Cadmium Removal from Contaminated Soil by Tunable Biopolymers

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An elastin-like polypeptide (ELP) composed of a polyhistidine tail (ELPH12) was exploited as a tunable, metal-binding biopolymer with high affinity toward cadmium. By taking advantage of the property of ELPH12 to undergo a reversible thermal precipitation, easy recovery of the sequestered cadmium from contaminated water was demonstrated as the result of a simple temperature change. In this study, batch soil washing experiments were performed to evaluate the feasibility of using ELPH12 as an environmentally benign strategy for removing cadmium from contaminated soil. The stability constant ($\log K_L$) for the cadmium−ELPH12 complex was determined to be 6.8, a value similar to that reported for the biosurfactant rhamnolipid. Two washings with 1.25 mg/mL of ELPH12 were able to remove more than 55% of the bound cadmium as compared to only 8% removal with ELP containing no histidine tail or 21% removal using the same concentration of EDTA. Unlike rhamnolipid from *Pseudomonas aeruginosa* ATCC 9027, which adsorbs extensively to soil, less than 10% of ELPH12 was adsorbed under all soil washing conditions. As a result, a significantly lower concentration of ELPH12 (0.036 mM as compared to 5−10 mM of biosurfactants) was required to achieve similar extraction efficiencies. However, cadmium recovery by simple precipitation was incomplete due to the displacement of bound cadmium by zinc ions present in soil. Owing to its benign nature, ease of production, and selective tailoring of the metal binding domain toward any target metals of interest, ELP biopolymers may find utility as an effective extractant for heavy metal removal from contaminated soil or ore processing.

Introduction

Soil contamination with heavy metals is a common and serious environmental problem at the majority of National Priority List (Superfund) sites (*1*). Commonly found heavy metals include lead, mercury, arsenic, chromium, cadmium, nickel, zinc, and copper (*2*). Unlike most other organic pollutants, heavy metals cannot be chemically or biologically degraded. Remediation of recalcitrant or sorbed metals in soil in an efficient and economic way is of increasing interest and importance worldwide.

Many techniques are available for decontaminating soils containing heavy metals and most of them fall under two major categories: immobilization (*3, 4*) and extraction (*5*). Immobilization involves fixation of heavy metals by solid-ifcation or stabilization, thereby, preventing their migration into the groundwater. These techniques render the treated soil unfit for future applications and are typically used as a last resort. Extraction procedures employ a combination of physical/chemical/biological methods for the actual removal of heavy metals from soil. Soil washing is a promising technique that involves the transfer of heavy metals into a wash solution either by desorption or solubilization (*6*). Different extractants such as acids, bases, chelating agents, oxidizing agents, and surfactants have been used for this purpose (*7*). The use of biological extractants is of particular interest because of their environmentally friendly and biodegradable nature (*8*). The feasibility of using biosurfactants, such as surfactin (*9*) from *Bacillus subtilis* ATCC 21332 and rhamnolipid (*10*) from *Pseudomonas aeruginosa* ATCC 9027, to enhance the removal of heavy metals form soil has been demonstrated. The major drawbacks of these biosurfactants are their extensive adsorption onto the soil and the high concentrations (in the mM range) typically required to successfully extract soil-bound metals.

One emerging alternative to biosurfactants is the use of metal-binding biopolymers based on elastin-like polypeptides (ELP) (*11*). ELPs are biopolymers consisting of a repeating pentapeptide VPGVG that undergo a reversible phase transition from water-soluble forms into aggregates upon increasing the temperature (*12*). The transition temperature (T_t) can be precisely controlled by varying the chain length and peptide sequence (*13*). Unlike the statistical nature of step and chain polymerization reactions, ELP biopolymers are specifically preprogrammed within a synthetic gene template that can be easily customized with the desired properties (*14*). More importantly, ELP has been successfully fused to metal-binding peptides or proteins while retaining the temperature responsive property as well as the functionality of the fusion partner (*11, 14*). They can be produced in large quantities by *E. coli* and easily purified to homogeneity by taking advantage of the temperature responsive characteristics of ELP. Previously, ELP-based biopolymers, containing a polyhistidine tail (ELPH12) as the metal chelating domain, were generated (*11*) demonstrating the possibility of easy recovery and regeneration of metal−biopolymer complex for many repeating cycles of cadmium removal. In this paper, we demonstrate the feasibility of using the ELPH12 biopolymer to enhance the removal of heavy metals from contaminated soil. The results presented here should pave the way for the use of ELP-based biopolymers for the environmental friendly cleanup of contaminated soil.

