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Cadmium detection by a thermally responsive elastin copolymer with metal-binding functionality

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ABSTRACT

Heavy metals are of great concern to environmental safety because of their adverse effects on the environment and human health, even at very low levels. In particular, cadmium and several cadmium-containing compounds are carcinogens and induce many types of cancer. Biological extracts of cadmium have been given greater attention recently because they are considered to be environmentally benign and economically acceptable. Among promising candidates, one emerging technology is the use of tunable, metal-binding biopolymers based on elastin-like polypeptides (ELPs). An ELP consists of the repeating pentapeptide of specific amino acids, Val-Pro-Gly-Xaa-Gly (where the "guest residue" Xaa is any amino except proline) that undergoes a reversible phase transition at a specific temperature (transition temperature, T_t). However, the ELP itself is relatively non-selective. A biopolymer with metal-binding domains that have stronger affinity, capacity, and selectivity would have distinct advantages. We investigated the use of a new generation of ELP biopolymers, EC_{18}-ELP containing synthetic phytochelatin (EC), which is a metal-binding protein with a repetitive motif $(Glu\text{-}Cys)_nGly$, as the metal-binding domain. In this study, an EC_{18}-ELP fusion protein was expressed in *Escherichia coli* and the metal binding ability of EC to cadmium was examined quantitatively. In addition, transition temperature variation was analyzed when the fusion protein bound to cadmium.

1. Introduction

Toxic metal contamination has become a major environmental concern in many parts of the world [1]. Unlike most other organic pollutants, heavy metals such as cadmium cannot be chemically or biologically degraded. In particular, cadmium and cadmium-containing compounds are carcinogens and induce many types of cancer. Therefore, efficient and economical remediation of recalcitrant cadmium in soil and water is of increasing interest and importance. Many treatment processes are available for contaminated cadmium, including chemical precipitation, membrane separation, conventional coagulation, ion exchange, reverse osmosis and other adsorbents such as polymer or carbon nanocomposites [2–5]. Although ion exchange and activated carbon have been generally used for this purpose [6,7], the higher cost and tedious regeneration process restrict its use. Therefore, the development of inexpensive and efficient methods for detecting and removing cadmium is highly demanded. In comparison with chemical methods, biological extractants have been given greater attention because they are environmentally friendly [8,9].

The feasibility of using biosurfactants, such as surfactin and rhamnolipid, to enhance the removal of heavy metals form soil has been demonstrated [10]. However, these biosurfactants generally lack the required affinity and specificity. The availability of genetic tools provides the possibility of specifically modifying biosurfactants with the required selectivity and affinity for cadmium. One attractive technique is the use of metal-binding biopolymers based on elastin-like polypeptides (ELPs). An ELP consists of a repeating pentapeptide (Val-Pro-Gly-Xaa-Gly, where the "guest residue" Xaa is any amino except proline) and is a new kind of a protein polymer showing interesting properties. Due to their thermally responsive properties (Fig. 1), ELPs undergo a reversible phase transition [11–13]. Due to their self-assembly, biocompatibility, biodegradation, and stimuli sensitive ability, ELPs have been applied to various fields including protein purification [14], tissue engineering scaffolds [15], and drug delivery systems [16]. Furthermore, due to their ease of production, and selective tailoring of the metal binding domain toward any target metal of interest, ELP-metal binding domain fusion biopolymers can be utilized as an effective extractant for heavy metal removal or detection [17]. Therefore, it is important to manipulate ELPs with the desired metal-binding residues to improve their affinity and specificity.

