analytical chemistry

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Single-Chain Variable Fragment (scFv) Antibodies Optimized for Environmental Analysis of Uranium

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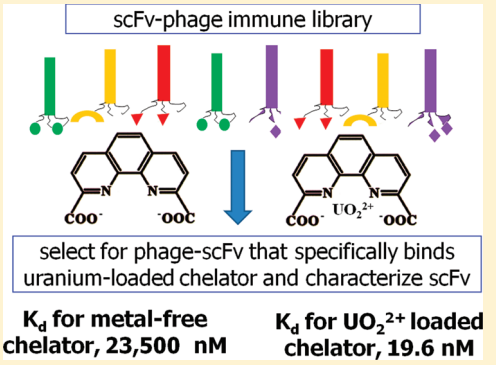
Supporting Information

ABSTRACT: Recombinant single-chain variable fragment antibodies (scFv) were specifically generated and selected for the measurement of environmental uranium with an antibody-based sensor. These sFvs, which recognized UO_2^{2+} complexed to 2,9-dicarboxyl-1,10-phenanthroline-acid (DCP), were produced using genetic material obtained from the spleen cells of rabbits immunized with UO_2^{2+}−DCP conjugated to keyhole limpet hemocyanin. Immunoglobulin light chain and heavy chain genes were amplified and cloned into the phagemid pSD3 for generation of a recombinant antibody library and phage-displayed antibodies. The screening process was designed to isolate antibodies that bound to a "loaded" noncovalent complex with high affinity, while selecting against binding to an "unloaded" complex. After five rounds of panning, individual positive scFv clones were used to infect *E. coli* TG1 and soluble scFv antibodies were purified and characterized. Binding studies showed that the best scFv bound tightly to the UO_2^{2+}−DCP complex (K_d, 19.6 nM). However, because of the depletion experiments performed on this library during the panning process, this scFv bound 1200-fold less tightly (K_d, 23.5 μM) to metal-free DCP. This scFv (clone 3A) was subsequently used to accurately determine the UO_2^{2+} concentrations in environmental water samples using a sensor based on kinetic exclusion analysis. The present studies demonstrate that recombinant scFvs with properties engineered for specific applications (i.e., biosensor-based measurement of metals in groundwater) can be prepared if the correct genetic material and techniques are employed. The phage display system permitted the generation of proteins with very specific binding properties (in this case, high affinity for a metal-chelate complex and low affinity for metal-free chelator). The recombinant scFvs isolated in these studies will be the basis for rapid and affordable assays for the detection of residual uranium in environmental water samples.



Almost all strategies for reducing global emissions of atmospheric carbon dioxide call for increasing the amount of electricity generated by nuclear power.[1,2] Nuclear power is a technologically mature, inexpensive source of electricity that is more reliable than that available from solar cells, wind turbines, or tidal/wave action.[3] Most long-term scenarios for reducing carbon emissions rely on nuclear power to provide electricity when these alternate sources of power are in abeyance. The primary fuel for nuclear power is uranium, and demand has already outstripped supplies.[4] As the worldwide reliance on nuclear power increases, more uranium will be required from the natural deposits in the earth's crust. Since uranium is usually found in relatively low levels in ores, most of the rock previously processed for uranium mining has been left behind as uranium mill tailings. Uranium mining activities in the 1940s and 1950s have resulted in massive quantities of mine tailings, which still contain trace amounts of uranium that have leached into ground and surface waters.[5,6] As mining activities increase in response to energy demands, ongoing and new operations must be conducted in ways that protect people and the environment and avoid the creation of new uranium mining legacies.

The detection and quantification of uranium is important both for monitoring remediation efforts at previously contaminated sites and for protecting personnel and the environment during current and future mining operations. Conventional analytical methods that measure uranium below the United States EPA action limit of 30 ppb (126 nM) include atomic absorption spectroscopy, inductively coupled plasma atomic emission spectroscopy (ICP-AES), inductively coupled plasma mass spectroscopy (ICP-MS) and kinetic phosphorescence analysis (KPA).[7−9] All of these techniques require well-trained analysts, expensive instrumentation and meticulous interpretation of data. Our laboratory has recently described immunosensors that can measure uranium at ppt levels.[10,11] When these sensors were deployed during a bioremediation experiment at a uranium mill tailings site, they provided on-site data in minutes to hours that were comparable to that determined offsite using KPA.[12]

The success of these immunosensors was largely dependent upon the binding properties of the monoclonal antibody used in the assay. The 12F6 antibody used in our previously published assays binds to chelated uranium (UO_2^{2+} in complex with 2,9-dicarboxyl-1,10-phenanthroline, DCP) with an

Received: January 19, 2011
Accepted: April 8, 2011
Published: April 08, 2011

equilibrium dissociation constant of 9.1×10^{-10} M.[13] The antibody also binds relatively tightly to the metal free chelator, DCP ($K_d = 7.5 \times 10^{-7}$ M). This relatively high affinity for the metal-free chelator meant that the DCP concentrations in our field assays had to be carefully controlled at a low concentration (200 nM). Decreasing the DCP concentration in the assays provided false negatives because there was not enough chelator to complex all of the uranium in the environmental sample; increasing the DCP concentration yielded false positives, because the DCP itself interacted with the 12F6 monoclonal antibody. The development of new antibodies less sensitive to the concentration of DCP will increase the precision of our environmental assays and make them easier for less experienced analysts to use in the field. The purpose of the current studies was to develop new antibodies with the specific properties required to improve the analytical method.