Experimental Methods

Soil Characterization. The soil used in this study was coarse, slightly acidic, subsurface soil obtained from the City of San Bernardino Rapid Infiltration and Extraction (RIX) wastewater treatment facility. Grain size distribution (Table 1) was performed according to the sieve and hydrometer ASTM D422 method (*15*). Soil was found to be mostly sand, 91%, with low amounts of silts and clay. Other soil characteristics were as follows: pH 6.5, 0.27% total organic carbon (TOC), and a cation exchange capacity of 10.1 mequiv 100 g^{-1}.

Artificially contaminated soil was prepared by soaking the soil in 1 mM cadmium nitrate for 3 months while maintaining the pH at 7.0. The soil was then rinsed with distilled water, recovered by centrifugation for 20 min at 17 700g, and dried in a vacuum oven at 120 °C overnight (Isotemp model 281A, Fisher Scientific). Dried cadmium-

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TABLE 1. Particle Size Distribution of the Sandy Soil

class name	particle size (mm)	fraction (%)
very coarse	1−2	41
coarse	0.5−1	36
medium	0.25−0.5	14
fine	0.25−0.1	7
very fine	0.1−0.05	1
silt and clay	<0.05	<1

contaminated soil was stored in closed plastic containers prior to experiments. The final cadmium content in the soil samples was determined to be 118 mg/kg soil using the USEPA Method 3050.

Production and Purification of ELPH12 Biopolymer. All cultivations were carried out in Luria Bertani (LB) media supplemented with $100\,\mu g$/mL of ampicillin. *E. coli* BLR (DE3) cells containing pET-Ela78h12 (*11*) coding for ELPH12 were grown in 3 L of medium in a BIOFLO 3000 fermentor (New Brunswick Scientific, Edison, NJ) until the late exponential phase. Cells were harvested, washed in 20 mL of 0.9% NaCl, and resuspended in 20 mL of 10 mM Tris pH 8.0 (Tb8) buffer. Cells were lysed with a French press, and cell debris was removed by centrifugation for 15 min at 30 000g. Purification of ELPH12 by repeated temperature transition was achieved by adding NaCl to the cell free extracts to a final concentration of 1 M. The sample was then heated to 30 °C and centrifuged at 30 000g at 30 °C. The pellet containing the ELPH12 protein was dissolved in ice-cold Tris buffer, pH 7 (Tb7). This temperature transition cycle was repeated two more times, and the pellet containing the biopolymer was finally dissolved in ice cold water. The purity of the protein was determined by SDS PAGE electrophoresis (*16*), followed by silver staining (Bio-Rad, Hercules, CA). The molecular weight of elastin was confirmed by MALDI-TOF mass spectroscopy. The mole ratios presented were calculated based on the theoretical molecular weight of 34369 Da for ELPH12 and 32368 Da for ELP (without the histidine tag).

Temperature Transition Profile of ELPH12. The concentration of ELPH12 in solution was determined by measuring the absorbance at 215 nm using a Beckman DU-60 spectrophotometer. An extinction coefficient of $64.1\,\mu g$/mL/A_{215} was used based on previous calibrations (*11, 14*) with known concentrations of ELPH12 biopolymer.

The transition temperature of ELPH12 was determined by measuring the solution absorbance at 300 nm between 10 and 60 °C using a Beckman DU-60 spectrophotometer. Measurements were conducted with 1 mL of ELPH12 solutions at a concentration of 1.5 mg/mL. The temperature was increased every 3 min at 2 °C increments. The transition temperature was determined as a temperature where the optical density reached half of the maximum.

Metal Binding Characteristics of ELPH12. Metal binding experiments were performed in $500\,\mu L$ of 50 mM Tris buffer, pH 7 containing 20 nmol of ELPH12 and varying concentrations of $CdCl_2$. After 2 h of incubation at room temperature (RT), the metal−biopolymer complex was precipitated by the addition of 1.5 M NaCl at 37 °C and centrifuged for 2 min at 14 000g. The resulting pellets were redissolved overnight in 1 mL of concentrated HNO_3. Prior to measurements, each sample was diluted by adding water to the appropriate dilutions. The amount of bound Cd^{2+} was analyzed by atomic flame absorption (AA) spectrometry (Shimadzu AA6701). ELP biopolymers without a histidine tail were used as a control.

