Published in final edited form as:

Biomacromolecules. 2013 July 8; 14(7): 2347–2353. doi:10.1021/bm400464s.

Calcium binding peptide motifs from calmodulin confer divalent ion selectivity to elastin-like polypeptides

Wafa Hassouneh, Michelle L. Nunalee, M. Coleman Shelton, and Ashutosh Chilkoti

Department of Biomedical Engineering, Campus Box 90281, Duke University, Durham, North Carolina 27708

Abstract

Calcium sensitive elastin-like polypeptides (CELPs) were synthesized by periodically interspersing a calcium-binding peptide sequence from calmodulin within an elastin-like polypeptide (ELP), with the goal of creating thermal and calcium responsive peptide polymers. The CELPs exhibit high sensitivity to calcium compared to monovalent cations but do not exhibit the exquisite selectivity for calcium over other divalent cations such as magnesium that is displayed by calmodulin. The CELPs were further used as a building block for the synthesis of calcium sensitive nanoparticles by fusing a hydrophilic, non-calcium sensitive ELP block with a CELP block that becomes more hydrophobic upon calcium binding. We show that addition of calcium at concentrations between 50–500 mM imparts sufficient amphiphilicity to the diblock polypeptide between 33 and 46 °C to trigger its self-assembly into monodisperse spherical micelles with a hydrodynamic radius of ~50 nm.

Keywords

Calcium sensitive; Elastin-like polypeptides; stimulus responsive peptide polymer; calcium triggered self-assembly; micelle

Introduction

ELPs are thermally responsive biopolymers consisting of the repeating amino acid sequence Val-Pro-Gly-X-Gly, where X, the guest residue, is any natural amino acid except proline¹⁻³. ELPs are soluble in water below a lower critical solution temperature (LCST) but become insoluble and aggregate above their LCST, referred to herein as their transition temperature (T_t). Given the ability to precisely engineer ELPs at the genetic level, additional functionality – beyond thermal responsiveness – can be directly embedded within the sequence through the choice of the guest residues^{4, 5}, by fusing a peptide or protein at one of the termini of an ELP^{6, 7}, or by periodically interspersing functional motifs between ELP repeats⁸. Using these methods, we and others have previously engineered ELPs to respond to multiple triggers such as pH, light and other extrinsic triggers^{4, 9, 10}. In this paper, we design an ELP with new function by embedding multiple, periodic copies of a peptide motif that binds calcium between ELP repeats.

This design was motivated by our goal of designing an ELP whose phase transition can be selectively triggered by calcium. Calcium is of interest as a trigger for biomedical applications because *in vivo*, its compartmental concentration can vary by over 4-orders of

magnitude. The extracellular concentration of calcium is maintained in the ~1-3 mM range while the intracellular concentration of calcium is rigidly maintained between 50-200 nM^{11, 12}. This large and spatially localized difference in calcium concentration could, in principle, provide a trigger to drive the phase transition of an ELP *in vivo*.

Despite the importance of calcium as a potential trigger of stimulus responsive behavior, to our knowledge, there are no examples of calcium-sensitive thermally responsive polymers. Previously published work employed calcium for gelation or mineralization and did not systematically show the sensitivity or selectivity of the system to calcium. Prieto *et al.* have engineered calcium phosphate binding sites into ELPs to create calcium phosphate-ELP precipitates as biomaterials for bone repair⁸. Alginate exhibits calcium-sensitive gelation¹³, and has been incorporated into synthetic thermally responsive polymers^{14, 15}, but alginate derived systems are intrinsically limited in their scope, as alginate is a naturally derived biopolymer that is isolated from sea weed¹³, so that its conjugates cannot be engineered at the molecular level.

This study was also motivated by a previous study, in which we synthesized a calmodulin-ELP fusion protein in which calmodulin acts as a calcium-binding allosteric actuator of the ELP transition.¹⁶ Calmodulin, one of the multitude of proteins that have evolved to respond to *in vivo* changes in calcium concentration, undergoes a large conformational change upon binding calcium¹⁷. This large conformational change alters the surface properties of calmodulin, leading to a change in the T_i of a calmodulin-ELP fusion. To our knowledge, this is the first example of the phase transition of a polymer that is triggered by a protein-ligand binding event. The transition is triggered by addition of CaCl_2 at millimolar concentrations as opposed to the 10^3 - 10^4 fold greater concentrations required for other chloride salts to trigger the phase transition of ELPs¹⁸. Furthermore, this behavior is highly selective for Ca^{2+} as compared to structurally similar divalent cations such as Mg^{2+} . Building upon this study, herein we sought to examine whether a minimal structural element –a calcium-binding loop of calmodulin– would be sufficient to impart calcium sensitivity to ELPs. To do so, we embedded multiple copies of a calcium-binding loop periodically into an ELP (herein termed CELP) and quantified the calcium-triggered modulation of the ELP T_i . We also fused a CELP to another ELP block that is not calcium sensitive with the goal of tuning the amphiphilicity of the diblock ELP by the addition of calcium with the goal of triggering its self-assembly as a function of calcium concentration.

