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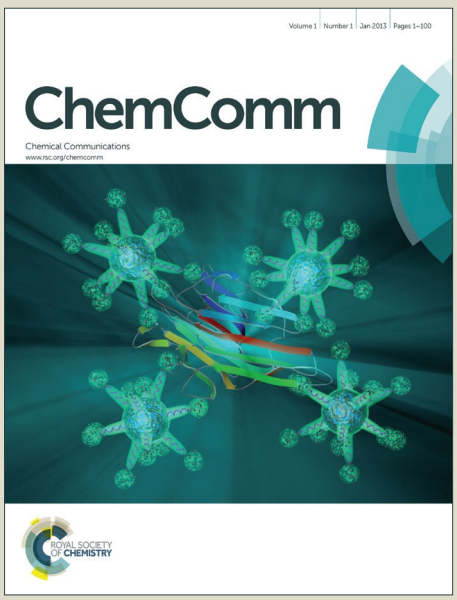

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Effects of Incorporation of Azido Moieties into Hydrophobic Core of Coiled Coil Peptides

Cite this: DOI: 10.1039/x0xx00000x

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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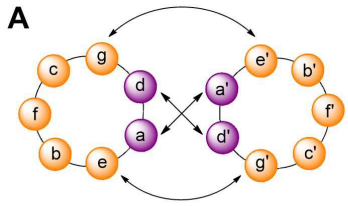
The secondary structure of the coiled coil peptides was regulated by altering the azido content at the hydrophobic core. These peptides were further investigated to form higher-order assemblies presumably via azido-mediated interactions.

Self-assembly of synthetic peptides has been a powerful strategy for in-depth analysis of protein-protein interactions as well as molecular design of biofunctional devices.[1] Among the known structural motifs in peptides and proteins, coiled coils have received a great deal of attention with their potential to program higher-order assembly processes via fine-tuning the peptide foldings.[1e,1f] Various relevant elements have been explored in this context to govern their secondary structures and oligomerization states, which have been further applied to regulate the hierarchical self-assembly into the supramolecular fibrils.[2] These investigations highlight that the morphology of the resulting assemblies can be controlled without specially designed self-assembly signals.[3] Nevertheless, a majority of previous studies have focused solely on the fibril formation and more efforts have yet to be paid to access assemblies with other morphologies.

Beyond applications in organic synthesis[4] and bioconjugation,[5] the azido moiety has gained significant attention with its potential to undergo a wide variety of molecular interactions including hydrogen bonding, electrostatic, and hydrophobic interactions. For instance, both hydrogen bonding and hydrophobic interaction involving the azido group were characterized in the crystal structure of azidothymidine (AZT)/HIV reverse transcriptase complex.[6] Its electrostatic interaction with Arg residues was also proposed from the computational modelling of cyclooxygenase-2 (COX-2)/inhibitor complexes.[7] More importantly, a recent crystallographic study on di-azido-α-cyclodextrin has established three different types of azido-mediated interactions; azido-hydrophobic, azido-azido dipolar, and azido-hydrogen bonding array interactions.[8] Based upon these advancements, we envision that this versatile azido functionality could serve as a potential handle to encode a folding information into coiled coil peptides with a rationally designed hydrophobic core and to form supramolecular self-assembled structures via diverse azido-mediated interactions.

Herein, we present a series of α-helical coiled coil peptides functionalized with azido groups at the core region and characterize their contribution towards peptide secondary structures. We further investigate whether this exquisite functionality can be integrated into a peptide scaffold to form higher-order assembled structures.



C

	b c d e f g a b c d e f g a b c d e f g a b c d e f g a b c
L8	Ac-NH-AQLKKE LQALKKE LAQLKWE LQALKKE LAQ-$CONH_2$
L6Z2	Ac-NH-AQLKKE LQAZKKE LAQZKWE LQALKKE LAQ-$CONH_2$
L4Z4	Ac-NH-AQZKKE LQAZKKE LAQZKWE LQAZKKE LAQ-$CONH_2$
L2Z6	Ac-NH-AQZKKE LQAZKKE ZAQZKWE ZQAZKKE LAQ-$CONH_2$
Z8	Ac-NH-AQZKKE ZQAZKKE ZAQZKWE ZQAZKKE ZAQ-$CONH_2$

Fig. 1 A) Helical wheel representation of a parallel coiled coil dimer. B) Structure of azidonorvaline (**Z**). C) Amino acid sequences of the designed peptides.

