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De novo design of orthogonal peptide pairs forming parallel coiled-coil heterodimers‡

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We used the principles governing the selectivity and stability of coiled-coil segments to design and experimentally test a set of four pairs of parallel coiled-coil-forming peptides composed of four heptad repeats. The design was based on maximizing the difference in stability between desired pairs and the most stable unwanted combinations using *N*-terminal helix initiator residues, favorable combinations of the electrostatic and hydrophobic interaction motifs and negative design motif based on burial of asparagine residues. Experimental analysis of all 36 pair combinations among the eight peptides was performed by circular dichroism (CD). On the basis of CD spectra, each peptide formed a high level of α-helical structure exclusively in combination with its designed peptide partner which demonstrates the orthogonality of the designed peptide pair set.

Keywords: design; peptide; coiled-coil; orthogonal; parallel; heterodimeric; circular dichroism

Introduction

Structures and functional devices in nature are predominantly based on polypeptides and are far more complex than those based on nucleic acids. Polypeptide design represents a route to new structures and functions with potential properties unseen in nature; however, it requires reliable rules that link polypeptide sequence to structure/function. *De novo* polypeptide design refers to the construction of completely new amino acid sequences with designed structures based on first principles. In *de novo* design, the polypeptide sequence is composed by the designer [1]. There are two important concepts of *de novo* polypeptide design: a positive and a negative design. In the positive design, sequence-to-structure rules are used to direct the formation of the most stable target structure. In comparison, the negative design aims to destabilize the competing unwanted structures, maximizing the energy gap, even at the cost of slightly destabilizing the target structure. Application of these principles has broad implications for the design of specific and therapeutically relevant peptide-based drugs, polypeptides able to act with minimal cross-talk to homologs or analogs, and for the nanobiotechnological design [2].

The coiled-coil is one of the simplest supersecondary-structure motif and one of the most ubiquitous facilitator of protein–protein inter- or intramolecular interactions. These interactions occur either between polypeptide chains or between domains of the same protein, respectively. α-Helical coiled-coil is an attractive choice for the peptide design because the rules governing its structure, oligomerization state and partner specificity are the most developed for any protein-folding motif [3]. Coiled-coils are characterized by a regular repeating unit of seven amino acids labeled with **a-g** (a heptad repeat), with a specific pattern of hydrophobic and hydrophilic residues (Figure 1). Nowadays, the rules of coiled-coil formation are known to a considerable detail [1,4]. Despite this apparent simplicity and similarity at the sequence level, coiled-coils display a considerable degree of structural diversity: helices may be arranged parallel or antiparallel and may form a variety of oligomeric states [5]. Many publications dealing with the design of coiled-coil polypeptides report on the specific and decisive role of each single amino acid residue at specific positions in the heptad repeat [6–9]. Energetically, the most important contribution comes from the coiled-coil hydrophobic core residues at positions **a** and **d.** In general, those positions are occupied by the aliphatic hydrophobic residues (Ala, Ile, Leu, Met, Val), rather than aromatic hydrophobic side chains (Phe, Trp, Tyr) [10]. However, positions **a** and **d** in coiled-coils are not exclusively occupied by the hydrophobic residues, sometimes other residues, like buried Asn [6,7,11,12] or buried Lys [13] can be used to impart dimerization and conformational specificity at the expense of stability. Electrostatic interactions between opposite charged residues at positions **e** and **g** are the second important contributor to the stabilization of coiled-coils, and the main handle to engineer selectivity between paired segments. The Lys residues at positions **g** in one chain and Glu residues at positions **e** in the other chain are expected to form interchain ion-pairs in the heterodimeric coiled-coil structure [14]. The solvent-exposed groups at the remaining positions **b, c** and **f** mainly have to support the helical propensity and can serve as the site to engineer the desired properties into the designed structure.