Experimental

ELP Nomenclature

ELPs are designated $\text{ELP}[X_a Y_b Z_c]\text{-}n$ where X, Y, and Z are the guest residues, a, b, and c are their relative ratios, and n is the number of VPGXG repeats. For example, $\text{ELP}[V_1 A_8 G_7]\text{-}96$ consists of 96 repeating pentapeptides, 6 of which are VPGVG, 48 are VPGAG, and 42 are VPGGG. $\text{ELP}[V]\text{-}90$ consists of 90 repeating pentapeptides, all of which have the guest residue valine. CELPs are designated $\text{CELP}[X\text{-}n] m$, where X-n denotes the guest residue and length of the ELP spacer that occurs after each calcium-binding sequence (for example, [V-10] means 10 repeats of VPGVG), and m denotes the number of times the calcium-binding sequence and ELP spacer monomer unit is repeated. For example, $\text{CELP}[V\text{-}10]\text{-}8$ has a monomer unit of one calcium binding sequence plus 10 repeats of VPGVG that is repeated 8 times. CELP block copolymers ($\text{CELP}_{B,C,S}$) are designated $\text{CELP}\text{-}n/m$, where n denotes the number of pentapeptide repeats in the hydrophilic ELP block with guest residue composition of V, A and G at a ratio of 1:8:7 respectively and m denotes the number of times the calcium-binding sequence and $\text{ELP}[V\text{-}20]$ spacer monomer unit is repeated. All ELP and CELP sequences are listed in Table S1.

Gene synthesis

Four single block CELPs were synthesized using recursive directional ligation (RDL) (Figure S1)³. The synthesis of CELP[V-10]-8 by RDL is detailed in the Supplementary Information. Block copolymer genes were obtained by the same procedure; ELP[V₁A₈G₇] genes previously synthesized in our group were inserted into CELP vectors at the N-terminus of the CELP gene.

Protein expression and purification

Genes encoding CELPs or CELP_{BC} were cloned into a modified pET25b expression vector, transformed into the BLR *E. coli* cell line, and grown in 1 L cultures using Terrific Broth dry medium according to standard protocols. The CELPs were then purified using inverse transition cycling (ITC) according to the method described by Meyer and Chilkoti¹⁹ with a few notable exceptions. While most ELPs are purified in phosphate-buffered saline (PBS), CELPs and CELP_{BC} were purified, stored, and characterized in a 10 mM HEPES buffer with 140 mM NaCl at pH 7.4 to avoid the precipitation of calcium phosphate that occurs upon the addition of calcium to PBS. In addition, a pH lowering step was used to help prevent impurities from binding to the acidic amino acids. Following the first cold spin, the pH was lowered to 4.0 before the first hot spin. The ELP pellets were resuspended in a 50 mM sodium succinate buffer at pH 4.0 after the first four hot spins. Before the fourth hot spin, the samples were flooded with a 20-fold excess of EDTA per calcium binding site to competitively bind any calcium ions. After the fourth hot spin, the pellets were resuspended in distilled deionized water to prepare for dialysis to remove all calcium ions. The samples were dialyzed against distilled, deionized water with a 10,000 Da molecular weight (MW) cutoff to remove all traces of calcium and other salts. Purity was verified by SDS-PAGE (Figure S2).

Transition temperature

The phase transition of CELPs and CELP_{BC} was characterized by monitoring the absorbance of an aqueous solution at 350 nm (OD₃₅₀) as a function of temperature (1 °C/min) on a UV-visible spectrophotometer equipped with a multicell thermoelectric temperature controller (Cary 300 Bio; Varian, Inc.). The T_i is defined as the temperature at the maximum of the derivative of OD₃₅₀ with respect to temperature.