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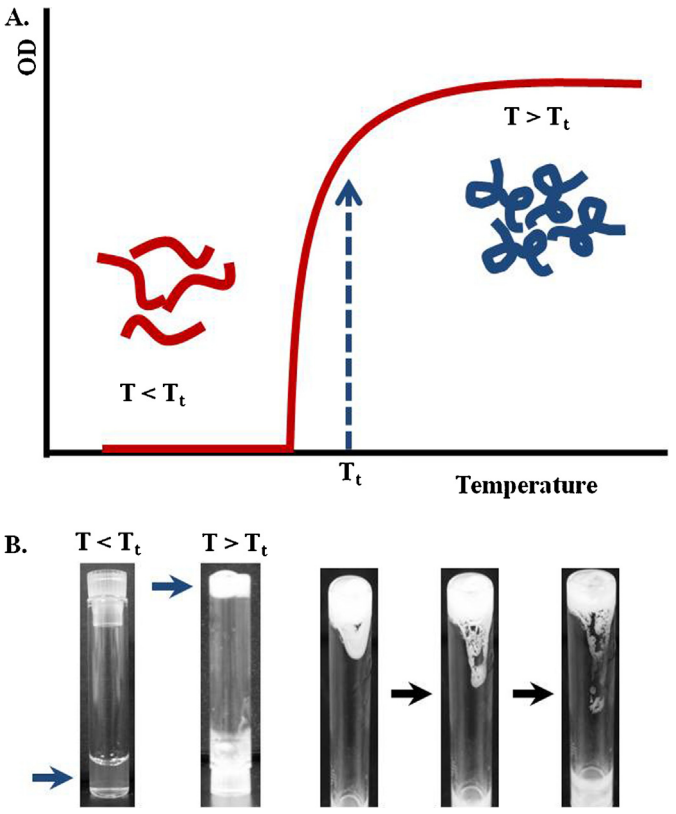

Fig. 1. (A) A reversible phase transition of elastin-like polypeptides (ELPs). Below a certain critical temperature (T_t), ELPs are highly soluble in aqueous solution through conformational changes. In contrast, they aggregate rapidly and turn into more 'rigid' form above the T_t. (B) Digital photographic images of an aqueous solution of ELP ($[VPGVG]_{128}$) in cold water (20 °C) and the physical gel formed in warm water (45 °C, left two pictures). The changes in sol formation are visualized with time at room temperature (right three pictures).

Phytochelatins (PCs) are naturally occurring metal-binding polypeptides and a detoxification biopolymer produced by some plants or fungi to sequester metal ions [18]. Phytochelatins, which consist of $(\gamma Glu\text{-}Cys)_nGly$, incorporate inorganic sulfide that results in an increase in Cd^{2+} binding efficiency of these peptides [19,20]. However, production of PCs in bacteria is almost impossible because factors that control PC chain elongation are not understood completely [21], and isolation of PCs from plants is difficult and time consuming [22]. Synthetic phytochelatins (ECs) are analogs of PCs that have an α-peptide bond instead of a γ-peptide bond. As a result, ECs have different bonding structure from that of PCs which provides a distinct advantage. Unlike PCs, it is possible to produce large quantities of ECs with any chain length of interest in bacteria using general recombinant DNA technology.

The purpose of this study was to investigate the utility of elastin copolymer with metal-binding functionality as an alternative method for detecting cadmium or cadmium-containing compounds. ECs have a cadmium-binding domain with a repetitive motif $(Glu\text{-}Cys)_nGly$, and expressed ELP-EC fusion proteins are used as a chelating agent to detect cadmium. We measured the phase transition temperature of ELP copolymers and analyzed cadmium binding affinity for them to demonstrate the utility of EC_{18}-ELPs with cadmium binding functionality.

2. Materials and methods

2.1. Materials

The *Escherichia coli* strains DH5α and BL21(DE3), a pUC18 plasmid, and a pET32a plasmid were purchased from Novagen (Madison, WI, USA). T4 DNA ligase, calf intestinal alkaline phosphatase (CIP), and the *Eco*RI, *Bam*HI, and *Nco*I restriction endonucleases were obtained from New England Biolabs (Beverly, MA, USA). The *Bve*I, *Bpi*I, *Bgl*II restriction endonucleases were purchased from Fermentas (Burlington, ONT, Canada). Plasmid DNAs were purified using Spin Miniprep kits from Promega (Madison, WI, USA). A Gel extraction kit and Ni-NTA spin columns were purchased from Qiagen Inc. (Valencia, CA, USA). Cyanogen bromide and formic acid were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Cobalt metal affinity resins and 10 mL disposable columns were purchased from Takara Bio Inc. (Shiga, Japan). Microcon filters and dialysis cassettes were purchased from Millipore (Billerica, MA, USA) and Thermo Scientific (Rockford, IL, USA), respectively. All other reagents were purchased from Sigma and used without additional purification.

