Designing Coiled Coils for Heterochiral Complexation to Enhance Binding and Enzymatic Stability

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ABSTRACT Coiled coils, commonly found in native proteins, are helical motifs important for mediating intermolecular interactions. While coiled coils are attractive for use in new therapies and biomaterials, the lack of enzymatic stability of naturally occurring L-peptides may limit their implementation in biological environments. D-peptides are of interest for biomedical applications as they are resistant to enzymatic degradation and recent reports indicate that stereochemistry-driven interactions, achieved by blending D- and L-peptides, yield access to a greater range of binding affinities and a resistance to enzymatic degradation compared to L-peptides alone. To our knowledge, this effect has not been studied in coiled coils. Here, we investigate the effects of blending heterochiral E/K coiled coils, which are a set of coiled coils widely used in biomaterials. We found that to achieve the unique and desirable properties of the heterochiral blend, we needed to redesign the coiled coils from a repeating pattern of seven amino acids (heptad) to a repeating pattern of eleven amino acids (hendecad) to make them more amenable to heterochiral complex formation. The redesigned hendecad coiled coils form both homochiral and heterochiral complexes, where the heterochiral complexes have stronger heats of binding between the constituent peptides and are more enzymatically stable than the analogous homochiral complexes. Our results highlight the ability to design peptides to make them amenable to heterochiral complexation, so as to achieve desirable properties like increased enzymatic stability and stronger binding. Looking forward, understanding how to design peptides to utilize the molecular tool of stereochemistry will be important to the design of next-generation therapeutics and biomaterials.

INTRODUCTION

Coiled coils are common helical structural motifs estimated to be found in approximately 10% of all eukaryotic proteins.[1,2] Specifically, coiled coils mediate interactions between proteins, operating, for example, in the regulation of DNA transcription and muscle contraction.[3,4] These functions are possible in complex biological environments as coiled coils have strong binding with a high degree of specificity.[4,5] Coiled coil specificity and affinity are derived from a combination of hydrophobic, electrostatic, and hydrogen bonding interactions, arising from conserved regions within coiled coil sequences. Typically, coiled coils are composed of repeating patterns of seven amino acids (*i.e.*, a heptad), labeled *abcdefg*, where amino acids in the *a* and *d* positions are hydrophobic and often those in the *e* and *g* positions are charged (**Figure 1A**).[3,6–9] While the strong affinity and high specificity of coiled coils make them attractive for use in biomaterials applications,[10–16] the lifetime of peptides *in vivo* is limited by poor enzymatic stability.[17–20]

One strategy to make peptide materials more stable is to alter stereochemistry, as D-amino acids resist degradation by enzymes.[21–25] While we could certainly improve the enzymatic stability of coiled coil biomaterials by making them entirely of D-amino acids, recent reports suggest that invoking stereochemistry-directed interactions between entirely L-peptides and entirely D-peptides gives rise to properties unique to those of naturally occurring L-peptides, including enhanced mechanics, stronger peptide-peptide interactions, and greater enzymatic stability. For example, 1:1 heterochiral blends of the D- and L-forms of the β-sheet forming peptide 'MAX1' result in hydrogels with a stiffness four times greater than those formed from homochiral D- or L-MAX1.[26,27] In another example, homochiral triple helices of the collagen-mimetic peptide $(PPG)_{10}$ are soluble but heterochiral mixtures precipitate, a result attributed to more favorable packing interactions for the heterochiral triple helices compared to homochiral.[28] Moreover, the enthalpy of interaction is stronger between heterochiral blends of peptides Ac-$(FKFK)_2$-NH_2 and Ac-(FEFE)2-NH2 as compared to homochiral peptide interactions.[29] With respect to enzymatic stability, the L-form of the peptide Ac-$(FKFE)_2$-NH_2 degrades within a day upon incubation with protease, while 1:1 blends of D- and L-$(FKFE)_2$ remain stable for at least 5 days, similar to the D-form of the peptide.[30] We sought to combine the enhanced interactions and enzymatic stability of heterochiral mixtures with the specific, strong binding of coiled coils into components of next-generation biomaterials.

Interest in heterochiral assemblies of coiled coil peptides stems back to early structural considerations for proteins.[31,32] Reports include a tetramer formed from heterochiral heptads,[33] yet more recent work by Gellman and coworkers highlighted that heptads may not be the most ideal sequence pattern for heterochiral assembly. Crystal structures revealed that side chain interactions between hydrophobic residues on heterochiral peptides occurred in a repeating pattern of residues spaced 3, 4, and 4 residues apart, rather than with a 3 and 4 residue spacing typical of the heptad repeating structure. The 3,4,4 spacing is consistent with a noncanonical repeating sequence pattern of eleven amino acids (*i.e.*, a hendecad), labeled *abcdefghijk*, where the hydrophobic amino acids are in positions *a*, *d*, and *h*.[34,35] In this case, the hendecad structure is preferred because coiled coils of opposing stereochemistry are unable to supercoil, a correction which aligns the hydrophobic faces of the coiled coils in conventional homochiral heptads.