Recombinant antibody technology has provided an alternative method to engineer low-cost antibodies with the desired specificity and affinity,[14] and a growing number of recombinant antibodies with specific binding properties have been produced for the analysis of food, clinical, biodefense and environmental samples.[15−18] Antibody phage display libraries sample a much higher portion of the immune repertoire than do hybridoma fusions, and can be subjected to multiple screening procedures to identify binding proteins with high specificity and affinity.[14,19] Competitive panning methods have been used by Love et al. to produce high affinity scFvs that could distinguish *B. anthracis* spores from those produced by *B. cereus* and *B. thuringiensis*[20] and by Krishmaswamy et al. to generate antibody fragments specific for *Candida albicans*.[21] Conway et al.[22] isolated a llama single domain antibody specific for specific botulinum serotype by employing a single round of panning in the presence of high concentrations of other serotypes. Subsequent panning steps were performed using decreasing concentrations of the serotype of interest. Subtractive panning of scFv libraries, used to isolate antibody fragments to specific protein conformational states, is also becoming an established method to generate reagents for the study of conformational changes in membrane proteins.[23]

Phage displayed antibody libraries can also allow investigators to isolate antibody fragments that will distinguish among haptens with very similar 3-dimensional structures. Moghaddam et al. used a naïve library to isolate scFvs that recognized the heroin metabolite 6-monoacetylmorphine (6MAM) but not morphine.[24] Their panning strategy involved the addition of high concentrations of the cross-reactive analyte (morphine) in the presence of decreasing concentrations of their analyte of interest (6MAM). They also shifted their scFv population toward those that bound the soluble antigen by using soluble antigen to elute bound phage particles. However, their best binders, even after chain shuffling to increase affinity, bound to analyte with K_d's of only 10^{-7} M. Fernanado et al.[25] used the Tomlinson J library to select scFvs that selectively recognized DNA G-quadruplex structures. Their strategy involved initial panning in the absence of competitor structures (duplex DNA) and then competitive panning using a fixed concentration of competitive duplex DNA or a G-quadraplex and decreasing concentrations of the G-quadraplex of interest.

In this study, we describe the generation of a phage display immune library and the development of a panning strategy that permitted the selection of single chain variable fragment (scFv) antibodies with binding properties tailored for use in our immunosensors; one of these antibodies was subsequently used to develop sensor-based assays for uranium in groundwater samples. To our knowledge, this is the first report of an scFv antibody that recognizes uranium, specifically UO_2^{2+} complexed to the chelator 2,9-dicarboxyl-1,10-phenanthroline.

MATERIALS AND METHODS

New Zealand white rabbits were obtained from Myrtle's Rabbitry, Thompson Station, TN. Animal experiments were reviewed and approved by Tulane University School of Medicine Institutional Animal Care and Use Committee. The 9E10 hybridoma was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA) and the 9E10 monoclonal antibody was purified using a Melon Gel IgG purification kit from Pierce (Rockford, IL). 2,9-Dicarboxy-1,10-phenanthroline (DCP) was purchased from Alfa Aesar (Heysham, U.K.). SuperScript III First-Strand Synthesis System and helper phage M13KO7 were products of Invitrogen (Carlsbad, CA). TiterMax Gold Adjuvant, HIS-Select Nickel Affinity Gel, keyhole limpet hemocyanin, and bovine serum albumin were products of Sigma Chemical Co. (Saint Louis, MO). GoTaq Hot Start Colorless Master Mix was obtained from Promega (Madison, WI), Quick Ligation Kit, *Bst*NI, *Sfi* I and *Pfl*MI were purchased from New England Biolabs (Ipswich, MA). ACS grade uranyl acetate was a product of Mallinckrodt Chemical Works (St. Louis, MO). SuperBlue TMB microwell peroxidase substrate was purchased from KPL (Gaithersburg, MD). UltraLink Biosupport beads (50−80 μm, diameter) were obtained from Pierce (Rockford, IL). HEPES-buffered saline (HBS, 137 mM NaCl, 3 mM KCl, 10 mM HEPES, pH 7.4) and phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) were prepared using reagents from Fisher Scientific (Pittsburgh, PA). All buffers were prepared using water purified with a Nanopure II water purification system (Barnstead International, Dubuque, IA).