The consensus is that three to four heptad repeats represent the minimal number of heptad repeats, required for a stable coiled-coil [15–19]. Several algorithms [5] and directed molecular network [20] for identifying and analyzing coiled-coil motifs were

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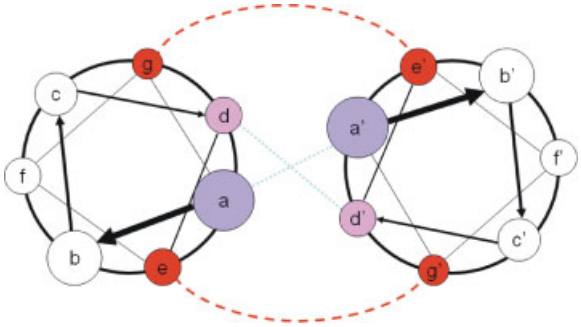

Figure 1. Helical wheel diagram for parallel coiled-coil. The sequence of seven amino acid residues (heptad repeat) is denoted **abcdefg**. Positions **a** and **d** are usually occupied by hydrophobic residues forming the hydrophobic core. Positions **e** and **g** are frequently occupied by charged residues which direct the parallel helix orientation forming interhelical electrostatic interactions. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

developed, and also a database of coiled-coil structures is freely available at http://coiledcoils.chm.bris.ac.uk/ [21]. Mostly, parallel coiled-coils have been designed and the parameters governing the stability of parallel coiled-coils are highly developed [22,23]. However, antiparallel orientation was also investigated and successfully designed [24–28]. In both parallel and antiparallel two-stranded α-helical coiled-coils interchain electrostatic interactions appear to play a major role in polypeptide folding by controlling the parallel or antiparallel alignment [24].

The potential of coiled-coils as building blocks for the assembly of protein structures has been recognized as well as the need to have available a set of orthogonal building blocks to provide the structural versatility. Bromley *et al*. [3] recently reported the design of a set of three orthogonal coiled-coil pairs, consisting of three heptads. As the three heptads are at the lower limit of coiled-coil stability, it would be advantageous to have a set of orthogonal coiled-coil building blocks consisting of four heptad repeats. Additionally, an increased number of heptads increases the potential number of orthogonal pairs.

In the present study, we have successfully designed a set of coiled-coil-forming peptides constituted of four heptad repeats. The negative design, the concept of which is a key to a successful polypeptide design, was applied to prevent the formation of unwanted peptide combinations and alternative coiled-coil topologies. The designed peptides were experimentally tested for pairing affinity as well as characterized in view of chemical and thermal stability using circular dichroism (CD).

Materials and Methods

Synthetic Peptides

Synthetic peptides P1, P2, P3, P4, P5, P6, P7 and P8 were purchased from W.M. Keck Biotechnology Resource Center (New Haven, USA). Stock peptide solutions were prepared by dissolving 5 mg of peptide in 1 ml of 0.1% ammonium bicarbonate with an exception of P2 solution. Peptide P2 was dissolved in distilled water. Stock solutions were diluted with buffer to the required concentration for CD measurements.

Circular Dichroism

CD measurements were performed on a Chirascan CD spectrometer equipped with a Peltier temperature controller (Applied Photophysics, Leatherhead, UK). Peptide solutions were prepared in 10 mM HEPES buffer, at pH 7.0. The concentration of peptides was 25 μM in all experiments. Spectra of individual peptides and peptide pairs were recorded in 1 mm quartz cuvettes (Hellma, Mullheim, Germany) at 20 °C using 1 nm step, 1 nm bandwidth and 1 s sampling. CD spectra of individual peptides in 50% TFE were also determined. CD spectra represented in results are the average of three scans. Chemical denaturation studies were performed at different concentrations of urea and guanidine hydrochloride (GdnHCl). The stability of orthogonal peptide pairs was determined from 0 to 8 M urea and from 0 to 6 M GdnHCl by measuring the ellipticity at 222 nm. Thermal unfolding curves were recorded for orthogonal peptide pairs without denaturant in solution, and in the presence of 1 M GdnHCl, at 222 nm through every 1 °C/min ramps.

Size Exclusion Chromatography

The individual peptides and equimolar mixtures of orthogonal peptide pairs at total concentration of 250 μM were applied to Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden). The separation was performed in 20 mM HEPES buffer, pH 7.0, at a flow rate of 0.5 ml/min, and A_{280} was followed.