Dynamic and static light scattering

The temperature triggered self-assembly of CELP_{BC} was monitored with dynamic light scattering (DLS) using a DynaPro Plate Reader (Wyatt Technology Corporation, Santa Barbara, CA) to determine the CELP_{BC}'s hydrodynamic radius (R_h). All CELP_{BC} solutions were prepared in HEPES buffer at a concentration of 10 μ M and filtered through an Anotop 10 Whatman 20 nm filter. Samples were contained in a 384 well plate with a sample volume of 35 μ L, and each sample was covered with a drop of mineral oil to prevent evaporation. DLS data were collected at a 90° angle (18 acquisitions, each 10 seconds) heated at 1 °C intervals from 20 to 60 °C. The autocorrelation function was analyzed using a cumulant and regularization algorithm for Rayleigh spheres provided by the manufacturer to determine R_h and mass fractions of the unimer, nanoparticles, and aggregate as the sample underwent its phase transition.

Simultaneous SLS/DLS measurement were obtained for angles between 30°-150° at 5° increments done in 3 runs each 15 seconds at each angle at 25°C and 40°C. Samples for the ALV/CGS 3 goniometer system were prepared by filtering solutions of 10 μ M CELP_{BC} with 500mM CaCl₂ through an Anotop 10 Whatman 100 nm filter into a 10 mm disposable

borosilicate glass tube (Fischer). The glass tube was pre-cleaned by washing three times with filtered ethanol (0.2 μm cellulose acetate filter).

Results and Discussion

CELP Design

The genetically encoded synthesis of ELPs provides precise control of their length, sequence and architecture. Herein, we sought to take advantage of these features to seamlessly embed calcium-binding peptide sequences into ELP sequences by recombinant DNA techniques to develop calcium responsive ELPs. The CELP design incorporates a periodic calcium binding peptide sequence along the polypeptide backbone separated by ELP segments as illustrated in Figure 1. The calcium-binding sequence was derived from the calcium-binding loop found in calmodulin (Figure S3). Of calmodulin's four calcium-binding sites, sites I and II have dissociation constants (K_d) of $\sim 10^{-5}$ M, while sites III and IV have slightly higher affinity with a K_d of $\sim 10^{-6}$ M.^{20, 21} Among these four calcium-binding sites in calmodulin, site II, with the peptide sequence DADGNGTIDFPE, has the fewest overall charged residues and no positive charges. This peptide sequence was used in the CELPs because its charge profile potentially minimizes the contribution of electrostatics to the behavior of the CELPs.

The CELPs consist of four or more copies of the site II calcium-binding sequence of calmodulin separated by the ELP spacer, roughly mimicking the primary sequence of calmodulin, and are listed in Table 1. The ELP chosen as a spacer for all CELPs was ELP[V], because valine is a hydrophobic guest residue with a low T_t , so that the expected increase in the T_t due to the inclusion of charged residues from the calcium-binding peptides would still place the T_t of the CELP in a temperature range where it could be conveniently detected.³ Two variables were systematically varied in our design of CELPs: 1) the number of calcium-binding sequences within a fixed CELP length, and 2) the overall CELP length. Two different ELP spacer lengths were used, ELP[V-10] and ELP[V-20] that consist of 10 repeats of VPGVG and 20 repeats of VPGVG between calcium binding sequences, respectively. CELP[V-10]-8 and CELP[V-20]-4 have the same total length of ELP (80 pentapeptides each), but CELP[V-10]-8 has twice as many calcium binding sequences (eight) as CELP[V-20]-4 (four). We varied the number of calcium-binding sequences, as we wished to examine its effect on the CELPs responsiveness to calcium. The effect of overall length of the CELP chain was examined for the ELP[V-20] spacer; 4 and 6 repeats of the calcium-binding sequence and ELP[V-20] spacer unit were synthesized and characterized with respect to their phase transition behavior.

CELP thermal behavior

The transition temperatures of the CELPs were determined as a function of calcium concentration by measuring the optical density at 350 nm (OD_{350}) and the T_t was determined to be the temperature at which the maximum of the derivative of OD_{350} with respect to temperature occurs (Figure S4). The T_t was also determined for a negative control, ELP[V]-90, which is an ELP with the same approximate amino acid length and guest residue composition as the ELP spacer in CELP, but is missing the calcium binding sequence. CELP[V-20]-4 shows greatly increased sensitivity to calcium in the 1 to 100 mM range as indicated by the large decrease in T_t with increasing CaCl_2 concentration as compared to the negative control ELP[V]-90 which shows no significant change in T_t (Figure 2A). In addition, the transition temperatures of CELP[V-20]-4 are well above the T_t expected for ELP[V]-90. ELP[V]-90 has a T_t of 32.7°C at an ELP concentration of 10 μM while CELP[V-20]-4 has a T_t of 74 °C at a CELP concentration of 10 μM and no calcium. This 41 °C increase in T_t is caused by the negative charges contributed by the calcium-

binding sequences; the four binding sequences in CELP[V-20]-4 contribute 16 amino acids that are negatively charged at pH 7.4. These negative charges greatly increase the overall hydrophilicity of the CELP molecule and cause the T_t to increase.

