Final Technical Report
Report/Product Number: DOE/ER/63459-1

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Title:	In-line uranium immunosensor

Abstract: In this project, personnel at Tulane University and Sapidyne Instruments Inc. developed an in-line uranium immunosensor that could be used to determine the efficacy of specific in situ biostimulation approaches. This sensor was designed to operate autonomously over relatively long periods of time (2-10 days) and was able to provide near real-time data about uranium immobilization in the absence of personnel at the site of the biostimulation experiments. An alpha prototype of the in-line immmunosensor was delivered from Sapidyne Instruments to Tulane University in December of 2002 and a beta prototype was delivered in November of 2003. The beta prototype of this instrument (now available commercially from Sapidyne Instruments) was programmed to autonomously dilute standard uranium to final concentrations of 2.5 to 100 nM (0.6 to 24 ppb) in buffer containing a fluorescently labeled anti-uranium antibody and the uranium chelator, 2,9-dicarboxyl-1,10-phenanthroline. The assay limit of detection for hexavalent uranium was 5.8 nM or 1.38 ppb. This limit of detection is well below the drinking water standard of 30 ppb recently promulgated by the EPA. The assay showed excellent precision; the coefficients of variation (CV's) in the linear range of the assay were less than 5% and CV's never rose above 14%. Analytical recovery in the immunosensors-based assay was assessed by adding variable known quantities of uranium to purified water samples. A quantitative recovery (93.75% - 108.17%) was obtained for sample with concentrations from 7.5 to 20 nM (2-4.75 ppb). In August of 2005 the sensor was transported to Oak Ridge National Laboratory, for testing of water samples at the Criddle test site (see Wu et al., *Environ. Sci. Technol*. 40:3978-3985 2006 for a description of this site). In this first on-site test, the in-line sensor was able to accurately detect changes in the concentrations of uranium in effluent samples from this site. Although the absolute values for the uranium concentrations were approximately 30% lower that what was determined with the ICP-MS at the site, the in-line sensor could correctly assess changes in the uranium concentrations in near real-time.

Detailed summary of accomplishments:

The specific aims of this project were as follows:

1) To construct an in-line immunosensor for hexavalent uranium after engineering discussions with the final users;

2) To incorporate reagents already developed for a handheld immunosensor into this device and to test its performance capabilities with hexavalent uranium spiked into buffer and groundwater samples;

3) To test the capabilities of the in-line sensor during field tests at the Field Research Center at Oak Ridge National Laboratories.

Specific Aim 1: Immunosensor construction. This portion of the project was subcontracted to a small company, Sapidyne Instruments Inc, located in Boise, ID. An alpha prototype of the in-line immmunosensor was delivered to the PI's laboratory in December of 2002 and the beta prototype (shown in Figure 1) was delivered in November of 2003. Preliminary validation of the sensors was performed using biotin and a fluorescently labeled anti-biotin antibody. These reagents allowed us to rapidly assess sensor performance using commercially available products and allowed researcher at Tulane to compare sensor performance characteristics with instrumental developers at Sapidyne.

Specific Aim 2: Development of a sensor assay for hexavalent uranium. Once our initial assessments demonstrated that the sensor was functioning correctly, all subsequent assay development was performed with reagents prepared at Tulane for the uranium assays. A brief explanation of how the sensor functions and preliminary data collected with the in-line instrument are presented in Figure 2. These preliminary data indicated that the sensor was functioning in accordance with our expectations. Subsequent experiments were carried out using the anti-uranium antibody 8A11 antibody, a capture reagent prepared at Tulane, and a hexavalent uranium standard obtained from the National Institute of Standards and Technology.

Detailed binding characterization of the 3 anti-uranium antibodies available in our laboratory (published in 2004 in *Bioconjugate Chemistry*) indicated that each could potentially be useful in sensor development. Sensor experiments were initiated with monoclonal antibody 12F6, because it bound to uranium with the highest affinity. Unfortunately, we discovered that covalent modification of the lysines residues of 12F6 destroyed its binding activity, and monoclonal antibody 8A11 was chosen for subsequent development work. We later discovered that both covalent and noncovalent modification of the 8A11 antibody could significantly enhance its affinity for chelated uranium. This unexpected result and our subsequent follow-up studies have revealed a fundamental property of antibody behavior that appears to be largely unrecognized. We plan to continue to study this binding behavior in a new DOE grant that was awarded in January of 2005.

After a series of experiments to optimize this portion of the assay procedure, we discovered that the labeling method that provided the best binding properties for the sensor was a non-covalent procedure, where the 8A11 antibody was mixed in a 1:3 molar ratio with a goat anti-mouse Fab fragment that had been covalently modified with the fluorophore, Cy5. This procedure was used to collect the sensor data presented below.