To determine the cadmium binding constant of ELPH12, Cd^{2+} complexation was studied with various ELPH12 to Cd^{2+} ratios (0.1 to 1.1) until all added Cd^{2+} was complexed. Binding was carried out in $200\,\mu L$ of Tris buffer at pH 7 with 50 nmol
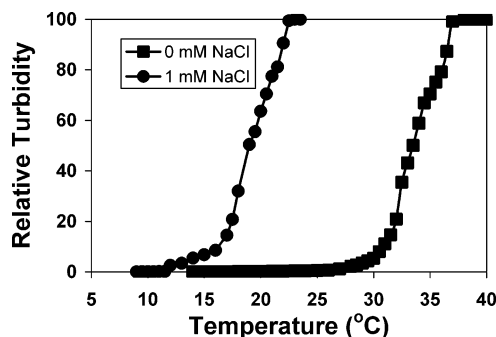

FIGURE 1. The transition temperature profiles of ELPH12 in the absence or presence of 1 mM NaCl. In both cases, the turbidity of solutions containing 1.25 mg/mL of ELPH12 dissolved in water was measured at 215 nm. Relative turbidity is defined as $[A_{300} - A_{300}$(min)]/$[A_{300}$(max) $- A_{300}$(min)] $\times$ 100.

of $CdCl_2$ at room temperature. Cadmium binding was determined as described above.

Soil−Biopolymer Adsorption Characteristics. The adsorption characteristic of ELPH12 was studied with uncontaminated soil. Experiments were performed with 4 g of soil using different soil-to-solution (wt/vol) ratios of 1:5, 1:10, and 1:20 and ELPH12 concentrations ranging from 0.65 to 5 mg/mL. Samples were mixed in a mechanical tumbler at 30 rpm for 24 h, and the soil pellet was recovered by passing the samples through a 0.22 μm filter. The ELPH12 concentrations remaining in the supernatants were determined. A control experiment with no soil was also performed. The minerals and other constituents released from the soil had no interference at the wavelength (215 nm) used to measure the ELPH12 content.

Biopolymer−Cadmium Extraction Studies. All batch soil washing experiments were carried out with 4 g of soil at a constant 1:10 (wt/vol) soil-to-solution ratio. Samples were collected after 24 h of tumbling in a mechanical tumbler (30 rpm) and centrifuged for 20 min at 17700g. Single batch washings were conducted with 1.25, 2.5, and 5 mg/mL of ELPH12. Two and three sequential batch washings were done with 1.25 mg/mL of ELPH12. Batch washings with 0.035 mM EDTA and 0.035 mM HCl were carried out at similar conditions above. Washed samples were dried in a vacuum oven at 120 °C overnight and analyzed for total cadmium removed. The percentage of metal removal was determined based on the initial metal content of 118 mg/kg of cadmium. All experiments were performed in triplicates, and the average results are presented.

Results and Discussion

Characteristics of ELPH12. A biopolymer (ELPH12) composed of 78 ELP repeats and a tandem hexahistidine cluster (His-tag) was used in this study to demonstrate the feasibility in soil washing applications. Since the transition behavior of ELP biopolymers is known to be dependent on ionic strength (*17*), the reversible phase transition temperature (T_t) of ELPH12 was investigated to determine the conditions required for easy aggregation and recovery. The turbidity profile was measured, and the onset of transition was determined at a temperature where the turbidity reached 50% of the maximum (Figure 1). In accordance to previous reports (*11, 18*), the transition temperature (T_t) decreased from 33.5 °C in the absence of salt to 19 °C in the presence of 1 M NaCl, Using this information, over 98% recovery of ELPH12 was obtained at all concentrations tested by precipitating at 37 °C in 1 M NaCl. Aggregation was reversible, and the precipitates resolubilized when the temperature was decreased below T_t.
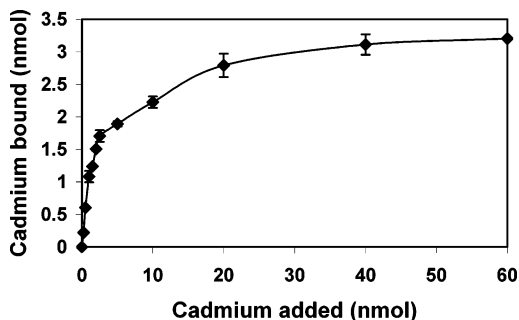
FIGURE 2. Cadmium binding stoichiometry of ELPH12 in 50 mM Tris buffer, pH 7. One nmol of ELPH12 was mixed with various amounts of cadmium. Results represented the average of three sets of experiment with error bars showing the standard deviations.

