12]. To minimize these changes in binding activity, the 8A11 antibody was non-covalently labeled via addition of a goat anti-mouse Fab fragment to which Cy5 had been covalently conjugated. A monovalent Cy5–Fab fragment was used in these studies in place of a divalent immunoglobulin to avoid cross-linking and precipitation of the primary antibody. In preliminary experiments, the optimal ratio of 8A11 to the Cy5–Fab was determined by varying the Fab concentration at a fixed concentration of primary antibody (data not shown). An additional control (a sample that contained the Cy5-labeled anti-Fab antibody but no primary 8A11 antibody) was included in all subsequent assays to ensure that there was minimal non-specific binding of the labeled Fab to the capture reagent.

3.2.3 Stability of the microbead capture reagent. Absorption coating of poly(methylmethacrylate) microbeads with capture reagent (UO_2^{2+}–DCP–BSA) did not provide an adequate signal in the uranium assay. A variety of other solid supports were tested for their ability to provide an acceptable signal-to-noise ratio in the assay (data not shown); an acrylamide microbead that permitted covalent attachment of the ligand to the solid support via an azlactone linkage had the best performance characteristics for this application. DCP–BSA was covalently conjugated to the azlactone

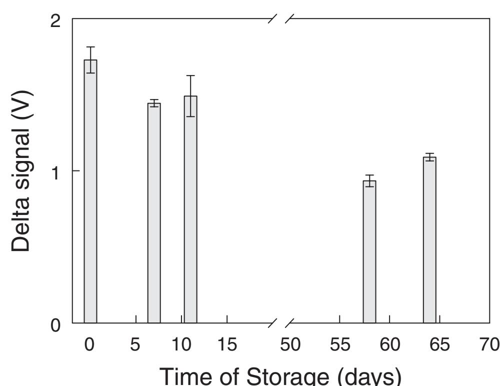

Figure 3. Stability of immobilized capture ligand. The capture ligand for the uranium antibody, BSA–DCP was covalently conjugated to azlactone beads as described in the experimental section. The beads were stored at 4°C in HBS containing 16 μM uranyl acetate and 0.03% NaN_3. After the indicated times of storage, the beads were loaded into the flow cell and excess uranyl ions were washed from the beadpack according to the timing routine shown in table 1. The ability of the microbeads to capture 8A11 antibody was assessed by drawing a 2 mL solution containing 8A11 antibody (0.83 nM), Cy5-labeled goat antimouse Fab (6.5 nM), and BSA (20 μM) through the flow cell. The delta signal was calculated as described in the legend to figure 2. Each bar represents the mean signal ±SD from three determinations.

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microbeads, and the blocked beads were stored at 4°C for increasing periods of time in the presence of the bactericide, 0.03% NaN_3. At various times after the beads had been prepared their ability to capture the anti-uranium antibody (8A11) was assessed, as shown in figure 3. This capture reagent retained greater than 50% of its activity after more than 2 months of storage. One normally tries to achieve a delta signal between 0.6 and 1.0 V in samples that contain antibody but no analyte, in order to accurately measure the extent of inhibition upon the addition of the analyte. Even after >2 months of storage, these microbeads provided a delta signal of approximately 1 V.

3.2.4 Calibration curve for UO_2^2+. The immunosensor assay developed for uranium was designed to accommodate the specific requirements of the 8A11 antibody, as outlined above. The flexibility provided by the sensor design (up to 11 sample lines were available for addition of different reagent solutions) and the ability to customize the timing routines for specific applications allowed us to design an assay method that could autonomous mix the following reagents prior to analysis: Cy5–Fab, 8A11 monoclonal antibody, DCP, HBS buffer, and NIST standard UO_2^{2+} (three different stock concentrations). Although we did not experience a problem with reagent carryover during the development of the biotin assay, we discovered that UO_2^{2+} was not being completely rinsed from the tubing and drive syringe during replicate analyses. This carryover led to inaccurate results and an apparent decrease in assay sensitivity. A wash step that included the addition of the DCP chelator to the wash buffers was included in the timing routine and this modification solved the problem of analyte carryover. The modifications in the assay required for the analysis of UO_2^{2+} were programmed into the in-line sensor by replacing line 20a–22h in table 1 with the lines shown in table 2.