With an increase in Ca^{+2} concentration, Cl^- concentrations rise as well, necessitating deconvolution of the effect of calcium binding from the salting-out effect of Cl^- as increasing the concentration of kosmotropic salt decreases the solubility of ELPs and enables their phase transition to be isothermally triggered²². This behavior is qualitatively described by the Hofmeister series, which predicts the effect of different cations and anions on protein –and ELP-solubility²³. The effect of salts on ELPs is similar to their effect on protein solubility, with the exception that ELPs are far more sensitive to salt than most proteins. For example, while it takes several molar concentration of $(\text{NH}_4)_2\text{SO}_4$ to “salt out” proteins, ELPs will phase separate in aqueous solution and aggregate by the addition of only several hundred mM of $(\text{NH}_4)_2\text{SO}_4$ ¹⁶. Hence, to delineate the specific effect of calcium binding on ELPs from a non-specific salting out effect, we compared the CELPs with equivalent ELPs that do not contain the calcium-binding sequence. In addition, we further determined the sensitivity and selectivity of CELPs by comparing their behavior in the presence of calcium versus other divalent and monovalent cations.

The T_t 's of CELP[V-20]-4 and ELP[V]-90 were determined from their temperature dependent turbidity profiles for a range of MgCl_2 , CaCl_2 , and NaCl concentrations (Figure 2A). CELP[V-20]-4 is significantly more sensitive to calcium at a concentration below 100 mM compared to ELP[V-90], as indicated by the large decrease in the CELP[V-20]-4 T_t as opposed to no change in the ELP[V]-90 T_t with increasing CaCl_2 concentrations below 100 mM. The observed effect of Ca^{+2} is therefore clearly due to the presence of calcium-binding sequences in CELP as opposed to a non specific salting out effect. However, above 100 mM, a decrease in ELP[V]-90 T_t is observed with increasing CaCl_2 concentration, which is due to the effect of Cl^- as predicted by the Hofmeister series, thus indicating that for CELP[V-20]-4, salting-out effects become relevant above 100 mM.

ELP[V]-90 shows no selectivity for Na^+ , Mg^{+2} , or Ca^{+2} in terms of their effect on its T_t , whereas each cation has a different effect on the T_t of CELP[V-20]-4. At a salt concentration between 0.1 and 10 mM (Figure 2B), CELP[V-20]-4 is highly sensitive to divalent cations such as calcium and magnesium, but not to a monovalent cation, such as sodium. We compared the sensitivity of CELP[V-20]-4 to calcium and magnesium by the slope of T_t vs. the log of salt concentration. The slopes for the two divalent ions are similar suggesting little selectivity towards calcium as compared to magnesium. However, at all salt concentrations, the effect of Ca^{2+} is larger than Mg^{2+} in decreasing the T_t of CELP, so that calcium is somewhat more effective than magnesium in interacting with the charged residues within the calcium-binding sequences and thereby decreasing the T_t .

The T_t of CELP[V-20]-4 at different CELP concentrations and calcium concentrations is shown in Figure 3A. With no CaCl_2 added and at a CELP concentration of 10 μM , the T_t of CELP[V-20]-4 is 74 °C. By adding just 1 mM CaCl_2 , the T_t drops to 62 °C, a 12 °C difference. At the increased CELP concentration of 100 μM , the T_t with no CaCl_2 added drops to 60 °C and upon the addition of 1 mM CaCl_2 drops to 54 °C, a 6°C difference. This indicates that the sensitivity to calcium decreases with increasing CELP concentrations for calcium concentrations below 1mM. This decrease in sensitivity could be a simple effect of reducing the ratio of calcium to binding sites available thereby shifting the equilibrium, leaving some calcium sites unbound in the CELP at the higher CELP concentrations.

A comparison of the transition temperatures of three different CELPs is shown in Figure 3B. An increase in the total length of the CELP by adding two additional repeat units to obtain

CELP[V-20]-6 lowers the T_t by $\sim 5^\circ\text{C}$ as compared to CELP[V-20]-4 which follows the expected trend for single segment ELPs, that is the ELP chain length inversely correlates with its T_t . CELP[V-10]-8 and CELP[V-20]-4 have the same total length of ELP (80 pentapeptides each), but CELP[V-10]-8 has twice as many calcium binding sequences (eight) as CELP[V-20]-4 (four). The increased number of negative charges contributed by the additional calcium binding sequences in CELP[V-10]-8 cause a significant increase in its T_t compared to that of CELP[V-20]-4. Because of the larger number of calcium binding sites, a calcium concentration of 10 mM must be added before the T_t of CELP[V-10]-8 is within the maximum temperature of $\sim 95^\circ\text{C}$ that the UV spectrophotometer can reach, while for CELP[V-20]-4, the T_t is below the maximum temperature, even in the absence of calcium. Furthermore, the sensitivity of CELP[V-10]-8 to calcium is greater than that of CELP[V-20]-4 as indicated by the slope of T_t versus log of CaCl_2 concentration for CELP[V-10]-8. This effect is consistent with the larger number of calcium-binding sites within the CELP[V-10]-8 sequence, as compared to CELP[V-20]-4.