FIGURE 3. Determination of the binding constant (K_L) of ELPH12 for cadmium. K_L was determined from the slope of a plot of the bound cadmium per mol of ELPH12 (q) vs free cadmium concentration (M).

FIGURE 4. Freundlich isotherm for adsorption of ELPH12 onto soil particles. x is the amount of ELPH12 adsorbed (in mg), m is the mass of soil (in g), and C_{eq} is the concentration of solute in solution after adsorption is complete (equilibrium).
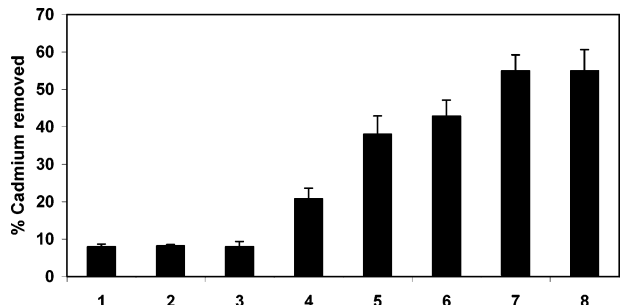
FIGURE 5. Comparison of batch soil washings by ELPH12 and various extractants: (1) a single batch washing with distilled water; (2) two sequential batch washings with distilled water; (3) a single batch washing with 1.25 mg/mL of ELP without the histidine tag; (4) a single batch washing with 0.036 mM EDTA; (5) a single batch washing with 1.25 mg/mL of ELPH12; (6) a single batch washing with 2.5 mg/mL of ELPH12; (7) a single batch washing with 5 mg/mL of ELPH12; (8) two sequential batch washings with 1.25 mg/mL of ELPH12.

Biopolymer−Metal Complexation. The maximum binding capacity of ELPH12 was investigated by incubating the biopolymers over a range of cadmium concentrations. After 1 h incubation, the biopolymer−cadmium complex was recovered by precipitation, and the amount of Cd(II) associated with the aggregates was measured. Binding increased rapidly at lower concentrations and reached a maximum value of 3.2 cadmium per biopolymer (Figure 2). This result is consistent with the reported coordination number of 4 for histidine−cadmium complexes (19), which translates into ~3 molecules of Cd(II) per 12 histidines.

The affinity of ELPH12 toward cadmium was investigated by determining the stability constant (K_L) of the biopolymer−metal complex in aqueous solution. After incubating different amounts of ELPH12 with 50 nmol of cadmium, the cadmium−biopolymer complex was recovered by precipitation, and the amount of bound cadmium was measured. The stability constant (K_L) was determined from the slope of a straight line relationship obtained by plotting the moles of cadmium bound per mol of ELPH12 vs the free metal concentration in solution (Figure 3). A log K_L value of 6.8 was obtained, which is comparable to that obtained with a rhamnolipid biosurfactant previously used successfully for cadmium removal from soil (8, 10). When comparing to the values for cyanide (5.3), citrate (5.0), and EDTA (18.2), all of which are considered to be strong chelators for Cd(II), our result indicates very strong complexation between ELPH12 and cadmium (20).

Soil−Biopolymer Sorption Characteristics. Extensive sorption of biosurfactants onto soil particles affects the effectiveness of the soil washing procedure. Higher adsorption implies lesser availability of the biopolymer for complexation to metals. The sorption behavior of the ELPH12 biopolymer to soil was investigated using a 1:10 soil-to-solution ratio and ELPH12 concentrations of 0.63, 1.25, 2.5, and 5 mg/mL, respectively. The sorption isotherm was nicely described using a Freundlich isotherm (Figure 4) as defined

$$\log x/m = \log K_f + 1/n \log C_{eq}$$

where x is the amount of ELPH12 adsorbed (in mg), m is the mass of soil (in g), K_f is the Freundlich adsorption coefficient, n is a measure of nonlinearity, and C_{eq} is the concentration of ELPH12 in solution after adsorption is complete (equilibrium).

