Evaluation of Steric Entanglement in Coiled-coil and Domain-swapped Protein Interfaces using 3D Printed Models

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July 20, 2022

Abstract

Oligomeric protein interfaces involve non-covalent attractive forces plus potential steric entanglement. 70 years ago, Crick proposed a "Knobs in Holes" model for coiled-coil protein interfaces. Subsequently, modifications to this model have been proposed, describing either a "leucine zipper", "jigsaw puzzle", or a "peptide Velcro" interface. These principally describe forms of steric entanglement that may enhance oligomer stability; however, such entanglement has not been rigorously evaluated since it is not possible to experimentally eliminate intrinsic noncovalent attractive forces. 3D printing provides a novel means to evaluate steric entanglement of protein interfaces in the absence of attractive forces. Surprisingly, quantitation of the energy required to dissociate various coiled-coil protein interfaces of 3D printed protein models suggests minimal steric entanglement. Conversely, evaluation of domain swapped interfaces of symmetric protein oligomers, differing by circular permutation, identifies extensive potential steric entanglement. Combined with available experimental data, the results suggest that steric entanglement of a protein interface can contribute to kinetic trapping of both folding and unfolding pathways. Steric entanglement of protein interfaces is therefore postulated to be an undesirable property for naturally evolved and designed protein oligomers.

INTRODUCTION

Crick proposed a "Knobs in Holes" model for the packing interface of adjacent α-helices in the coiled-coil oligomeric assembly of keratin, noting that an ~20° rotation of the axis of one helix would intercalate its side chains ("knobs") into surface cavities ("holes") that exist between the side chains in the other helix[1,2] (Fig. 1). This interaction results in left-handed supercoiling of the duplex, reducing the residues per turn from 3.6 to 3.5, and altering the periodic repeat of amino acids in the helix from 18 to 7. Thus, supercoiling yields a heptad repeat of amino acids forming the coiled-coil interface. This interface is largely solvent-excluded, and hydrophobic side chain patterning conforming to a heptad repeat would therefore promote coiled-coil assembly. Structural details of the knobs and holes described by Crick were limited to a description involving simple cylindrical shapes; however, a potential "systematic interlocking" of the knobs in holes was noted by Crick (although it is unclear whether this referred to molecular complementarity or steric entanglement). A model for coiled-coil interactions was provided by Crick for a hypothetical three-stranded coil, but indicated no obvious steric entanglement [2].

Richmond and Richards [3] undertook a geometric analysis of α-helical packing in sperm whale myoglobin. An area of interest was in protein unfolding; specifically, that if the reaction coordinate of unfolding is the reverse of folding, then understanding possible movements of interacting α-helices can identify the most plausible folding/unfolding reaction coordinate. They reported that steric interactions between adjacent side chains oppose shear and torsional movement of packed helices; however, separation of helices in a direction normal to the helix axis is not restricted by any interlocking of side chains (such movement is opposed by non-covalent attractive forces). Thus, the folding/unfolding reaction coordinate was postulated to be principally associated with translational movement normal to the helical axes.

A heptad hydrophobic repeat is the quintessential feature shared by all proteins that adopt a coiled-coil structure [4-6]. Due to the γ-branched nature of leucine side chains Landschulz and coworkers proposed that the leucine sidechains from one helix interdigitate with those of the second helix, forming a "molecular zipper"[7]. This interdigitation of leucine was postulated to "lock" the two helices together in a form of steric entanglement referred to as a "leucine zipper" (Fig. 2).

Kim and coworkers [8] explored the role of electrostatic charges in the promotion of heterodimeric coiled-coil α-helices. They presented evidence that design of favorable heterodimeric assembly can be achieved by destabilization of specific homodimeric interactions. The resulting heterodimeric helices were described as "peptide Velcro" since the "individual peptides have little self-affinity, but high affinity for each other". However, this study pointed out that affinity for the heterodimeric peptides can be negatively affected by unfavorable electrostatics. This result suggests a limited role for steric entanglement, and a greater role for molecular complementarity and favorable charge interactions. Thus, use of the term "Velcro" appears inappropriate, since the basis of Velcro interactions is exclusively steric entanglement and does not involve any attractive forces (Fig. 2).