CELP Diblock Copolymers

Several examples of diblocks formed from an ELP with a hydrophobic block with a low T_t (T_{t1}) and hydrophilic block with a high T_t (T_{t2}) have been shown to form spherical micelles, while other block copolymer design have also been shown to form vesicles²⁴⁻²⁷. Above a critical micellization temperature (CMT), ELP diblocks self assemble into spherical micelles in which the hydrophobic block forms the core of the micelle while the hydrophilic block forms the corona, presumably because the more hydrophobic block (T_{t1} block) preferentially desolvates with an increase in temperature. We synthesized a diblock ELP (CELP_{BC}) with a hydrophobic: hydrophilic block length ratio of 1:1, with a CELP as the hydrophobic block to sensitize the ELP's self-assembly to calcium. At low calcium concentrations, both blocks are hydrophilic and the CELP_{BC} is soluble. We hypothesized that as the calcium concentration is increased, binding of Ca^{2+} to the negatively charged residues in the CELP block should shield the negative charges and increase the hydrophobicity of this block, so that at a critical calcium concentration, the newly acquired amphiphilicity of the diblock drives self-assembly of the CELP_{BC} into micelles (or other self-assembled nano-meso scale structures). A schematic of a CELP_{BC} and its self-assembly into spherical micelles triggered by calcium binding is shown in Figure 4. The hydrophobic segment in our design of the CELP_{BC} is a CELP[V-20]-6 segment. In order to impart sufficient amphiphilicity to a CELP_{BC} upon calcium-binding, the hydrophilic segment of the diblock was chosen to be ELP[V₁A₈G₇]-128, a 128 pentapeptide ELP composed of guest residues valine, alanine, and glycine at a ratio of 1:8:7 and with a high T_t . At a concentration of 25 μM this segment has a T_t of 78°C . This CELP_{BC}, composed of the ELP[V₁A₈G₇]-128 block fused to CELP[V-20]-6, has a total MW of 106 kDa, and will be referred to herein as CELP128/6.

Nanoparticle formation by CELP_{BC}'s was successfully demonstrated by temperature-programmed turbidimetry measurements and DLS as a function of solution temperature. Turbidity measurements for 10 μM CELP-128/6 are shown in Figure 5A with increasing concentration of calcium. In the absence of calcium, CELP-128/6 transitions from a unimer to a polydisperse, micron size aggregate at a T_t of 55°C . With the addition of 10 mM CaCl_2 , the CELP-128/6 undergoes a transition from unimer to aggregates at a lower T_t of 47°C , but no self-assembly into nanoparticles is observed. At calcium concentrations of 50 mM and greater, however, evidence of nanoparticle formation at intermediate temperatures can be seen in the turbidity profiles, as in our experience, a region of low OD (~ 0.1 - 0.5) preceding aggregation is indicative of nanoparticle formation. At 50 mM CaCl_2 , the nanoparticle region extends from 41°C to 45°C . The temperature range over which nanoparticles form increases with increasing CaCl_2 concentrations, as at 500 mM CaCl_2 , nanoparticles are observed from 32°C to 49°C . This extension of the micelle formation temperature range

with increasing CaCl_2 concentrations can be attributed to two effects: 1) an increase in the calcium bound to the CELP block thereby increasing the block's hydrophobicity and 2) the salting-out effect of Cl^- . To separate the specific effect of Ca^{2+} binding from that of a non-specific salting out effect, we examined the thermal behavior of a control ELP diblock that lacks the Ca^{2+} binding sequence, as a function of CaCl_2 (Figure S5). This ELP diblock is composed of a hydrophilic block of the same composition as CELP_{BCS} , $\text{ELP}[\text{V}_1\text{A}_8\text{G}_7]$ -128, and an $\text{ELP}[\text{V}]$ -60 hydrophobic block that has been previously shown to self-assemble into micelles with an increase in temperature²⁵. The temperature at which the unimer self-assembles into micelles decreases with increasing salt concentrations (i.e. the temperature at which a small increase in O.D. is first observed). Therefore, the extension of nanoparticle formation temperature range for CELP_{BCS} could be a result of the effect of Cl^- on T_t as well as calcium binding.