Rather, the hydrophobic faces of hendecads align without a need for supercoiling, making them amenable to any combination of stereochemistry. While these reports provide a good basis for the structural considerations of heterochiral coiled coils, the potentially unique properties of the resulting heterochiral complexes have not yet been studied.

Here, we redesign the complementary glutamic acid/lysine (E/K)-rich coiled coil sequences (**Figure 1B**) ubiquitously employed as components in previously reported biomaterials[10,13,14] to promote heterochiral complexation and compare the intermolecular interactions and enzymatic stability of the resulting complexes to those of analogous homochiral coiled coils. We found that, to allow for heterochiral complexation and the possibility of accessing unique heterochiral blend properties, we needed to redesign the original heptad repeat sequences of the E/K coils as hendecad repeat sequences. The heterochiral hendecad repeat complexes exhibit higher binding strength and greater enzymatic stability than analogous all L hendecad complexes. Unlocking the benefits of stereochemistry-directed interactions in widely used biomaterial motifs such as coiled coils has the potential to greatly extend the lifetime of and tailor intermolecular interactions for next-generation materials.

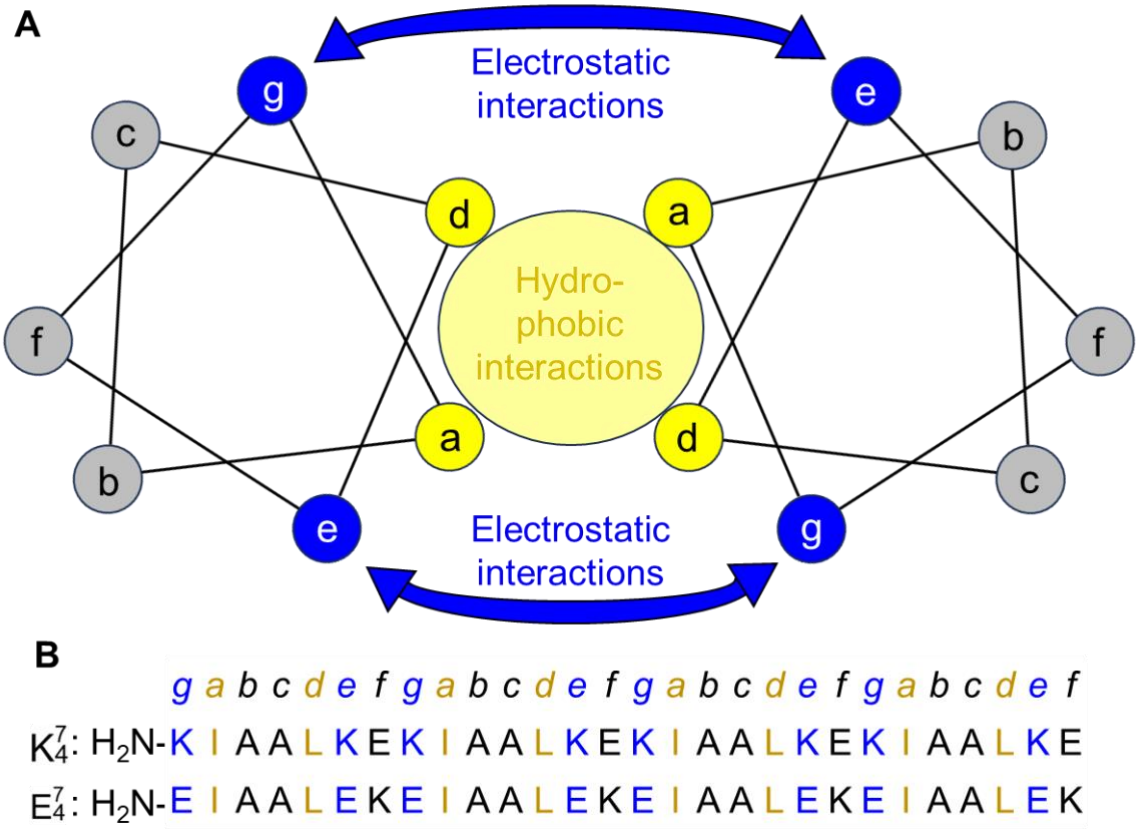

Figure 1. (A) Helical wheel diagram of the heptad repeat *abcdefg*, with hydrophobic interactions between complementary coils highlighted in yellow and electrostatic interactions between complementary coils highlighted in blue. (B) Sequences of K_4^7 and E_4^7 aligned with the heptad *abcdefg* registry.

RESULTS AND DISCUSSION

Here we investigate the effects of stereochemistry-driven interactions on the affinity and stability of coiled coil complexes. Using isothermal titration calorimetry and high performance liquid chromatography-based enzymatic stability measurements, we compare the affinities and enzymatic stabilities of analogous homochiral and heterochiral coiled coils composed of either repeating heptads or hendecads. This study demonstrates the advantages of heterochiral coiled coil complexes and provides a template for modifying existing heptad coiled coils to accommodate heterochiral coiled coil formation.