Efimov [9] evaluated the knobs in holes helical interface model using a purely mathematical perspective. Efimov used the term "jigsaw puzzle" to describe the structural complementarity of the hydrophobic amino acids comprising the packing interface between helices, stating "There is an exquisite complementarity between the hydrophobic stripes of the α-helices that fit together like pieces of a jigsaw puzzle". Jigsaw puzzle pieces have no attractive forces and are sterically entangled through a dove-tail type interface (Fig. 2). The relative contribution of non-covalent attractive interactions versus steric entanglement intended by the jigsaw puzzle descriptor was not provided (although it is suggestive of principally steric entanglement).

One of the original reports of "domain-swapping" protein interfaces describes how monomeric diphtheria toxin converts to a stable dimeric oligomer in response to freezing in acidic pH [10]. The dimer has a swapped subdomain of 15 kDa and is described as "intertwined" and "entangled". It was postulated that the domain-swapped form may be less stable than the monomer, but kinetically trapped at neutral pH due to physical entanglement. The dimerization of bovine seminal ribonuclease involves similar swapping of a relatively short region of 15 residues; while this may not comprise a structural domain, it meets the definition of domain-swapping[11,12]. Tawfik and coworkers [13]described the de novo design of β-propeller lectins by tandem duplication of repetitive units termed "blades" (a four-stranded antiparallel β-sheet). The repeated modules comprise three strands of one "blade", plus one strand of the following blade. The resulting N- and C-termini interactions meet the definition of domain-swapping (as defined by Eisenberg) and were described as "Velcro-like interactions", suggesting a steric entanglement. Tame and coworkers[14] reported the computational design of a symmetric β-propeller protein based upon the sensor domain of a protein kinase from Mycobacterium tuberculosis. This protein architecture is a 6-bladed propeller where the last β-strand completes the first propellor motif in a domain-swapped arrangement that was characterized as a "Velcro strap".

Overall, in studies of both coiled-coil and domain-swapped protein oligomeric assemblies, commonly utilized descriptors for the interface include "zipper", "Velcro", and "jigsaw". These terms fundamentally describe steric entanglements (Fig. 2), which can potentially oppose shear, rotation, and translational movement of interacting surfaces with no reliance upon any attractive force. Despite their widespread adoption, the precise meaning of such terms as regards the relative importance of non-covalent attractive forces versus steric entanglement is ambiguous. If non-covalent attractive forces could be "switched off", the potential role of entanglement alone could be evaluated. However, it is not possible to experimentally eliminate attractive forces; furthermore, the elimination of attractive forces would promote loss of structure essential for assembly. However, 3D printed models offer the possibility of accurate molecular models that enable evaluation of steric entanglement in the absence of any attractive forces.

In the present report we describe 3D printing of various coiled-coil structures as well as different domain-swapped permutations of a de novo designed oligomer form of a symmetric β-trefoil protein. The kinetic energy required to dissociate these complexes is also quantified. While the coiled-coil complexes exhibit a structural complementarity at the interface, which opposes shear and rotation, there is minimal evidence of

steric entanglement to prevent translation normal to the axis of the helix. In circular permutations of the oligomeric β-trefoil protein, there are varying degrees of steric entanglement depending upon the details of the domain-swapped region. There is evidence of an inverse correlation between extent of entanglement, level of protein expression, and folding cooperativity. The results suggest that the presence of steric entanglement is a barrier to both folding and unfolding, consistent with a kinetically trapped intermediate, and may be largely eschewed in natural protein oligomeric interfaces.