The anti-uranium antibodies recognize U(VI) in complex with 2,9-dicarboxyl-1,10-phenanthroline (DCP). An immobilized version of this U(VI)-DCP complex was used as a capture reagent in the microcolumn of the in-line sensor. The sensor was programmed to autonomously dilute NIST standard uranium to final concentrations of 2.5 to 100 nM (0.6 to 24 ppb) in buffer containing fluorescently labeled 8A11 antibody and 200 nM DCP. The sensor was programmed to perform 7 replicates of each concentration, and the resulting calibration curve is presented in Figure 3. The assay sensitivity, defined as the slope in the middle of the calibration

curve (0-10 nM) was -0.0349. The assay limit of detection, was determined by identifying the lowest measurable concentration of U(VI) that could be distinguishable from zero concentration ± 2SD. On the basis of 7 replicates, the **lowest limit of detection with the immunosensor was 5.8 nM or 1.38 ppb**. This limit of detection is well below the drinking water standard of 30 ppb recently promulgated by the EPA. The assay precision profile, obtained from the results of the calibration standards, is also shown in Figure 2. The assay showed excellent precision; the coefficients of variation (CV's) in the linear range of the assay were less than 5% and CV's never rose above 14%. 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 obtained with the in-line sensor demonstrate that all of these variables are under good control in the in-line immunosensor assay for U(VI).

Analytical recovery in the immunosensors-based assay was assessed by adding variable known quantities of uranium to purified water samples. Each sample was subsequently assayed in triplicate for its uranium content on the in-line sensor. The sensor was programmed to run autonomously; it first executed a standard curve in triplicate, then diluted the water samples with reagents for the analysis of 3 replicates. The mean analytical recovery was calculated as the ratio between the U(VI) concentration found and the concentration added, as shown in Table 1, below. A quantatative recovery (93.75% - 108.17%) was obtained for sample 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 (0.4 ppb) were somewhat low.

Table 1. Analytical recovery of U(VI) added to water samples

Added (UVI), nm	Found U(VI), nM	Recovery, (%)
4.0	3.107±0.203	77.67±6.53
7.5	7.032±0.241	93.75±3.43
12.5	13.008±0.339	104.06±2.60
15.0	15.821±1.881	105.48±11.9
18.0	19.064±2.136	105.91±11.2
20.0	21.634±1.435	108.17±6.63

These data indicate that 1) the antibody and other reagents used for testing of the immunosensors are behaving as expected, based on our previous characterization studies; 2) the performance of the in-line sensor is more than adequate to deliver the precision and sensitivity required for the measurement of uranium in surface and groundwater. These data were published in 2005 in the *Journal of Environmental Analytical Chemistry*.

Specific Aim 3: Testing the capabilities of the in-line sensor during field tests at the Field Research Center at Oak Ridge National Laboratories. In August of 2005 the sensor was transported to Oak Ridge National Laboratory, for testing of water samples at the Criddle test site (see Wu et al., *Environ. Sci. Technol*. 40:3978-3985 2006 for a description of this site). In this first on-site test, the in-line sensor was able to accurately detect changes in the concentrations of uranium in the effluent from this site. The absolute values for the uranium concentrations were approximately 30% lower that what was determined with the ICP-MS at the site.

Number of students trained: none

Number of postdocs trained: one

Publications that have resulted from this award:

1. R.C. Blake II and D.A. Blake (2003) "Kinetic exclusion assay to study high-affinity binding interactions in homogeneous solutions", *Methods in Molecular Biology, Vol. 248: Antibody Engineering: Methods and Protocols,* (Benjamin K.C. Lo, Ed.) Humana Press, Totowa, New Jersey, pp 417-430.

2. T.R. Glass, N. Ohmura, H. Saiki, D.A. Blake, R.C. Blake II, and S.J. Lackie (2004) "Use of excess solid phase capacity in immunoassays: Advantages for semi-continuous, near real time measurements and for analysis of matrix effects", *Anal. Chem*. **76**:767-772.

3. R.C. Blake II, A.R. Pavlov, M. Khosraviani, H.E. Ensley, G.E. Kiefer, H. Yu, X. Li, and D.A. Blake (2004) "Novel monoclonal antibodies with specificity for chelated uranium(VI): Isolation and binding properties", *Bioconjugate Chemistry* **15**:1125-1136.

4. H. Yu, R.M. Jones, and D.A. Blake (2005) "An immunosensor for autonomous in-line detection of heavy metals: Validation for hexavalent uranium", *Int. J. Env. Anal. Chem*. **85**:817-830.