Five peptides have been designed by implementing azido-containing amino acids at the hydrophobic core (*a* and *d* positions) on the basis of a previously reported α-helical coiled coil peptides[9] (Fig. 1). The design specifies the self-association entirely in a parallel fashion through a network of charge pair interactions at *e* and *g* positions.[10] In general, this hydrophobic core is highly conserved and expansive, conferring the major structural determinant for coiled coil helices. As such, we hypothesized that deploying azido groups at the core would facilitate the control of peptide conformations. Particularly in **L6Z2** and **L4Z4**, we incorporated azidonorvaline (**Z**) exclusively at *d* positions in such a way that these residues remain open to interactions throughout the whole hydrophobic interface upon parallel self-association. In addition, charged residues were excluded from the core region to eliminate any other interfering factors for the core interaction. One

tryptophan residue was introduced for an accurate measurement of peptide concentrations by a specific absorbance at 280 nm.

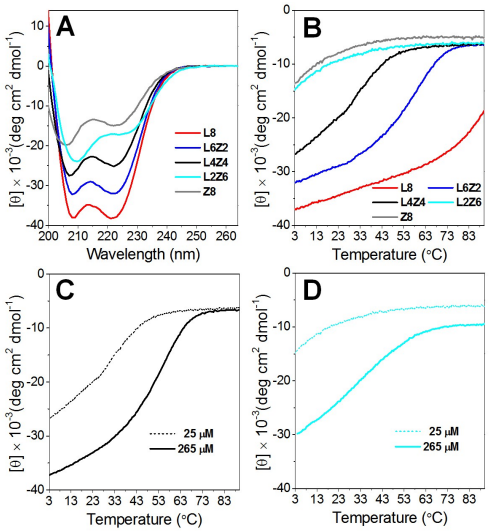


Fig. 2 (A) CD spectra of all peptides at 25 μM (3 °C). (B) Thermal denaturation profiles of all peptides at 25 μM. Thermal denaturation profiles of (C) **L4Z4** and (D) **L2Z6**.

Although organic azides share the similarities with organic halides in respect to electronegativity and hydrophobicity,[11] it remains elusive how substantially they contribute to the overall hydrophobicity and core packing capacity of the α-helical coiled coils. Hence, circular dichroism (CD) measurements were carried out to characterize the secondary structures of the peptides. The CD traces at 3 °C indicated that **L8**, **L6Z2**, and **L4Z4** (c = 25 μM) adopted the typical α-helical profiles with maximum helical contents of 100, 84, and 70 per cent, respectively (Fig. 2A). Cooperative thermal unfolding transitions were further identified with distinct melting temperature (T_m) values of 89, 52, and 25 °C for **L8**, **L6Z2**, and **L4Z4**, respectively (Fig. 2B). On the other hand, **L2Z6** and **Z8** displayed significantly disrupted α-helices with a limiting helical content of 44 and 35 per cent, respectively, and their T_m values were not measurable within the temperature range of the experiment due to low thermal stabilities (Fig. 2A and 2B). Although these CD studies showed that replacing leucine (**L**) with **Z** at the hydrophobic core caused a loss of α-helical stability, it is noteworthy that even four units of **Z** can be accommodated to retain the folding of amphiphilic coiled coil peptides, highlighting its considerable hydrophobic nature. Collectively, these results shed light on the premise of **Z** residue to enable systematic variation in α-helical stability.