Bacterial Strains and Plasmids. *E. coli* TG1 was used as a host for cloning and phage production and *E. coli* Top10F' cells were used as a host for expression. TG1 and Top10F' were grown in 2 × TY media or on TYE agars at 37°. Plasmid transformations were performed by electroporation using a BTX ECM 399 Electroporation system (Harvard Apparatus, Holliston, MA) under conditions of capacitance at 2500 V; the resulting bacterial cells were recovered in SOC medium. The pSD3 vector was a kind gift of Professor Garry Whitelam, University of Leicester, Leicester, U.K. This vector contains sites for the insertion of antibody heavy- and light-chain variable regions at the N-terminal of the coat protein III (ΔgIII) of bacteriophage M13. Between the cloning gene fragments and Δ gIII are inserted three features encoding a c-myc tag for detection, a $(His)_6$ peptide for purification and an amber codon to facilitate soluble scFv production.[26]

Rabbit Immunization. Conjugates of DCP coupled to BSA and KLH were synthesized and loaded with UO_2^{2+} according to previously published methods.[13] A multiple injection immunization technique was employed.[27] Details of the immunization protocol can be found in the Supporting Information.

Construction of scFv Library. Total RNA was prepared using the RNAeasy kit (Qiagen, Valencia, CA). A total of 5 μg of splenic RNA was used for cDNA preparation. First-strand cDNA synthesis was performed with SuperScript III Reverse Transcriptase using either hexamer or oligo $(dT)_{23}$ primers and the resulting cDNA was split equally into 20 tubes (for 10 heavy chain (HC) and 10 light chain (LC) PCR amplification reactions)

Table 1. Selection Strategy during the Panning Process

round of panning	coating conjugate[a] (μg/mL)	DCP used in preincubation (μM)	incubation time (hour)	number of washes
1	100	0.05	2	5
2	100	0.1	1.5	10
3	10	0.1	1	10
4	10	0.5	1	15
5	1	0.5	1	20

[a] Phage-displayed scFvs were selected against a UO_2^{2+}−DCP−BSA conjugate coated onto immunotubes. After elution, the phage were reinfected into *E. coli* TG1.

and subjected to amplification. Primers used for amplifying the variable regions of the heavy chains (HC) and light chains (LC) were adapted from a previous report.[26] The library size was calculated by multiplying the total number of clones generated per ng of DNA by the fraction of unique clones, as determined by restriction digests of plasmid DNA from representative colonies, as described previously.[28]

Selection of Antibodies that Selectively Recognized the UO_2^{2+}DCP Complex. *E. coli* TG1 carrying the scFv gene in the phagemid vector were grown for phage production according to standard methods.[29] Several selection strategies were tested for their ability to isolated scFv that recognized the UO_2^{2+}−DCP complex but not metal-free DCP. The most successful strategy employed first loose and then increasingly stringent panning conditions to enrich the population of scFv-phage that bound to UO_2^{2+}−DCP. Details of the panning procedure are available in the Supporting Information. An aliquot of the of phage library (10^{13} cfu/mL) was preincubated for 0.5−1 h at room temperature in HBS containing 3% BSA and increasing concentrations of DCP, as shown in Table 1. The preincubated phage were then added to the immunotubes and incubated at 37° for 2 h with rotation. Phage not binding the antigen-coated tubes were removed by washing with PBST (PBS containing 0.05% Tween 20), followed by washing with PBS. The bound phage were eluted with 1 mL of 100 mM triethylamine (TAE) at room temperature with rotation for 10 min and the solution was immediately neutralized by adding 0.5 mL of Tris-HCl (1M, pH 7.4). A further 200 μL of Tris−HCl was added to the immunotube to neutralize any remaining phage. An 0.75 mL aliquot of the eluted phage and 0.2 mL of those remaining in the tube were used to infect a 9.25 and 2 mL culture of log-phase *E. coli* TG1 cells, respectively. The infected bacteria were pooled, and a 100 μL aliquot was used in serial dilutions to titrate the number of phage eluted. This value was designated "phage output". The remaining infected TG1 cells were collected by centrifugation, resuspended in 2 mL of 2 × TY and plated onto a 150 mm culture dish containing 2 × TY-AG overnight at 37 °C. Phage were prepared from the recovered colonies and titered by infection. This value was used in determining the 'phage input' for later rounds of biopanning.[30]

Phage ELISA and Monoclonal Phage ELISA. The resultant phage pool at each round was tested by phage ELISA to evaluate the success of the panning experiment. In brief, 96-well microtiter plates were coated with UO_2^{2+}−DCP−BSA (2 μg/mL), blocked with 3% BSA and washed. Phage solution was subjected to a 3-fold serial dilution into 3% MPBS in the wells of the coated plate. After incubation for 1 h at room temperature, plates were washed and the bound antibodies were detected by horseradish peroxidase (HRP) labeled antimyc antibody (9E10) (Bethyl Laboratory, Montgomery, TX), followed by incubation with SuperBlue TMB microwell peroxidase substrate. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Following the last selection round, 150 individual colonies were randomly picked from plates for phagemid rescue and characterization of the binding properties of the monoclonal phage. The procedure was as described above, only the phage solution was prepared from single clone. The ELISA reading obtained with a single clone was evaluated by S/N ratio (sample/negative). The cut off value was S/N > 3. Strongly positive colonies in the monoclonal phage ELISA were carried forward for further study. The diversity of selected positive clones was determined using the DNA fingerprinting assay.[28] Positive scFv clones that gave a unique *BstN*1 fingerprint were analyzed further by nucleic acid sequencing. Phagemid DNA was purified with the Qiagen kit (Qiagen, Valencia, CA) and sequenced by a commercial facility (acgt Inc. Wheeling, IL). Two sequencing primers were used: pSDFor: TATTTCAAGGAGACAGTC; pSDRev: ATTGGCCTTGATATTCAC. The resulting VH and VL sequences were searched against the Genebank (http://www.ncbi.nlm.nih.gov/Genbank/) and Canonicals-Chothia Canonical Assignment (http://www.bioinf.org.uk/abs/chothia.html) for sequence homology analysis with known antibody genes.