2.2. Preparation of EC-ELP genes

For synthesis of ELP genes, a single-stranded synthetic oligonucleotide (5′-ATATAGAATTCACCTGCAATAGTCCCGGGTGTTGGTGTGCCAGGCGTTGGTGTGCCGGG-TGTTGGTGTGCCAGGCGTTGTCCATGTCTTCGGATCCAATAT-3′) was amplified via PCR with two oligonucleotide primers (TP1-5′-ATATAGAATTCACCTGCAATA-3′, TP3-5′-ATATTGGATCCGAAGACAT-3′). The PCR products and pUC18 cloning vector were double-digested with *Eco*RI and *Bam*HI restriction enzymes and ligated to produce a $[VPGVG]_2$ gene. In order to obtain a $[VPGVG]_4$ gene, the ELP monomer gene was multimerized by the previously reported strategy termed the "recursive directional ligation" (RDL) method [14,23,24]. By using this method, two ELP genes, $[VPGVG]_{64}$ and $[VPGVG]_{128}$, were prepared and finally transferred to a pET32a expression vector.

EC was designed to encode $(Glu\text{-}Cys)_{18}$-Gly. The DNA sequence of the EC gene is shown in Fig. 2A. A single-stranded synthetic oligonucleotide (131 bp) was amplified via polymerase chain reaction (PCR) with two oligonucleotide primers EEC-F (5′-TTTAGATCTTGAGTGCGAGTGCGAGTGCGAGTGCGAGTGCGAGTGCGAGTGCGAGTGC-GAGTGCGAATGTGAATGCGAGTGT-3′), and EEC-B (5′-TTTCCATGGCGCCACACTCG-CACTCGCATTCGCACTCACACTCACATTCACACTCGCATTCACATTC-3′). EEC-F primer contained a *Bgl*II recognition site (-GAATTC-), and the EEC-B primer contained an *Nco*I recognition site (-GGATCC-). As a result, the PCR products could be double digested with *Bgl*II and *Nco*I restriction enzymes. Then, the pET-32a expression vector with ELP genes was also double digested overnight with *Bgl*II and *Nco*I at 37 °C. PCR products with cohesive termini were then ligated with the linearized pET-32a expression vector with ELP genes at 25 °C for 4 h, and the ligation mixture was transformed into the expression strain BL21(DE3). The EC_{18}-ELP $[V_{64}]$ gene, and EC_{18}-ELP $[V_{128}]$ gene (16-mer, and 32mer, respectively) were synthesized using a conventional ligation method (Fig. 2B). To identify the ligation between the EC_{18} gene segment and the pET-32a expression vector with ELP genes, transformants were isolated and each plasmid obtained from the isolated transformants was screened for the presence and size of EC_{18}-ELP fusion gene segments by double digestion with *Bgl*II and *Eco*RI and analyzed via 1.5% agarose gel electrophoresis.

2.3. Expression and purification of the EC-ELP genes

Transformed cells were used to inoculate Luria broth medium (3 mL) containing ampicillin (50 μg/mL) and incubated for 2 h at 30 °C. Each seed culture was then divided into three aliquots, which were used to inoculate 100 mL of the same medium in a 500 mL culture flask (total 300 mL). The main culture was incubated with shaking for several hours at 30 °C until an OD_{600} of 0.6 was reached. At that time, isopropyl-β-galactoside was added at a final concentration of 1 mM to initiate recombinant protein production. Cells were cultured for an additional 4 h, harvested by centrifugation at 6280 rpm for 15 min at 4 °C, and resuspended in 8 mL of TE buffer (1 M Tris-Cl, 10 mM EDTA; pH 7.4). Resuspended cells were lysed by several freeze (−80 °C)/thaw (50 °C) cycles and subsequently sonicated three times for 35 s. The cell lysates were centrifuged at 15,000 × *g* for 20 min at 4 °C to remove insoluble cell debris, and NaCl was added to the bacterial lysates (final concentration: 2 M) to trigger the inverse transition. Then, these mixtures were centrifuged at 15,000 × *g* for 12 min at 40 °C. After the supernatant was discarded and TE buffer was added, the mixtures were re-centrifuged 15,000 × *g* for 17 min at 40 °C. These steps were repeated three times, and protein purification was performed by nickel-nitrilotriacetic (Ni-NTA) metal-affinity chromatography. The purified target proteins were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After analysis, each target protein was dialyzed against deionized water by a slide-a-lyzer dialysis cassette (7000 MWCO) for two days, and lyophilized to a dry powder.