While turbidity measurements suggest the formation of nanoparticles, DLS measurements are necessary to confirm their presence. The hydrodynamic radius (R_h) of CELP -128/6 was determined by DLS as a function of temperature. Figure 5B shows a comparison of R_h with the turbidity measurements for 10 μM CELP -128/6 with 500 mM CaCl_2 as a function of solution temperature. From 20 °C to 32 °C, the R_h is ~8 nm, which corresponds to the size expected for a soluble 106.3 kDa polypeptide with a largely random coil structure. From 33 °C to 46 °C, the R_h is between 45 and 49 nm. At temperatures above 47 °C, micron-sized aggregates form. These results correspond closely with the turbidity measurements. In addition, the diffusion coefficient (D_{app}) was measured at scattering angles from 30° to 150° (Figure S6A). The change in D_{app} with scattering angle was found to be insignificant suggesting the nanoparticles formed are monodisperse and spherical. The radius of gyration (R_g) was determined from the slope of the Zimm plot obtained from static light scattering and was found to be 23.8 nm giving a ratio of R_g/R_h of 0.5 indicating a spherical particle topology, consistent with the formation of spherical micelles (Figure S6B). The aggregation number was determined by dividing the y-intercept of the Zimm plot obtained for the unimer measured at 25°C by that for the micelle measured at 40 °C. These micelles were found to have an aggregation number of 155 unimers/micelle.

It is interesting to note that while large changes in T_t are observed in single block CELPs with the addition of small amounts of calcium, higher amounts of calcium must be added to a diblock in solution before nanoparticles form. The nanoparticles require such a high calcium level to form because in the absence of calcium, both blocks are quite –and similarly– hydrophilic, as seen by their T_t s, the value of which correlates with the mean hydrophobicity of the ELP. The T_t of $\text{CELP}[\text{V}-20]$ -6 at 10 μM in HEPES buffer is ~70 °C, and is close to the T_t of $\text{ELP}[\text{V}_1\text{A}_8\text{G}_7]$ -128. In order to drive self assembly of the CELP 128/6 diblock ELP formed by fusion of these two blocks, enough calcium must be added to selectively drive binding of Ca^{2+} to the CELP block to increase the hydrophobicity of this block relative to $\text{ELP}[\text{V}_1\text{A}_8\text{G}_7]$ -128 block. Micelles begin to form when the polymer attains a critical level of amphiphilicity that is imparted by the selective binding of Ca^{2+} to the CELP block and by the selective desolvation of this block with an increase in temperature. For example, for CELP -128/6 at a solution concentration of 10 μM , 100 mM CaCl_2 has to be added and the solution temperature raised above 38 °C (the CMT of this diblock at 10 μM) to trigger its self-assembly into spherical micelles. These results also suggest that to create CELP_{BCS} that are more sensitive to calcium, the hydrophobicity of the CELP block should be increased by incorporating more hydrophobic residues at the guest position, so that self-assembly can be triggered at a lower calcium concentration and lower temperature.

Calcium selectivity of CELP_{BC}

We next investigated the selectivity and sensitivity of CELP_{BC} to calcium relative to magnesium and sodium. Figure 5 shows turbidity profiles for 10 μM CELP -128/6 with

increasing amounts of three different salts. NaCl, does not trigger nanoparticle formation even at a concentration of 1.5 M at any temperature between 20 and 70 °C but instead transitions from unimer to aggregate at 24 °C (Figure 5C). MgCl₂, in contrast, triggers nanoparticle formation, but only at a concentration of 100 mM (Figure 5D) while CaCl₂, triggers nanoparticle formation at a lower, 50 mM concentration (Figure 5A). These results demonstrate that the self-assembly of CELP-128/6 is divalent ion selective, and that the self-assembly shows a 2 fold selectivity for calcium as compared to magnesium.