Expression and Affinity Purification of Soluble scFv. Unique clones were used to infect *E. coli* Top10F′ to produce soluble scFv antibody. Details of expression and purification procedures are available in Supporting Information. The purity of the scFvs was checked by SDS gel electrophoresis and the protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). The purified scFv antibody samples were used for study or stored at −70 °C in 50% glycerol/50% HBS.

Equilibrium Binding Studies and Specificity Analyses. Kinetic exclusion analysis on the KinExA 3000 (Sapidyne Instruments, Boise, ID) was used to determine the equilibrium dissociation constant (K_d) for the interaction of scFv antibody 3A and PLH3B with metal-free DCP and the UO_2^{2+}−DCP complex. The principles and details of these experiments have been reported elsewhere[12,31−33] and details of the procedure can be found in Supporting Information. Briefly, equilibrium mixtures of the scFv and ligand (DCP−UO_2^{2+} or metal free DCP) were allowed to flow over a flow/capillary cell that contained beads with immobilized UO_2^{2+}−DCP−BSA. Antibody with no ligand in its binding site was captured by the beads and subsequently quantified with Cy5-labeled 9E10,[34] made in-house using a Cy5 Ab labeling Kit (GE Healthcare, Piscataway, NJ). The signals (delta) at varying ligand concentrations were fit using SlideWrite software (Advanced Graphics Software, Carlsbad, CA) and the following binding equation: $y = a_0 - (a_1 \times x)/(a_2 + x)$, where y is the delta at any given ligand concentration, x is the ligand concentration, a_0 is the delta when no ligand was present (y intercept), a_2 is the equilibrium dissociation constant (K_d), and a_1 is the total change in the value of delta as the x goes from zero to infinity.

Other metal ions (Zn^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Cd^{2+} and Cu^{2+}) were tested to determine the specificity of the selected scFv antibodies for the UO_2^{2+}−DCP complex. The method was essentially as described above; in the reaction mixtures, uranium was replaced with other metal ions. The cross reactivity (CR) was

Table 2. Enrichment of Phage Clones during the Panning Process and Measurement of Polyclonal PhAb Binding to the UO_2^{2+}−DCP−BSA Conjugate

round of panning	input phage	output phage	ELISA response, Abs 450nM (fold over background signal)
1	1.40×10^{13}	5.80×10^{4}	ND[a]
2	3.90×10^{12}	8.00×10^{4}	0.212 (1.00)
3	2.18×10^{12}	6.00×10^{5}	0.409 (1.93)
4	1.30×10^{12}	7.90×10^{5}	0.682 (3.21)
5	1.97×10^{12}	4.12×10^{6}	0.968 (4.56)

[a] Not determined.

calculated by dividing the K_d determined for other metal-DCP complexes by the K_d of UO_2^{2+}−DCP complex.

Analysis of Environmental Water Samples. Acidified environmental groundwater samples were available from a previously published study.[12] The KinExA 3000 was used to construct standard curves for UO_2^{2+} in HBS and in diluted Rifle Artificial Ground Water (AGW) made as previously described;[12] the final concentrations in the calibration assay mixtures applied to the flow cell were as follows: scFv antibody, 0.833 nM; Cy5-labled antimyc antibody (9E10), 10 nM; BSA, 50 μg/mL; DCP, 1 μM; UO_2^{2+} standard solutions, 0, 1, 10, 25, 50, and 100 nM. After the calibration curve was established, Rifle groundwater samples were diluted 30, 40, and 50 fold in HBS containing 2 μM DCP. These samples were diluted an additional 2 fold by the addition of the rest of the assay components, so that the final concentrations of scFv, Cy5-labeled 9E10, BSA and DCP were identical to those used to generate the calibration curve. Data analysis was performed by the sensor software, which calculated a binding curve using deltas from the UO_2^{2+} standards and automatically compared deltas from the environmental samples to this curve to determine the UO_2^{2+} concentration in the unknowns.[12]

The minimal level of detection (MDL) for each experiment was determined by calculating the mean and SD for the delta values obtained from samples without UO_2^{2+}. This SD was multiplied by 3, and subtracted from the mean zero value. The UO_2^{2+} on the *x*-axis of the standard curve that corresponded to this 0−3SD calculated delta was designated as the MDL.[35]