2.4. Characterization of phase transition behaviors

The thermal properties of the EC_{18}-ELPs were characterized with a spectrophotometer by measuring the OD_{350} of the ELP solutions. Lyophilized polypeptides were dissolved in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, and 1.4 mM KH_2PO_4; pH 7.4) to a 5 mM solution, and NaCl was added to the solution, if necessary. EC_{18}-ELP solutions were analyzed with a Cary Bio 300 UV visible spectrophotometer with a temperature scanning function to monitor transition temperature (Varian Instruments, Walnut Creek, CA, USA). The heating rate was 1 °C min^{-1} and data were collected at 1 °C intervals from 10 to 70 °C.

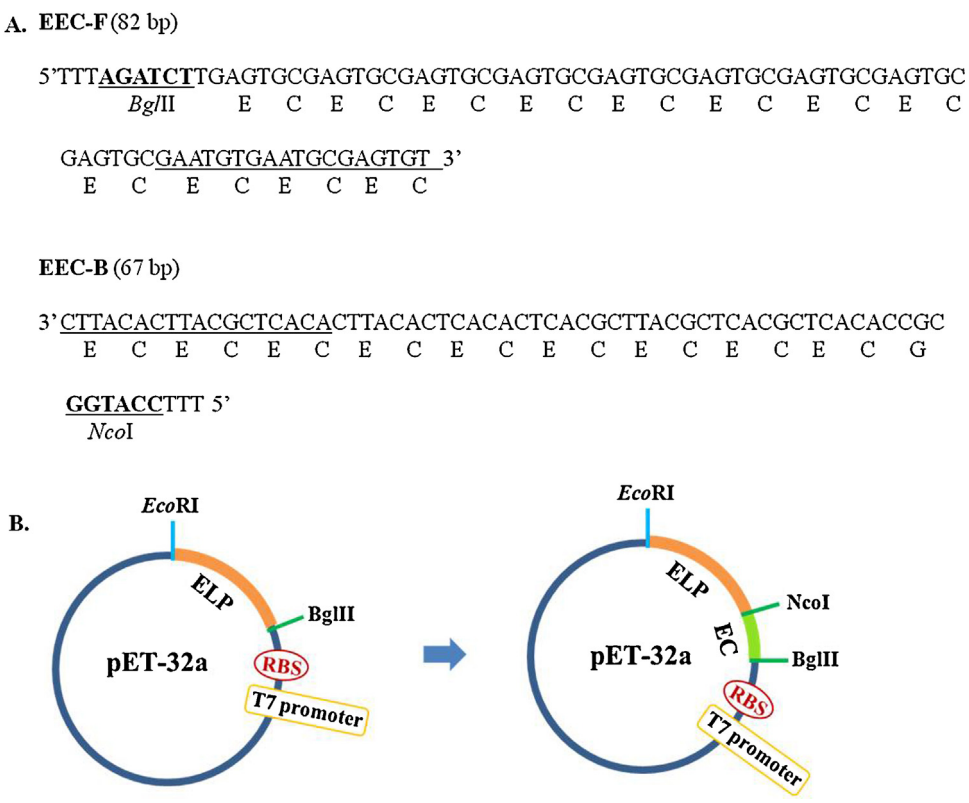

Fig. 2. (A) The EEC-F and B primer sequences for synthesizing the synthetic phytochelatin (EC) gene, (B) The schematic diagram of EC_{18}-elastin-like polypeptides (ELP) genes in the pET-32a expression vector.