A uranium calibration curve, generated autonomously as reagents from sample lines 2–7 were mixed and then analysed by the in-line sensor, is shown in figure 4. The sensor

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Table 2. Timing routine for construction of a UO_2^{2+} calibration curve using the in-line immunosensor.[a]

				Draw		Dispense			
Line	Fluid movement	Sample line[b]	Time (s)	Vol (μL)	Rate (μL min^{-1})	Vol (μL)	Rate (μL min^{-1})	Loop	Stir
20	*Addition of cy5-labeled anti-mouse Fab fragment*								
20a	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20b	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20c	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20d	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20e	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20f	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20g	From tube 2 to DS	2	12	600	3.0	–	–	Yes	No
20h	From tube 2 to DS	2	12	600	3.0	–		Yes	No
21	*Non-specific binding control (a) or addition of anti-uranium antibody*								
21a	From tube 6 to DS	6	12	600	3.0	–	–	Yes	No
21b	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21c	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21d	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21e	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21f	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21g	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No
21h	From tube 3 to DS	3	12	600	3.0	–	–	Yes	No

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21	*Controls (a and b) or addition of UO_2^{2+}*								
21a	From tube 4 to DS	4	0	0	3.0	–	–	Yes	No
21b	From tube 4 to DS	4	0	0	3.0	–	–	Yes	No
21c	From tube 4 to DS	4	24	1200	3.0	–	–	Yes	No
21d	From tube 4 to DS	4	12	600	3.0	–	–	Yes	No
21e	From tube 4 to DS	4	5	240	3.0	–	–	Yes	No
21f	From tube 7 to DS	7	24	1200	3.0	–	–	Yes	No
21g	From tube 7 to DS	7	12	600	3.0	–	–	Yes	No
21h	From tube 7 to DS	7	6	300	3.0	–	–	Yes	No
22	*Addition of DCP Chelator*								
22a	From tube 5 to DS	5	24	1200	3.0	–	–	Yes	No
22b	From tube 5 to DS	5	24	1200	3.0	–	–	Yes	No
22c	From tube 5 to DS	0	0	0	3.0	–	–	Yes	No
22d	From tube 5 to DS	5	12	600	3.0	–	–	Yes	No
22e	From tube 5 to DS	5	19	960	3.0	–	–	Yes	No
22f	From tube 5 to DS	5	0	0	3.0	–	–	Yes	No
22g	From tube 5 to DS	5	12	600	3.0	–	–	Yes	No
22h	From tube 5 to DS	5	18	1200	3.0	–	–	Yes	No

[a] Execution of the command lines in table 2 will lead to the following solution conditions: lines 20a–22a (non-specific binding of Cy5–Fab to capture ligand), Cy5–Fab, 1 : 2000; DCP, 200 nM in HBS containing 12.5 $\mu g\,mL^{-1}$ BSA. Lines 20b–22b (no uranium control) Cy5–Fab, 1 : 2000; 8A11 mAb, 0.5 $\mu g\,mL^{-1}$; DCP, 200 nM in HBS containing 25 $\mu g\,mL^{-1}$ BSA. Lines 20–22c–h, Cy5–Fab, 1 : 2000; 8A11 mAb, 0.5 $\mu g\,mL^{-1}$; DCP 200 nM; UO_2^{2+}, 100–2.5 nM in HBS containing 25 $\mu g\,mL^{-1}$ BSA.

[b] The solutions supplied to sample lines 2–7 were as follows: 2, Cy5-labeled anti-mouse Fab (1 : 500 dilution) in HBS containing 50 $\mu g\,mL^{-1}$ BSA); 3, anti-uranium antibody 8A11 (2 $\mu g\,mL^{-1}$) in HBS containing 50 $\mu g\,mL^{-1}$ BSA; 4, 200 nM NIST standard uranium in HBS containing 400 nM DCP; 5, 400 nM DCP in HBS; 6, HBS buffer; 7, 20 nM NIST standard uranium in HBS containing 400 nM DCP.