RESULTS AND DISCUSSION

Previous work in our laboratory had shown that monoclonal antibodies that bound to metal-chelate complexes lost their binding activity when expressed as scFv fragments.[36,37] ScFvs that recognized metal-chelate complexes could be isolated from naïve libraries, but their binding affinities were too low to be useful in environmental analysis.[38,39] Immune libraries, while smaller than naïve libraries, often allow the isolation of high affinity scFv fragments with equilibrium dissociation constants in the low nanomolar range.[40,41] An immune library was therefore generated from genetic material derived from rabbits immunized with the UO_2^{2+}−DCP hapten covalently conjugated to keyhole limpet hemocyanin. Rabbits were chosen for library production because (1) a relatively small number of V-region-specific primers are required for the generation of rabbit antibody fragment libraries;[42] (2) hapten and peptide epitopes that do not elicit a good immune response in mice often show enhanced immunogenicity in rabbits;[43,44] and (3) rabbit antibodies often have higher specificity and affinity than do mouse antibodies.[45] Two rabbits were therefore immunized until their sera showed strong reactivity with the UO_2^{2+}−DCP complex in competitive ELISA (data not shown).

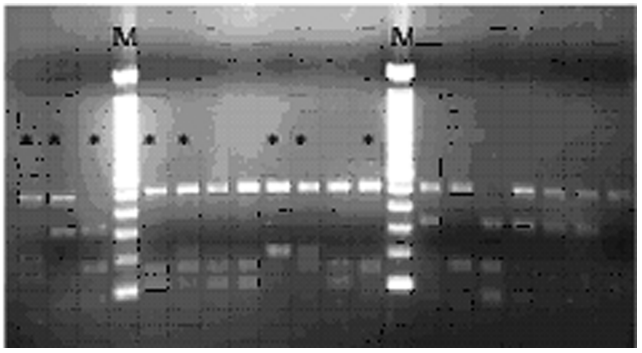


Figure 1. *BstN* I fingerprint analysis of scFv genes present in clones with binding activity. Eight different banding patterns (marked with * above the bands) were detected among the 18 clones that bound to UO_2^{2+}−DCP−BSA in ELISA. M, DNA ladder.

Library Construction and Selection of Phage Antibodies. Total RNA was isolated from pooled spleen tissue of two immunized rabbits for the synthesis of cDNA. A set of degenerate primers was designed for isolation of rabbit variable (V) genes, which proved to be effective for use with both VL and VH libraries.[26] VH and VL genes were directly amplified by PCR from first-strand cDNA. Amplification of VH generated a major DNA fragment approximately 400 bps in length, while VL amplification generated the expected 340 bps fragment. The gel-purified VH and VL gene fragments were digested and assembled into phagemid pSD3,[26] and primers used to amplify the complete scFv produced a product of 900 bps (data not shown). Multiple aliquots of the assembled pSD3 plasmid were transformed into *E. coli* TG1, an amber suppressor strain, and the transformants were pooled. PCR analysis of 16 randomly selected clones from this primary library showed 95% of the library carried a full length of scFv insert (data not shown). Library diversity was estimated by determining the percentage of unique *Bst1N1* fingerprints in a selected number of clones. On the basis of the total amount of DNA used for transformation, the calculated transformation efficiency, and estimates of library diversity, the library size was calculated to be $\sim 1 \times 10^7$.

An aliquot of the library was subsequently grown for phage production and selection of antibodies with the desired binding properties. The screening strategy ultimately employed for these experiments is outlined in Table 1. The goal of this selection process was to isolate antibodies that bound to a "loaded" noncovalent complex of UO_2^{2+}−DCP with high affinity while binding with much lower affinity to an "unloaded" complex of metal-free DCP. The selection procedure included gradually decreasing conjugate coating concentrations and incubation times coupled with increasing washes during successive rounds of panning. Preincubation with high concentrations of the carrier protein BSA (3%) and increasing concentrations of metal-free DCP (0.5−5 μM) prior to the panning process was used to select against phage that bound to these moieties. At the beginning of the selection process, very few phage particles were able to bind in the phage-ELISA (data not shown). However, after the first round of panning, PhAbs binding to UO_2^{2+}−DCP−BSA were

Table 3. CDR Amino Acid Sequences[a] for scFvs 3A and PLH3B

		clone 3A	clone PLH3B
Light Chain	CDR1	QASQNVWNKNALA	QASQSVASNNYLS
	CDR2	KASTLAS	FASKLAS
	CDR3	AGYKNYNNDDFA	AGDYGGSTDNHV
Heavy Chain	CDR1	GFDLSSSYWIC	GIDFNAYSYIC
	CDR2	CMHSGNSDSAYYASWAK	CIHLKSDSTGYASWAKG
	CDR3	ADSGSSWSFDL	NYYADL

[a] Amino acid sequences were deduced from DNA sequences. CDRs were selected as described in ref 38.