Heptad coiled coil formation: homochiral vs. heterochiral. The anion-rich coiled coil $(EIAALEK)_n$ (E_n^7, where n is the number of heptad repeats and the 7 superscript indicates a heptad repeat) and cation-rich coiled coil $(KIAALKE)_n$ (K_n^7) are known to form homochiral complexes when $n \geq 3$.[14] The secondary structure of these coiled coils was confirmed to be α-helical by circular dichroism (CD) spectroscopy (**Figure S27**). We used isothermal titration calorimetry (ITC), a label-free, solution-based technique used to study interactions between biomolecules,[36] to assess the thermodynamics of heptad coiled coil complex formation. The homochiral titration of L-K_4^7: L-E_4^7 in 1X PBS at pH 7.4 results in heats of interaction that are initially exothermic (with a maximum binding heat of -98 ± 4 kJ/mol) until reaching a molar

ratio of ~0.8:1 L-K_4^7:L-E_4^7, after which endothermic heats of interaction (with a maximum of binding heat of 63 ± 0.6 kJ/mol) are observed (**Figure 2A**). This may indicate that coiled coil interactions are initially exothermic due to enthalpically favorable electrostatic interactions between complementary coils, but as binding partners are consumed, molecular rearrangements which result in the endothermic (entropically-favorable) release of ordered water molecules dominate the heats of interaction.[37–39] These thermograms were reproducible (**Figure S21**), yet did not fit well to single-site or multisite binding models that would allow us to obtain a binding constant to compare to other pairs. The 1:1 mixture of L-K_4^7 and L-E_4^7 yields blends that are also helical, having a stronger helical CD signal than the individual coiled coils (**Figure S28A**). In contrast to the homochiral titration, the heterochiral titration of D-K_4^7 (designed by simply switching the chirality of all amino acids in the peptide from L to D) into L-E_4^7 produces only endothermic binding heats smaller in magnitude (having a maximum binding heat of 48 ± 0.3 kJ/mol) than the corresponding homochiral pair (**Figure 2B**). This finding may indicate that interactions between the heterochiral heptad coils are dominated by endothermic hydrophobic interactions, with little contribution from electrostatic interactions. To test this, we repeated the homochiral titration of L-K_4^7 into L-E_4^7 in 10X PBS, where we expected the excess salt to screen electrostatic interactions. Supporting our hypothesis, we found the heats of interaction in 10X PBS to be endothermic in contrast to the exothermic and endothermic heats of interaction that we observed in 1X PBS (**Figure 2C**). Additionally, the CD signal of the 1:1 mixture of D-K_4^7 into L-E_4^7 is very close to zero at all wavelengths between 200 nm and 250 nm, indicating that the CD is simply the sum of the equimolar D- and L-coils (**Figure S28C**). Together, these findings indicate that simply inverting the stereochemistry of one peptide in heptad-based coiled coils disrupts complex formation to some degree, ablating any potential for stronger interactions in heterochiral complexes compared to homochiral complexes in this heptad configuration, which is consistent with previous reports of non-ideality for heterochiral complexation of heptad-based coiled coils.

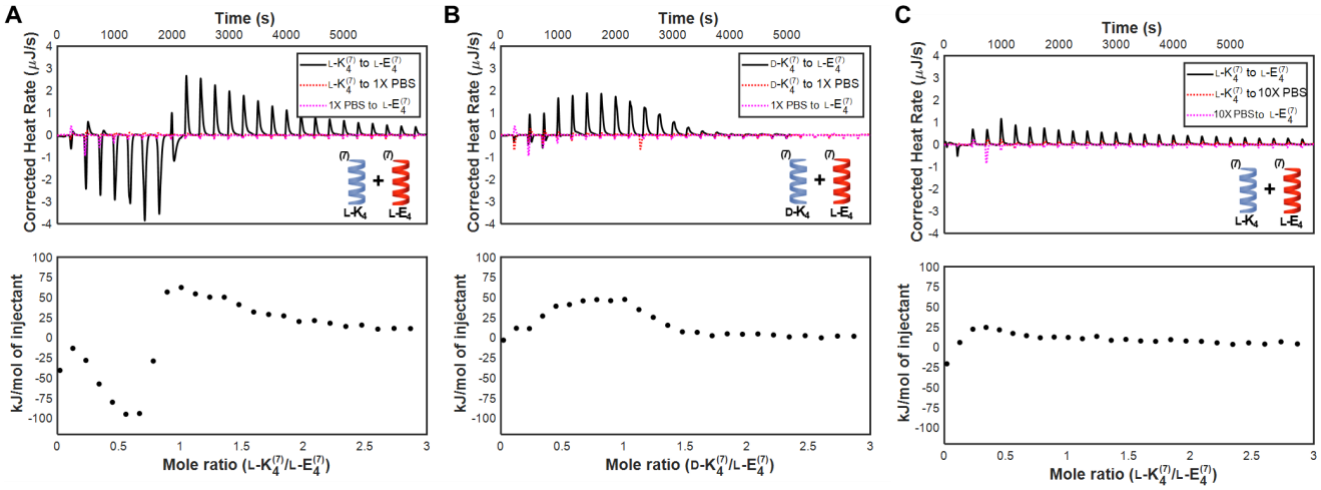

Figure 2. Thermograms and integrated binding heats of: (a) homochiral heptad coiled coils L-K_4^7 and L-E_4^7 in 1X PBS; (b) heterochiral heptad coiled coils D-K_4^7 and L-E_4^7 in 1X PBS; and (c) homochiral heptad coiled coils L-K_4^7 and L-E_4^7 in 10X PBS. While both exothermic and endothermic binding heats are observed for interactions between homochiral coils in 1X PBS, only endothermic binding heats are observed for interactions between heterochiral coils in 1X PBS and interactions between homochiral coils in 10X PBS.