5. R.C. Blake II and D.A. Blake (2005) "Quantitative analysis of antibody-antigen interactions using immobilized ligands: Kinetic exclusion assays are more accurate than surface plasmon resonance", *Progress in Monoclonal Antibody Research,* (M.A. Simons, ed.) Nova Science Inc., Hauppauge, New York, pp 109-135.

6. R.C. Blake II, N. Ohmura, S.J. Lackie, X. Li, J.B. Delehanty, I.A. Darwish, and D.A Blake (2005) "Monoclonal antibodies that exhibit allosteric binding behavior", In *Monoclonal Antibodies: New Research,* (M.A. Simons, ed.) Nova Science Publishers, Inc., Hauppauge, New York, pp 1-36.

Conference Presentations:

1. D.A. Blake, R.C. Blake II, and G.E. Keifer (2003) "Epitope Mapping Using Solution-based Equilibrium and Kinetic Binding Measurements with Structurally Related Ligands", 19th International Congress of Biochemistry and Molecular Biology, Toronto, Canada, July 20-24.

2. D.A. Blake, X. Li, H. Yu, and R.C. Blake, II (2004) "New Insights into the Functional Behavior of Antibodies as Revealed by Binding Studies on an Anti-Uranium Monoclonal Antibody", DOE-NABIR PI Workshop, Warrenton, VA, Mar. 15-17.

3. D.A. Blake, H. Yu, and R.M. Jones (2004) “In-line Uranium Immunosensor”, DOE-NABIR PI Workshop, Warrenton, VA, Mar. 15-17.

4. H. Yu, X. Li, R.C. Blake II, R.M. Jones, and D.A. Blake (2004) “An Automated Immunosensor for Autonomous In-line Detection of Heavy Metals: Validation for Hexavalent Uranium”, The 6th Workshop on Biosensors and BioAnalytical µ-Techniques in Environmental and Clinical Analysis, Rome, Italy, October 8-12.

5. H. Yu, D.A. Blake, R.C. Blake II, and R.M. Jones (2004) “Adaptation of the KinExA Flow Fluorimeter to Develop an Autonomous In-Line Immunosensor for the Detection of Environmental Contaminants”, First Annual KinExA Symposium, Boise, ID, October 19-20.

Figure Legends.

Figure 1. Picture of the beta prototype handheld sensor.

Figure 2. The immunosensor method. **Panel A**, 1: Fluorescently labeled antibody (Y*) is incubated with its specific ligand (shown here as a chelated metal ion) until the binding reaction has come to equilibrium (usually less than 10 minutes at room temperature). 2: The incubation mixture is passed rapidly through a microcolumn of beads that contain an immobilized version of the ligand. 3: A portion of the antibody with no bound ligand interacts with the ligand immobilized on the microcolumn 4: The fluorescent antibody left after a buffer wash is inversely proportional to the amount of ligand in the original equilibrium binding reaction. **Panel B**, Fluorescent signal (in volts) is monitored as the antibody flows through the microcolumn. The amount of antibody bound to the microcolumn can be determined as the difference (delta) in the average signal (measured as volts) in the background portion of the trace (t=100-200 seconds) and the signal after the automated wash step (t=1340-1370 second). Traces a, b, c, and d are from reaction mixtures to which have been added increasing amounts of free ligand. **Panel C**, When this delta signal is replotted against ligand concentration (biotin in this example) a standard curve is generated.

Figure 3. Calibration curve and precision profile for the analysis of U(VI) with the in-line sensor. The 8A11 monoclonal antibody, Cy5-Fab, DCP, and UO_2^{2+} were mixed according to the timing routine shown in Tables 1 and 3. The delta signal (average instrument response from 1245-1250 sec minus average instrument response from 5-10 seconds) was determined, Because the experiment was performed over 3 days and with 2 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 $\pm$ SD of 7 determinations.

Figure 1.

Figure 2.

