

Tunable Biopolymers for Heavy Metal Removal

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ABSTRACT: Tunable biopolymers were synthesized for the removal of heavy metals from dilute waste streams. Protein–protein interaction was tailored specifically into tunable, metal-binding biopolymers using elastin-like polypeptides composed of either one or two hexahistidine clusters. These tunable biopolymers retained the functionality of the elastin domain, undergoing a reversible phase transition above the transition temperature. Aggregation could be tuned within a wide range of temperatures by controlling the chain length of the biopolymers. The presence of the histidine clusters enabled Cd^{2+} to bind strongly to the biopolymers. Recovery of biopolymer– Cd^{2+} complexes was easily achieved by triggering aggregation either by raising temperature or by salt addition. Regenerated biopolymers could be reloaded with Cd^{2+} and reused for repeated cycles with similar efficiency. This system is very flexible as both domains can be engineered to respond to various phase transition conditions and to provide various levels of metal-binding selectivity. The ability to modulate the properties of the biopolymers simply by tuning process conditions should open up new opportunities for the separation and recovery of other environmental contaminants.

Introduction

Because of their intrinsically persistent nature, heavy metal ions are major contributors to pollution of the biosphere.^{1,2} These metals when discharged or transported into the environment may undergo transformations and can have a large environmental, public health, and economic impact.³ Heavy metals such as Pb^{2+} , Hg^{2+} , and Cd^{2+} are currently ranked second, third, and seventh, respectively, on the EPA's priority list. Conventional physiochemical technologies are often inadequate to reduce metal concentration to acceptable regulatory standards.⁴ The increasingly restrictive federal regulation of allowable levels of heavy metal discharge and accelerated requirements for the remediation of contaminated sites necessitate the development of highly efficient technologies to selectively remove dilute metal wastes to sub-part-per-billion levels.

The metal complexation behavior of polymeric ligands has attracted much attention in recent years. "Polymer filtration" is an emerging cleanup technology, which employs water-soluble polymers for the chelation of heavy metals and ultrafiltration membranes to concentrate the polymer–metal complex and produce an effluent free of target metals.⁵ Although this technology is highly effective, the cost of ultrafiltration and the toxic nature of the synthetic polymer make it undesirable for large-scale operation. Preferably, recovery of polymer–metal complexes can be achieved by simple changes in process condition, and environmentally benign materials can be employed. One alternative to synthetic polymers is the use of protein-based biopolymers.⁶ Advances in genetic engineering now enable us to specifically design metal-binding biopolymers with tunable properties that can be used to selectively remove heavy metals from dilute solutions.

Elastin (Ela)-based biopolymers consisting of the repeating pentapeptide (VPGVG) are structurally simi-

lar to the mammalian protein, elastin, that undergo a reversible phase transition from water-soluble forms into aggregates as the temperature increases.⁷ The transition from polymer solution into aggregates occurs over a wide range of transition temperatures (T_t) and can be tuned by controlling the chain length and peptide sequence.⁸ The value of T_t has also been shown to be sensitive to pH, ionic strength, pressure, and covalent modifications of the residues.⁹ Elastin oligomers with up to 180 repeats have been exploited for the thermally stimulated phase separation of enzymes.¹⁰ Elastin fusions exhibit a similar reversible, soluble–insoluble phase transition, and enzyme activity was unaffected even after repeated resolubilization. Moreover, Wang et al.¹¹ have recently demonstrated that a genetically engineered His-tag coiled-coil protein domain can undergo repeated phase transitions without any effect on metal-binding via the histidine tag. These results suggested that thermally stimulated aggregation has no significant impact on the fusion domain. Because phase transition properties can be maintained as protein fusions and the fusion functionality can be maintained even after repeated phase transition, it is easy to envision that tunable biopolymers with metal-binding function could be similarly designed. This is a highly desirable property because the sequestered metals can be easily recovered as precipitates by inducing aggregation of the biopolymer–metal complex. In this paper, our goal was to develop tunable, metal-binding biopolymers and to investigate their metal-binding and reversible phase transition properties. Our results demonstrate the flexibility in engineering the phase transition and metal-binding functions independently for a wide range of applications.