2.5. Cadmium binding of EC_{18}-ELPs

After the purified EC_{18}-ELPs were dissolved in 50 mM Tris–HCl buffer at pH 7.4, Cd^{2+} was subsequently added to the solutions and incubated for 1 h at room temperature. The final EC_{18}-ELPs and Cd^{2+} concentrations were adjusted to be 10 mM, 1.72 mM, respectively. The sample was precipitated by adding 2 M NaCl at 40 °C followed by a 10 min centrifugation at 12,000 × *g*. The resulting pellets were redissolved in 100 μL HNO_3 and diluted with water. The amounts of Cd^{2+} in pellets and the supernatant were analyzed by flame atomic absorption spectrophotometer (Shimadzu AA7000). These processes were repeated three times with the same manner to confirm the reproducibility.

3. Results and discussion

3.1. Characteristics of the EC-ELPs

EC was prepared by PCR with the EEC-F and EEC-B primers, and the PCR products were identified by 2% agarose gel electrophoresis. The ELP [V_{64}] and ELP [V_{128}] genes (16-mer, and 32mer, respectively) were synthesized as stated in a previous study [23,24]. As shown in Fig. 3A, the ELP [V_{64}] and ELP [V_{128}] genes were identified. Finally, EC genes digested with *Bgl*II and *Nco*I were ligated with the linearized pET-32a expression vector with the ELP [V_{64}] and ELP [V_{128}] genes. The resulting cloned genes were identified by *Bgl*II and *Eco*RI digestion. Each size of the EC_{18}-ELP genes was consistent with calculated values (Fig. 3A). The EC_{18}-ELP fusion proteins were produced in *E. coli* BL21(DE3) strain, and 12% SDS-PAGE analysis indicated that the size of each EC_{18}-ELP fusion protein was consistent with the calculated values (47.78 kDa and 73.99 kDa each) (Fig. 3B). EC_{18}-ELP fusion proteins were then purified by nickel-nitrilotriacetic (Ni-NTA) metal-affinity chromatography. Matrix-assisted laser desorption ionization mass spectrometer analysis indicated that the expressed proteins had the expected molecular weight (data not shown).

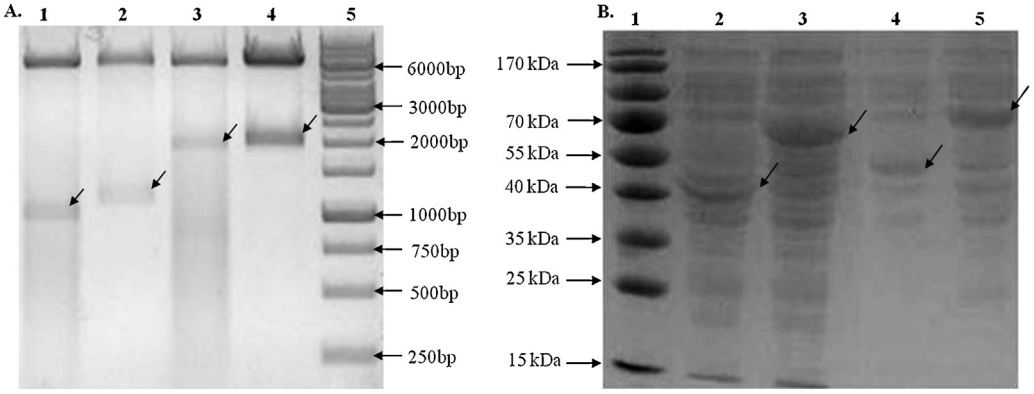

Fig. 3. Synthesis of EC_{18}-elastin-like polypeptide (ELP) genes and expression. (A) Agarose gel electrophoresis (1.5%) results of EC_{18}-ELP genes digested with *Bgl*II and *Eco*RI: Lane 1, ELP [V_{64}] gene (1027 bp); lane 2, EC_{18}-ELP [V_{64}] gene (1117 bp); lane 3, ELP [V_{128}] gene (1987 bp); lane 4, EC_{18}-ELP [V_{128}] gene (2077 bp); lane 5, 1 kb standard DNA ladder, (B) SDS-PAGE (12%) results of expressed EC_{18}-ELPs: lane 1, standard protein marker, lane 2, ELP [V_{64}]; lane 3, ELP [V_{128}]; lane 4, EC_{18}-ELP [V_{64}]; lane 5, EC_{18}-ELP [V_{128}].