Enzymatic degradation of heptad coiled coils. While we did not observe stronger binding for heterochiral heptads, knowing that stereochemistry-driven interactions are expected to provide both binding strength and enzymatic stability advantages, we next examined whether these heptads would exhibit more enzymatic stability in a heterochiral mixture compared to homochiral. Solutions of K_4^7 and E_4^7 (200 μM in 1X PBS at pH 7.4) were blended as homochiral (L-K_4^7:L-E_4^7) or heterochiral (D-K_4^7:L-E_4^7) mixtures. These blends were then incubated with 5 μg/mL Proteinase K, an enzyme known for its broad-spectrum activity and expected to cleave after the I, A, L, and E residues of these peptides. Immediately after adding Proteinase K, as well as after 1, 3, 6, 12, and 24 h incubations, we used high performance liquid chromatography (HPLC) to monitor the degradation of the coiled coils. In the HPLC eluent (low pH and in the presence of an organic solvent, acetonitrile), the coiled coil complex does not remain bound, resulting in two peaks corresponding to intact, K_4^7 (eluting at 5.7 min) and intact E_4^7 (eluting at 6.9 min). In the absence of Proteinase K, K_4^7 and E_4^7 exhibit little to no degradation over 24 h in the homochiral or heterochiral blends (**Figure S31**). In the presence of Proteinase K, we observe that ~50% of the coiled coils degrade after 6 h and none of the intact coiled coils remain after just 24 h (**Figure 3A**). For the heterochiral blend, the D-K_4^7 coil experiences little to no degradation, as expected. However, the L-E_4^7 coil in the heterochiral complex degrades more rapidly than it did in the homochiral complex, as the peak at 6.9 min disappears completely within 12 h (**Figure 3B**). This is consistent with the lower heats of binding that we observe in heterochiral heptads, as the L-coil in the homochiral complex is better protected from the protease, perhaps due to shielding from the complex. The mere presence of a D-peptide in the

material is insufficient to slow enzymatic degradation. These results further motivated us to design coiled coils with a hendecad repeating pattern and repeat these experiments.

