Interfacing Native and Non Native Peptides: Using Affimers to Recognise α-Helix Mimicking Foldamers

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Selection methods are used to identify Affimers that recognise α-helix mimicking N-alkylated aromatic oligoamides thus demonstrating foldamer and natrual α-amino acid codes are compatible.

Proteins adopt specific three-dimensional compact conformations comprising helices, sheets, loops, turns and disordered domains to orient distinct groups for function e.g. molecular recognition and catalysis. Inspired by the diversity of natural protein structure, foldamers are defined as sequences of non-natural monomers designed to adopt well defined secondary and tertiary or quaternary structures and ultimately 3D architectures with novel, enhanced or emergent function.

This goal aligns closely with efforts to build functional proteins de novo in synthetic biology. Although a major challenge in supramolecular chemical biology, considerable progress has been made in the de novo or “bottom-up” design of tertiary foldamers (Fig. 1a) whilst efforts to understand and control their dynamic topology have broadened potential applications (e.g. PPI inhibition, Fig. 1b). An alternative strategy for the design of functional foldamers is to replace segments of protein sequence with non-natural foldamers (Fig. 1c), an approach termed “protein-prosthesis” and leading to “bionic proteins” (Fig. 1c). A third related approach would be to exploit the potential of combinatorial biology to identify natural biomacromolecule sequences (comprised of amino-acid or nucleotide building blocks) that recognise a single synthetic foldamer (Fig. 1d). In identifying compatible natural and non-natural components driven by complementary molecular recognition, such an approach could be used to identify potential biological targets of a given foldamer, a potentially more rapid route to ligand discovery than the painstaking construction of libraries using synthetic foldamer assembly strategies. Alternatively, the biological selection approach may generate non-covalent foldamer-biomacromolecule complexes that serve as starting points for the construction of foldamer-biomacromolecule hybrid tertiary structures (making an assumption that the former differs the later only in respect of chain entropy).

Figure 1: Schematics for elaborating functional foldamer structures (a) bottom-up foldamer construction (b) PPI inhibition using foldamers (c) protein-prosthesis (d) combinatorial selection of peptides for foldamers (e) N-alkylated aromatic oligoamide proteomimetic illustrating α-helix mimicry (f) structure of an Affimer (PDB ID: 4N6U)

Our group recently developed a series of proteomimetic aromatic oligoamide foldamers designed to mimic the α-helix and discovered selective inhibitors of α-helix mediated protein-protein interactions (PPIs) in the proteomimetic approach,
a scaffold replicates the spatial and angular projection of essential recognition groups that represent ‘hot-spot’ residues at the PPI interaction (Fig. 1e)\textsuperscript{35, 36} Given this known compatibility of our proteomimetic scaffolds with protein structure, we were keen to explore the extent to which biological selection methods might generate peptide sequences for future exploitation in construction of foldamer-peptide hybrids. We used a non-antibody-based scaffold termed an Affimer (Fig. 1f) in tandem with phage display screening.\textsuperscript{37} The Affimer scaffold\textsuperscript{38} is a consensus optimised pyrophycocyanin protein with high stability comprising four β-strands and an α-helix with randomisation at the loops connecting each pair s of β-strands and has been successfully used in a number of discovery settings.\textsuperscript{38-41} Using this scaffold and trimeric aromatic oligoamide foldamers, we identified selective peptide-foldamer interactions using phage display screening.

![Figure 2: Structures of N-alkylated trimeric aromatic oligoamide foldamers 1-2 and their biotin/fluorescein derivatives that represent the primary focus of this study.](image)

In this preliminary study we used our previously described N-alkylated aromatic oligoamide foldamer series\textsuperscript{34-36} these oligomers are amenable to solid-phase peptide synthesis\textsuperscript{44} and act as effective inhibitors of α-helix mediated PPIs\textsuperscript{16} A series of foldamers was prepared following established methods (See ESI, Scheme S1-2)\textsuperscript{42, 43} In total, six trimers (1-6) and their biotinylated equivalents (Biotin-1-6) were synthesised (Fig. 2 and ESI, Scheme S2 for additional structures).

Biotinylated trimers Biotin-1-6 were immobilised alternatively on: streptavidin-coated wells, neutravidin-coated wells, and streptavidin-coated magnetic beads. Immobilized trimers were incubated against an Affimer library (diversity of $3 \times 10^{10}$)\textsuperscript{17} The phage library was pre-panned three times on each respective surface before panning against the immobilized targets. Bound phage were eluted and used to infect ER2738 E. coli cells in the presence of M13K07 helper phage over four panning rounds (see ESI Fig. S1). Following the final round of panning, 48 monoclonal Affimers for each trimer were analysed by ELISA; bound phage were detected using Anti-Fd-Bacteriophage-horse radish peroxidase (HRP) and SeramunBlau\textsuperscript{®} fast TMB (Fig. 2 and Fig. S2). We assessed the extent of binding and selectivity for all 48 Affimers: for Biotin-1, there were 25 Affimers which bound, 12 of which were fully selective for Biotin-1; for Biotin-2, 41 Affimers, 30 of which were selective; 7 Affimers for Biotin-3, 2 of which were selective; 19 Affimers for Biotin-4, none of which were selective; 18 Affimers for Biotin-5, none of which were selective; and, 25 Affimers for Biotin-6, 4 of which were selective (see further discussion in ESI). These preliminary data establish that N-alkylated aromatic oligoamide foldamers can interact with natural α-polypeptide/protein structures in an effective and highly selective manner i.e. the natural and non-natural foldamer “codes” are compatible.