Conclusion

Calcium sensitive elastin-like polypeptides (CELPs) were designed as thermally sensitive calcium-binding calmodulin analogs with the goal of generating thermal and calcium responsive peptide polymers. These artificial polypeptides consist of ELP spacer segments separated at regular intervals by the calcium-binding amino acid sequence found at the second calcium-binding site of calmodulin. CELPs are extremely sensitive to Ca²⁺ compared to monovalent cations. They do not however, retain selectivity for calcium over other divalent cations such as Mg²⁺ that is displayed by calmodulin, indicating that the tertiary structure of calmodulin is critical to the selectivity displayed by calmodulin for Ca²⁺ as compared to its closest divalent homolog, Mg²⁺. The CELPs were further used to engineer calcium sensitive nanoparticles by synthesis of a diblock polypeptide composed of a hydrophilic non-calcium sensitive ELP block and a CELP block that becomes more hydrophobic upon binding calcium. We show that addition of calcium at concentrations between 50–500 mM imparts sufficient amphiphilicity to the diblock polypeptide between 33 °C and 46 °C to trigger its self-assembly into monodisperse spherical micelles with a hydrodynamic radius of ~50 nm. Future design of CELPs to enhance their calcium sensitivity will explore other calcium binding sequence with a higher calcium affinity and lower net charge, as well as increasing the hydrophobicity of the ELP segments in the calcium binding domain.^{21, 28-34}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to acknowledge financial support from NIH Grants GM61232, and EB001630 and support from NSF's Triangle MRSEC DMR-1121107

References

1. Urry DW. Prog. Biophys. Mol. Biol. 1992; 57(1):23–57. [PubMed: 1549698]
2. Urry DW, Luan CH, Parker TM, Gowda DC, Prasad KU, Reid MC, Safavy A. J. Am. Chem. Soc. 1991; 113(11):4346–4348.
3. Meyer DE, Chilkoti A. Biomacromolecules. 2002; 3(2):357–367. [PubMed: 11888323]
4. Callahan DJ, Liu WE, Li XH, Dreher MR, Hassouneh W, Kim M, Marszalek P, Chilkoti A. Nano Lett. 2012; 12(4):2165–2170. [PubMed: 22417133]
5. Trabbic-Carlson K, Setton LA, Chilkoti A. Biomacromolecules. 2003; 4(3):572–580. [PubMed: 12741772]
6. Trabbic-Carlson K, Meyer DE, Liu L, Piervincenzi R, Nath N, LaBean T, Chilkoti A. Protein Eng. Des. Sel. 2004; 17(1):57–66. [PubMed: 14985538]
7. Massodi I, Bidwell GL, Raucher D. J. Controlled Release. 2005; 108(2-3):396–408.
8. Prieto S, Shkilnyy A, Rumlach C, Ribeiro A, Arias FJ, Rodriguez-Cabello JC, Taubert A. Biomacromolecules. 2011; 12(5):1480–1486. [PubMed: 21438535]

9. Alonso M, Reboto V, Guiscardo L, Mate V, Rodriguez-Cabello JC. *Macromolecules*. 2001; 34(23): 8072–8077.
10. Strzegowski LA, Martinez MB, Gowda DC, Urry DW, Tirrell DA. *J. Am. Chem. Soc.* 1994; 116(2):813–814.
11. Carafoli E. *Annu. Rev. Biochem.* 1987; 56:395–433. [PubMed: 3304139]
12. Barry WH, Bridge JHB. *Circulation*. 1993; 87(6):1806–1815. [PubMed: 8389258]
13. Kuo CK, Ma PX. *Biomaterials*. 2001; 22(6):511–521. [PubMed: 11219714]
14. Shi J, Alves NM, Mano JF. *Macromol. Biosci.* 2006; 6(5):358–363. [PubMed: 16671051]
15. Wang J-Y, Jin Y, Xie R, Liu J-Y, Ju X-J, Meng T, Chu L-Y. *J. Colloid Interface Sci.* 2011; 353(1): 61–68. [PubMed: 20932528]
16. Kim B, Chilkoti A. *J. Am. Chem. Soc.* 2008; 130(52):17867–17873. [PubMed: 19055326]
17. Finn BE, Evenas J, Drakenberg T, Waltho JP, Thulin E, Forsen S. 1995; 2(9):777–783.
18. Cho YH, Zhang YJ, Christensen T, Sagle LB, Chilkoti A, Cremer PS. *J. Phys. Chem. B*. 2008; 112(44):13765–13771. [PubMed: 18842018]
19. Meyer DE, Chilkoti A. *Nat. Biotechnol.* 1999; 17(11):1112–1115. [PubMed: 10545920]
20. Wang CLA. *Biochem. Biophys. Res. Commun.* 1985; 130(1):426–430. [PubMed: 4026838]
21. Strynadka NCJ, James MNG. *Annu. Rev. Biochem.* 1989; 58:951–998. [PubMed: 2673026]
22. Cho Y, Zhang Y, Christensen T, Sagle LB, Chilkoti A, Cremer PS. *J. Phys. Chem. B*. 2008; 112:13765–13771. [PubMed: 18842018]
23. Hofmeister F. *Arch. Exp. Pathol. Pharmacol.* 1888; 25(1):1–30.
24. Lee TAT, Cooper A, Apkarian RP, Conticello VP. *Adv. Mater. (Weinheim, Ger.)*. 2000; 12(15): 1105–1110.
25. Dreher MR, Simnick AJ, Fischer K, Smith RJ, Patel A, Schmidt M, Chilkoti A. *J. Am. Chem. Soc.* 2008; 130(2):687–694. [PubMed: 18085778]
26. Simnick AJ, Lim DW, Chow D, Chilkoti A. *Polym. Rev.* 2007; 47(1):121–154.
27. Martin L, Castro E, Ribeiro A, Alonso M, Rodriguez Cabello JC. *Biomacromolecules*. 2012; 13(2):293–298. [PubMed: 22263638]
28. Bertini, I.; Sigel, A.; Sigel, H. *Handbook on Metalloproteins*. Marcel Dekker; New York: 2001.
29. Stuart DI, Acharya KR, Walker NPC, Smith SG, Lewis M, Phillips DC. *Nature*. 1986; 324(6092): 84–87. [PubMed: 3785375]
30. Kim SB, Lim JW. *Asian-Australas. J. Anim. Sci.* 2004; 17(10):1459–1464.
31. Kroger N, Bergsdorf C, Sumper M. *EMBO J.* 1994; 13(19):4676–4683. [PubMed: 7925309]
32. Hofmann SL, Goldstein JL, Orth K, Moomaw CR, Slaughter CA, Brown MS. *J. Biol. Chem.* 1989; 264(30):18083–18090. [PubMed: 2808365]
33. Fliegel L, Ohnishi M, Carpenter MR, Khanna VK, Reithmeier RAF, MacLennan DH. *Proc. Natl. Acad. Sci. U. S. A.* 1987; 84(5):1167–1171. [PubMed: 3469659]
34. Zhao H, Waite JH. *J. Biol. Chem.* 2006; 281(36):26150–26158. [PubMed: 16844688]