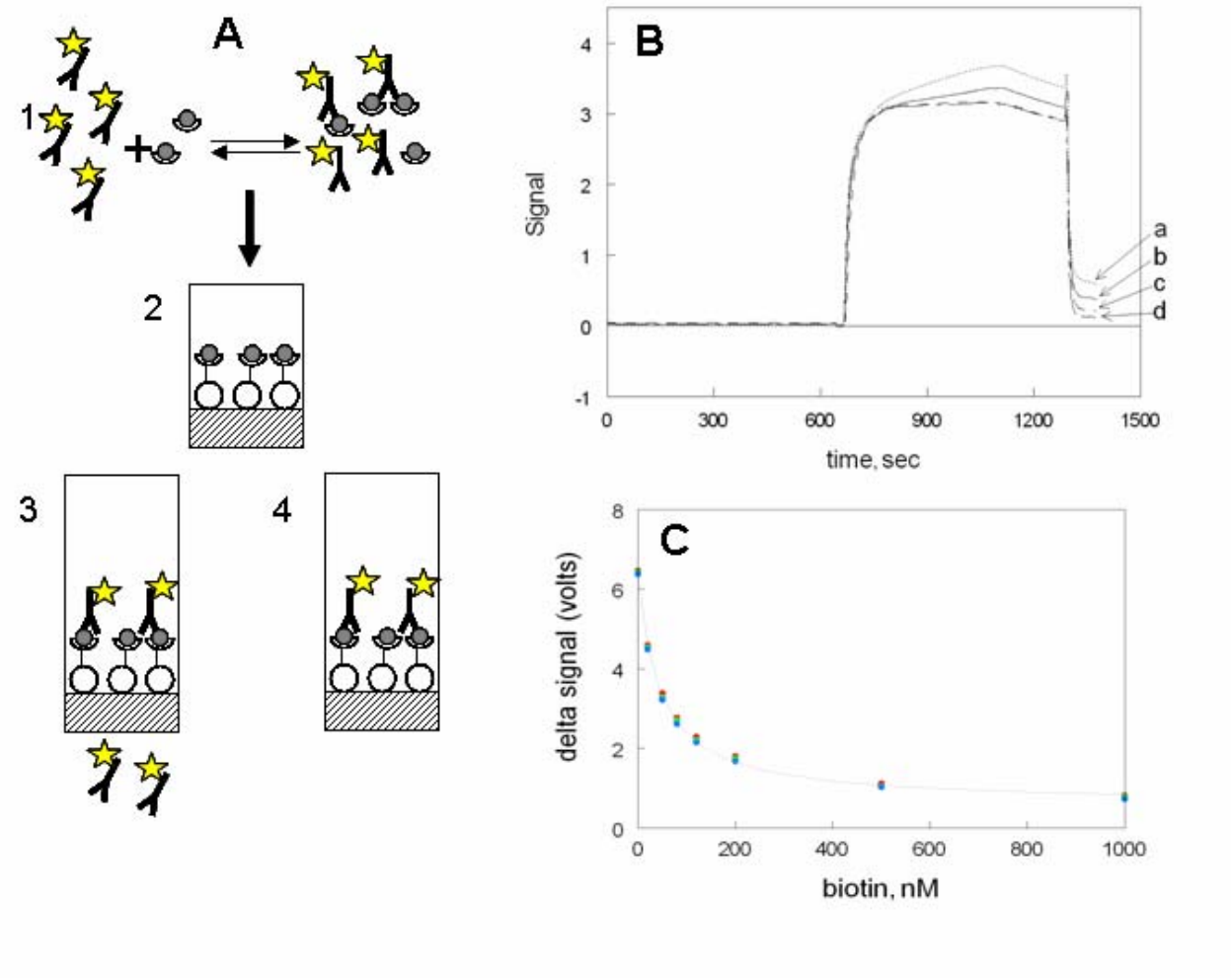
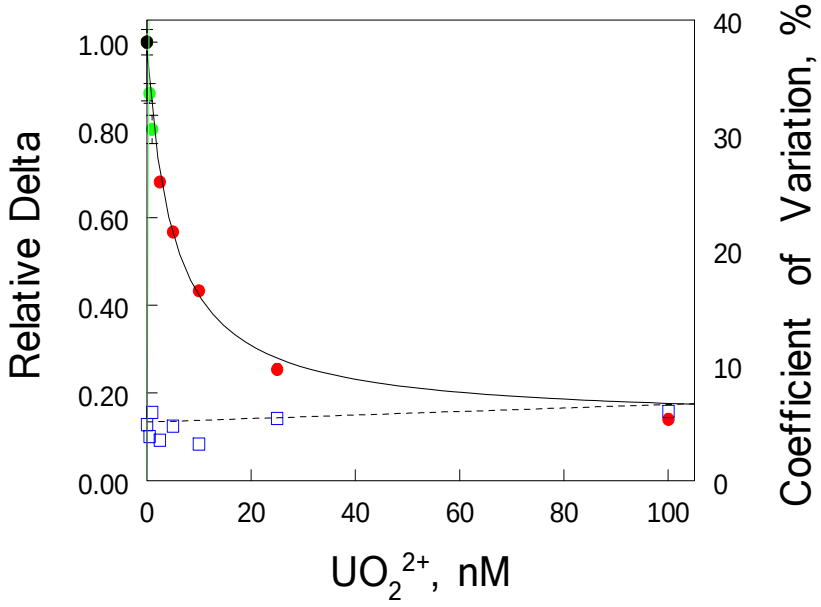


Figure 3.


Relative Delta
Coefficient of Variation, %
1.00
0.80
0.60
0.40
0.20
0.00
40
30
20
10
0
0
20
40
60
80
100
UO2 2+, nM