A sufficient peptide concentration is required to adopt a defined conformation of α-helices[12] and thereby to trigger the self-assembly process. As such, the peptides were further inspected at higher concentration (265 μM) by CD. The effects of concentration on folding and oligomerization of peptides were significant. **L6Z2**, **L4Z4,** and **L2Z6** exhibited markedly higher α-helicities and thermal stabilities in comparison with the values at 25 μM: T_m values of 69, 49, and 25 °C for **L6Z2**, **L4Z4,** and **L2Z6**, respectively (Fig. 2C and 2D, Fig. S8 and S10 in SI). It is important that the ratios of mean residue ellipticities (MREs) at 222 to 208 nm ($\theta_{222}/\theta_{208}$) at 3 °C were higher than 1 (1.04, 1.06, and 1.1 for **L6Z2**, **L4Z4**, and **L2Z6**, respectively), indicating highly interacting coiled coil conformations (see SI, Fig. S9, S12, and S13).[10,13] In contrast, CD spectra of **Z8** displayed a red shifted minimum at 225 nm and complete disappearance of the band at 208 nm, which is a characteristic CD profile for meso- or macroscale assembly systems as a result of the light scattering (See SI, Fig. S11).[14] Nevertheless, as indicated by CD, the wholesale replacement of **L** with **Z** (**Z8**) resulted in a substantial loss of pre-organized peptide secondary structure with a minimal α-helical content even at 265 μM, which would limit the control over the morphology of resulting ensembles. Hence, we chose **L6Z2**, **L4Z4,** and **L2Z6** as suitable candidates for further investigation, as well as **L8** as a control for comparison.

Dynamic light scattering (DLS) experiments were employed to facilitate the real-time analysis of aggregation states in solution (Fig. 3A, 3B, and see SI, Fig. S14). With the concentration of 265 μM, **L8**, **L6Z2**, and **L4Z4** were incubated respectively at 3 °C for 7 days. Measurements for **L8** suggested the assembly of two peptide strands with a mean hydrodynamic radius (D_h) of 4.8 nm, which is in good agreement with the previous report in that **L8** was designed to favor exclusively dimeric coiled coils.[9] DLS measurements for **L6Z2** also showed a major peak at 4.8 nm, indicating dimeric coiled coils. Interestingly, we also observed a secondary peak with mean D_h of 162 nm, highlighting the potential of azido group in higher-order self-assembly. Meanwhile, two particle size distribution (PSD) peaks were identified in the case of **L4Z4** with mean D_h values of approximately 125 nm and 1.7 μm, indicating the coexistence of small and large aggregates. Prolonged incubation up to 30 days resulted in the disappearance of a peak at 125 nm and the convergence to the larger aggregates, implying a sequential aggregation pathway: the formation of primary aggregates followed by formation of large particles extending out to 1.7 μm. The differences between **L8, L6Z2** and **L4Z4** in the assembly behavior clearly illustrates the ability of azido groups to assemble the individual coiled coil modules into higher-order constructs by its interaction with other components.

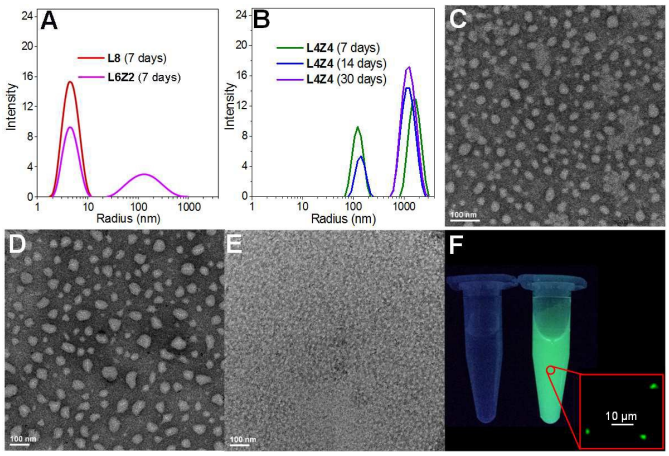


Fig. 3 (A) Hydrodynamic size distribution by DLS of (A) **L8** and (B) **L4Z4**. TEM images of (C) **L4Z4** (3 °C, 7 days), (D) **L4Z4** (3 °C, 14 days) and **L4Z4** (14 days at 3 °C and then 15 hr at 70 °C) from different incubation conditions. (F) The aggregates from **L4Z4** before (left) and after (right) coupling to FITC under UV light (inset: confocal microscopy image of FITC-conjugated **L4Z4**).

