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Title

Incorporation of Short, Charged Peptide Tag Affects Temperature Responsiveness of Positively-Charged Elastin-Like Polypeptides

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Abstract

Elastin-like polypeptides (ELPs) are recombinant protein domains exhibiting lower critical solution temperature (LCST) behavior. This LCST behavior not only is controlled by intrinsic factors including amino acid composition and polypeptide chain length but also is affected by non-ELP fusion domains. Here, we report that the presence of a composite non-ELP sequence that includes both His and T7 tags or a short Ser-Lys-Gly-Pro-Gly (SKGPG) sequence can dramatically change the LCST behavior of a positively-charged ELP domain. Both the His and T7 tags have been widely used in recombinant protein design to enable affinity chromatography and serve as epitopes for protein detection. The SKGPG sequence has been used to improve the expression of ELPs. Both the composite tag and the SKGPG sequence are <15% of the total length of the fusion ELP proteins. Despite the small size, the incorporation of the composite tag introduced pH-sensitive LCST behavior to the positively-charged ELP fusion protein. This pH sensitivity was not observed with the incorporation of the SKGPG sequence. The pH sensitivity results from both electrostatic and hydrophobic interactions between the composite tag and the positively-charged ELP domain. The hydrophobicity of the composite tag also alters the ELP interaction with Hofmeister salts by changing the overall hydrophobicity of the fusion protein. Our results suggest that incorporation of short tag sequences should be considered when designing temperature-responsive ELPs and provide insight into utilizing both electrostatic and hydrophobic interactions to design temperature-responsive recombinant proteins as well as synthetic polymers.

Keywords

Inverse transition cycling; LCST; Electrostatic interactions; Recombinant proteins

Introduction

Because of applications in various fields such as tissue engineering,[1] biosensors,[2] and drug delivery,[3] there is great interest in understanding factors that affect the responsiveness of temperature-sensitive polymers. Poly(*N*-isopropylacrylamide), or PNIPAM, is a representative temperature-responsive polymer exhibiting lower critical solution temperature (LCST) behavior with a transition temperature (T_t) of 32 °C.[4] The PNIPAM network is extended and hydrated below its T_t but collapses at temperatures above the T_t due to hydrophobic interactions. Based on this property, PNIPAM-coated surfaces have been used to harvest cultured cell sheets without disturbing the secreted extracellular matrix or the cells themselves.[5] The volume change as the PNIPAM network transitions below and above the T_t has also been harnessed to fabricate optical sensing platforms for temperature, pH, and salts in solution.[6] Variants of synthetic polymers based on PNIPAM have also been developed with different T_t values or hydrophobicities for different on-demand drug delivery applications.[4]

Certain natural polymers also display temperature responsiveness. For example, resilin-like polypeptides derived from *Drosophila melanogaster* have been reported to have temperature-triggered phase separation.[7, 8] Elastin, which is found in mammalian extracellular matrix, also undergoes hydrophobic collapse above 37 °C.[9] Elastin-like polypeptides (ELPs) are recombinant protein domains derived from the elastin amino acid sequence. ELPs are composed of the pentapeptide repeats Val-Pro-Gly-Xaa-Gly where Xaa is referred to as a guest residue that can be any amino acid except proline.[10, 11] ELPs also exhibit LCST behavior and form coacervates in aqueous solution.[12] Below the T_t, the ELP chain is unstructured and hydrated.[13, 14] When the solution temperature is above the T_t, the ELP chain collapses and expels some of its waters of hydration. The collapsed ELP chains further aggregate due to hydrophobic interactions, and there is macroscopic phase separation into ELP-poor and ELP-rich phases. Upon cooling to the T_t, the solution goes back to a single phase, and the collapsed ELP chains become extended and hydrated. The T_t of an ELP has a strong dependence on the guest residue composition and the resulting hydrophobicity.[15] Hydrophobicity can be tuned by increasing the mole fraction of hydrophobic guest residues or by incorporating amino acids with higher hydrophobicity. Increasing the hydrophobicity lowers the T_t.

Other factors can influence ELP LCST behavior as well, including ELP chain length, protein concentration, salt identity, salt concentration, and pH of the solution.[16] For a fixed guest residue composition, both longer ELP chains and higher ELP concentrations result in lower T_t values.[17] Hofmeister salts also affect the LCST behavior of ELPs, and both the type of salt and its

concentration determine the effect on the T_t.[18, 19] The LCST behavior of ELPs is sensitive to pH when ionizable amino acids are incorporated at guest residue positions. For example, MacKay and coworkers constructed two series of ELPs containing either Glu or His as guest residues and showed that the LCST behaviors were pH sensitive near their respective pKa values.[20] This pH sensitivity is a result of protonation or deprotonation of the ionizable amino acids. When the ionizable amino acids bear charges, the ELPs exhibit higher T_t values due to a decrease in the overall hydrophobicity. At the pH where the ionizable residues are uncharged, the increased hydrophobicity leads to lower T_t values. Variants of PNIPAM with carboxylic acid groups also show similar changes in hydrophobicity that result in pH-sensitive LCST behavior due to the deprotonation of the carboxylic acid groups.[21, 22]

ELP-based fusion proteins exhibit LCST behavior, which has been utilized for facile purification.[23] However, the T_t observed with the fusion protein can be different from the T_t of the ELP domain alone. Trabbic-Carlson and coworkers compared the T_t values of different ELP fusion proteins and found that the shift in T_t correlated with the surface hydrophobicity of the fusion partners.[24] In a follow-up study, the surface-exposed residues on the fusion partners were categorized into nonpolar, polar, and charged groups to compare their effects on the T_t shift.[25] The results showed that the charged amino acids have the most significant impact on the shift in the fusion protein T_t. In recent studies by Qin *et al.*, the relative position between the ELP domain and the fusion partner was found to have an effect on the LCST behavior and self-assembly of mCherry-ELP fusion proteins.[26, 27]

In this paper, we investigated a series of ELP-fusion proteins based on the same positively-charged ELP sequence with two commonly-used N-terminal non-ELP sequences. One non-ELP sequence is a widely-used composite tag composed of a T7 tag (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly), a His tag (7xHis), and an enterokinase cleavage site (Asp-Asp-Asp-Asp-Lys), and the other one is a short Ser-Lys-Gly-Pro-Gly (SKGPG) sequence used to promote expression of ELPs.

The composite tag contains two epitope tags, the T7 and His tags, that are frequently used in constructing recombinant proteins, particularly those that are expressed in the widely-used pET system.[28] In particular, the T7 tag enhances the protein expression level in *E. coli*.[29, 30] The T7 tag also enables confirmation that the desired protein has been expressed through antibody detection when performing a Western blot. Although ELP-based proteins can generally be purified through inverse transition cycling (ITC),[31] incorporation of a His tag, which enables protein purification via immobilized metal affinity chromatography (IMAC), can be beneficial for applications where

higher purity is required (e.g., producing ELPs with low endotoxin levels so that the ELPs can be used in animal studies).[32]

Although there are benefits to the T7 and His tags, the presence of these tags can be undesirable because of potential alterations to the structure or function of the protein of interest.[33, 34] Instead, the SKGPG sequence has been used as a leading sequence to improve the expression level of ELPs in *E. coli*.[35, 36] ELPs with the SKPGPG sequence have also been used to study the LCST behaviors with different guest residue compositions,[20, 37] with different ELP chain lengths,[17] and with different Hofmeister ions.[18, 19]

Based on the attributes of the tags, one tag may be preferred over the other for a specific application; however, very few studies have examined whether changing the leading sequence significantly affects the LCST behavior of ELPs. A previous study by Trabbic-Carlson and coworkers showed that a leading sequence containing the His tag lowered the T_t of a non-charged ELP by 3 °C in phosphate buffered saline (PBS).[24] In this study, we examine the effects on the LCST behavior of a positively-charged ELP sequence when the N-terminal non-ELP sequence is either the composite tag, which includes the T7 and His tags, or the short SKGPG sequence that enhances protein expression. The positively-charged ELP contains lysine residues in the guest residue positions. The amine groups on the lysine residues can be used for further protein modifications such as crosslinking or molecular conjugation.[38-40] We focus on a pH range of 5.5 to 8, which includes physiological pH and spans the isoelectric point of the histidine residues in the His tag. The two non-ELP sequences have different charge states in this pH range and therefore change the overall pH sensitivity of the LCST behavior of the positively-charged ELP sequence. We also report that non-ELP sequences can alter the ELP sequence interaction with Hofmeister salts. These results can provide insight into harnessing electrostatic and hydrophobic interactions to vary the pH- and temperature-response of ELPs as well as other temperature-responsive recombinant proteins and synthetic polymers.