Results and Discussion

The aim of the present study was to design a set of peptide pairs that form heterodimeric parallel coiled-coils. Each of the peptides had to satisfy two requirements: it must form coiled-coil exclusively with its designed pairing partner, while any other combination of pairing should be significantly disfavored. In this way the selected set of coiled-coil-forming pairs is called orthogonal peptides. It is recommended to base the design on several different interactions to ensure the specificity of pairing. Therefore, the negative design which made incorrect peptide combinations energetically less stable than the correct combinations, was also applied for the selection of peptide sequences.

We based our design of peptides on the rules that govern the α-helical coiled-coil structure, oligomerization state and partner specificity. Additionally, we also took into consideration the requests for our long-term aim of constructing polypeptide nanostructures, where polypeptides consist of three coiled-coil-forming peptide segments.

Design of Peptide Heptad Interaction Pairs

The construction of designed peptides is shown in Table 1. Peptides are comprised of four heptads with an additional *N*-terminal Ser-Pro-Glu-Asp (SPED) extension and a Gly (G) residue at the *C*-terminus. Leu (L) residue is fixed at position **d** in all heptads, as it favors the formation of coiled-coil dimers and Tyr (Y) is fixed at position **f** of the last heptad to facilitate the peptide detection and quantitation by absorbance measurement.

The *N*-terminal part of coiled-coil has special properties as the capping interactions at the ends of α-helices are important determinants of the stability of the protein's secondary and tertiary structure. The residues stabilizing the *N*-terminus of α-helices have been studied [29,30]. The most frequent residues at the *N*-terminal positions either have special dihedral angles, stabilize helical dipole or they form a hydrogen bond between its side chain and the polypeptide backbone. We selected the tetrapeptide SPED,

Table 1. Sequences of designed orthogonal peptides which form parallel coiled-coils P1-P2, P3-P4, P5-P6 and P7-P8

	Sequence[a]					Hydophobic pattern at positions **a**[b]	Electrostatic pattern of heptads[c]
	SPED	gabc**L**ef	gabc**L**ef	gabc**L**ef	gabc**L**e**Y**	**G**		
P1	SPED	**E**IQAL**E**E	**E**<u>**N**</u>AQL**E**Q	**E**<u>**N**</u>AAL**E**E	**E**IAQL**E**Y	G	I <u>**N**</u> <u>**N**</u> I	EEEE
P2	SPED	**K**IAQL**K**E	**K**<u>**N**</u>AAL**K**E	**K**<u>**N**</u>QQL**K**E	**K**IQAL**K**Y	G	I <u>**N**</u> <u>**N**</u> I	KKKK
P3	SPED	**E**IQQL**E**E	**E**IAQL**E**Q	**K**<u>**N**</u>AAL**K**E	**K**<u>**N**</u>QAL**K**Y	G	I I <u>**N**</u> <u>**N**</u>	EEKK
P4	SPED	**K**IAQL**K**Q	**K**IQAL**K**Q	**E**<u>**N**</u>QQL**E**E	**E**<u>**N**</u>AAL**E**Y	G	I I <u>**N**</u> <u>**N**</u>	KKEE
P5	SPED	**E**<u>**N**</u>AAL**E**E	**K**IAQL**K**Q	**K**<u>**N**</u>AAL**K**E	**E**IQAL**E**Y	G	<u>**N**</u> I <u>**N**</u> I	EKKE
P6	SPED	**K**<u>**N**</u>AAL**K**E	**E**IQAL**E**E	**E**<u>**N**</u>QAL**E**E	**K**IAQL**K**Y	G	<u>**N**</u> I <u>**N**</u> I	KEEK
P7	SPED	**E**IQAL**E**E	**K**<u>**N**</u>AQL**K**Q	**E**IAAL**E**E	**K**<u>**N**</u>QAL**K**Y	G	I <u>**N**</u> I <u>**N**</u>	EKEK
P8	SPED	**K**IAQL**K**E	**E**<u>**N**</u>QQL**E**Q	**K**IQAL**K**E	**E**<u>**N**</u>AAL**E**Y	G	I <u>**N**</u> I <u>**N**</u>	KEKE

[a] The sequences are written in the one-letter amino acid code. Asp residues (N) at positions **a** are bolded and underlined. Charged residues of glutamic acid (E) and lysine (K) are bolded.
[b] N residue is inserted twice in peptide, at the equivalent **a** positions of the same peptide pair.
[c] At positions **g** and **e** of each heptad repeat either acidic E or basic K is inserted.

because it is formed as a consensus of residues at positions N1 – N4 of α-helices. Inclusion of Pro residue at position 2 of the *N*-terminal part and an additional Gly residue as the ultimate residue of each peptide creates the Gly-Ser-Pro tripeptide when we link together the three peptide segments forming the polypeptide building block for self-assembling. We anticipate that this sequence breaks the helical folding and allows the flexibility between coiled-coil segments of the building block.