Figure 1.

Design of calcium sensitive elastin-like polypeptides (CELPs). CELPs contain negatively charged calcium binding sequences spaced at regular intervals along the ELP backbone. The calcium ions bind to the calcium binding sequences, which partially neutralizes the charge, decreases the CELP's transition temperature below solution temperature, and thereby isothermally triggers the phase transition of CELP.

Figure 2.

A. Transition temperature of 10 μM CELP[V-20]-4 and ELP[V]-90 at different concentrations of CaCl_2 , MgCl_2 , and NaCl . **B.** Transition temperatures of 10 μM CELP[V-20]-4 at salt concentrations between 0.1 and 10 mM, the physiological relevant range.

Figure 3.

A. Transition temperature (T_t) as a function of CaCl_2 concentration for CELP[V-20]-4 at concentrations of 10, 25, 50, and 100 μM in HEPES buffer. **B.** Transition temperature as a function of CaCl_2 concentration for 10 μM CELP[V-10]-8, CELP[V-20]-4, and CELP[V-20]-6 in HEPES buffer.

Figure 4.

Schematic illustrating micelle formation by CELP block copolymers consisting of one CELP block and one hydrophilic, high T_t ELP block. When the calcium concentration is increased, calcium binding to the CELP block depresses its T_t leading to self-assembly into spherical micelles.

Figure 5.

A. Turbidity measurements for 10 μ M CELP-128/6 as a function of temperature at increasing concentrations of CaCl₂. Turbidity profiles were obtained by monitoring optical density (OD) at 350 nm in HEPES buffer as the solution was heated at a rate of 1 °C/min. The increase in OD indicates formation of ELP nanoparticles larger than an ELP unimer. **B.** Hydrodynamic radius (R_h) and turbidity as a function of temperature for 10 μ M CELP-128/6 in HEPES buffer with 500 mM CaCl₂. **C.** Turbidity measurements for 10 μ M CELP-128/6 as a function of temperature in HEPES buffer with increasing amounts of NaCl, and **D.** MgCl₂.

Table 1

Sequences of the three CELPs. Underlined amino acids denote the calcium-binding sequence adapted from calmodulin.

CELP	Amino acid sequence	Molecular weight (kDa)
CELP[V-10]-8	[VGD <u>ADGNGTIDFPEG</u> -[VPGVG] ₁₀] ₈	44.5
CELP[V-20]-4	[VGD <u>ADGNGTIDFPEG</u> -[VPGVG] ₂₀] ₄	38.6
CELP[V-20]-6	[VGD <u>ADGNGTIDFPEG</u> -[VPGVG] ₂₀] ₆	57.9