Experimental methods

Materials. All materials and reagents were purchased from Sigma-Aldrich unless otherwise stated. Milli-Q water was used in all experiments except for protein expression and dialysis where reverse-osmosis purified water was used.

Protein Construction. In this work, an ELP sequence with a guest residue composition of Tyr, Lys, and Val (named the YKV domain) was studied. Two different leading sequences were fused to the N-terminus of the YKV domain (Fig. 1). The leading sequence that made the LCST behavior

sensitive to pH was named S-tag (for Sensitive-tag). The other leading sequence resulted in pH-insensitive LCST behavior and was designated as I-tag (for Insensitive-tag). The fusion ELP proteins were named based on the identity of the leading sequence and the number of the pentapeptides in the YKV domain. For example, S-tag[YKV-48] had the pH-sensitive S-tag followed by a YKV domain with 48 pentapeptides.

S-tag[YKV-48] was previously characterized for its adhesive properties and was referred to as ELY_{16} or ELP[$K_2Y_2V_2$-48].[41, 42] S-tag[YKV-72] and S-tag[YKV-96] were cloned using the encoding fragment of the YKV domain, recursive directional ligation,[43] and a cloning scheme previously developed by our lab.[44] To construct I-tag[YKV-48], pET21b plasmid (a gift from Dr. Chongli Yuan, Purdue University) was first modified by removing the T7-tag and His-tag followed by inserting a DNA oligo encoding a leading sequence with the amino acid sequence Ser-Lys-Gly-Pro-Gly. The previously cloned YKV-encoding DNA fragment was then inserted into the modified pET21b after the leading sequence. Amino acid sequences of the above proteins can be found in Fig. S1A.

Protein Expression and Purification. S-tag[YKV] proteins and I-tag[YKV-48] were produced as described previously.[41] Briefly, both proteins were transformed into the Rosetta2(DE3)pLysS *E. coli* expression host (EMD Chemicals, Gibbstown, NJ). 2xYT medium was inoculated with appropriate antibiotics. For the S-tag[YKV] proteins, 50 μg/mL kanamycin and 35 μg/mL chloramphenicol were used, and, for the I-tag[YKV-48] protein, 200 μg/mL ampicillin and 35 μg/mL chloramphenicol were used. Cells were grown for 16-18 h at 275 rpm and 37 °C. The overnight culture was used to inoculate 0.5-1 L of 2xYT medium with appropriate antibiotics in a 4-L flask at 1:100. The culture was grown at 300 rpm and 37 °C and induced with 1.25 mM isopropyl β-$_D$-1-thiogalactopyranoside (IPTG, EMD Chemicals) when the optical density at 600 nm was between 0.6 to 0.8. The cells were harvested 5 h after induction by centrifugation at 5380*g* for 15 min at 4 °C. Cell pellets were resuspended in a denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, pH 8.0) at 2 mL/g wet pellet; the resuspension was stored at -80 °C.

Cells were lysed by 1-2 freeze-thaw cycles and sonication (Misonix XL-2000, Qsonica, Newtown, CT) at 15 watts with 30 sec on/off cycles on ice. After 30 cycles, the lysate was centrifuged at 10000*g* for 25 min at 4 °C to remove insoluble debris. Next, 10 (w/v)% ammonium sulfate was added to the supernatant, and the solution was incubated on ice for 10 min before centrifugation at 10000*g* for 25 min at 4 °C. After centrifugation, another 10 (w/v)% ammonium sulfate was added to the supernatant before a second centrifugation. The pellet was collected and

resuspended in water at 200 mg wet pellet/mL. The resuspension was heated to 75 °C for 5 min with constant stirring, vortexed, and heated again as before. The heated resuspension was cooled on ice until ice cold and centrifuged at 10000*g* for 25 min at 4 °C. The supernatant was diluted by half with ice-cold water before being dialyzed against reverse osmosis water at 4 °C. The water was changed at least 3 h apart from the previous change until the total reverse osmosis water volume exceeded 10^6 of the supernatant volume. The dialyzed solution was centrifuged at 10000*g* for 25 min at 4 °C, and the supernatant was frozen at -80 °C for overnight before lyophilization.

Protein expression and purification was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250, and purity was quantified by densitometry using ImageJ software (National Institutes of Health, NIH, Bethesda, MD). The molecular weights of the proteins were confirmed using electrospray ionization mass spectrometry (Research Instrumentation Center, Purdue University) (Fig. S2). The amino acid compositions of I-tag[YKV-48] was confirmed by amino acid analysis (Table S1) by the Molecular Structure Facility at the University of California, Davis.

T_t **Measurement**. A tri-buffer system (APT buffer) was prepared by combining 10 mM acetate buffer, 10 mM phosphate buffer (at pH 7), and 10 mM Tris buffer. Stock APT buffers with different salts were prepared similarly with the addition of 1 M NaCl, 2 M NaI, or 2 M Na_2SO_4. APT buffers with different salt concentrations were prepared by mixing the stock solutions.

Proteins were dissolved in ice-cold APT buffer, and the solutions were incubated on ice for at least 10 min to ensure complete solubilization. To prepare solutions for T_t measurements at different protein concentrations, a 400 μM stock solution of the protein was prepared first. Solutions with lower protein concentrations were then prepared by sequential dilution. The solutions were warmed to room temperature before adjusting to the desired pH with concentrated acids and bases. Acids and bases were chosen based on the identity of the salt in the buffer and were HCl and NaOH for solutions with NaCl, acetic acid and NaOH for solutions with NaI, and sulfuric acid and NaOH for solutions with Na_2SO_4.

The T_t values of the solutions were determined using a Crystal16 (Technobis Group, Alkmaar, the Netherlands) by measuring transmission at 645 nm over a temperature range. Solutions were held at least 15 °C lower than estimated T_t values for 10 min, and the temperature was increased at 1 °C/min until temperatures at least 20 °C higher than the estimated T_t values were reached. For solutions with T_t values higher than 75 °C, the temperature ramp was terminated at 95

°C to avoid the boiling point of water. After the temperature ramp, solutions were cooled back to 4 °C to check the reversibility of the LCST behavior. For solutions that had T_t values lower than 15 °C, the solutions were held at 4 °C for 10 min to avoid the freezing point of water. The T_t was determined as the temperature at which the minimum of the first derivative of the transmission curve Δ(transmission)/ΔT occurred. Three samples were prepared independently and measured separately to report an average T_t for each condition. All numerical calculation and regression were performed using MATLAB (R2018a, MathWorks, Natick, MA).