Further, we designed sequences for four heptads composing a peptide. In order to restrict the number of possible combinations to manageable levels, we limited the variability at defined positions to a limited set of residues that have been previously shown to stabilize the interactions in heterodimers. Our design was based on the rule that hydrophobic residues at position **a** or **d** and oppositely charged residues at positions **e** and **g** between the two helices stabilize, whereas burial of polar residues (Asn) at positions **a** or **d** and the same charge at positions **e** and **g** destabilize the coiled-coil dimer. In this simplified model, we considered a limited number of variable residues only at positions that significantly affect the stability (**a, d, e** and **g**), neglecting the effect on other positions (positions **b, c** and **f**).

The combination of Ile [18] or Asn [1,31] at position **a** and Leu at position **d** appears to be the most favorable for the formation of parallel coiled-coil dimer. Thus, Leu residue was set at position **d** in all heptads, while either Ile or Asn residue was used for position **a**. Pairing of Asn with Asn at position **a** of the target paired heptad acts stabilizing, although not as much as pairing two Ile residues. On the other hand, pairing between Asn and Ile is strongly penalized, which provides an important mechanism of securing the correct pairing. In addition to destabilizing the incorrect parallel pairs, this design feature also prevents the formation of antiparallel heptads, where the Asn at position **a** would be paired with Leu at position **d**. Electrostatic interactions between heptads in the parallel orientation are guided by the interaction between residues at positions **g** and **e** of the matching heptads. The oppositely charged residues stabilize the correct pair, while the residues with same charge at those positions would be unfavorable for the pair formation. At the same time, this design strategy favors heterodimer formation as a result of electrostatic destabilization of the homodimers [31]. Positions **b, c** and **f** are occupied by Gln, Glu or Ala, which have the greatest helix-forming propensity [32] and provide sufficient solubility of the peptide, as well as prevent the formation of aggregates above dimers, which can also be influenced by residues at those positions [1,4].

Identification of a Set of Orthogonal Coiled-Coil-Forming Peptide Pairs

On the basis of the design rules described above, four different arrangements are possible for each heptad – two based on different charged residues at positions **g** and **e**, (same or opposite charge) multiplied by two choices depending on the residue at position **a** (either Asn or Ile). This bases set gave us the combinatorial sequence space for four heptads, which can be selected among the four electrostatic combinations and seven combinations comprising two Asn and two Ile residues. Among the possible combinations, we selected the set of four peptide pairs with the greatest degree of orthogonality in terms of maximizing the energy gap between the designed sequence and any other undesired combinations. However, negative design tends to reduce the intensity of the interaction between two peptides, but significantly more for the undesirable combinations and orientations. Therefore, the energy difference between the desired and undesired combinations is increased.

Accordingly, we designed a set of four coiled-coil-forming pairs and their sequences are presented in Table 1. Each peptide is composed of four heptads. Asn (N) is inserted twice in each peptide at the equivalent **a** positions of the same pair of coiled-coil-forming peptides, differing from the positions in other orthogonal pairs and contributing to the pairing specificity. Thus each peptide at positions **a** of four heptads contains two Asn (N) and two Ile (I) residues, with the arrangement INNI, IINN, NINI and ININ for pairs P1-P2, P3-P4, P5-P6 and P7-P8, respectively. The electrostatic coding used the following arrangement of acidic Glu (E) and basic Lys (K) residues: EEEE, EEKK, EKKE and EKEK for peptides P1, P3, P5 and P7, respectively, and the oppositely charged residues for their complementary pairing partners P2, P4, P6 and P8, respectively.