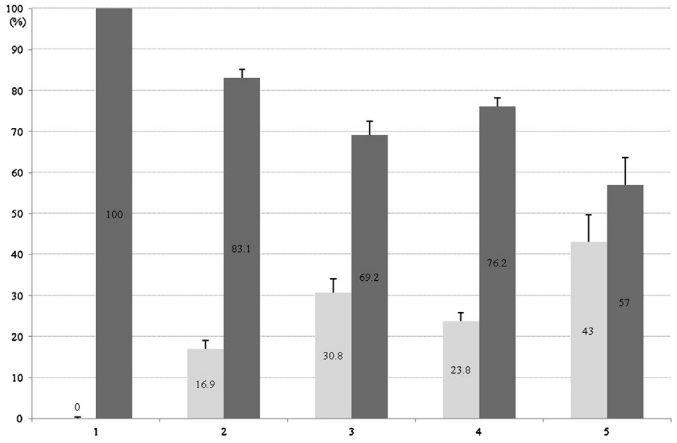

Fig. 4. Cadmium binding affinity of elastin-like polypeptides (ELPs) and EC_{18}-ELPs. The amounts of bound Cd^{2+} to ELPs or EC_{18}-ELPs are indicated in light gray, and free Cd^{2+} ions in the supernatant are indicated in dark gray: lane 1, control (no addition of ELPs or EC_{18}-ELPs); lane 2, addition of ELP [V_{64}]; lane 3, addition of EC_{18}-ELP [V_{64}]; lane 4, addition of ELP [V_{128}]; lane 5, addition of EC_{18}-ELP [V_{128}].

3.2. Cadmium binding affinity of EC_{18}-ELPs

The purified EC_{18}-ELP fusion proteins were incubated in 50 mM Tris–HCl buffer in the presence of Cd^{2+} (initial Cd^{2+} concentration: 1.72 mM) to test the cadmium binding affinity of EC_{18}-ELPs. Significantly increased binding affinity was observed in EC_{18}-ELPs compared with that of pure ELPs. The amounts of Cd^{2+} bound to EC_{18}-ELP [V_{64}] and EC_{18}-ELP [V_{128}] were 30.8% and 43% of the initial sample, respectively, in the presence of 2 M NaCl, whereas those of Cd^{2+} bound to pure ELP [V_{64}] and ELP [V_{128}] were 16.9% and 23.8% of the initial sample under the same conditions (Fig. 4). Fig. 4 shows that some cadmium ions were bound to pure ELPs in an unspecific manner during the phase transition, and that the ELP chain length strong influenced cadmium binding. The amount of Cd^{2+} bound to EC_{18}-ELP [V_{128}] increased almost one and a half times compared to that of EC_{18}-ELP [V_{64}]. The ratio of Cd^{2+} bound to the EC domain of the total Cd^{2+} bound to EC_{18}-ELPs were calculated, and we found that 45.1% [= (30.8 − 16.9)/30.8] of Cd^{2+} in EC_{18}-ELP [V_{64}] and 44.7% [= (43.0 − 23.8)/43.0] of Cd^{2+} in EC_{18}-ELP [V_{128}] were bound do this domain. This low ratio may be due to the relatively short length of the EC domain in the whole fusion protein (The portion of EC domain of EC_{18}-ELP was < 8%), and we expect that this value can be increased if EC is elongated. However, these results demonstrated that incorporating metal-binding peptides such as EC into ELPs can enhance cadmium affinity significantly.

3.3. Thermal behaviors of EC_{18}-ELPs and EC_{18}-ELP-Cd^{2+} complexes

The transition behaviors of EC_{18}-ELPs were investigated to determine the working conditions required for easy aggregation and detection of cadmium binding. While the transition temperatures of EC_{18}-ELP [V_{64}] and EC_{18}-ELP [V_{128}] were observed at 34.3 °C and 24.2 °C, the transition temperatures changed to 27.93 °C and 19.8 °C respectively, when Cd^{2+} was bound to the EC_{18}-ELPs (Fig. 5). These shifts may be correlated with conformational changes in the EC_{18}-ELP-Cd^{2+} complexes. The ELP transition temperature is affected by their molecular weight (MW), concentration, composition of the amino acids in the ELPs, and ionic and strength of the aqueous solution [23]. In this case, bound Cd^{2+} increased overall hydrophobicity of the EC_{18}-ELP-Cd^{2+} complexes, and consequently the transition temperature shifted to a lower value.