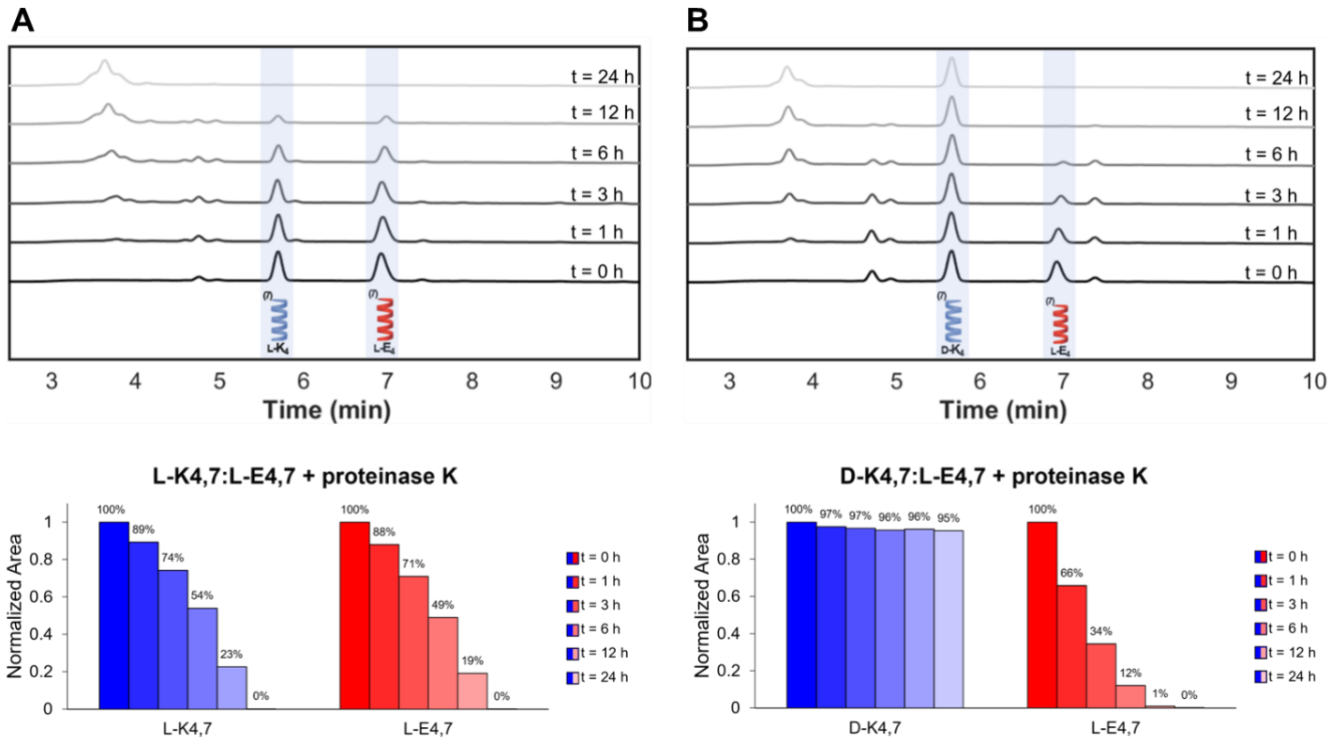

Figure 3. Enzymatic stability of heptad coiled coils in the presence of 5 μg/mL Proteinase K. HPLC chromatograms and percent intact K_4^7 and E_4^7 by peak area immediately after addition of and upon incubation for 1, 3, 6, 12, and 24 h with Proteinase K in A) a homochiral blend and B) a heterochiral blend.

Design of hendecad coiled coils. From Gellman and coworkers' work, we know that a hendecad repeating structure (*abcdefghijk*), where *a*, *d*, and *h* are hydrophobic residues, is favorable for heterochiral coil formation.[34,35] To assist with the redesign of the heptad coiled coils to a repeating hendecad structure based on E_n^7 and K_n^7, we employed helical wheel diagrams to visualize potential sequences. We observed that, as expected, the hydrophobic residues in the *a* and *d* positions of the heptad are gathered on one face of the helix, with charged residues, in the case of K_4^7, cationic residues specifically, flanking on either side (**Figure 4A**). With this knowledge, we used the same amino acids from K_n^7 to design a coiled coil with hendecad repeat structure. We first placed isoleucines and leucines in the *a*, *d*, and *h* positions, then cationic lysine residues in the *e*, *g*, and *k* positions to place them on either side of the hydrophobic face of the helix (**Figure 4B**), matching the placement of amino acids around the helix that we observed in the heptad repeat structure. The *b*, *c*, *i*, and *j* positions were filled with alanines, similar to the heptad structure, and the *f* position was filled with glutamic acid, serving the same role as in the heptad to provide solubility while being on the opposite side from the interacting face of the helix. For the E-rich hendecad, we used the same sequence except with all lysines exchanged for glutamic acids and vice versa. Using this newly designed hendecad-based coiled coil sequence, we again investigated complex formation and enzymatic stability of homochiral and heterochiral coils.