To predict the coiled-coil interactions of four selected peptide pairs, we examined two algorithms [33,34] and selected the parameters of Hagemann *et al*. [34]. We implemented the parameters of the algorithm to calculate the interaction energy and the stability of pairs depending on the phase within the heptad repeat in both the parallel and antiparallel orientations. Parameters were known only for the parallel orientation; however, the

Journal of PeptideScience

Table 2. Predicted melting temperatures in°C for (A) parallel and (B) antiparallel coiled-coil peptide pairs. T_m for the orthogonal parallel pairs are bolded

(A)

	Parallel P1	P2	P3	P4	P5	P6	P7	P8
P1	33	**100**	29	27	31	32	30	29
P2	–	−6	10	7	11	12	11	9
P3	–	–	10	**93**	19	20	19	17
P4	–	–	–	5	17	18	17	15
P5	–	–	–	–	13	**101**	−15	−16
P6	–	–	–	–	–	16	−13	−15
P7	–	–	–	–	–	–	12	**96**
P8	–	–	–	–	–	–	–	9

(B)

	Antiparallel P1	P2	P3	P4	P5	P6	P7	P8
P1	−62	5	−30	−33	−28	−27	−29	−30
P2		−100	−49	−52	−47	−46	−48	−49
P3			1	−87	−40	−38	−41	−42
P4				−3	−42	−41	−43	−44
P5					−81	7	−39	−40
P6						−78	−37	−38
P7							3	−84
P8								1

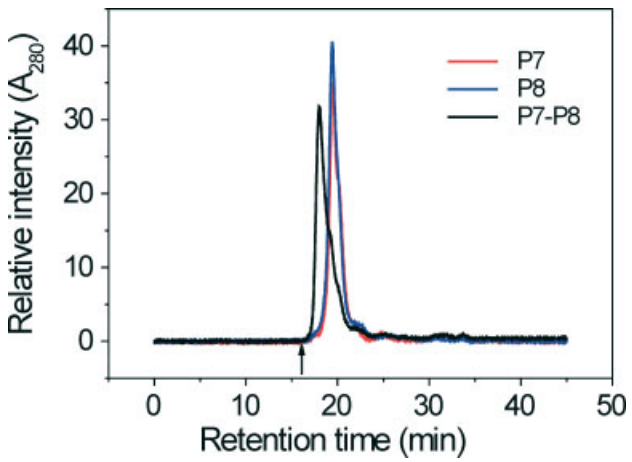

Figure 2. Size exclusion chromatography. Peaks eluting at 19.5 min represent individual peptides P7 and P8, respectively. Peak eluting at 18.0 min represents a heterodimer P7-P8. The elution time of standard lysozyme with molecular weight of 14.4 kDa is labeled with an arrow.

interactions in both orientations are very similar and we expected that the relative stability between the antiparallel arrangements are also reflected from the estimation of stability using the additive contributions of each of the heptads. This allowed us to generate a ranking list based on the calculated interaction energy between all possible combinations of coiled-coil-forming peptides composed of four heptads in both parallel and antiparallel orientation. Predicted melting temperatures based on the parameters from Hagemann *et al.* [34] are presented in Table 2. The result verified the design of the set of orthogonal pairs as the most stable are peptide pairs P1-P2, P3-P4, P5-P6 and P7-P8. The difference in melting temperatures between the least stable orthogonal pair and the most stable undesired pair was predicted to be >60°C. The result also confirmed that our design favored parallel over the antiparallel orientation.

Experimental Characterization of Orthogonal Peptide Pairs

The properties of designed coiled-coil orthogonal pairs were examined for synthetic peptides with respect to the formation of the secondary structure, binding specificity and stability. We used size exclusion chromatography and CD measurements for the analysis of the secondary structure, and in combination with the temperature or chemical denaturation we analyzed the stability of coiled-coils formed by the orthogonal peptides.

Coiled-coil dimers are favored by sequences enriched for Ile at **a** sites and Leu at **d** sites as well as oppositely charged pairs of residues at neighboring **g** and **e** positions [10,32,35,36]. We examined the oligomerization state of equimolar mixtures of designed orthogonal pairs by performing size exclusion chromatography. The results revealed that individual peptides exist as monomers but orthogonal pairs form heterodimers. The chromatogram for P7, P8 and P7-P8, representative for other combinations is shown in Figure 2.