A K_f value of 1.3 was obtained, and the isotherm was nonlinear with $1/n = 0.85$. A maximum of 19% of the added ELPH12 was adsorbed to the soil particles. This is significantly lower than the sorption values of 50−75% observed with surfactin (9) and rhamnolipid (10). Since there is very little adsorption of the biopolymer to soil, we expect that the use of ELPH12 would afford a similar extraction efficiency at much lower biopolymer concentrations than those required for the biosurfactants.
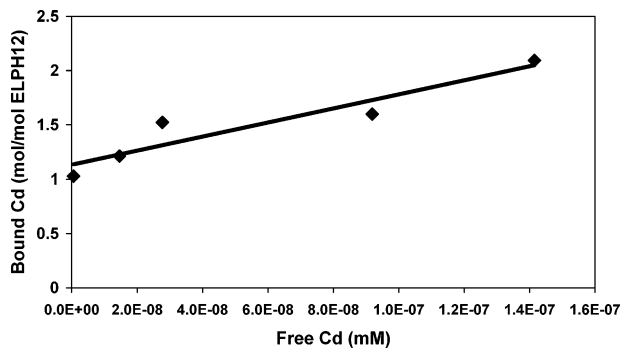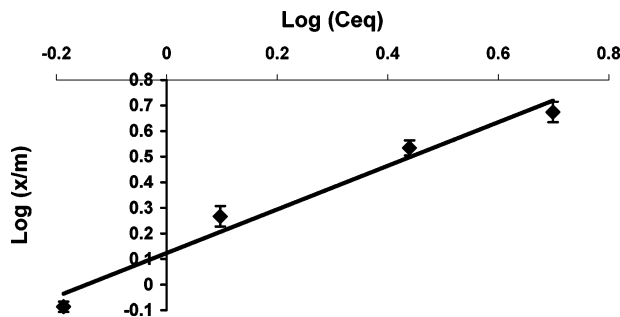
Batch Soil Washing Studies. Artificially contaminated soil was prepared by soaking a sandy soil in 1 mM cadmium nitrate for 3 months. The final cadmium content was determined to be 118 mg/kg soil. To evaluate the feasibility of extracting soil-bound cadmium with the ELPH12 biopolymers, contaminated soil was incubated overnight with various concentrations of ELPH12 dissolved in water. The pH of the wash solution remained near neutral even without the addition of buffer. As shown in Figure 5, cadmium removal increased with increasing ELPH12 concentrations; 38%, 44%, and 55% of the soil-bound cadmium was removed by 1.25, 2.5, and 5 mg/mL of ELPH12 solutions, respectively. For comparison, soil washing with distilled water or with 1.25

mg/mL (0.036 mM) of ELP biopolymers containing no His-tag removed only 8% of the bound cadmium, indicating that the histidine cluster on ELPH12 is mainly responsible for the improved metal chelation. More importantly, the fraction of ELPH12 adsorbed onto soil was less than 10% in all soil washing experiments, a value slightly lower than soil not contaminated with cadmium. This reduction in sorption is important as a significantly lower concentration of ELPH12 (0.036 mM as compared to 5−10 mM of biosurfactants) was required to achieve similar extraction efficiencies when comparing to biosurfactants (10).

To compare the efficiency of the ELPH12 biopolymers with a commonly used chelating agent—ethylenediamine-tetraacetic acid (EDTA), batch washing experiments were also performed using 0.036 mM EDTA. The amount of cadmium removed was only 50% of the value obtained using a similar concentration of ELPH12, suggesting that the ELPH12 biopolymer may be more efficient as a chelator for soil washing than EDTA on a molar basis.

To explore the possibility of sequential extraction using the minimum amount of biopolymers, three batches of soil washing with 1.25 mg/mL of ELPH12 were performed. The extent of cadmium removed increased to 55% in the second wash to a level comparable with the removal by 5 mg/mL of ELPH12 in a single batch washing (Figure 5). Sequential washes with water, in contrast, did not improve the removal efficiency. Any additional wash, however, did not yield any higher level of cadmium removal. This may be due to the presence of cadmium inside the pores of soil particles minimizing complexation with the biopolymers.