Linear Regression of T_t Data as a Function of Protein Concentration. The concentration dependence of the LCST behavior of ELPs can be described by an empirical equation[17, 20]

$$Tt = Tt|_{1\,\mu M,\,pH} - b_{pH}\ln[ELP] \quad (1)$$

where $Tt|_{1\,\mu M,\,pH}$ is the fitted T_t at a protein concentration of 1 μM at a given pH, b_{pH} is the concentration dependence of the LCST behavior at a given pH, and [ELP] is the concentration of ELPs in μM. The linear regression was performed on the whole data set using the regstats function in MATLAB. The coefficient covariance matrix was reported using the regstats function. The square root of the diagonals of the coefficient covariance matrix was reported as the standard error of each fitted parameter.

Results and Discussion

Production of the S-tag and I-tag[YKV] Proteins. We are interested in the effect of two widely-used non-ELP sequences on the LCST behavior of the overall ELP-based protein. The SKGPG tag and composite sequence, which is composed of His and T7 tags, are commonly used to promote protein expression and/or facilitate purification and identification of ELP-based proteins. Researchers may prefer to have one tag or the other based on the specific application; however, it was not well understood whether changing the leading sequence would have significant effects on the ELP behavior (e.g., T_t). Thus, we examined a set of recombinant proteins based on the same ELP sequence with a guest residue composition of Tyr, Lys, and Val (YKV domain).

An SKGPG sequence was inserted on the N-terminal side of a YKV domain with 48 pentapeptide repeats (Fig. 1A). The SKGPG sequence has been reported to improve the expression yield of other ELP-based recombinant proteins[35, 36] and has been incorporated in many ELP-based recombinant proteins used to investigate LCST behavior.[20, 37] The charge state of this short sequence was expected to be insensitive to changes in pH near neutral and was named I-tag. The protein was designated as I-tag[YKV-48].

Another recombinant protein, S-tag[YKV-48], which had a different N-terminal amino acid sequence compared to I-tag[YKV-48], was examined. Specifically, the non-ELP leading sequence of S-tag[YKV-48] was composed of a T7 tag, a 7x His tag, and an enterokinase cleavage site (Fig. 1A). We were interested in investigating these tags because of their broad application in recombinant protein design and their widespread use with ELPs. The T7 tag is a short epitope tag that has been shown to enhance recombinant protein expression in *E. coli*.[29, 30] It also allows protein detection via Western blot as well as purification through antibody affinity column.[29] The His tag has been widely used as a purification tag for IMAC. Although ELP-based recombinant proteins can be purified by the ITC method utilizing the LCST behavior,[31] chromatographic purification is beneficial for applications where removal of endotoxin is necessary.[45-48] The enterokinase site is often harnessed to remove epitope and purification tags. The advantage of the enterokinase site is that the cleavage occurs after the Lys position and leaves no trace of the cleavage site in the protein of interest.[49] This leading sequence was expected to have pH-sensitive charge states because of the abundance of ionizable amino acids and was thus named S-tag. In addition to S-tag[YKV-48], we examined two more S-tag proteins with longer YKV domains (i.e., S-tag[YKV-72] and S-tag[YKV-96] composed of 72 and 96 pentapeptide repeats, respectively).

Both the I-tag and S-tag[YKV] proteins were successfully purified using a previously described salting and heating method (Fig. 1B).[41] Based on densitometry analysis of the SDS-PAGE gels, the purity of the lyophilized product was >95% for all proteins. Western blot using an anti-T7 tag antibody confirmed the presence of the T7 tag on the purified S-tag[YKV] proteins (Fig. 1B). Western blot was not applicable for detecting I-tag[YKV-48] because there was no epitope tag for antibody recognition. The typical yield was 100-120 mg/L culture for the S-tag[YKV] proteins and 60-80 mg/L culture for I-tag[YKV-48]. The difference in yields is consistent with previous reports that the T7 tag can improve recombinant protein expression in *E. coli*.[29, 50]

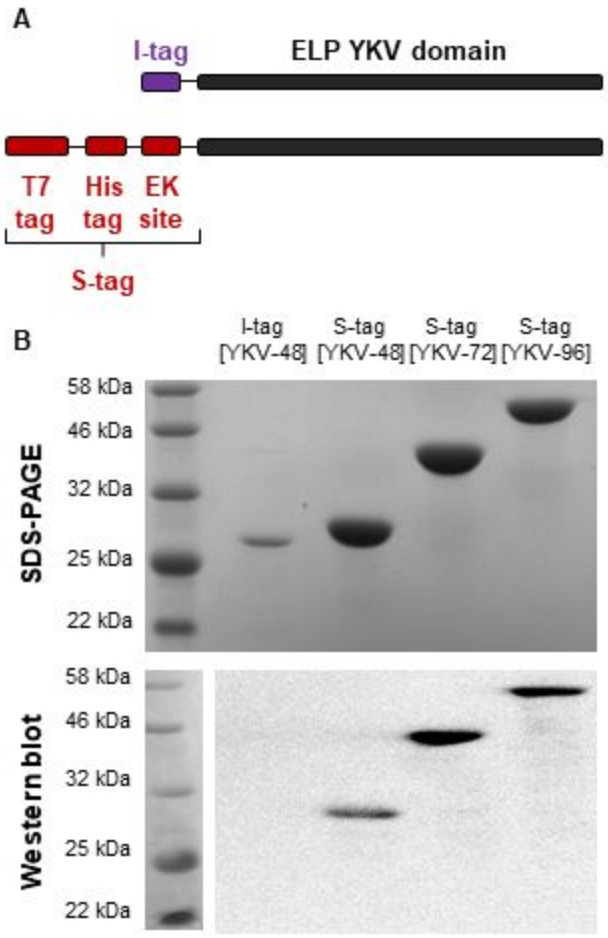

Figure 1. Production of the I-tag and S-tag[YKV] proteins. (A) Illustration of the differences between the I-tag and S-tag[YKV] proteins. All proteins shared a common ELP YKV domain. I-tag included the sequence of SKGPG. The S-tag was composed of a T7 tag, a 7x His tag, and an enterokinase cleavage site. (B) SDS-PAGE and Western blot images of purified I-tag and S-tag[YKV] proteins. All proteins on the SDS-PAGE gel were close to their expected molecular weights (I-tag[YKV-48]: 21 kDa, S-tag[YKV-48]: 25 kDa, S-tag[YKV-72]: 28.5 kDa, and S-tag[YKV-96]: 32 kDa). Western blot confirmed the presence of the T7 tag on the S-tag[YKV] proteins. Western blot was not applicable for I-tag[YKV-48] due to a lack of the T7 tag epitope used for detection.

Short Non-ELP Sequences Introduced pH Sensitivity to the LCST Behavior. To assess the effect of pH on LCST behavior of I-tag[YKV-48] and S-tag[YKV-48], we first determined the T_t by varying the pH from 5.5 to 8.0 in increments of 0.5 pH units and focused on one protein concentration (50 μM protein solutions in APT buffer with 0.2 M NaCl). Note that both I-tag and S-tag were relatively short compared to the YKV domain. I-tag constituted ~9.8% of the total sequence length of I-tag[YKV48], and S-tag was ~14% of the total length of S-tag[YKV48]. Despite the small change in sequence composition due to the tag sequences, there were dramatic differences in the LCST behavior of the two proteins under the same conditions. At a concentration

of 50 μM, the difference between T_t values of I-tag[YKV-48] was within 2 °C across the whole pH range (Fig. 2A). On the other hand, the T_t values of S-tag[YKV-48] resulted in a much wider distribution than that of I-tag[YKV-48] (Fig. 2B). The T_t of S-tag[YKV-48] was 76.3 °C at pH 5.5 and decreased to 48.1 °C at pH 8.0. Altogether, at a 50 μM concentration, S-tag[YKV-48] showed pH-sensitive LCST behavior, whereas I-tag[YKV-48] did not.