METHODS

Protein Data Bank (.pdb) format files for poly leucine α-helices (28-mer), with either all *trans* or all *gauche* + rotamers, were generated using the BIOVIA Discovery Studio Visualizer software (Dassault Systèmes, San Diego). PDB files for GCN4 leucine zipper (1ZIK), 14-3-3 protein (2BQ0), Symfoil (3O4D) and Monofoil (3OL0) are from the RCSB database [15]. All files were stripped of HETATOMs (i.e., waters and ligands). In the case of 14-3-3 the interface domain region relevant to the A domain was identified as residue positions A145-A206, and those for the B domain were identified as B3-B54/B98-B127 and these were the 3D printed regions (the B domains being monolithic). All residues for the GCN4 leucine zipper 30-mer were retained, as was the 42-mer region of the Monofoil polypeptide in the trimer oligomer forming a β-trefoil fold. Domain-swapped permutants of Monofoil [16] were generated using corresponding internal 42-mer regions of the Symfoil (i.e., intact β-trefoil) structure (Fig. 3).

Standard Tessellation Language (.stl) files were generated for all structures using the Chimera software package [17]. Corresponding G-code files were generated using IdeaMaker software (Raise3D, Irvine CA) using standard van der Waals radii and a scale of 2.8 mm/Å. Molecules were 3D printed using a Raise3D Pro2 printer (Raise3D, Irvine CA) and 1.75 mm Flexmark 9 (Sigma Aldrich, St. Louis MO) thermoplastic polyurethane (TPU) filament (Durometer hardness of 90, elongation at break of 600%, and tensile strain of 55 MPa). Models were printed with 25% infill (grid pattern), three surface shells, and removable supports for regions of overhang. Printed models were stripped of supports, and weighed (with duplicate printed models typically exhibiting a mass difference within 1-2 g). Weight was used for quality control as printing nozzle obstruction yielded models of 5-10 g lower mass, weakened integrity, and reduced kinetic energy upon impact. An example of a 3D printed model, with supports trimmed, is given in Fig. 4.

Kinetic energy was imparted to models via an essentially elastic collision by fall from defined height onto a concrete surface. Ten repetitions were performed at each evaluated height, with essentially random model orientation. Drop heights varied from 5.0 cm to 12.0 m. At each height the number of repetitions resulting in model dissociation was noted and the fraction folded calculated. Drop heights were increased until the fraction folded yielded 0.0 (i.e., dissociation observed in all 10 trials) for each model. Kinetic energy was calculated as mass*g *height (g =9.8 m/s^2) and the imparted energy (J) was normalized for total model mass (J/Kg).

RESULTS

3D printed model properties

Standard polylactic acid (PLA) filament used in 3D printing proved too brittle to enable assembly of Monofoil (and domain-swapped) oligomers without fracturing. Thermoplastic urethane (TPU) filament was therefore used for 3D printing of all molecular models. The flexibility of TPU permitted interface adjustments that tended to promote structural complementarity of all models. Analysis the printed models shows that for the scale (2.8 mm/Å) and printing properties utilized, the models had an average mass of 1.79±0.11 g/amino acid (Table 1).

Cooperativity of model dissociation

For models that demonstrated steric entanglement, plots of the fraction folded versus kinetic energy exhibited cooperative unfolding behavior (Fig. 5). The midpoint of the unfolding was determined at the fractional folding value of 0.5.

Πολψλευσινε α-ηελιξ σοιλεδ-σοιλ ιντερφασε

Molecular complementarity was discernable for all coiled-coil interfaces, with 20° rotation, for each of the polyleucine α-helical models (confirming knobs in holes interface). Local steric interactions resisted shear parallel to the helical axes and rotation normal to the helical axes; however, there was no discernable steric entanglement preventing translation normal to the helical axis. Even the lowest drop height evaluated (5 cm) resulted in complete unfolding (i.e., fractional folding = 0.0) (Fig. 5). The exceptions to this general characteristic were observed only for the antiparallel *gauche+/gauche+* rotamer pair (dissociation midpoint of ˜1 J/Kg), and the antiparallel *trans/trans* rotamer pair (dissociation midpoint of ˜4 J/Kg).

GCN4 and 14-3-3 coiled-coil oligomer interfaces

Molecular complementarity was discernable for the GCN4 and 14-3-3 coiled-coil oligomeric interfaces. Local steric interactions resisted shear parallel to the helical axes and rotation normal to the helical axes; however, there was no discernable steric entanglement preventing translation normal to the helical axis (i.e., separation occurred under the effect of their own weight).