When the amount of cadmium extracted by ELPH12 was calculated for the batch washing experiments, a ratio of 1.3, 0.7, and 0.5 Cd/ELPH12 was obtained for the 1.25, 2.5, and 5 mg/mL of ELPH12 solutions, respectively. These values are significantly lower than the maximum binding capacity of 3.2, indicating potentially empty binding sites on the ELPH12 biopolymers. However, when the actual cadmium associated with ELPH12 was analyzed, only 25% of the extracted cadmium was associated with the biopolymers while the remaining was found in solution. Apparently, the soil-bound cadmium was first extracted by the histidine cluster and subsequently desorbed probably due to the presence of other competing agents in the wash solution.

To determine whether stronger complexing agents presented in the wash solution were responsible for displacing cadmium from the biopolymers, ELPH12 was first removed from the washing solution by precipitation. Additional ELPH12 (5 mg/mL) was added, and essentially all cadmium left in the wash solution was removed by coprecipitation with ELPH12. This result suggests that most of the cadmium was indeed free in solution and not bound to other complexing agents. To address whether other cations extracted from soil were completing with cadmium for the histidine cluster, the mineral content of the wash solution was analyzed by Inductively Coupled Plasma (ICP). Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cd^{2+}, and Al^{3+} were identified as the major constituent metals that were coextracted into the wash solution. To identify which cations were responsible for lowering the cadmium affinity of ELPH12, competitive binding experiments were conducted with equal concentrations of these cations presented along with cadmium. After incubation for 1 h, cadmium bound to the ELPH12 was analyzed. While calcium, magnesium, iron, and aluminum had negligible effects on cadmium binding when added separately, zinc caused a significant drop in cadmium binding to a ratio of 0.17 mol of Cd^{2+} per mol of ELPH12 as compared to a ratio of 1 mol in the control containing only cadmium (Figure 6). A metal mixture containing 100 μM of Ca^{2+}, Mg^2, Fe^{2+}, Al^{2+}, Zn^{2+}, and Cd^{2+} reduced the binding ratio to 0.26 mol of Cd^{2+} per mol of ELPH12, while the metal mixture
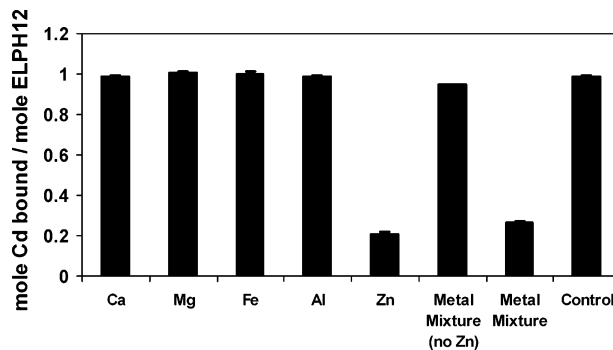

FIGURE 6. Cadmium binding by ELPH12 in the presence of other divalent cationic metals. In the control experiment, 100 μM of Cd^{2+} and ELPH12 was added. For the competitive binding experiments, 100 μM of the corresponding metal was also added. Metal mixture with no Zn^{2+} contained 100 μM of Ca^{2+}, Mg^2, Fe^{2+}, Al^{2+}, and Cd^{2+} in 100 μM of ELPH12. Metal mixture contained 100 μM of Ca^{2+}, Mg^2, Fe^{2+}, Al^{2+}, Zn^{2+}, and Cd^{2+} in 100 μM of ELPH12.

taken in the same ratio in the absence of Zn^{2+} did not have any significant effect. Analysis of zinc revealed that 2.5 mol of Zn^{2+} were bound to each mol of ELPH12 in all Zn^{2+}-containing samples. This indicates that zinc, even if present in low quantities, would affect cadmium binding due to its higher affinity toward the histidine cluster. This observation is not surprising as histidine clusters are known to bind nonspecifically to many different cations (19). However, the good news is that cations that are common in soils such as Fe, Mg, Ca, and Al did not interfere with cadmium binding. Because of the affinity of the histidine cluster toward other heavy metals such as lead and mercury, this His-tag based ELP biopolymer may be useful for the removal of soil contaminated with mixed-metal wastes.