Experimental Methods

Construction of Elastin-Based Biopolymers. DNA manipulations were performed according to standard methods.¹² The high fidelity *Pfu* polymerase (Promega, Madison, WI) was used in PCR reactions. All cloning steps were carried out in *E. coli* JM109. DNA sequencing was carried out after each cloning step for verification.

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A. Basis (VPGVG)₁₀-H₆ sequence

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CCC GAA TTC
      IEcoRI
V   P   G   V   G   V   P   G   V   G   V   P   G   V   G
GTT CCG GGT GTT GGT GTA CCG GGT GTT GGT GTG CCG GGT GTT GGT

V   P   G   V   G   V   P   G   V   G   V   P   G   V   G
GTT CCG GGT GTA GGC GTA CCG GGC GTA GGC GTG CCG GGC GTT GGC

V   P   G   V   G   V   P   G   V   G   V   P   G   V   G
GTT CCG GGC GTG GGC GTA CCG GGC GTG GGC GTG CCG GGT GTT GGC

V   P   G   V   G   H   H   H   H   H   H   H   H
GTC CCG GGT GTA GGT CAC CAC CAC CAC CAC CAC CAC TAA GGA TCC GGG
      ISmaI                                     IBamHI

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B. Linkers used in the subcloning procedures

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TATGG           GGGTGTAGGTTAAG           GTCACCATCACCATCACCATGGTCTGCAGG
  ACCTTAA       CCCACATCCAATTCCTAG       GTAGTGGTAGTGGTACCAGACGTCCCAGTG

  LIN1           LIN2           LIN3

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C. Amino acid sequence of the biopolymers

Biopolymer	Protein sequence	Size (Da)	Amino acids
Ela38H6	MEF(VPGVG) ₃₈ H ₆	16810	199
Ela48H6	MEF(VPGVG) ₄₈ H ₆	20905	249
Ela58H6	MEF(VPGVG) ₅₈ H ₆	25001	299
Ela68H6	MEF(VPGVG) ₆₈ H ₆	29096	349
Ela78H6	MEF(VPGVG) ₇₈ H ₆	33191	399
Ela78H12	MEF(VPGVG) ₇₈ H ₆ GLQGH ₆	34369	409
Ela78	MEF(VPGVG) ₇₈	32368	393

Figure 1. DNA and amino acid sequences: (A) DNA sequence of the basic building block of the biopolymers; (B) linker sequences for subcloning; (C) amino acid sequences of the biopolymers.

The artificial genes were constructed as follows. Two oligonucleotides (Loma Linda University, CA), CCCgaattcGTTC-CGGGTGTTGGTGTACCG-GGTGTTGGTGTGCCGGGTGT-TGGTGTTCGGGTGTAGGCGTACCGGGCGTAGGCGTGCCGGGCG and CCCggatccTTAGTGGTGGTGGTGGTGGTGACCT-ACAC-CCGGGACGCCAACACCCGGCAGCCCCACGCCCGGT-ACGCCACGCCCGGAACGCCAACGCCCGGCACGCCTA-CGCC, were annealed using a 20-base complementary region at the 3' ends and extended with the *Pfu* polymerase to create the synthetic gene coding for (VPGVG)₁₀-H₆. The 189 bp fragment was digested with *EcoRI* and *BamHI* and inserted into the corresponding sites of plasmid pMAL-c2x (New England Biolabs, Beverly, MA). The resulting plasmid pEla10h6 allows expression of (VPGVG)₁₀-H₆ as a fusion to the maltose-binding protein (MalE). Proteins with higher number of VPGVG repeats were obtained by successive insertions of the (VPGVG)₁₀ repeating units. The VG(VPGVG)₁₀-H₆ sequence was PCR amplified using primers AGGCGTAGGCGTTC-CGGGTGTTGGTGTGTA and CCCGGATCCTTAGTG-GTGGTGTG and plasmid pEla10h6 as the template. After digestion with *BamHI*, the PCR product was inserted between the *SmaI* and *BamHI* sites located at the 3' end of the MalE-(VPGVG)-H₆ fusion. This insert introduced 10 additional repeats of VPGVG and recreated the 3' terminal sequences including the H₆ tag. This extension step was repeated two times. DNA sequencing revealed that the resulting fusion gene was actually coding for MalE-(VPGVG)₃₈-H₆, instead of 40 repeats,

which probably happened due to an error during PCR reactions.