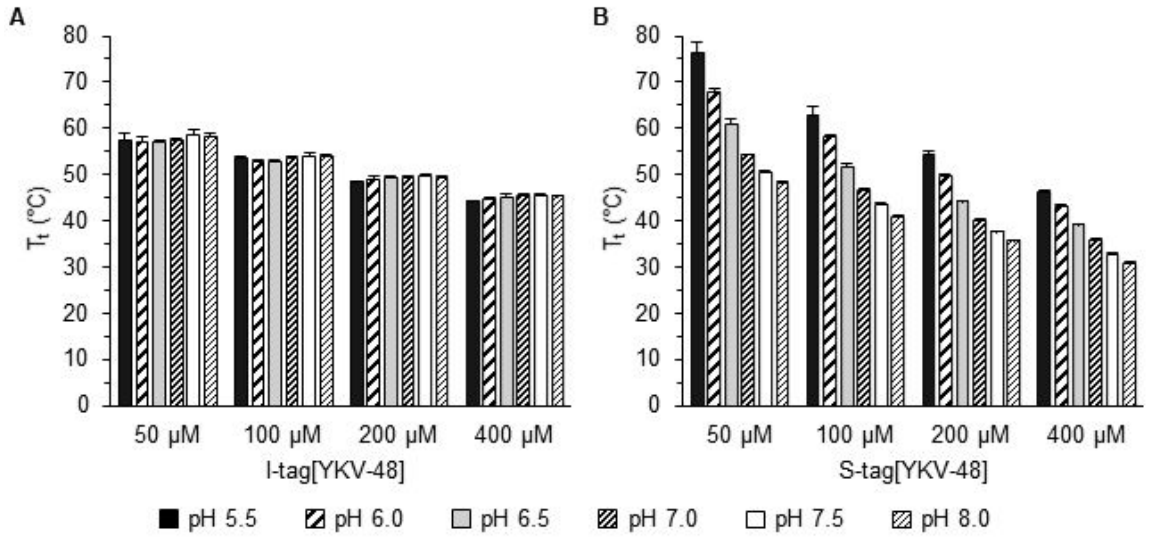

Figure 2. The T_t values of I-tag[YKV-48] are insensitive to pH, whereas the T_t values of S-tag[YKV-48] decrease drastically with increasing pH. T_t measurements of (A) I-tag[YKV-48] and (B) S-tag[YKV-48] at protein concentrations ranging from 50 to 400 μM and pH values ranging from 5.5 to 8.0. Both proteins had lower T_t values with increasing protein concentration. At a given concentration, the T_t values of I-tag[YKV-48] were similar at all pH values. On the other hand, S-tag[YKV-48] showed pH-sensitive T_t values. NaCl was added to the solution for all groups at a final concentration of 0.2 M. The T_t values were presented as the average with standard deviation of three independent samples.

Trabbic-Carlson *et al.* showed that hydrophobic proteins fused to ELP sequences affect the LCST behavior of the overall fusion proteins.[24] The authors reported that such an effect was not observed when the hydrophobic proteins and the ELP sequences were not in molecular proximity (i.e., not on the same polypeptide chain). Because the S-tag was on the same polypeptide chain as the YKV sequence, it is possible that the observed pH sensitivity introduced by the S-tag requires the same molecular proximity.

Protein Concentration Affected LCST Behavior and pH Sensitivity. To investigate the effect of protein concentration on LCST behavior and pH sensitivity, T_t values were quantified at concentrations of 100, 200, and 400 μM for both I-tag and S-tag[YKV-48]. Similar to the results for 50 μM, pH-insensitive LCST behavior for I-tag[YKV-48] was observed for all concentrations

tested (Fig. 2A). However, the overall T_t decreased as protein concentration increased. This concentration dependence is characteristic of LCST behavior of ELPs.[17]

For S-tag[YKV-48], the LCST behavior was pH sensitive for all concentrations tested (Fig. 2B). Furthermore, as observed with I-tag[YKV-48], the T_t of S-tag[YKV-48]at a given pH value decreased as the protein concentration increased. Examining the pH sensitivity of the LCST behavior in more detail revealed that there was a relationship to protein concentration. For example, at the lowest protein concentration of 50 μM the difference between T_t values at pH 5.5 and at pH 8.0 was 28.2 °C whereas at the highest concentration of 400 μM the difference was 15.6 °C. Thus, as the protein concentration increased, the LCST behavior of S-tag[YKV-48] became less sensitive to pH.

The concentration dependence of the LCST behavior of ELPs can be quantitatively described by an empirical equation (equation 1). The T_t results at a given pH were fitted to equation 1 and were plotted with the regression line generated using the best-fit b_{pH} and $LCST|_{1\ \mu M,\ pH}$ (Table S2) as shown in Fig. 3. Both I-tag[YKV-48] and S-tag[YKV-48] measurements were described well by equation 1. Lyons and coworkers used equation 1 to fit the LCST behavior of an uncharged ELP in PBS and reported a b_{pH} value of 11.0 °C/ln[μM], which is similar to our fitted values.[51] The similar b_{pH} values suggest that equation 1 can be used to describe the LCST behavior of ELPs with different charges as well as with different short leading sequences.

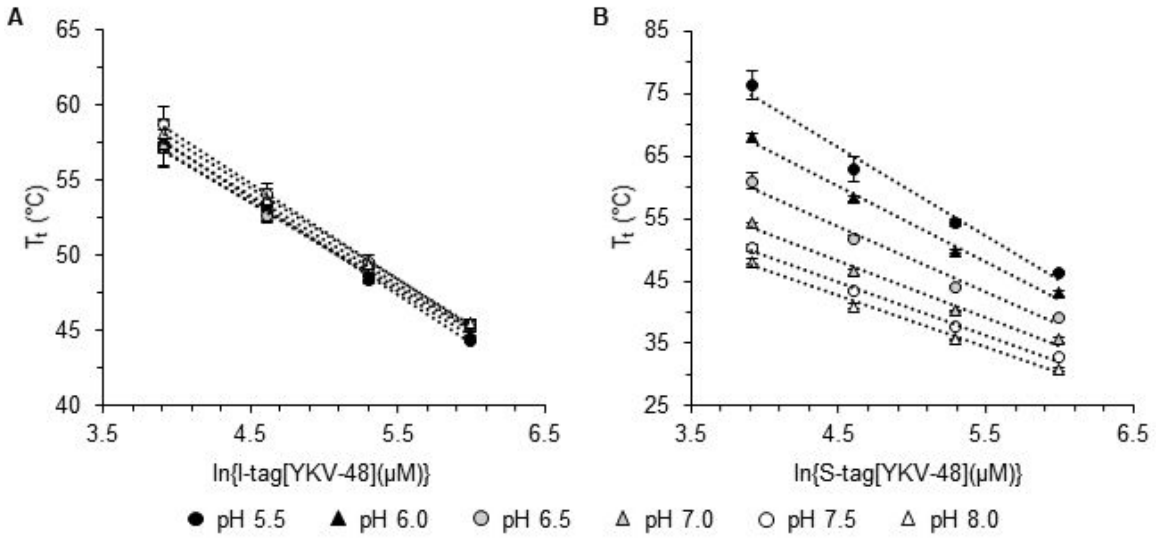

Figure 3. T_t values of I-tag[YKV-48] and S-tag[YKV-48] were well described by a two-parameter equation. T_t measurements with best-fit lines from equation 1 of (A) I-tag[YKV-48] and (B) S-tag[YKV-48]. The T_t values were presented as the average with standard deviation of three independent samples.