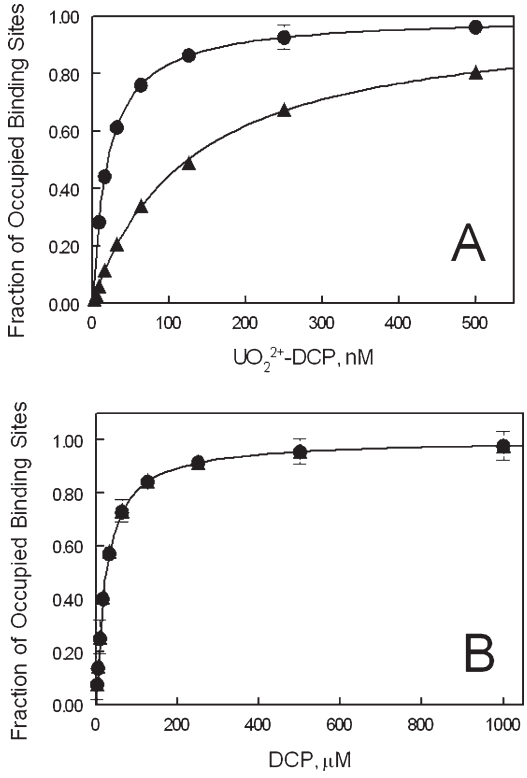


Figure 2. Binding of the scFv antibodies to the UO_2^{2+}−DCP complex and metal free DCP. (A) scFv 3A (●) and PLH3B (▲) binding to the UO_2^{2+}−DCP complex. (B) scFv 3A (●) and PLH3B (▲) binding to metal-free DCP. Experiments to determine the affinity for the UO_2^{2+}−DCP complex were performed by varying the concentration of UO_2^{2+} in a background of 1 μM DCP. Plotted points represent the mean ± SD (n = 3).

detected by ELISA, and a marked increase in antiuranium binding activity was found during the course of the panning process, as shown in Table 2. All rounds of panning were efficient, as evinced by an increase in the number of output phage after each round of selection and the increased binding of the polyclonal phage to immobilized UO_2^{2+}−DCP−BSA in the ELISA.

Approximately 150 individual clones were picked randomly from the output phage of the fifth round of panning and grown in 96-well plates. After a small-scale phage rescue, activity against UO_2^{2+}−DCP−BSA was tested by monoclonal phage ELISA, and 50% of the colonies were positive (data not shown). The clones that showed the strongest binding to the immobilized UO_2^{2+}−DCP−BSA were carried forward for further study. The scFv gene of these selected positive clones was reamplified by PCR and subjected to *BstN* I restriction analysis. Eight different banding patterns were detected in the 18 clones used for this analysis as shown in Figure 1. The presence of unique scFv genes in 11 of the 18 clones was confirmed by sequencing of the VH and VL regions and subsequent sequence alignment. Competitive inhibition ELISA was used to further narrow the clones chosen for additional study. The competitive ELISA results indicated that scFv antibodies 3A and PLH3B had highest affinity for soluble UO_2^{2+}−DCP, with IC_{50}s of 140 and 190 nM, respectively. The sequences of these two clones were analyzed by aligning them to the Canonicals-Chothia Canonical (http://www.bioinf.org.uk/abs/chothia.html).[46] Results revealed that the VL gene in these molecules belonged to the V lambda Subgroup.[47] As shown in Table 3, both sequences had unique amino acid substitutions, and as expected, most of the differences were in the complementarity-determining regions (CDRs).[48] While the light chain CDR2 of 3A and PLH3B differ by only one amino acid, all other CDRs have unique sequences. The heavy chain CDR3 of scFv 3A is 11 residues in length and contains no tyrosine residues, whereas PLH3B is five residues shorter and contains one tyrosine residue. Information from the other 9 clones sequenced in this study demonstrated that a single light chain could combine with unique heavy chains to generate scFvs that bound to the UO_2^{2+}−DCP complex with a wide variety of affinities. Thus, the ability of these scFvs to bind to UO_2^{2+}−DCP appeared to be controlled primarily by amino acid variations in the heavy chains. These data are available in Supporting Information (Table 1S) and are in agreement with a previous study,[49] that reported the dominance of the heavy chain in the antibody−antigen interaction.

Binding Activity of Purified scFvs. The monoclonal phage form of 3A and PLH3B were used to infect the nonsuppressor *E. coli* Top10F cells to produce soluble proteins containing both myc- and His-tags at the carboxy termini. The scFv-His fusion proteins were affinity-purified from periplasmic extracts and analyzed by SDS-PAGE. The electrophoretograms of both scFvs were similar. The molecular mass of the major band was close to 30 kDa (data not shown), which is consistent with the molecular weight of 30 325 calculated from the amino acid sequence.