Although the results demonstrated here using a tandem hexahistidine cluster as the metal-binding domain was promising, the percentage removal was relatively modest. Moreover, the idea of inexpensively recovering the bound cadmium by simple precipitation was not complete due to stronger displacement by the zinc ions present in the soil and the need of NaCl for precipitation. It should be noted that the flexibility of tailoring the desired metal-binding domain in the ELP biopolymer is a unique property that could be easily exploited for improved affinity and specificity for the target metals (14, 21). Metal-binding peptides such as metallothioein (22) and synthetic phytochelatins (23) have been shown to have much improved affinity for cadmium than polyhistidine. Incorporation of these cysteine-rich peptides into the ELP biopolymer could significantly enhance its ability to extract heavy metals from soil. For real-world applications, biopolymers could be designed to enable precipitation in the absence of NaCl. This is what makes the ELP technology so flexible because of the ability to control the transition properties precisely. These feasibilities are currently under investigation.

Acknowledgments

Literature Cited

(1) Ellis, W. D.; Payne, J. R.; McNabb, G. D. *Treatment of Contaminated Soils with Aqueous Surfactants;* EPA/600/2-85/129; U.S. EPA: Washington, DC, 1985.
(2) Peters, R. W. *J. Hazard. Mater.* **1999**, *66*, 151−210.
(3) Singh, B. R.; Oste, L. *Environ. Rev.* **2001**, *9*, 81−97.
(4) Barbara, L.; Furrer, G.; Schaerli, H.; Schulin, R. *Environ. Sci. Technol.* **1999**, *33*, 2945−2952.

(5) Rampley, C. G.; Ogden, K. L. *Environ. Sci. Technol.* **1998**, *32*, 987−993.

(6) Semer, R.; Reddy, K. *J. Hazard. Mater.* **1996**, *45*, 45−57.

(7) Griffiths, R. A. *J. Hazard. Mater.* **1995,** *40*, 175−189.

(8) Tan, H.; Champion, J. T.; Artiola, J. F.; Brusseau, M. L.; Miller, R. M. *Environ. Sci. Technol.* **1994**, *28*, 2402−2406.

(9) Mulligan, C. N.; Yong, R. N.; Gibbs, B. F.; James, S.; Bennett, H. P. J. *Environ. Sci. Technol.* **1999**, *33*, 3812−3820.

(10) Herman, D. C.; Artiola, J. F.; Miller, R. M. *Environ. Sci. Technol.* **1995**, *29*, 2280−2285.

(11) Kostal, J.; Mulchandani, A.; Chen, W. *Macromolecules* **2001**, *34*, 2257−2261.

(12) Urry, D. W. *J. Phys. Chem. B* **1997**, *101*, 11007−11028.

(13) Urry, D. W.; et al. *Biopolymers* **1992**, *32*, 1243−1250.

(14) Kostal, J.; Mulchandani, A.; Gropp, K. E.; Chen, W. *Environ. Sci. Technol.* **2003**, *37*, 4457−4462.

(15) Special procedure for testing soil and rocks for engineering purposes. (D422-54). *1970 Annual Book of ASTM Standards*; ASTM: West Conshohocken, PA, pp 101−103.

(16) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.

(17) Urry, D. W.; Luan, C.-H.; Parker, T. M.; Gowda, D. C.; Prasad, K. U.; Reid, M. C.; Safavy, A. *J. Am. Chem. Soc.* **1991**, *113*, 4346−4348.

(18) Meyer, D. E.; Chilkoti, A. *Nat. Biotechnol.* **1999**, *17*, 1112−1115.

(19) Kidambi S. S.; Lee, D.-K.; Ramamoorthy, A. *Inorg. Chem.* **2003**, *42*, 3142−3151.

(20) Schecher, W. D. MINEQL+: Version 4.5, Environmental Research Software, Hallowell, ME, 2001.

(21) Bae, B.; Wu, C.; Kostal, J.; Mulchandai, A.; Chen, W. *Appl. Environ. Microbiol.* **2003**, *69*, 3176−3180.

(22) Sousa, C.; Kotrba, P.; Ruml, T.; Cebolla, A.; de Lorenzo, V. *J. Bacteriol.* **1998**, *180*, 2280−2284.

(23) Bae, W.; Mehra, R.; Mulchandai, A.; Chen, W. *Appl. Environ. Microbiol.* **2001**, *67*, 5335−5338.