Fig. 4 shows the fitted b_{pH} parameters of S-tag[YKV-48] and I-tag[YKV-48] as a function of pH. I-tag[YKV-48] showed a nearly constant b_{pH} across the pH range. On the other hand, S-tag[YKV-48] showed a decreasing trend in b_{pH} as pH increased. This trend indicated that S-tag[YKV-48] exhibited a pH-sensitive concentration dependence of LCST behavior, and the LCST behavior of S-tag[YKV-48] was more sensitive to changes in protein concentration at lower pH values than at higher pH values. As a result, the T_t distribution of S-tag[YKV-48] was narrower at higher protein concentrations (Fig. 2B). We also noted that there were smaller changes in b_{pH} at higher pH values (7.0 to 8.0) compared to the large changes in b_{pH} at a lower pH range (5.5 to 7.0). This change suggested that the concentration dependence of S-tag[YKV-48] became less sensitive to pH at high pH values.

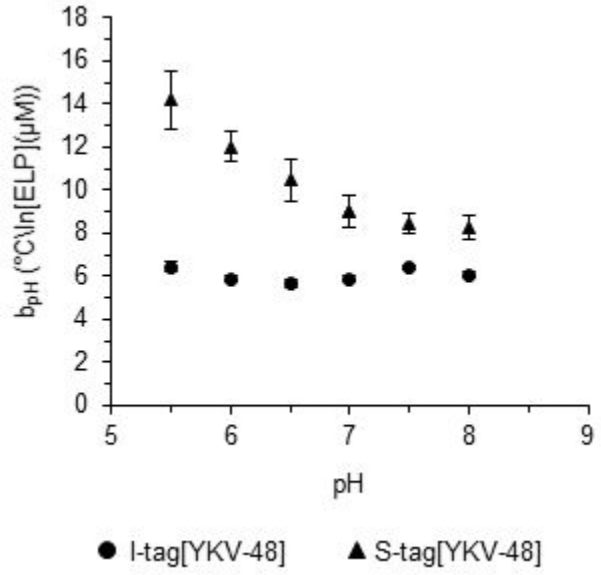

Figure 4. Different pH sensitivity in concentration dependence (b_{pH}) between I-tag[YKV-48] and S-tag[YKV-48]. Fitted b_{pH} of I-tag and S-tag[YKV-48] with standard error were plotted as a function of pH. The b_{pH} values for I-tag[YKV-48] yielded a nearly horizontal line, and b_{pH} was not sensitive to pH. On the other hand, the b_{pH} values for S-tag[YKV-48] were sensitive to pH and exhibited two different trends below and above pH 7. Larger changes in the b_{pH} values were observed from pH 5.5 to 7.0, whereas smaller changes were seen from pH 7.0 to 8.0.

pH Sensitivity Can Be Attributed to the Protonation State of Histidine. To explain different trends in b_{pH}, we analyzed the protein charge as a function of pH. The charge of the amino acid sequence was calculated using the Henderson-Hasselbalch equation by assuming that the pKa value of each residue was the same as the isolated amino acid and was not affected by the protein structure. The calculations were performed separately on the YKV domain and the N-terminal sequences as indicated in Fig. S1A. The YKV domain had a nearly constant charge from pH 5.5 to pH 8.0 (Fig.

S1B). This constant charge was expected because the most abundant ionizable residues were Lys with a pKa of 10.7 and Tyr with a pKa of 10.1. The charge on the I-tag was also nearly constant over the same pH range, whereas the S-tag showed a pH-dependent charge because of the ionizable His residues in the His tag. Histidine had a pKa of 6.0 and was positively charged below pH 6.0. On the other hand, Asp residues in the enterokinase cleavage site carried negative charges when the pH was above the Asp pKa of 3.7. As a result, the charge on the S-tag transitioned from positive to negative at approximately pH 6.3 as His residues became deprotonated and uncharged, and this pH value was close to where the b_{pH} trend changed at a pH of approximately 7.0 (Fig. 4).

Previous studies have utilized histidine at the guest residue position to construct ELPs with pH-sensitive LCST behavior over the same pH range.[20] Deprotonation of His resulted in increased hydrophobicity and decreased T_t. Placing histidine adjacent to ELP domains was also reported to lower the T_t of an uncharged ELP at pH 7.4.[24] However, it has not been shown whether the change in hydrophobicity and charge state of His outside of an ELP domain can affect the pH-sensitive LCST behavior. Our S-tag[YKV-48] demonstrated that His, when not incorporated as a guest residue, can still influence the ELP LCST behavior and can be harnessed to introduce pH sensitivity to the LCST behavior.

The Extent of pH sensitivity Depended on ELP Domain Length. We next examined to what extent the pH sensitivity introduced by the S-tag persisted when the ratio of the lengths of the non-ELP sequences to the ELP was varied. Such information can be useful when designing ELP-based fusion proteins in order to minimize the effect from non-ELP sequences on the overall LCST behavior or to utilize the effect as way to introduce expanded responsiveness to the LCST behavior. To that end, the YKV domain was elongated to 72 (S-tag[YKV-72]) and 96 repeats (S-tag[YKV-96]). All proteins were prepared at 200 μM in APT buffer with 0.2 M NaCl, and the T_t values are shown in Fig. 5. As the percentage of S-tag of the whole sequence decreased from 14% for S-tag[YKV-48] to 7.5% for S-tag[YKV-96], the T_t became less sensitive to pH (Fig. 5). Furthermore, at a given pH, T_t values decreased with longer YKV domains. This trend was consistent with previous work in which longer ELPs with the same guest residue composition exhibited lower T_t values.[17, 50]

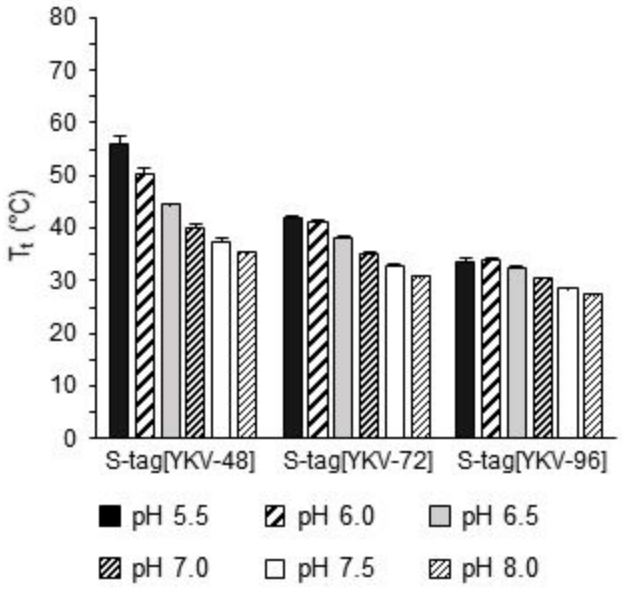

Figure 5. Longer YKV domain lengths decreased the extent of pH sensitivity derived from the S-tag sequence. At 200 μM protein and 0.2 M NaCl, the change in pH sensitivity due to the extension of the YKV domain was more prominent at a lower pH region (pH 5.5 to 6.5). The data set of S-tag[YKV-48] is replotted from Fig. 2B. The T_t values were presented as the average with standard deviation of three independent samples.