We measured the CD spectra of each individual peptide in buffer (Figure 3A). In both shape and magnitude, these spectra indicated that individual peptides from P1 to P8 are predominantly unfolded and do not form a defined secondary structure, which could occur from homodimers. The CD profiles for individual peptides in 50% TFE (data not shown) acquired the characteristics of α-helical structure since TFE acts as α-helix-inducing solvent. Further, we tested all 28 possible equimolar mixtures of two different peptides. CD spectra of designed orthogonal pairs P1-P2, P3-P4, P5-P6 and P7-P8 confirmed the formation of coiled-coil heterodimers and the minima at 208 and 222 nm indicated that these equimolar mixtures are highly helical (Figure 3B). On the other hand, for the majority of the undesired pairs the molar ellipticity of the mixture was simply the sum of the molar ellipticities for individual peptide (Figure 3C). Only peptide P2 showed weak interactions with other peptides, probably due to its high positive net charge. Maximal CD signal measured for the most stable undesired pair P2-P4 was $<50\%$ of that for the designed pair P1-P2. Therefore, partner specificity have been demonstrated, and nonspecified peptide combinations revealed very little tendency for the formation of coiled-coils.

Urea and GdnHCl denaturation profiles are used to determine the conformational stability of proteins. As shown before, urea and GdnHCl denaturations may give significantly different results of polypeptide stability, depending on the importance of electrostatic interactions for the structural stability of the investigated polypeptide [37]. The reason is in fact that the ionic nature of GdnHCl screens the electrostatic interactions in comparison to the uncharged urea. The urea denaturation profiles in Figure 4A show the highest stability of P1-P2 heterodimer, followed by P3-P4 with midpoint around 6 M, and P5-P6, P7-P8 with midpoints around 3.5 M. The GdnHCl denaturation decreases the differences in the stability between four different pairs, with P1-P2 coiled-coil retaining the highest stability with midpoint of transition at 2.5 M followed by the other pairs with midpoints around 1.5 M (Figure 4B). From these results, we can conclude that electrostatic interactions contribute most interaction energy for the pair P1-P2 followed by P3-P4.