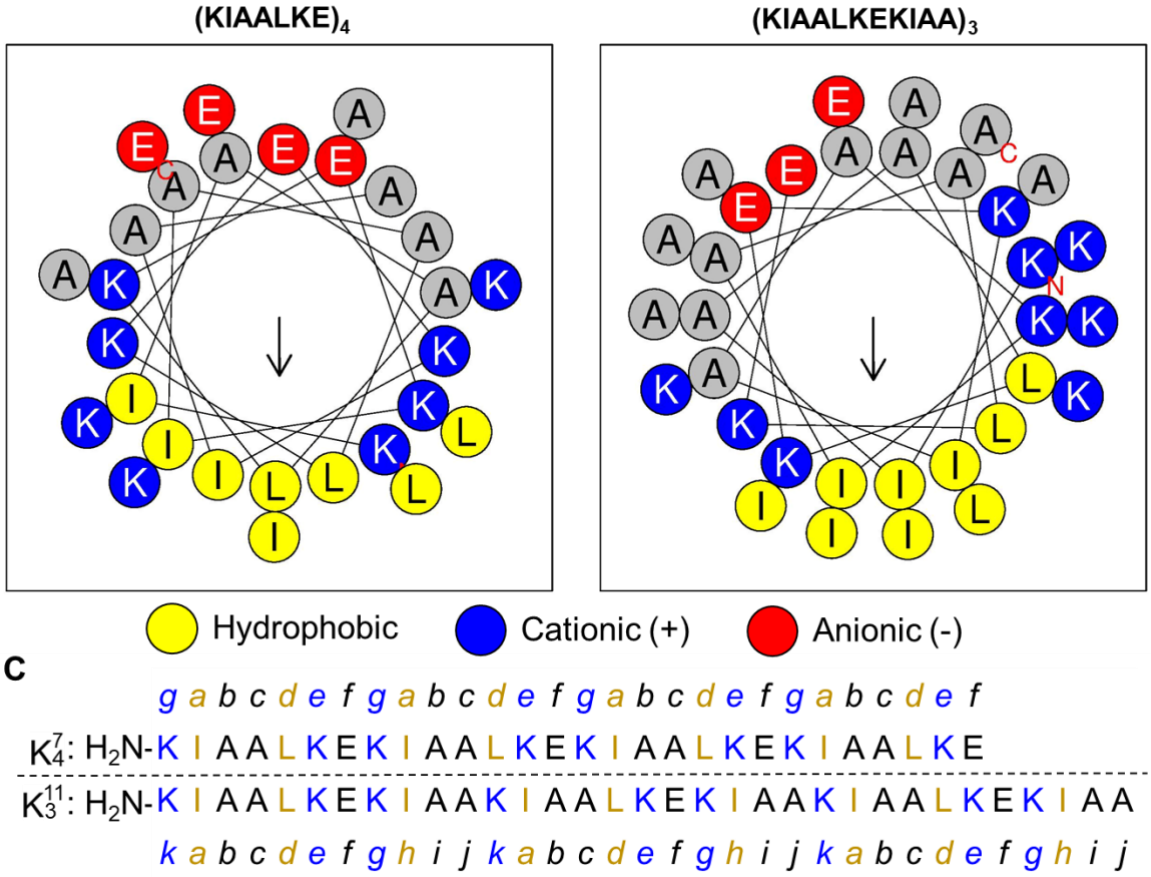

Figure 4. Helical wheel diagrams of: (A) K_4^7; and (B) K_3^{11}. Helical wheels generated using HeliQuest (https://heliquest.ipmc.cnrs.fr/). (C) Sequences of K_4^7 and K_3^{11} aligned with the heptad *abcdefg* and hendecad *abcdefghijk* registers.

Hendecad coiled coil formation: homochiral vs. heterochiral. To elucidate whether the change from a heptad to a hendecad repeating structure affords stronger heterochiral complex affinity compared to homochiral, we examined hendecad complex formation using ITC. We investigated hendecad coiled coils with a length of three repeats (K_3^{11} and E_3^{11}) as they have a similar number of amino acids to the heptad coiled coils we used previously (33 amino acids for three repeats of the hendecad and 28 amino acids for four repeats of the heptad). First, we confirmed the helical secondary structure of these newly designed hendecad coiled coils using CD spectroscopy (**Figure S27B**). The homochiral titration of L-K_3^{11} into L-E_3^{11} in 1X PBS at pH 7.4 results in an interaction that is initially exothermic (with a maximum binding heat of --46 ± 8 kJ/mol) and becomes endothermic (with a maximum binding heat of 26 ± 1 kJ/mol) at a molar ratio of ~0.5 L-K_3^{11}:L-E_3^{11} (**Figure 5A**), similar to the profile observed for the homochiral interaction of the heptad coils L-K_4^7 and L-E_4^7. The binding heats then trend to zero (after subtracting the dilution heats of injectant into buffer and buffer into titrand) for all molar ratios > 1.6 L-K_3^{11}: L-E_3^{11}, indicating no further interaction. Blending these coiled coils at a 1:1 ratio yields a mixture with a stronger α-helical signal by CD spectroscopy than either individual coiled coil (**Figure S28B**). The heterochiral titration also begins with exothermic binding heats that become endothermic, but this titration has a second exothermically dominated domain at molar ratios > 1.2 D-K_3^{11}: L-E_3^{11} and the binding heats don't trend to zero until molar ratios > 2.5 D-K_3^{11}: L-E_3^{11} (**Figure 5B**). While the maximum exothermic binding heats for the homochiral titration and the two exothermic domains of the heterochiral interaction are similar in magnitude (-46 ± 8 kJ/mol for the homochiral interaction and -46 ± 1 kJ/mol and -44 ± 0.7 kJ/mol for the first and second exothermic domains of the heterochiral interaction), the maximum endothermic binding heats for the heterochiral interaction are much greater than the homochiral interaction (26 ± 0.7 kJ/mol for the homochiral titration and 88 ± 5 kJ/mol for the heterochiral titration). This indicates a stronger binding affinity for the heterochiral interaction of these hendecad coiled coils that mirrors what has been previously reported for Ac-(FKFE)$_2$.[29] Similarly to heterochiral heptad coiled coils, the CD signal for 1:1 D-K_3^{11} : L-E_3^{11} (**Figure S28D**) is close to zero for the wavelength range from 200 nm to 250 nm, due to opposing stereochemistries of the equimolar blend of peptides.