Monofoil and permutant domain-swapped interfaces

Varying degree of steric entanglement was exhibited by Monofoil and circularly permuted forms. Monofoil exhibited the least steric entanglement, with a dissociation midpoint of ˜1.0 J/Kg (like the antiparallel *gauche+/gauche+* coiled coil). Permutant P3 exhibited an entanglement midpoint of ˜4 J/Kg (like the antiparallel *trans/trans* coiled coil). Permutant P1 exhibited a greater entanglement midpoint of ˜7 J/Kg. Permutant P2 was remarkable in having an entanglement energy that prevented dissociation even at the highest evaluated energy of 120 J/Kg) (Fig. 5).

CONCLUSIONS

Evaluation of the dissociation of 3D printed models of polyleucine coiled-coils provides scant evidence for steric entanglement of the interface, with measurable entanglement occurring only with antiparallel coiled-coils. Parallel coiled-coils are more common than antiparallel coiled-coils and formation of antiparallel coiled-coils has tended to rely upon designed cysteine mutations and enforced disulfide bonds, or specific charged/polar interactions in antiparallel vs. parallel coiled-coil orientations [18]. The analysis of 3D printed coiled-coils supports the geometric analysis of Richmond and Richards [3] in that steric interactions between adjacent side chains oppose shear and torsional movement of packed helices, but separation normal to the helical axis is unimpeded by any steric consideration (it would be opposed principally by non-covalent attractive forces). Thus, the "zipper", "jigsaw", and "Velcro" descriptors (implying steric entanglement, Fig. 2) for the polyleucine coiled-coil interface are unsupported by these results.

Our initial expectation in evaluating potential entanglement associated with alternative domain-swapped definitions of the trefoil motif trimer was that the wild-type Monofoil definition of N- and C-termini would yield the greatest entanglement. This was based upon the hypothesized role of enhanced stability afforded by domain swapped entanglement in symmetric protein architecture [14,19-21]. However, the wild-type Monofoil definition yielded the least entanglement. Expression and characterization of the stability and folding properties of Monofoil and the domain-swapped polypeptides, described herein, have previously been reported [16]. We note there is a general decrease in the level of protein expression upon increased steric entanglement (Table 2). The inability of the permutant #2 3D model to disassemble despite input of substantial kinetic energy indicates a high energy barrier to unfolding, and therefore, the potential for kinetic trapping of both unfolding and folding (Fig. 6). The inhibition of efficient expression in response to increased entanglement is consistent with this interpretation.

The general avoidance of interface entanglement with 3D printed models of polyleucine helices GCN4 and 14-3-3 coiled-coils, and the lowest entanglement being exhibited by the natural termini definition of the Monofoil trimeric oligomer (compared to all domain-swapped alternatives), indicates that entanglement at oligomeric interfaces is likely to be selected against in protein evolution. Thus, the present work suggests

that the de novo design of oligomeric interfaces should avoid steric entanglement as a strategy to enhance stability due to the potential negative consequences of kinetic trapping of the folding/unfolding pathway.

SUPPLEMENTARY MATERIAL

N/A

ACKNOWLEDGEMENTS

Research support from Trefoil Therapeutics Inc. and FSU College of Medicine is acknowledged.

CONFLICT OF INTEREST STATEMENT

MB is a cofounder and has equity ownership in Trefoil Therapeutics Inc.

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TABLES

Table 1. Mass of 3D printed models

Model [a]	**Amino acids**
(g)	**Mass/amino acid (g)**
Polyleucine *trans* Helix	28
Polyleucine *gauche+* Helix	28
GCN4 (1ZIK) A	30
GCN4 (1ZIK) B	30
14-3-3 (2BQ0) A145-A206	62
14-3-3 (2BQ0) B3-B54, B98-B127	82
Monofoil (3OL0)	41
Permutant #1	41
Permutant #2	41
Permutant #3	41
[a] RCSB accession indicated in parentheses. Models were printed in triplicate.	[a] RCSB accession indicated in parentheses.