Kinetic exclusion analysis was used to determine the equilibrium dissociation constants for both metal-free DCP and the UO_2^{2+}-loaded DCP complex, as shown in Figure 2. Experiments to determine the affinity for the UO_2^{2+}−DCP complex were performed by varying the concentration of UO_2^{2+} in a background of 1×10^{-6} M DCP. Since the K_d of the DCP chelator for UO_2^{2+} is 3.5×10^{-9} M,[13] the addition of DCP at a concentration ~300 fold above this K_d ensured that all of the UO_2^{2+} added to the reaction mixtures existed as the DCP complex. Both purified scFv antibodies bound to the UO_2^{2+}−DCP complex with much higher affinity than to metal-free DCP. The scFv antibody 3A had a K_d of 1.96×10^{-8} M for UO_2^{2+}−DCP and 2.35×10^{-5} M for metal-free DCP, a ~1200 fold difference in affinity for the

dx.doi.org/10.1021/ac200159x |*Anal. Chem.* 2011, 83, 3717–3724

Table 4. Cross Reactivity of scFv 3A with Other Metal Ions in the Presence and Absence of DCP

metal ion (2 μm)	in the presence of 2 μM DCP[a]	in the absence of DCP[a]
Ca^{2+}	–	–
Cd^{2+}	–	–
Ni^{2+}	–	–
Cu^{2+}	–	–
Zn^{2+}	±*	–
Mg^{2+}	–	–

[a] –, less than 1% cross reactivity in both KinExA and competitive ELISA. ±, less than 1% cross reactivity in KinExA, but 6.8% in competitive ELISA.

uranium-loaded versus the metal-free chelator. The scFv PLH3B antibody bound to the UO_2^{2+}–DCP complex and metal-free with K_ds of 1.25×10^{-7} and 2.48×10^{-5} M, respectively, for a difference in affinities of ~180 fold. The clone that showed the least difference in affinity for loaded and unloaded DCP was 5B (see Table 1S in Supporting Information). In this clone, the K_ds for DCP and UO_2^{2+}–DCP were 4 μM and 169 nM, respectively (a 24-fold difference).

Other metal ions were also examined for their ability to bind to the 3A and PLH3B scFv antibodies. As shown in Table 4, neither the DCP-metal complexes nor the metals alone showed measurable cross reactivity to scFv antibody 3A when the KinExA was used for analysis. The 3A antibody exhibited approximately 7% cross reactivity with Zn^{2+} when tested in competitive ELISA (data not shown).

Determination of Uranium in Groundwater using the 3A Antibody. The purified 3A scFv antibody was subsequently formatted into an antibody-based assay for uranium using immunosensors already available in the laboratory.[10,12] Environmental water samples available from a previous study at a uranium mine tailing site in Rifle, CO[12] were analyzed using the new scFv antibody. In preliminary experiments, uranium standard curves were generated in samples containing 10% artificial groundwater and compared to those prepared with buffer alone. The two standard curves were within experimental error of each other (data not shown), which indicated that the scFv antibody could be used for the analysis of groundwater from the Rifle, CO site. A standard curve and precision profile for the environmental assay is shown in Figure 3. The immunoassay showed acceptable precision (<20%) at all points on the standard curve; at most points, the coefficient of variation was much less than 10%. The spikes into groundwater were done multiple times, with an n of 3 for each experimental point. The error for any single spike was always below 20% and when the CV's at each point on a standard curve were averaged, the average CV ranged from 4.7 to 6.2% in replicate experiments. The reproducibility in assays with the recombinant antibody was similar to that obtained previously using monoclonal antibodies.[12]

The minimal level of detection, defined as 3SDs below the zero value,[35] was 2.2 nM, well below the US EPA action limit of 126 nM.[50] Analytical recovery of UO_2^{2+} was determined both for spiked buffer containing 10% Rifle artificial groundwater and for 2 environmental samples collected at the Rifle site, as shown in Table 5. The recovery for the environmental samples was calculated based on analysis of the samples by kinetic phosphorescence analysis,[7] performed by an independent DOE contractor. The recovered concentrations of uranium by the immunosensor assay were well correlated both to the spiked uranium added to the samples and with the concentrations determined by KPA. Sample

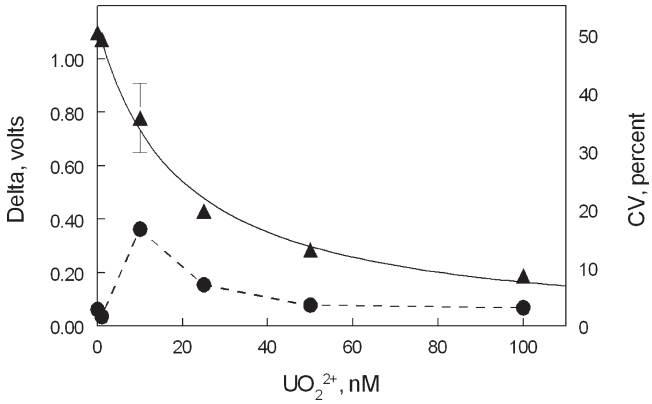


Figure 3. Standard curve (▲) and precision profile (●) for uranium assay using the 3A scFv. The plotted points on the calibration curves represent the mean ± SD (n = 3).