The malE sequence was subsequently replaced with a short linker LIN1 (Figure 1B), resulting in a gene fusion coding for the MEF(VPGVG)₃₈H₆ biopolymer. This fragment was subsequently excised with *NdeI* and *BamHI* and cloned into plasmid pET3a (Novagen, Madison, WI) under control of the T7 promoter. Further extension to 48, 58, 68, and 78 VPGVG repeats was accomplished using the same strategy described above. Modifications to the longest MEF(VPGVG)₇₈H₆ fusion were made to construct biopolymers with either no histidine or 12 histidines. For the Ela78 biopolymers without histidine, plasmid pET-Ela78h6 was digested with *SmaI*-*BamHI* and ligated with the linker LIN2 (Figure 1B). For the Ela78H12 biopolymers containing two hexahistidines, plasmid pET-Ela78h6 was digested with *BstEII* and ligated with the linker LIN3 (Figure 1B), resulting in the production of MEF-(VPGVG)₇₈H₆GLQGH₆ fusions.

Production and Purification of Biopolymers. All cultivations were carried out in Luria-Bertani (LB) media supplemented with 100 µg/mL ampicillin. For protein expression, expression plasmids were introduced into *E. coli* BLR(DE3) (Novagen, Madison, WI). Cells were grown in 3 L of medium in a BIOFLO 3000 fermentor (New Brunswick Scientific, Edison, NJ) until late exponential phase when 1 mM IPTG was added. After 3 h, cells were harvested, washed in 0.9% NaCl, and resuspended in 10 mM Tris pH 8.0 (Tb8) buffer.

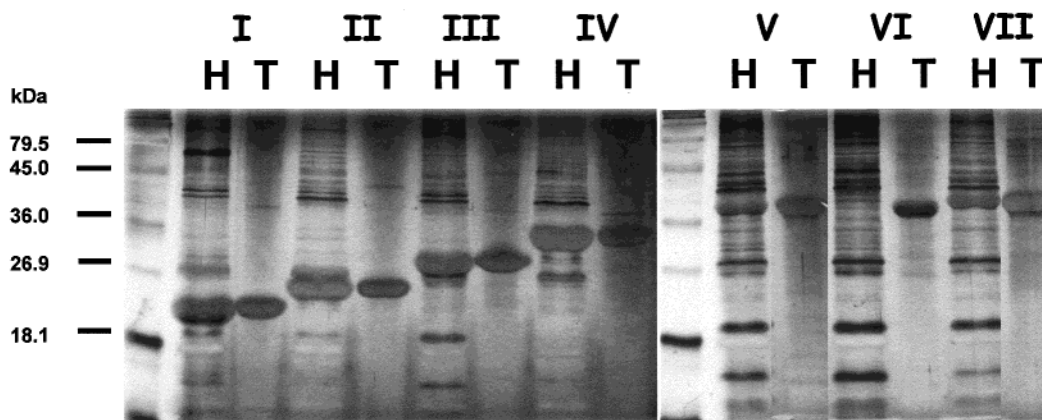


Figure 2. Purification of biopolymers either by Ni-NTA affinity chromatography (H) or by reversible temperature transition (T): I, Ela38H6; II, Ela48H6; III, Ela58H6; IV, Ela68H6; V, Ela78H6; VI, Ela78; VII, Ela78H12. A 3 μ g sample of protein was loaded in each well on a 15% SDS-PAGE gel.

Cells were lysed with a French press, and cell debris was removed by centrifugation for 15 min at 30 000*g*. Purification of biopolymers by Ni-NTA agarose resin (QIAGEN, Valencia, CA) was done in small scale as described in the QIAGEN manual. Purification of biopolymers by repeated temperature transition was achieved by modifying the procedures of McPherson et al.¹³ To the cell free extracts, 5 M NaCl was added to bring a final concentration of 1 M. The sample was then heated to 30 °C and centrifuged at 30 000*g* at 30 °C. The pellet containing elastomeric protein was dissolved in cold Tb8 buffer. This temperature transition cycle (precipitation of the elastomeric protein with 1 M NaCl at 30 °C, centrifuging at 30 °C) was repeated two more times, and the pellet containing the biopolymers was finally dissolved in ice cold water. The protein concentration was determined as absorbance at 215 nm. The purity of the protein preparation was determined by SDS PAGE electrophoresis,¹⁴ followed by silver staining (Bio-Rad, Hercules, CA).