Table 2. Entanglement energy versus expression level and folding for domain-swapped variants of Monofoil trimer oligomer

Protein	**Entanglement**		
(J kg^{-1})	**Expression**		
(mg L^{-1})	**Folding** [a]		
Monofoil	0.48	10.0	Cooperative
Permutant #3	3.7	5.0	Non-cooperative
Permutant #1	6.6	<0.2	N/A
Permutant #2	>120	0.0	N/A
[a] Tenorio et al. [16]	[a] Tenorio et al. [16]	[a] Tenorio et al. [16]	[a] Tenorio et al. [16]

FIGURE LEGENDS

Figure 1

The "knobs in holes" coiled-coil interface of Crick . Parallel α-helices are represented as flattened sheets, with the red helix on the bottom (with side chains pointing towards the viewer) and the blue helix on the top (with side chains pointing away from the viewer). If the top helix is rotated 20° counterclockwise its side chains ("knobs") juxtapose with the spaces ("holes") between side chains in the bottom helix. This rotation leads to left-handed supercoiling that alters the helical twist from 3.6 to 3.5 residues per turn, resulting in a heptad repeat (right). Hydrophobic side chain patterning conforming to a heptad repeat thereby promotes the coiled-coil interaction [1,2]

Figure 2

Hypothesized leucine "Zipper", "jigsaw", and "Velcro" coiled-coil interface . The interdigitation of the γ-branched leucine side chain (panel A) was postulated to "lock" coiled-coil α-helices together in a form of steric entanglement (i.e., the "leucine zipper") (panel B) (after Landschultz, 1988) [7]. Panels C-D illustrate the steric entanglement of physical interfaces of a zipper, jigsaw pieces and Velcro (hook and loop interface), respectively. Each of these objects have been invoked to describe the coiled coil interface.

Figure 3

The Monofoil homotrimer and circular permutants . The Symfoil protein is a de novo designed symmetric β-trefoil protein having three exact repeats of a 42-mer "trefoil" motif [22,23]. Expression of the isolated trefoil motif ("Monofoil") yields a stably folded trimeric oligomer regenerating an intact β-trefoil fold. Circular permutation at each turn position in the trefoil motif yields three different permutants (#1-3).

Figure 4

3D printed model of the Monofoil permutant P2 trimer oligomeric assembly . The 40-mer Monofoil permutant P2 polypeptide was 3D printed in white, black and orange TPU. The individual peptide models were assembled to form an intact β-trefoil architecture. The view is down the threefold axis of rotational symmetry. This structure exhibits extreme entanglement (Fig. 5).