Interestingly, the range of the transition temperature drop was not constant after Cd^{2+} binding when we compared EC_{18}-ELP [V_{64}] with EC_{18}-ELP [V_{128}]. While the EC_{18}-ELP [V_{64}] transition temperature drop was 6.4 °C, that of EC_{18}-ELP [V_{128}] was 4.4 °C. Because many cadmium ions bound to the EC domain intensively and the portion of EC domain in EC_{18}-ELP [V_{64}] was relatively larger than that of EC_{18}-ELP [V_{128}], it was assumed that the change in overall hydrophobicity after EC_{18}-ELP [V_{64}] Cd^{2+} binding was much greater than that in EC_{18}-ELP [V_{128}]. If longer EC polypeptides fused with ELPs are produced successfully, these EC-ELPs can be used as effective cadmium ion detection tools in water.

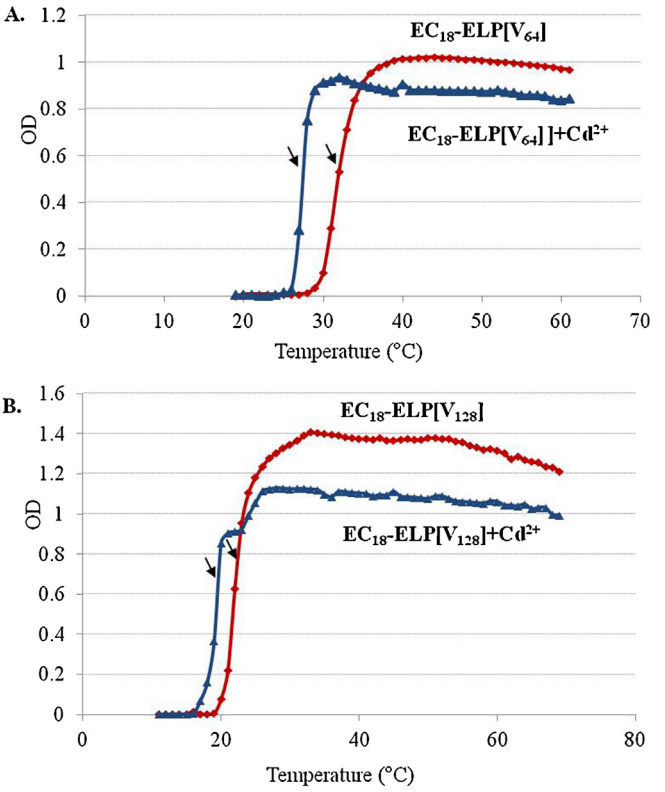

Fig. 5. Turbidity profiles of EC_{18}-elastin-like polypeptides (ELPs) and EC_{18}-ELPs-Cd^{2+} complexes. The profiles were observed in PBS buffer with 2 M NaCl. (A) Turbidity profiles of EC_{18}-ELP [V_{64}] and the EC_{18}-ELP [V_{64}]-Cd^{2+} complex. The observed T_t (black arrow) was 34.3 °C and 27.9 °C, respectively. (B) Turbidity profiles of the EC_{18}-ELP [V_{128}] and EC_{18}-ELP [V_{128}]-Cd^{2+} complex. The observed T_t (black arrow) was 24.2 °C and 19.8 °C, respectively.

In conclusion, this study demonstrated the possibility of using ELPs with high cadmium binding affinity for detecting and removing heavy metals. We incorporated an EC_{18} domain into the ELP biopolymer, and the metal binding ability of EC_{18} to cadmium was examined quantitatively. The results illustrated that the expression of ELPs accumulated cadmium in sol–gel phase transition and that EC_{18}-ELPs were more effective for bioaccumulation. Therefore, these techniques can be the basis for a biosensor to detect cadmium or other heavy metals if the advantage of the improved affinity to cadmium by using EC-ELPs applied to detect heavy metal contamination. Furthermore, it will be an excellent candidate to eliminate noxious heavy metals in the environment.

Acknowledgments

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