Characterization of Metal-Binding Biopolymers. The transition temperature of the biopolymers was measured in a 96-well microplate. A 200 μ L aliquot of protein solution was added in each well, and the optical density was followed at 655 nm in a microplate reader (BIO-RAD 3550-UV) with temperature control. The temperature was raised from 20 to 40 °C, in 2 °C increments each 5 min. The transition temperature was determined as the temperature at which the optical density reaches half of its maximum.^{13,15}

Metal-binding experiments were performed in 1 mL of Tb8 buffer at a final protein concentration of 44.5 μ M and a final CdCl₂ concentration of 100 μ M. After incubating for 30 min at room temperature, the biopolymers were precipitated by raising the temperature to 30 °C and by NaCl addition to 1 M. The solution was then centrifuged at 14 000*g* for 1 min in a benchtop microcentrifuge to separate the pellet. The pellet was washed with Tb8 buffer with the same NaCl concentration, redissolved in 1 mL of ice cold Tb8 buffer, and diluted in 2% HNO₃. The amount of Cd²⁺ bound to the biopolymers was analyzed by flame atomic absorption spectrophotometry (Shimadzu AA6701). In the isothermic or isotonic mode, metal binding was carried out in Tb8 buffer, and precipitation was achieved either by the addition of NaCl to a final concentration 1 M or by addition of 0.1 M NaCl and raising the temperature to 37 °C.

For the metal-binding experiments, 5 μ g of cadmium was added in each cycle. After the precipitated biopolymers were recovered by centrifugation, they were incubated with a "stripping buffer", which was composed of either 50 mM sodium acetate pH 4 with 0.5 M NaCl (acid stripping) or Tb8 buffer containing 0.1 M NaCl and 100 mM EDTA (EDTA stripping). The regenerated biopolymers were recovered by centrifugation and redissolved in ice cold water. Subsequent cycles were repeated using the same procedure.

Results and Discussion

Design and Production of Tunable Biopolymers.

Since the chain length of the elastin repeats has been shown to affect the transition properties,⁶ initial efforts were focused on designing tunable biopolymers with varying VPGVG repeats. Only shorter biopolymers ranging from 30 to 80 repeats with predicted transition phase temperatures⁸ around 30 °C in water were investigated because aggregation and recovery of biopolymer-metal complexes can be achieved with only a small increase in process temperature.

The strategy used for constructing the tunable biopolymers was similar to that reported by McPherson et al.¹⁵ The basic biopolymer was comprised of repeating VPGVG and a hexahistidine tail as the metal-binding moiety. The synthetic gene coding for (VPGVG)₁₀-(H)₆ was generated by annealing two synthetic oligos (Figure 1). Repeating VPGVG units were added until a chain length of 78 repeats was achieved. A *Sma*I site was specifically designed at the end of the elastin repeats to enable the easy replacement of different metal-binding moieties. Six different biopolymers consisting of a N-terminal 38-, 48-, 58-, 68-, or 78-repeating VPGVG domain and either one hexahistidine or two hexahistidines at the C-terminal were constructed (Figure 1C). A similar Ela78 biopolymer without the hexahistidine tail was also constructed. Using this iterative approach of cloning repetitive elastin sequences, a series of specially designed biopolymers with precisely defined chain length and metal-binding properties were obtained.