Affimers showing differential values between test and negative control by ELISA were sequenced using a T7P primer – mixed colonies were removed from the list. Each Affimer was named using the format X-AFY, where X is the number of the trimer and Y the number of the Affimer out of 48 (see ESI, Table S1 for further details). We focussed on Biotin-1 and Biotin-2 since they exhibited promising results in the ELISA assay (Fig. 3) Biotin-1 and Biotin-2 generated Affimers enriched predominantly in hydrophobic amino acids (see ESI for further details). For each trimer, two selective (1-AF8, 2-AF17, 2-AF23, 2-AF25) and one non-selective Affimer (1-AF26 and 2-AF1) were chosen (Fig. 3, yellow arrows) to be subcloned, expressed as his-tagged proteins and finally purified on a Ni-NTA resin.

To establish the affinity of interaction between foldamer and Affimer, we performed a direct ELISA based titration of the Affimers against immobilised trimer (Fig. 4a and ESI, Fig. S3); here, anti-6X his-tag HRP and SeramunBlau\textsuperscript{®} fast TMB were
used for detection. Well-defined dose-response behaviour was observed for 1-AF8 and 2-AF25, which could be fit using a logistic model to obtain EC_{50} values of 4.8 (± 0.6) and 0.98 (± 0.08) μM respectively. The remaining Affimers gave noisy data which made curve fitting challenging (see ESI for data); we attribute this to the hydrophobic nature of the side chains and consequent amplification of hydrophobic Affimers.

Figure 4: a) Direct titration (ELISA) for 1-AF8 (n = 3) and 2-AF25 (n = 2) against the corresponding immobilized foldamer. For clarity, binding curves for the remaining affimers are shown in the ESI. b) Competition assay (ELISA) using foldamers to displace 1-AF8 and 2-AF25 (n = 3) from the corresponding immobilized foldamer. For clarity, binding curves for the remaining foldamers are shown in the ESI.

To obtain evidence of a specific non-covalent interaction between foldamer and Affimer, we performed competition experiments. The extent to which immobilized trimers Biotin-1 and Biotin-2 recruited their complementary Affimers in the presence of competing quantities of unlabelled unfunctionalized 1 or 2 was assessed at a single competitor. Based on the results of the direct ELISA, we chose to work at the following concentrations: 10 μM for 1-AF8 and 1-AF17, 1 μM for 1-AF26, 20 μM for 2-AF1, 2 μM for 2-AF23 and 5 μM for 2-AF25. We also attempted to compete for Affimer binding with Biotin-1 or Biotin-2, streptavidin or a complex of Biotin-1 and streptavidin. The normalised average for each of the four conditions (n = 3 for each) is given in the ESI (Fig. S4). Under single concentration conditions, we observed a general trend for the biotinylated trimer to more effectively compete for the Affimer than the unlabelled trimer. For clarity, binding curves for the remaining competitors are shown in the ESI.

In agreement with the data obtained using ELISA, Flu-2 was shown to bind to 2-AF25 with a K_{d} = 146 ± 11 nM (Fig. 5a). Gratifyingly, binding was not observed for Flu-2rev (Fig. 5b and ESI, Fig. S7), which attests to the high selectivity of the Affimer for the exact sequence/order and composition of side chains. We also tested Flu-1 against 1-AF8 (Fig. 5b), but were unable to obtain a binding curve; this may arise due to the introduction of the fluorescein motif interfering with the molecular recognition features of 1.

In conclusion, we used Affimer display to identify for the first time, peptide sequences with high affinity and selectivity for aromatic oligoamide foldamers. The selected Affimers are specific for a given foldamer sequence (e.g. 2 vs 2rev) demonstrating that the α-amino acid and N-alkylated aromatic amino acids “codes” are compatible in terms of molecular recognition. Moreover a diversity of binding modes can be selected for using this approach including Affimers that recognise the foldamer, or the foldamer and its biotin immobilisation linker. Rather than considering this later selection product as undesirable, the results emphasise the potential to elaborate complex 3D architectures comprising natural and non-natural parts. Thus, the work highlights more broadly, the potential of using phage display to identify natural amino acid sequences that bind to foldamers and adds to the synthetic biology “toolkit”. Our own future efforts will focus on using this approach in tandem with chemo/bioinformatic analyses to identify potential disease relevant peptide/protein targets for foldamers, and on the study of the interaction between foldamer and the recognition determining sequences excised from the Affimer from which they are derived.

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References