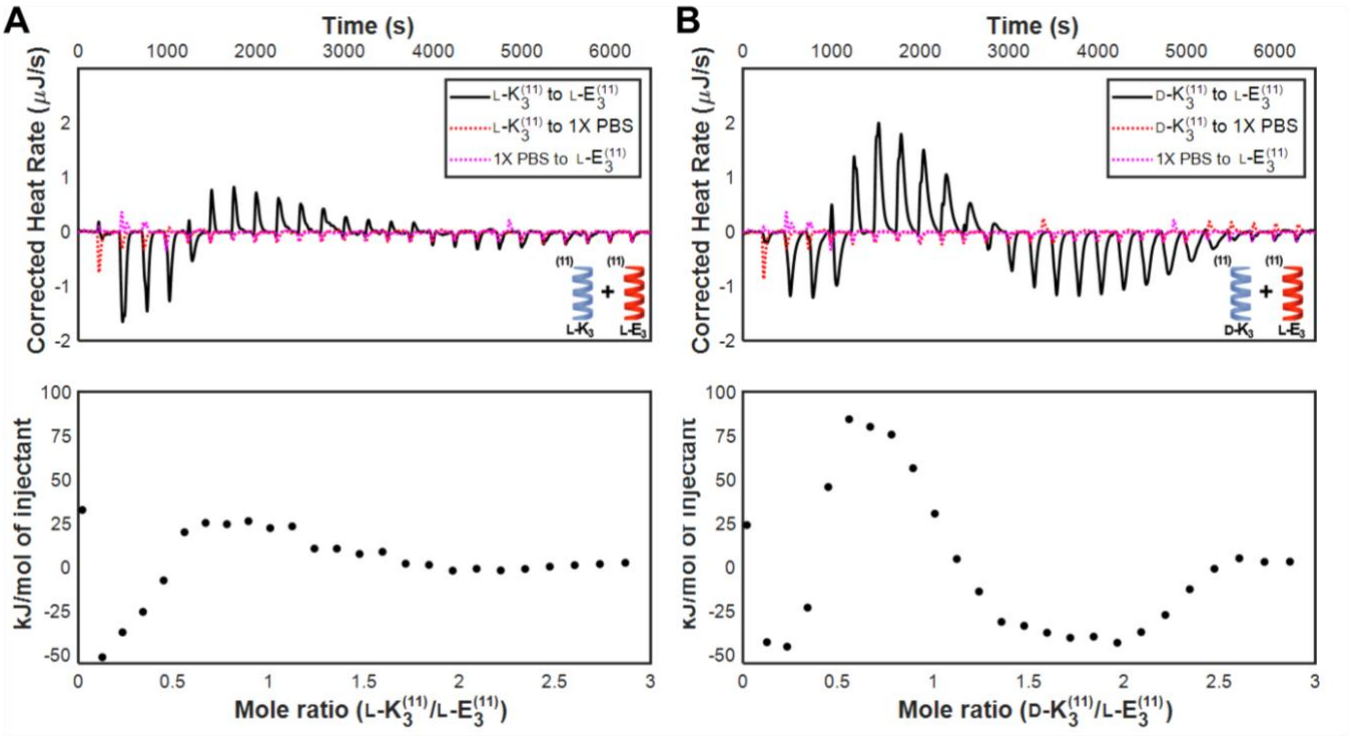

Figure 5. Thermograms and integrated binding heats of: (a) homochiral hendecad coiled coils L-K_3^{11} and L-E_3^{11} in 1X PBS; and (b) heterochiral hendecad coiled coils D-K_3^{11} and L-E_3^{11} in 1X PBS. Larger heats of interaction are observed for the heterochiral complex than for the homochiral complex.

Enzymatic degradation of hendecad coiled coils. Encouraged by the stronger binding affinity we observed for heterochiral hendecad coiled coils compared to homochiral, we next investigated the enzymatic stability of our designed hendecad coiled coils. We incubated solutions of homochiral (L-K_3^{11}:L-E_3^{11}) or heterochiral (D-K_3^{11}:L-E_3^{11}) hendecad coiled coils with 5 μg/mL Proteinase K as we did for the heptad coiled coils (200 μM each in 1X PBS at pH 7.4). Similar to the heptad coiled coils, the hendecad coiled coils elute as two separate peaks, one for K_3^{11} (eluting at 5.9 min) and one for E_3^{11} (eluting at 7.9 min). When incubated in buffer alone without Proteinase K, both the homochiral and heterochiral complexes remain stable over 30 h (**Figure S32**). In the presence of Proteinase K, both L-K_3^{11} and L-E_3^{11} in the homochiral complex degrade completely in under 6 h, with only 11% and 7% of the peak area for each, respectively, remaining after 3 h (**Figure 6A**). On the other hand, 44% of the L-E_3^{11} in the heterochiral complex remains after 30 h of incubation (**Figure 6B**), and we found that even after 7 days of incubation, L-E_3^{11} did not completely degrade as 23% of the peptide still remains (**Figure S33**). As expected, the D-K_3^{11} in the heterochiral complex remains largely stable over the 30 h incubation with Proteinase K. These results demonstrate that forming a heterochiral complex using our designed hendecad coiled coils is an effective strategy to protect a coiled coil in the natural L stereochemistry from degradation.