Transmission electron microscopy (TEM) was used to visualize the shape of the assembled **L4Z4**. After 14 days of incubation at 3 °C, spherical globules were identified with a diameter of 80 nm (Fig. 3D), which was smaller than those from DLS measurements presumably due to the different

specimen states, *e.g.* dried vs. hydrated. Nevertheless, these results are noteworthy because most of coiled coils tend to self-assemble into fibers unless they are designed to form globules with specific assembly signals.[3] We assume that this morphological feature arises from the radial association of α-helical strands via the combination of azido-azido interactions and azido-mediated hydrogen bonding with potential hydrogen bonding donors at peripheral positions, such as glutamine and lysine. To further clarify the size discrepancy between TEM and DLS measurements, the aggregates obtained from incubation for 14 days at 3 °C were labeled with fluorescein isothiocyanate (FITC) and examined by confocal fluorescence microscopy (Fig. 3F). Interestingly, the globules remained intact and the particle sizes were identified with diameters of approximately 1–2 μm, consistent with DLS measurements. In addition, the peptide solution was stood at 70 °C for 1 day to investigate the thermal stability of these globules (Fig. 3E). The globules were completely deformed upon heating. Notably, similar to the observations for **L8** and **L6Z2** (see SI, Fig. S15 and S16), no structural transition to β-sheet but simple unfolding was observed upon heating and prolonged incubation, as indicated by CD spectra, highlighting the α-helical conformational stability of **L4Z4** (see SI, Fig. S17).

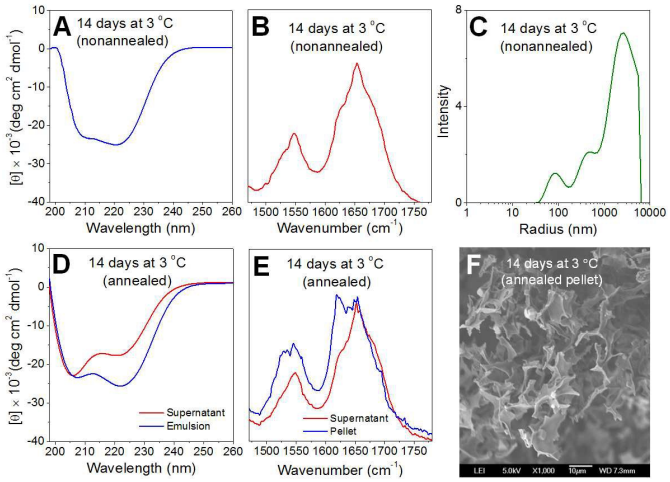


Fig. 4 (A) CD spectrum, (B) FT-IR spectrum, and (C) hydrodynamic size distribution by DLS of **L2Z6** without preheating, (D) CD spectrum, (E) FT-IR spectrum, and (F) SEM image of **L2Z6** with preheating.

We next evaluated the assembly of **L2Z6**, which possesses less helical content but more azido content than **L4Z4**. After 14 days of incubation at 3 °C, despite preservation of initial α-helical character as indicated by CD and Fourier transformed infrared (FT-IR) analyses (Fig. 4A and 4B), a wide particle size distribution by DLS measurements indicated relatively disordered aggregates in distinction from those of **L4Z4** (Fig. 4C). The observed dissimilarity in assembly behavior between **L4Z4** and **L2Z6** may arise from the difference in α-helical content at 3 °C: 98 and 68 per cent for **L4Z4** and **L2Z6**, respectively and/or difference in azido content. When the peptide solution was heated at 90 °C for 30 min followed by 14 days of incubation at 3 °C, white precipitates were formed during the incubation. As depicted in Fig. 4E, the precipitates were further characterized by FT-IR analysis as mixed conformational aggregates of α-helix (1650 cm^{-1}) and β-sheet (1621 cm^{-1}).[15] Notably, the scanning electron microscopy (SEM) analysis of these aggregates after lyophilization revealed the unique morphological feature of 2-dimensional sheet-like microstructure (Fig. 4F). These results show that the azido groups at the hydrophobic core of the coiled coils modulate the degree of α-helical stability and lead to different types of aggregates presumably via azido-mediated intermolecular interactions. However, the heterogeneity in peptide conformation and morphology has limited the mechanistic understanding underlying this assembly process.