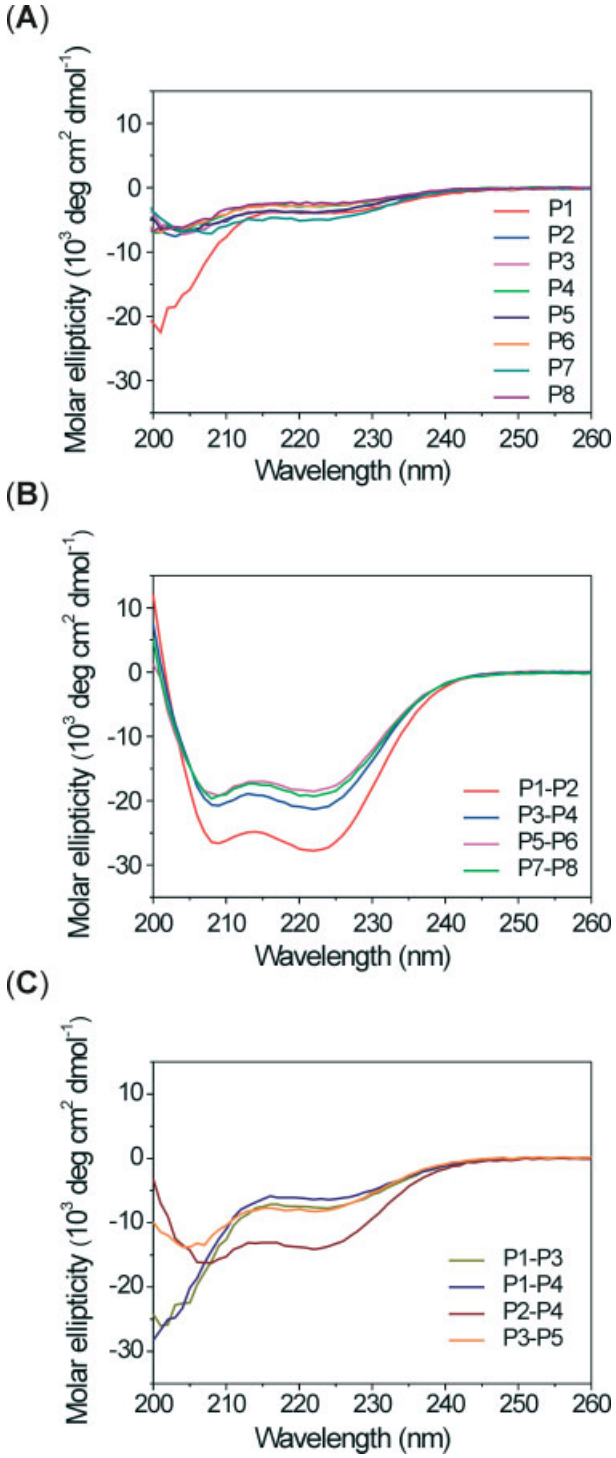

Figure 3. CD spectra of (A) individual-designed peptides, (B) equimolar mixtures of orthogonal peptide pairs P1-P2, P3-P4, P5-P6 and P7-P8, and (C) some nonspecified pairs. The spectra from all the other nonspecified pairwise combinations are intermediate between the curves P1-P4 and P2-P4. Spectra were recorded at 25 μM of each peptide in 10 mM HEPES, pH7.0, at 20 °C.

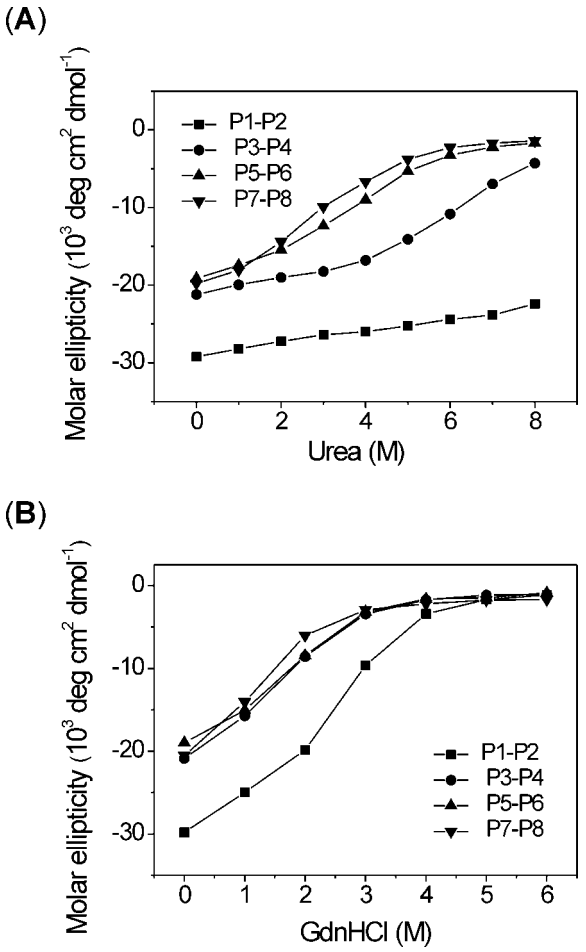

Figure 4. Chemical denaturation curves for the designed peptide pairs. CD signal at 222 nm and 20 °C was determined when peptide pairs were unfolded by adding increasing concentrations of (A) urea or (B) GdnHCl in equimolar peptide mixture in 10 mM HEPES buffer, pH 7.0.

The temperature denaturation curves demonstrate that thermal stability of the orthogonal peptide pairs varies quite considerably. P1-P2 coiled-coil where the thermal transition was not completed at 90 °C is the most stable, and pairs P5-P6, P7-P8 are the least stable with T_m of around 40 °C (Figure 5A). A difference in peptide pair stability was significantly decreased when temperature denaturation was performed in the presence of 1 M GdnHCl (Figure 5B), which again confirms the large contribution of electrostatic interactions for the P1-P2 pair, followed by P3-P4. Therefore, the results from temperature denaturation study are consistent with the chemical denaturation experiments.