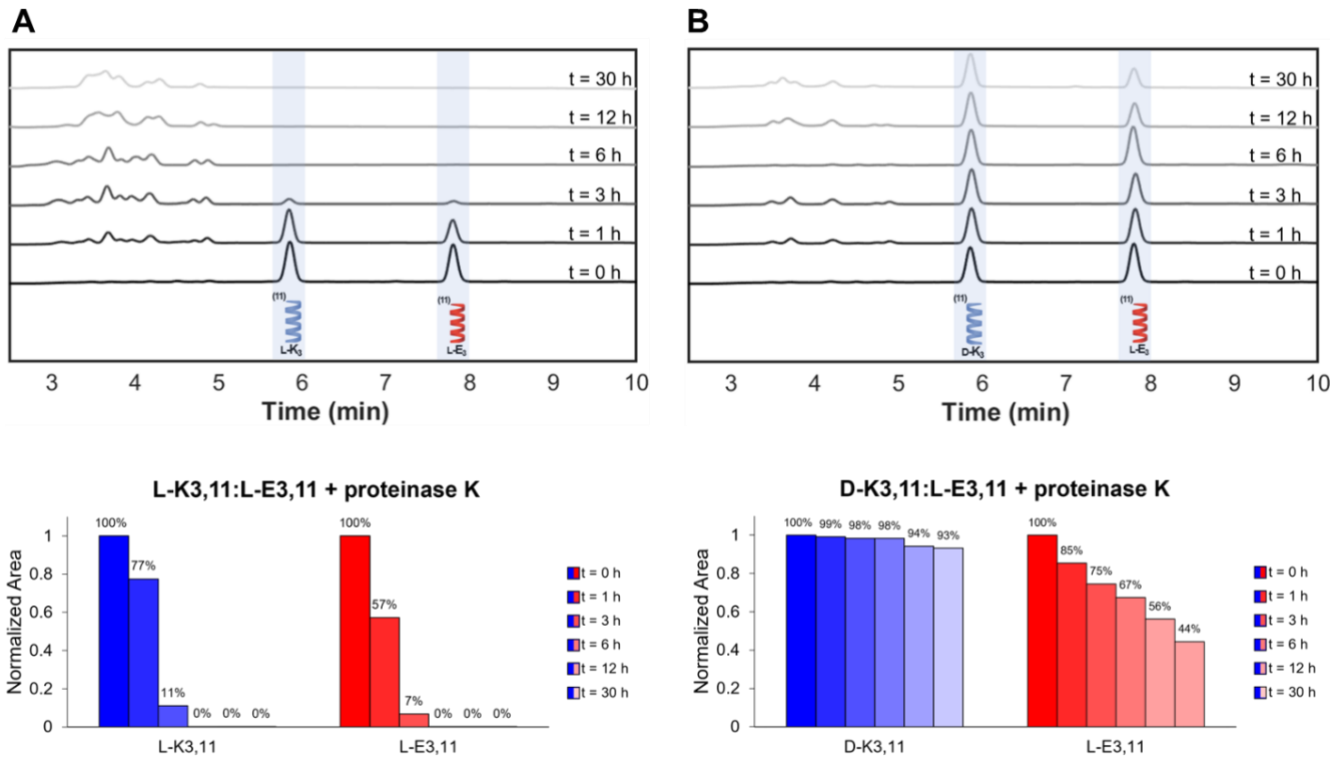

Figure 6. Enzymatic stability of hendecad coiled coils in the presence of 5 μg/mL Proteinase K. HPLC chromatograms and percent intact K_3^{11} and E_3^{11} by peak area immediately after addition of and upon incubation for 1, 3, 6, 12, and 30 h with Proteinase K in A) a homochiral blend and B) a heterochiral blend.

CONCLUSION

This work highlights that peptides can be rationally designed to undergo heterochiral interactions and thereby unlock a larger range of binding affinities and better control over enzymatic stability. Experiments with coiled coils featuring the canonical heptad repeat pattern reveal that they bind stronger as homochiral compared to heterochiral mixtures, but with limited enzymatic stability. Redesigning these heptad coiled coils into noncanonical hendecad repeat patterns enables complexation from both homochiral and heterochiral mixtures, with greater binding strength and enzymatic stability observed for the latter. The consistency between the binding heats observed in ITC and the enzymatic degradation profiles from HPLC throughout the manuscript corroborated the ITC results we observed despite not fitting the ITC data to binding models. We observed that, in cases where the binding heats for one complex were smaller than another, the L-peptides in that complex degraded more quickly in the presence of enzyme, suggesting that such enzymatic stability measurements are a useful tool to assess intermolecular interactions. Going forward, while the design rules for homochiral coiled coils with heptad repeating patterns are relatively well known, continuing to correlate peptide sequence design in both homochiral and heterochiral mixtures of hendecad coiled coils to properties of the resulting complexes will provide important insights that enrich our molecular toolkit for engineering tunable materials.

ASSOCIATED CONTENT

Supporting Information. Materials, experimental details, peptide characterization, additional ITC thermograms, circular dichroism spectroscopy, and additional HPLC degradation experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

V.G. and R.L. conceived the idea and designed the experiments. V.G. conducted experiments and associated analysis.

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ABBREVIATIONS

ITC, isothermal titration calorimetry; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline

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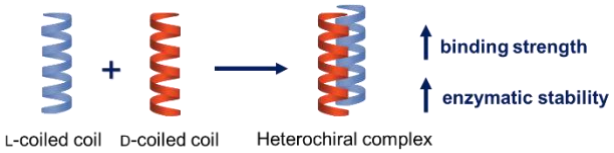
TOC:


binding strength
enzymatic stability
L-coiled coil
D-coiled coil
Heterochiral complex