All biopolymers listed in Figure 1C were easily produced in *E. coli* BLR(DE3) and purified taking advantage of their inherent temperature responsive properties (Figure 2). The apparent molecular mass of the biopolymers was slightly higher than the calculated size, a phenomenon observed for other elastin-based proteins.¹⁵ In addition, except for Ela78, which has no histidines (Figure 2, VI), all other biopolymers were also purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography, demonstrating the presence and functionality of the polyhistidine tags (Figure 2). These results clearly demonstrate that the metal-binding domain and the elastin domain are fully functional without interfering with each other. Selected biopolymers were produced in larger scale and purified by repeated temperature transition, with yields ranging from 148 to 236 mg in a 3 L culture. Shorter biopolymers were obtained in larger

Table 1. Transition Temperatures (°C) of Biopolymers at Different NaCl Concentrations^a

biopolymer	NaCl (M)				
	0.0	0.25	0.5	0.75	1.0
Ela38H6	>40	>40	>40	34	26
Ela58H6	>40	37	35	27	<20
Ela78H6	>40	33	29	22	<20
Ela78	>40	35	29	23	<20
Ela78H12	35	30	22	<20	<20

^a Biopolymers were dissolved in 10 mM Tris (pH 8.0) buffer.

quantities as was reported for the production of other elastin-based proteins in *E. coli*.^{10,16}

Phase Transition Properties. The reversible phase transition behaviors of the biopolymers were investigated. The temperature profile for turbidity formation was used to measure the onset of folding and aggregation of the biopolymers.¹⁷ The value of T_t , which is defined as the temperature at which 50% turbidity occurred, was used to indicate the phase transition properties of the different biopolymers. In agreement with other reports,^{6,8,10} the transition temperature decreased with increasing protein concentration (data not shown), protein size, and NaCl concentration (Table 1). The Ela38H6 biopolymers exhibited a T_t value >40 °C in Tris (pH 8) buffer with 0.25 M NaCl, while the value of T_t decreased to 30 °C for biopolymers with 78 elastin repeats. The transition profile was also dependent on the polyhistidine tails; small differences in T_t values were detected between the Ela78, Ela78H6, and Ela78H12 biopolymers, indicating that the presence of a small metal-binding moiety has some effect on the phase transition behavior of the elastin domain. Aggregation of the biopolymers was reversible, and the precipitates were resolubilized completely when the temperature was decreased below T_t . These data were used to select appropriate experimental conditions for the metal-binding experiments.

The phase transition behavior could be influenced by other process parameters such as ionic strength.¹⁸ As shown in Table 1, the value of T_t can be tuned from >40 to 26 °C for the Ela38H6 biopolymers simply by adjusting the salt concentration. Similar behaviors were observed with the other biopolymers. In addition to temperature, this result suggests that biopolymer-metal complexes could be recovered by salt addition under isothermal conditions. This operation may be preferred for large-scale processes.

Heavy Metal Removal by Tunable Biopolymers.

The metal-binding capability of the biopolymers was demonstrated by incubating the biopolymers with excess cadmium. After 30 min incubation, the elastin biopolymers were recovered by precipitation, and the amount of Cd²⁺ bound to the aggregates was measured (Table 2). Independent of the number of elastin repeats, all biopolymers containing a hexahistidine domain bound Cd²⁺ at a 1:1 ratio, an observation in line with the binding affinity of the hexahistidine tag.¹⁹ Increasing the concentration of biopolymers resulted in a corresponding increase in Cd²⁺ removal (data not shown). In contrast, a similar elastin biopolymer (Ela78) without the hexahistidine tag was unable to remove Cd²⁺ from the solution even after induced precipitation (Table 2). This result demonstrates that the elastin moiety itself does not bind or entrap Cd²⁺. To demonstrate the possibility of fine-tuning the metal-binding capability of the biopolymers, metal-binding experiments were

Table 2. Binding of Cadmium in Tb8 Buffer^a

biopolymer	Cd ²⁺ binding (mol protein: mol Cd ²⁺ ± SD) ^b	biopolymer	Cd ²⁺ binding (mol protein: mol Cd ²⁺ ± SD)
Ela38H6	1:1.04 ± 0.04	Ela78H12	1:1.50 ± 0.02
Ela58H6	1:0.97 ± 0.01	Ela78H12 ^c	1:1.39 ± 0.01
Ela78H6	1:0.95 ± 0.01	Ela78H12 ^d	1:1.50 ± 0.03
Ela78	1:0.01 ± 0.00		

^a A 44.5 nmol sample of biopolymers was incubated with excess cadmium (100 nmol of Cd²⁺). Aggregation of the biopolymers was induced by raising the temperature to 30 °C and by NaCl addition to 1 M. The amount of Cd²⁺ bound to the precipitates was measured. ^b SD = standard deviation. ^c Precipitation by increasing temperature only. ^d Precipitation by salt addition only.