The experimental results demonstrate that we succeeded in designing the set of orthogonal parallel coiled-coil dimmers. However, we observed some differences in peptide pairs P1-P2, P3-P4, P5-P6 and P7-P8, which could be used for the assembly of polypeptide nanostructures and to refine the parameters for the prediction of coiled-coil dimer stability. Although the hydrophobic and electrostatic patterns among four heptads in orthogonal pairs possess the equal number of Asn and Glu/Lys residues, respectively, their arrangement appears to be very important as well. We propose that differences observed in the stability of peptide pairs mainly originate in the differences in the electrostatic motif among peptides P1 through P8. The net charge difference between peptides P1 and P2 is by far the largest among all pairs as they are composed of either all acidic or all basic residues at positions **g** and **e** of P1 and P2, respectively. Peptide pair P1-P2 was demonstrated to be the most stable, followed by pair P3-P4 which contains blocks of two basic and two acidic heptads (KKEE pattern). In pairs P5-P6 and P7-P8 heptads border to a greater extent with

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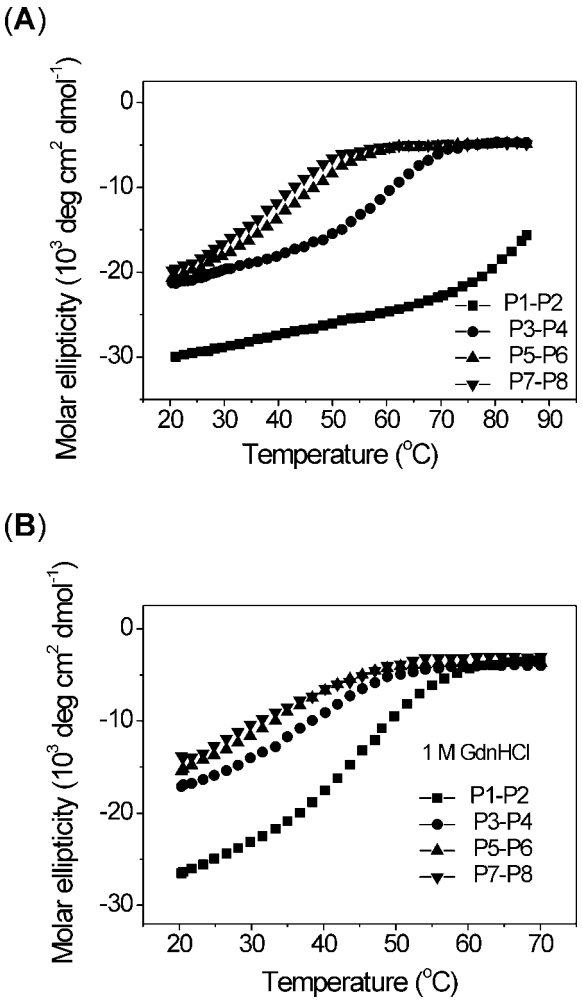

Figure 5. Thermal stability for four designed orthogonal peptide pairs determined from the CD signal at 222 nm. Stability curves were obtained from equimolar mixtures (A) in buffer only and (B) in the presence of 1 M GdnHCl, at 25 μM of each peptide in 10 mM HEPES buffer, pH 7.0.

heptads of the opposite charge (EKKE and EKEK pattern) and these pairs showed to be the least stable. On the other hand, the peptide pair is more stable when the hydrophobic pattern at positions **a** among the four heptads contains Asn residues in close proximity, as it is the case of P1-P2 and P3-P4 (I**NN**I and II**NN** pattern). Results indicate that parameters of the algorithm, whose results are presented in Table 2, must be improved as the stability of pairs P5-P6 and P7-P8 with intermixing charged and hydrophobic heptads is significantly overestimated. In summary, the contribution of each heptad cannot be regarded in complete isolation, because electrostatic as well as hydrophobic interactions of the neighboring heptads also influence the stability of coiled-coils.

In conclusion, we presented a strategy for the design of artificial set of coiled-coil-forming peptide pairs and experimental verification of their characteristics. The peptide set showed the desired partner specificity. This set can therefore be used for the design of self-assembled polypeptide nanostructures comprising parallel heterodimeric coiled-coils. Results of the effect of ionic strength provide experimental conditions for the selection of stability of different coiled-coil pairs.

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