Increasing Ionic Strength Shielded pH-Sensitive LCST Behavior Introduced by the Tag Sequence. Because both the S-tag and the YKV domain are charged, we hypothesized that interactions between the S-tag and the YKV domain involved electrostatic interactions. Increasing the ionic strength (i.e., salt concentration) was expected to shield the interactions between the S-tag and YKV domain. As a result, the LCST behavior of S-tag[YKV-48] was expected to become less pH sensitive as salt concentration increased.

T_t values were measured with increasing NaCl concentration from 0.1 to 0.8 M at a fixed protein concentration of 200 μM. As shown in Fig. 6A, the T_t values of I-tag[YKV-48] depended on NaCl concentration but remained pH insensitive. This result reflected the constant charge on both the I-tag and YKV domain over this pH range. On the other hand, the LCST behavior of S-tag[YKV-48] remained pH sensitive over all the NaCl concentrations (Fig. 6B). It should be noted that as NaCl concentration increased, the T_t became less sensitive to pH. For example, the differences in T_t values between pH 5.5 and pH 8.0 were 5.6 and 4.4 °C at 0.6 and 0.8 M NaCl, respectively. These results supported the hypothesis that the electrostatic interactions between the S-tag and the positively-charged YKV domain contributed to the observed pH-sensitive LCST behavior of the S-tag[YKV] proteins.

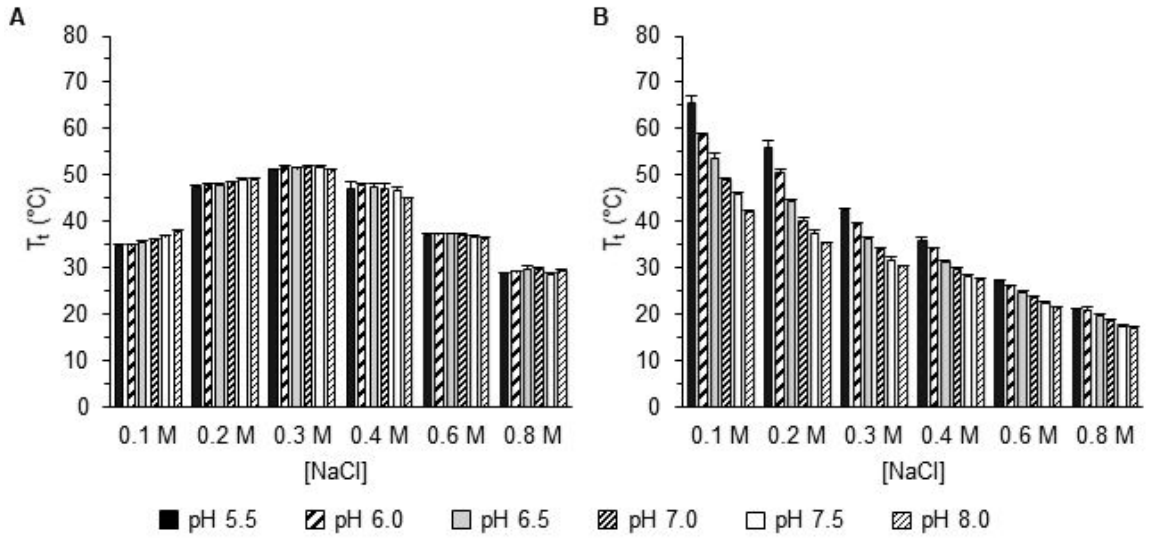

Figure 6. High ionic strength shielded the electrostatic interactions between S-tag and the YKV-48 domain. T_t measurements of (A) I-tag and (B) S-tag[YKV-48] at different NaCl concentrations with 200 μM protein. The LCST behavior of I-tag[YKV-48] remained insensitive to pH over all NaCl concentrations. On the other hand, the LCST behavior of S-tag[YKV-48] showed decreasing pH sensitivity with increasing NaCl concentration. In addition, the two proteins responded differently to the increasing NaCl concentration. The T_t values of I-tag[YKV-48] initially increased until 0.3 M NaCl then decreased. In contrast, the T_t values of S-tag[YKV-48] decreased monotonically with increasing NaCl concentration. T_t values were presented as the average with standard deviation of three independent samples.

Electrostatic interactions play an important role in other temperature-responsive materials as well. For example, the temperature responsiveness of a charged resilin-like polypeptide was proposed to involve both electrostatic and hydrophobic interactions.[7] Another example is the use of copolymers of *N*-isopropylacrylamide (NIPAM) and acrylic acid or methacrylic acid to achieve pH-dependent LCST behavior.[21, 52, 53] When the solution pH was above the pKa of the carboxylic acid groups on the acrylic acid segments, more carboxylic acid groups became deprotonated.[21, 52] As a result, the increased electrostatic repulsion between the charged carboxylic acid groups hindered the collapse of the polymer chains and led to elevated T_t values. Based on our results, other temperature-responsive materials, such as resilin-like polypeptides or PNIPAM, could be conjugated to peptides that are rich in ionizable amino acids, such as histidine, to achieve different ranges of pH-responsiveness, which could be varied based on the pKa of the peptide sequence.

Hydrophobicity of the Tag Affected LCST Behavior. In addition to the electrostatic interactions identified using NaCl solutions, the hydrophobicity of ELP domains and the non-ELP sequences also affected the LCST behavior of ELP proteins. To examine the hydrophobicity difference between the two tags as a function of pH, we used an estimation method proposed by Trabbic-

Carlson *et al*.[24] This estimation was based on the hydrophobicity scale established by Urry[15] that was originally used to predict the T_t of an ELP with a known guest residue composition. It should be noted that this estimation had no direct physical meaning and was only used to demonstrate the difference in the relative hydrophobicity between the tags in an ELP-fusion protein context. Similar to its charge state, the I-tag had a nearly constant relative hydrophobicity, and the S-tag had a pH-dependent relative hydrophobicity (Fig. S3). The increase in hydrophobicity of the S-tag can also be attributed to the deprotonation of His above its pKa. We thus hypothesize that the pH-dependent charge and hydrophobicity states of the His tag contributed to the pH responsiveness observed with the S-tag[YKV] proteins and that the T7 tag in the S-tag changed the hydrophobicity of the S-tag[YKV] proteins in a pH-independent fashion.

The S-tag was relatively more hydrophobic than the I-tag from pH 5.5 to 8.0, and thus S-tag[YKV-48] was expected to have lower T_t values than I-tag[YKV-48] under the same conditions. However, this finding based on the hydrophobicity analysis was in conflict with the results shown in Fig. 2 and 6. By plotting the difference in T_t values between the S-tag[YKV-48] and I-tag[YKV-48] proteins, it was shown that S-tag[YKV-48] had higher T_t values than I-tag[YKV-48] from pH 5.5 to 6.5 at a protein concentration of 50 μM, from 5.5 to 6.0 at protein concentrations of 100 and 200 μM, and only at pH 5.5 at a protein concentration of 400 μM (Fig. 7A). With increasing ionic strength (≥0.3 M NaCl), S-tag[YKV-48] had lower T_t values than I-tag[YKV-48] at all pH values (Fig. 7B).