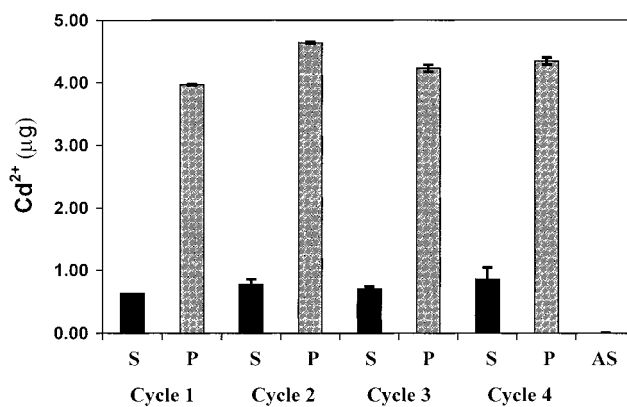


Figure 3. Repeating cycles of metal binding with Ela78H12 biopolymers. A 5 µg sample of cadmium was added in each cycle. S = Cd²⁺ remaining in solution; P = Cd²⁺ removed by biopolymers; AS = average amount of Cd²⁺ remaining in biopolymers after stripping.

performed with the Ela78H12 biopolymer. The addition of six extra histidines increased the Cd²⁺ binding capacity of the Ela78H12 biopolymers to a ratio of 1.5:1 (Table 2). Clearly, the metal-binding capability of the biopolymers can be tailored specifically for any target metal of interest simply by employing the appropriate metal-binding domain.

Beside temperature, another easy way to achieve precipitation of the biopolymers is by increasing the salt concentration at constant temperature (isothermal precipitation). On the basis of the temperature transition profiles reported in Table 1, the possibility of using either isotonic or isothermic conditions in the metal-binding removal process was investigated. Table 2 shows that both conditions were almost equally effective for Cd²⁺ binding and removal by the Ela78H12 biopolymers.

Regeneration of the metal-binding sites for repeated usage is an important criterion for any successful application. Previous reports have demonstrated that sequestered metals can be removed from polyhistidine either by lowering the pH²⁰ or by treating with chelators such as EDTA.^{21,22} To demonstrate this possibility, precipitated Ela78H12-Cd²⁺ complexes were removed from solution and treated with a "stripping buffer", which was composed of either a pH 4.0 acetate buffer or a solution containing 100 mM EDTA. Although both stripping buffers were effective in removing virtually all the bound Cd²⁺, only the acidic stripping conditions could be used for repeated cycles (Figure 3). Traces of EDTA prevented further binding of Cd²⁺ to the biopolymers (data not shown). The regenerated biopolymer aggregates were resolubilized below 25 °C and remained fully functional even after four repeating cycles.

Conclusions

The tunable biopolymers presented here extended on ideas from nature toward entirely new objectives. Protein–protein interaction is tailored specifically into tunable metal-binding biopolymers. The biosynthetic approach is environmentally friendly and allows precise and independent control of the length, composition, and charge density of the interacting end blocks and metal-binding domains. The net result is the flexibility in designing tunable biopolymers that can undergo a transition from water-soluble forms into aggregates under a wide range of conditions, and such precise control is valuable to satisfy the needs of different process conditions. In addition, these tunable biopolymers offer the advantages of being easily regenerated and reused for many repeating cycles. Production and purification of biopolymers are based on the same phase transition principle and could be easily scaled up to kilogram quantity, therefore providing a low-cost and environmentally benign technology for heavy metal removal.

Although the results reported here are for biopolymers with polyhistidine as the metal-binding moiety, other metal-binding domains with high affinity toward heavy metals may be similarly applied. The ability to incorporate multiple binding domains with different selectivity for different metals within a single polypeptide may prove to be a versatile strategy for the removal of mixed metal wastes.

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