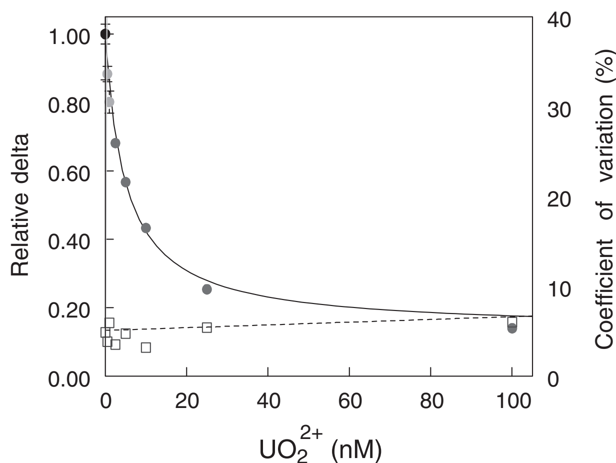

Figure 4. Dose–response curve (●) and precision profile (□) for UO_2^{2+}. The 8A11 monoclonal antibody, Cy5–Fab, DCP, and UO_2^{2+} were mixed according to the timing routine shown in table 2. The delta signal (average instrument response from 1245 to 1250 s minus average instrument response from 5 to 10 s) was determined. Because the experiment was performed over 3 days and with two different batches of capture beads, the delta value for the 8A11 sample with no UO_2^{2+} was set to 1.0 and all data were recalculated as relative delta. Each point represents the mean ±SD of seven determinations. In some cases, the error in the analysis was less than the diameter of the plotted point.

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was programmed to analyse UO_2^{2+} at concentrations from 2.5 to 100 nM (0.6–24 ppb). The assay limit of detection was subsequently determined by identifying the lowest measurable concentration of UO_2^{2+} that could be distinguishable from zero concentration, ±2 SD. On the basis of seven replicates, the lowest limit of detection with the immunosensor was 5.8 nM or 1.4 ppb. This limit of detection is well below the drinking water standard of 30 ppb recently promulgated by the U.S. Environmental Protection Agency [23]. The precision profile for the assay (reported as coefficient of variation, CV) is also shown in figure 4. CVs in the assay ranged from 3.5 to 5.9%, with an average of 4.6%. In general, precision in an immunoassay depends upon accuracy in the dispensing of reagents, control of the time of incubation, and uniformity in the quantity and quality of the capture reagent. The precision data presented herein demonstrate that all of these variables are under good control in the in-line immunosensor assay for UO_2^{2+}.

Analytical recovery in the immunoassay was assessed by adding varying known concentrations of UO_2^{2+} to purified water samples. Each sample was subsequently assayed in triplicate for its UO_2^{2+} content on the in-line sensor. The sensor was programmed to run autonomously; it first executed a standard curve in triplicate, and then diluted the water samples with reagents for analysis of three replicates. The mean analytical recovery was calculated as the ratio between the UO_2^{2+} concentration found and the concentration added, as shown in table 3. A quantitative recovery (93.75–108.17%) of the added UO_2^{2+} was obtained for samples with concentrations from 7.5 to 20 nM (2–4.75 ppb). As expected from our limit of detection predictions, analytical recoveries for the 4 nM sample (0.5 ppb) were somewhat low. The percent recoveries from samples with the higher UO_2^{2+} concentrations were systematically higher and experiments are underway to elucidate the basis for this finding. The average percent recovery in these experiments was 99.18%.

Table 3. Analytical recovery of UO_2^{2+} in the immunosensor assay.[a]

Added UO_2^{2+} (nM)	Found UO_2^{2+} (nM)	% Recovery
4.0	3.11 ± 0.20	77.67
7.5	7.03 ± 0.24	93.76
12.5	13.00 ± 0.34	104.06
15.0	15.82 ± 1.88	105.48
18.0	19.06 ± 2.14	105.91
20.0	21.63 ± 1.44	108.17
Average		**99.18**

[a] Values are the mean of three determinations ± SD.

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4. Conclusions

A new automated immunosensor has been developed that can collect a sample from a process line, amend the sample with assay reagents, and determine the amount of a specific contaminate based on comparisons to an instrument-generated standard curve. Sensor function has been verified in two different assay systems: a model assay for biotin that utilized commercially available reagents and an antibody-based analysis of uranium that employed a monoclonal antibody previously isolated and characterized in our laboratory [16, 17]. The sensor's multiple sample lines facilitated assay development and the associated software was easily modified to accommodate the requirements of specific antibodies and/or analytes. Once the timing routines have been developed for a specific analyte, these programs can be loaded into the sensor to run automatically, with no further input by the end-user. Our previous studies [16, 17] and the experiments described herein demonstrate that UO_2^{2+} assay has the required sensitivity and specificity for environmental analysis. Studies are currently underway to field test this sensor for groundwater monitoring at several US Department of Energy sites.

Although the present application is for UO_2^{2+}, the sensor could be easily reconfigured and reprogrammed for the analysis of any low molecular analyte for which a suitable antibody and capture reagent can be identified.

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