Table 5. Analytical Recovery of UO_2^{2+} Added to Samples Containing 10% Artificial Groundwater and Analysis of U(VI) in Environmental Water Samples

sample[a]	UO_2^{2+} in sample (nM)	UO_2^{2+} found by immunoassay (nM)	% recovery
Spike 1	10	8.83	88.3
Spike 2	25	31.26	124.5
Spike 3	50	53.87	107.7
Spike 4	100	84.93	84.93
U02–6/27/08	90.5[b]	87.2	96.42
D06–8/31/08	70.9[b]	64.9	91.46
Average			**98.89**

[a] Spiked samples were prepared in HBS containing 10% Rifle artificial groundwater. The 2 environmental samples (U02 and D06, available from a previous study[12]) were diluted into HBS containing DCP before analysis. [b] These 2 environmental samples were analyzed for uranium by an independent contractor using kinetic phosphorescence analysis.[7]

recovery ranged from 84.9 to 124.5% and average sample recovery was 98.89%.

CONCLUSIONS

Recombinant antibodies that can be expressed and purified from bacterial cultures offer significant advantages over monoclonal antibodies. The mass production of recombinant antibodies is less expensive and the scFv format is much easier to manipulate at a molecular level than expression systems for intact IgGs or Fabs. The data presented herein demonstrate that one can produce single chain Fv antibodies that bind to a metal chelate complex with high affinity. The nanomolar affinities of the scFvs isolated in the selection process suggest that affinity maturation of antibodies specific to DCP–UO_2^{2+} complex was dominated by somatic hypermutation.[51,52] The differences in the amino acid residues among the 11 scFvs isolated in this study (shown in Table 1S, Supporting Information) may allow us to deduce the residues that interact with the uranium–chelate complex and thereby permit us to further modify antibody affinity and specificity through site-directed mutagenesis.

The highest affinity scFv isolated in this study, 3A, had significant advantages for environmental analysis when compared

to the monoclonal antibodies against chelated UO_2^{2+} previously described by our laboratory.[13] The 12F6 monoclonal antibody used in our previous field studies[12] bound to the UO_2^{2+}−DCP complex with a K_d of 0.91 nM.[13] The 3A scFv bound to the UO_2^{2+}−DCP with a higher K_d of 19.6 nM; however, the affinity of this scFv is well suited for the analysis of uranium in environmental samples, where the EPA action limit for uranium is 126 nM.[50] The scFv antibody 3A bound to metal-free DCP with a K_d of 23.51 μM, compared to a K_d of 0.75 μM for the 12F5 monoclonal antibody. The increased tolerance for DCP (31-fold higher than that of the 12F6 monoclonal) allowed us to use 5-fold higher concentrations of the UO_2^{2+}-specific chelator in our environmental assays; these higher chelator concentrations will facilitate extraction of UO_2^{2+} from its natural complexants in groundwater and increase assay precision. In our previous studies,[12] precision was compromised unless the DCP concentration was tightly controlled at 200 nM.

The higher concentrations of metal-free chelator will also make this environmental immunoassay faster and easier to use in the field. In our previous studies with the 12F6 monoclonal antibody, groundwater samples required a pretreatment regimen that included acidification and dilution/neutralization in the presence of DCP.[12] Preliminary studies with field samples currently available in our laboratory has indicated that groundwater samples assayed with the 3A antibody can be diluted directly into buffers containing higher concentrations of chelator, thus obviating the need for additional sample pretreatment. As uranium mining increases in response to global warming, sensors based on these recombinant antibodies could provide on-site, near real-time data on uranium contamination during all phases of mine operations and postoperation monitoring. Such antibodies could also be of use in the development of rapid tests for uranium in serum, hair or urine that could be used to monitor worker safety and/or identify persons who have recently worked with uranium-containing materials.

The selection strategies developed in this study may also prove useful to investigators who wish to tailor the binding characteristics of their antibodies for specific applications. One of the most common methods for isolation of antibodies with the ability to differentiate among haptens with similar structures is to perform the panning experiments in the presence of high concentrations of the cross-reactive haptens.[24,25,53] This procedure was not effective in the present study, because the chelator is an integral part on the hapten structure. A gradual increase in the concentration of the metal-free chelator coupled with a simultaneous decrease in the coating concentration of the metal-chelate complex proved successful in generation antibody fragments with the ability to differentiate a loaded versus unloaded complex. Strategies similar to those described herein could also be used to develop scFvs that recognize a receptor−ligand complex with significantly higher affinity than a receptor with no ligand in its binding site. Hybridoma technology is much less likely to produce such binding proteins because processes involved in *in vivo* antigen processing would lead to the dissociation of the receptor−ligand complex. Studies are underway in our laboratory to test the wider applicability of the novel approaches developed in this study.

■ ASSOCIATED CONTENT

Supporting Information. Supplementary materials and methods and Table 1S. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This study was supported by grants from the Office of Science, Department of Energy (DE-SC0004959) and the Office of Naval Research, Department of Defense, (N00014-10-1-0270). Christopher Boustany was supported by the National Science Foundation/EPSCoR under Award No. (EPS-0701491).The authors gratefully acknowledge the gift of the pSD3 vector from Dr. Garry Whitelam, University of Leicester, Leicester, U.K.

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