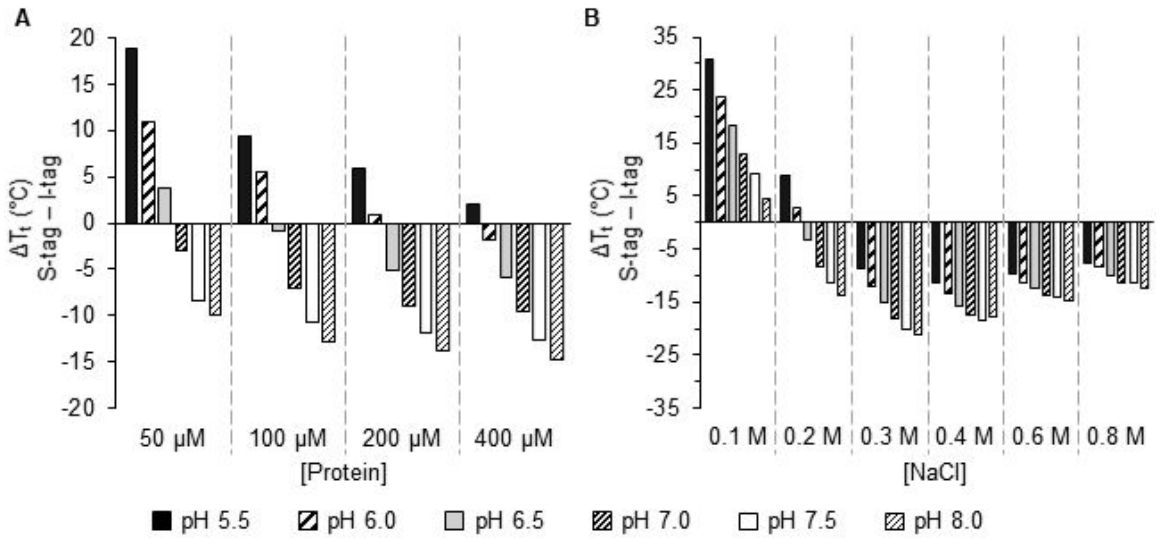

Figure 7. High protein concentration and high ionic strength decreased the T_t values of S-tag[YKV-48] more significantly than those of I-tag[YKV-48]. LCST differences between S-tag and I-tag[YKV-48] with different (A) protein concentrations and (B) NaCl concentrations. S-tag[YKV-48] had higher T_t values than I-tag[YKV-48] at low pH values and low protein concentrations. As

the protein concentration increased, S-tag[YKV-48] only had a higher T_t at pH 5.5. With varying NaCl concentration, S-tag[YKV-48] only had higher T_t values at NaCl concentrations ≤0.2 M. The plotted differences were based on the averaged T_t values reported in Fig. 2 and 6.

Based on the charge estimation (Fig. S1B), both the S-tag and the YKV domain were positively charged from pH 5.5-6.5 and oppositely charged above pH 6.5 where the S-tag became negatively charged. We therefore hypothesized that the interaction between the S-tag and the YKV domain is a combination of electrostatic and hydrophobic interactions, and the dominant type of interaction depended on pH. This type of balance between electrostatic and hydrophobic interactions has also been proposed to play an important role in the LCST behavior of PNIPAM-based materials.[53, 54] At low ionic strengths and low pH values, the repulsive charge interactions between the S-tag and the YKV domain hindered the collapse of the protein chain and thus led to higher T_t values. At low ionic strengths and high pH values, the attractive charge interactions between the S-tag and the YKV domain worked synergistically with the increasing hydrophobicity of the S-tag and resulted in lower T_t values. The electrostatic interactions were shielded by increasing ionic strength, and the hydrophobic interactions dominated the LCST behavior. As a result, S-tag[YKV-48] exhibited lower T_t values than I-tag[YKV-48] at higher pH values and ≥0.3 M NaCl, and the T_t differences between the two proteins became smaller as the NaCl concentration increased above 0.3 M (Fig. 7B).

It should be noted that the LCST behavior observed at 0.1 M NaCl was not fully explained by our hypothesis. Specifically, S-tag[YKV-48] had higher T_t values than I-tag[YKV-48] from pH 7.0 to 8.0. Based on our hypothesis, under this low ionic strength, both attractive electrostatic interactions between the S-tag and the YKV domain and the increased hydrophobicity on the S-tag should lead to T_t values that are lower than those of I-tag[YKV-48]. This inconsistency suggested that there were more factors that needed to be considered when explaining the difference in the LCST behaviors reported here. It has been proposed that the effect of the interfacial tension caused by ions at the water-protein interface can be critical when describing LCST behavior of charged systems, and both cations and anions contributed to the effect.[55] Therefore, we further explored the effects from the tags on changing the water-protein interfacial tension by determining T_t values of I-tag[YKV-48] and S-tag[YKV-48] with different cosolutes.

Tag Identity Affected the Interactions between ELP Proteins and Hofmeister Ions. In Fig. 6, it was shown that the LCST behavior of I-tag[YKV-48] and S-tag[YKV-48] responded differently to an increased NaCl concentration. S-tag[YKV-48] showed a monotonic decreasing trend, whereas I-tag[YKV-48] exhibited an increasing T_t below 0.3 M NaCl and a decreasing trend above 0.3 M NaCl. The initial increasing T_t trend is similar to the salting-in effect on ELP LCST behavior

reported with chaotropic Hofmeister anions.[18] However, Cl^- is generally considered to be in the middle of the Hofmeister series,[18, 56] and previous reports found that, over a concentration range that included the one used in this work, Cl^- did not show a salting-in effect but instead showed a salting-out effect on both neutral and negatively-charged ELPs.[18, 19, 57] ELP LCST behavior is influenced by the interaction among Hofmeister ions, water molecules, and ELP protein chains.[58] Because both I-tag[YKV-48] and S-tag[YKV-48] were positively charged over the pH range investigated, we assumed the interactions with anions was the major contributor to the overall electrostatic interactions between the proteins and the ions in the solution. However, it was unexpected to observe a strong salting-in trend with I-tag[YKV-48] from 0.1 to 0.3 M NaCl (Fig. 6A). Since Cl^- exerted a typical salting-out effect on S-tag[YKV-48] as the NaCl concentration increased from 0.1 to 0.8 M, we hypothesized that the salting-in trend from Cl^- arose from the distinct tag sequences and not from the YKV domain.

To assess the effect of the tags on interactions with Hofmeister anions, T_t measurements were taken in the presence of Na_2SO_4, which introduces a kosmotropic anion SO_4^{2-}, or NaI, which has the chaotropic anion I^-. We note the presence of negatively-charged regions in our protein sequences (e.g., S-tag at pH ≥ 6.5) and that these regions can interact with cations and could affect the overall LCST behavior of the proteins. As a result, we chose the same cation (i.e., Na^+) for the sulfate and iodide salts to minimize the differences in the potential interaction between the cations and the negatively-charged regions of the proteins. Fig. 8A shows that Na_2SO_4 had a strong salting-out trend on S-tag[YKV-48]. The T_t decreased by 19.4 °C from 0.1 to 0.2 M Na_2SO_4 and by 43.5 °C from 0.2 to 0.6 M Na_2SO_4. Generally, SO_4^{2-} is considered to be one of the strongest kosmotropes in the Hofmeister series. Somewhat surprisingly, Na_2SO_4 showed a slight salting-in effect on I-tag[YKV-48] with an increase of 4.3 °C from 0.1 to 0.2 M Na_2SO_4. Further increasing the Na_2SO_4 concentration to 0.6 M resulted in a decrease in the T_t of I-tag[YKV-48] by 39.3 °C. Note that, unlike the data with NaCl, the measurements were taken at a protein concentration of 50 μM instead of 200 μM and at a pH of 5.5 because, at Na_2SO_4 concentrations of ≥200 μM, the T_t was <4 °C or the protein was insoluble.