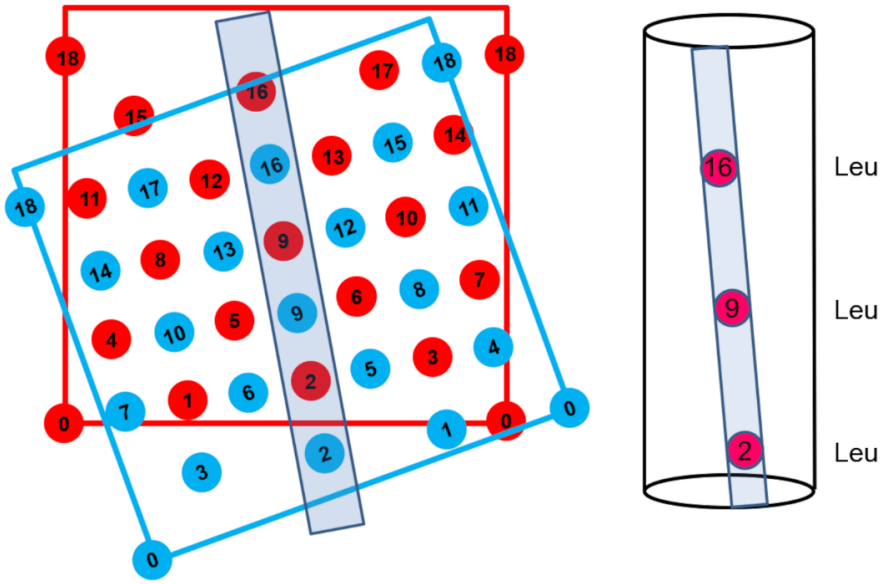
Figure 5

Kinetic energy of dissociation for 3D printed protein oligomers . Upper panel: Dissociation of coiled-coils of 28-mer polyleucine in either all *trans* or all *gauche+* rotamers, and either parallel or anti-parallel orientations. Lower panel: Dissociation of trimeric assemblies of the Monofoil trefoil motif (forming a β-trefoil) and with different circular permutations of the domain-swapped interface (see Fig. 3).

Figure 6

Protein interface entanglement and kinetic trapping . Entanglement of a protein oligomerization interface can result in an increased energy barrier to unfolding (red). This barrier can lead to kinetic trapping of the unfolding pathway. The reaction coordinate diagram also illustrates how a high energy intermediate will also lead to similar kinetic trapping of the folding pathway.

Figure 1



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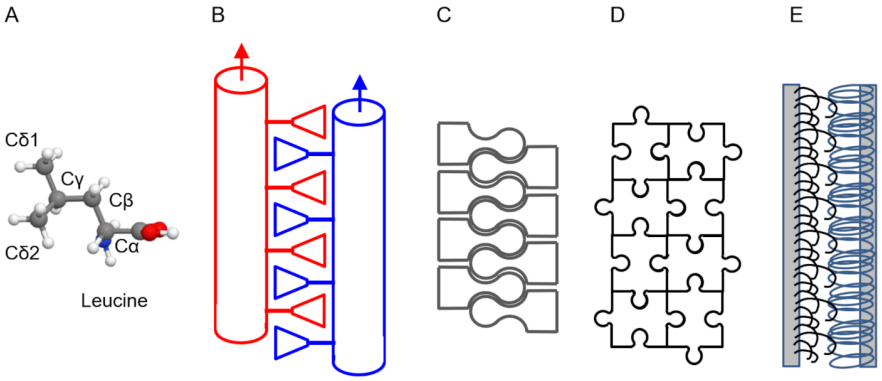

Figure 3

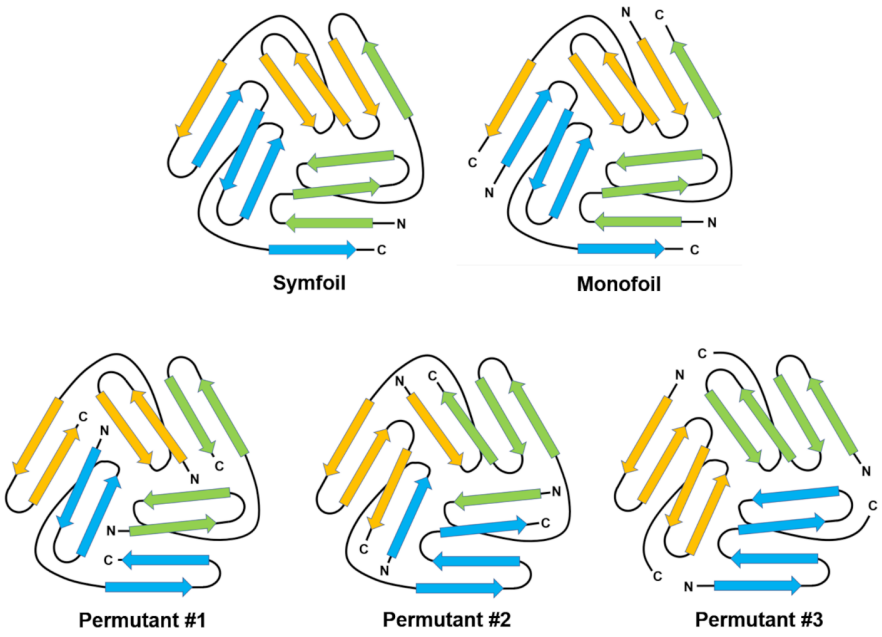

Figure 4

Figure 5

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Figure 6