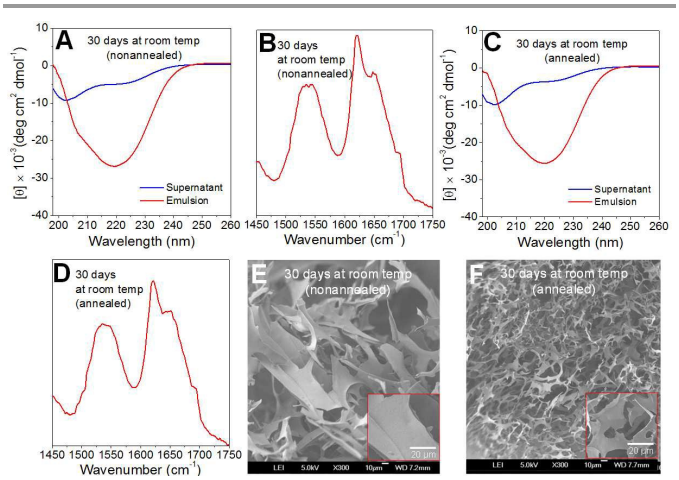


Fig. 5 (A) CD spectrum and (B) FT-IR spectrum of **L2Z6** without preheating, (C) CD spectrum and (D) FT-IR spectrum of **L2Z6** with preheating, (E) and (F) SEM images of **L2Z6** without preheating.

The transition from α-helix to β-sheet has been previously observed in the case of a long-term incubation at ambient temperatures.[16] Therefore, we investigated how significantly the β-sheet conformation could affect the morphology of the aggregates. When **L2Z6** was subject to incubation for 30 days at room temperature with or without preheating for 30 min at 90 °C, SEM images revealed the formation of 3-dimensional interconnected network for both annealed and non-annealed samples (Fig 5E and 5F). Importantly, further inspection of these microstructures demonstrated larger and wider 2-dimensional sheets than those at 3 °C (Fig. 5E and 5F). CD spectra of the suspended sample solutions displayed a minimum at 220 nm as well as the complete disappearance of a band at 208 nm (Fig. 5A and 5C). Although it was a typical CD profile for β-aggregates, we could not completely rule out the possibility of highly aggregated α-helical systems due to their spectral similarities.[3c,14] Hence, we further examined the freeze-dried **L2Z6** pellet by FT-IR to elucidate its secondary structure. The spectrum showed a distinct peak at 1621 cm^{-1} that is characteristic of intermolecular β-aggregates, along with only minimal band at 1650 cm^{-1} associated with α-helices.[15] It is clear that the observed 2-dimensional morphology of the assembled **L2Z6** was mainly attributed to β-structure organization (Fig. 5B and 5D). The observed morphological feature is distinct from the previous reports that the conformational transition from the α-helical structure into a β-sheet-rich isoform tends to trigger the formation of amyloid fibers.[16,17] Importantly, these results demonstrate that the variation of the azido content at the hydrophobic core of coiled coils can affect α-helical stability and secondary structure formation, which leads to the distinct modes of peptide aggregation.

In summary, we investigated the self-assembly of coiled coil peptides by controlling the azido content at the hydrophobic core. The increase of the azido content modulated the secondary structure of coiled coils involving conformational transition from α-helix to β-sheet. The azido functionality also directed a self-assembly process, presumably due to its ability to facilitate intermolecular interactions. Notably, we observed two morphologically distinct self-assembled structures, spherical globules (**L4Z4**) and 2-D sheets (**L2Z6**) depending on the number of azido groups in peptides. We envision that our findings facilitate further explorations of the azido functionality in the field of supramolecular assemblies.

We gratefully acknowledge financial support by the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore).

Notes and references

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† Electronic Supplementary Information (ESI) available: General peptide synthesis, procedures for CD measurements including spectra, DLS, TEM, FT-IR, and SEM measurements, confocal microscopy measurements including labelling of **L4Z4** aggregates with FITC. See DOI: 10.1039/b000000x/

‡ These authors equally contributed to this work.

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