Fig. 8B shows the T_t of 200 μM S-tag and I-tag[YKV-48] with 0.1 to 1.2 M NaI at pH 7.5. Both proteins had elevated T_t values at higher NaI concentrations (>0.2 M with S-tag[YKV-48] and >0.4 M with I-tag[YKV-48]). This salting-in trend was expected because the I^- anion is on the chaotropic end of the Hofmeister series. However, the decreasing trend in T_t before the salting-in concentration was surprising. Because we observed the decreasing trend with both S-tag and I-tag[YKV-48], the identity of the tag is probably not a major contributor to the decreasing trend in

T_t observed with low NaI concentrations. To the best of our knowledge, only one similar trend has been reported with another positively-charged ELP with a guest residue composition of one Lys and six Val.[59] We also note that a salting-out effect has been reported with a neutral ELP at high concentrations of NaI.[18] It is possible that a similar salting-out effect will occur with our positively-charged ELP at higher NaI concentrations beyond the concentration range tested here.

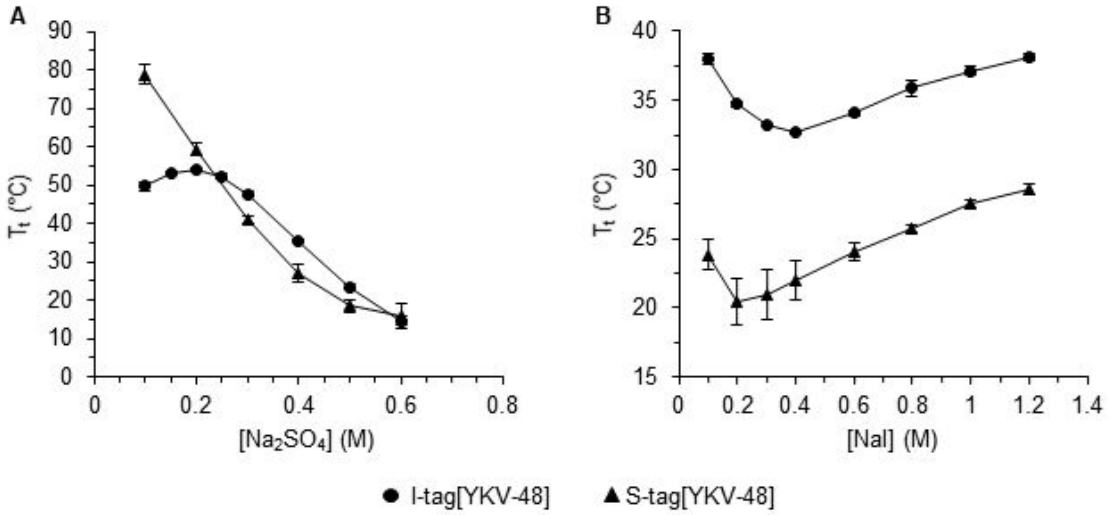

Figure 8. Non-ELP tag identity affected the interactions between ELPs and Hofmeister ions. T_t measurements of I-tag[YKV-48] and S-tag[YKV-48] with (A) Na_2SO_4 and (B) NaI. Both I-tag[YKV-48] and S-tag[YKV-48] had decreasing T_t values at >0.2 M Na_2SO_4. I-tag[YKV-48] showed an increasing trend in T_t from 0.1 to 0.2 M Na_2SO_4 even with a strong kosmotropic ion such as SO_4^{2-}. With NaI, both proteins exhibited a similar trend in which T_t values decreased <0.2 M NaI and increased at higher concentrations. T_t values with Na_2SO_4 were determined at a protein concentration of 50 μM at pH 5.5. T_t values with NaI were determined at a protein concentration of 200 μM at pH 7.5. The presented T_t values were averages of three independent samples with standard deviation.

The observed responses of the LCST behavior in response to three Hofmeister anions suggested that a broader range of anions could exert a salting-in effect on I-tag[YKV-48] compared to S-tag[YKV-48]. Different mechanisms have been proposed to explain the salting-in effect induced by chaotropic anions. Examples include electrostatic interactions between anions and backbone amides[18] and hydrophobic interactions between chaotropic anions and hydrophobic pockets in the protein.[60] In the electrostatic interaction hypothesis, direct binding of anions to backbone amides is enthalpically favorable toward weakly hydrated anions (i.e., chaotropic anions), and the binding increases the net charge on the protein and thus prevents hydrophobic collapse. Because of the sequence differences between I-tag and S-tag, the enthalpy gains from binding of

anions to backbone amides could be different and result in the different responses to the Hofmeister ions observed between I-tag[YKV-48] and S-tag[YKV-48]. In the hydrophobic interaction hypothesis, binding of weakly hydrated anions is entropically favorable. This binding competes with the interaction between two hydrophobic surfaces and therefore destabilizes the protein by increasing solubility of hydrophobic pockets. As shown in Fig. S3, I-tag was more hydrophilic than S-tag based on the relative hydrophobicities. Therefore, I-tag[YKV-48] should have had weaker hydrophobic interactions, which resulted in a broader range of hydrated anions in the Hoffmeister series that exerted a salting-in effect on the LCST behavior of I-tag[YKV-48].

Conclusions

The T7 tag, His tag, and enterokinase cleavage sites are useful sequences commonly used in recombinant protein design and in ELP-fusion proteins. However, their effects on the LCST behavior of the ELP-fusion proteins have not been fully investigated. In this work, we examined an S-tag composed of the above three sequences and investigated its effect on the LCST behavior of a positively-charged ELP YKV domain. The resulting S-tag had pH-dependent charge and hydrophobicity over the tested pH range. The pH dependence of charge and hydrophobicity on S-tag introduced additional pH sensitivity into the LCST behavior of the S-tag[YKV] proteins. On the other hand, an I-tag with no pH dependence in charge and hydrophobicity did not show similar effects on the LCST behavior of the I-tag[YKV] protein. Our results showed that pH sensitivity was introduced to the LCST behavior by interactions between the pH-dependent S-tag and the positively-charged YKV domain. We hypothesized that the interaction involved both electrostatic and hydrophobic interactions, and the two interaction types affected the LCST behavior differently. Increasing the ionic strength shielded the electrostatic interactions and resulted in hydrophobic interactions being the main determinant of LCST behavior. The identity of non-ELP sequences also changed the response to different Hofmeister anions. The more hydrophilic non-ELP sequence, I-tag, resulted in a broader range of anions capable of causing a salting-in effect on the LCST behavior. Our results suggested that both electrostatic and hydrophobic interactions between non-ELP and ELP sequences participated in LCST behavior. Overall, our work demonstrates that the identity of the tag sequences should be an integral part of the design consideration when constructing positively-charged ELPs and that short tags can be harnessed to engineer pH-responsiveness of the LCST behavior. Furthermore, the lessons learned from our work about using tags with charged groups to alter the balance between electrostatic and hydrophobic interactions between polymer chains can be extended to the design of other temperature-responsive recombinant proteins and synthetic polymers.

Associated Content

Supporting information

Fitted values of $Tt|_{1\ \mu M,\ pH}$, ESI-MS of I-tag[YKV-48], S-tag[YKV-72] and S-tag[YKV-96], amino acid analysis of I-tag[YKV-48], and estimations of protein charge and relative hydrophobicity.

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Notes

The authors declare no competing financial interest.

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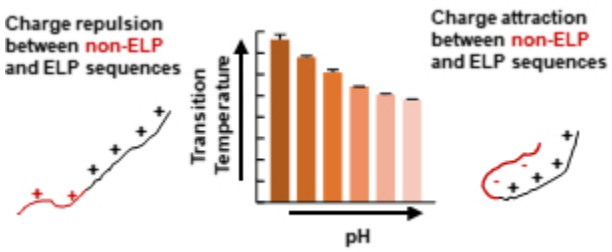

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