Due to the ongoing evaluation of a potential patent application related to the scaffold utilised in this study, specific technical details, methodologies, and particular design aspects have been deliberately omitted or generalised. The findings and conclusions presented aim to provide an overview of the project's outcomes while ensuring necessary confidentiality to protect intellectual property rights.

Advancing Biosensor Design: Exploring the Applicability of a Novel Engineered Protein Scaffold for Biosensing

James Ratcliffe

Protein-based biosensors are a class of analytical tools that exploit the unique properties of proteins to detect and quantify biological, chemical, or environmental substances[1]. Robust and biologically neutral protein scaffolds have emerged as promising alternatives to immunoglobulin based proteins (e.g. antibodies) that are traditionally used, offering new possibilities in biosensing applications[2].

In this study, we aimed to establish the applicability of a promising new protein scaffold for detecting protein-protein interactions. The scaffold of interest demonstrates significant potential for biosensing applications due to its high natural thermal stability, exceptional resilience to harsh conditions such as extreme pH, high salt concentrations, detergents, and denaturants, and its tolerance for extensive engineering and chemical modification. We specifically sought to characterise the interaction between a rationally designed variant of the scaffold and a model test protein (an SH3 domain). We did this by designing two constructs, referred to as Construct A and Construct B, each of which contained two tandemly arrayed scaffold domains (Figure 1). Construct B has a native scaffold sequence while Construct A has an SH3 binding motif inserted into a permissible loop.

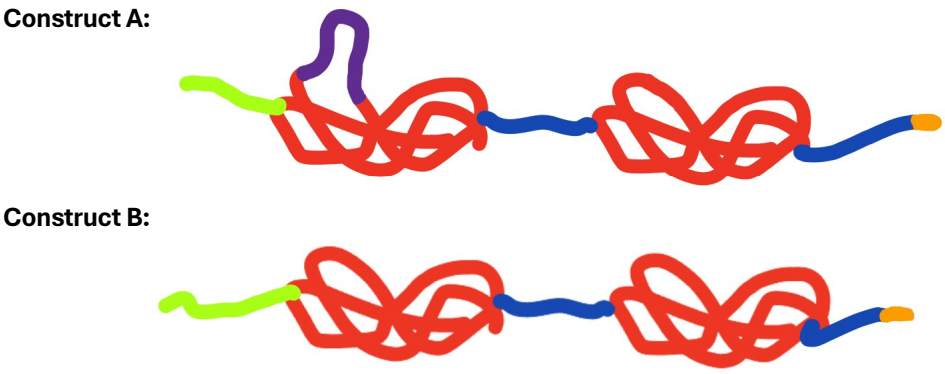

Figure 1. Schematic representation of Constructs A and B. *The scaffold domains are depicted in red, with Construct A incorporating the SH3 binding peptide shown in purple within the N-terminal scaffold domain, while Construct B consists of two unmodified scaffolds. A Gly-Ser linker, represented in navy, connects the tandemly arrayed scaffold domains. Both constructs feature an N-terminal His tag (green) for purification purposes and a C-terminal cysteine tag (orange) for covalent immobilization in binding experiments.*

Samples of the two constructs were produced via expression from recombinant *E. coli* and purified using standard chromatograph approaches. The thermal stability of purified Constructs A and B were assessed using Differential Scanning Fluorimetry (DSF). Construct B exhibited a melting temperature (T_m) of 87.1°C, while Construct A had a T_m of 82.8°C (Figure 2). Our results suggest that both Constructs are folded in solution, with these data being consistent with results from other scaffold constructs characterised by my host group. A small reduction in T_m was seen in Construct A compared to the native sequence, indicating that the modification in Construct A did not significantly perturb the

overall stability. The modification in Construct A comprises an insertion of approximately 20 residues into the scaffold and the observation that the modification only causes a relatively modest reduction in T_m is an important demonstration of the capacity of the scaffold to be engineered for biosensing applications[2] (Figure 2).

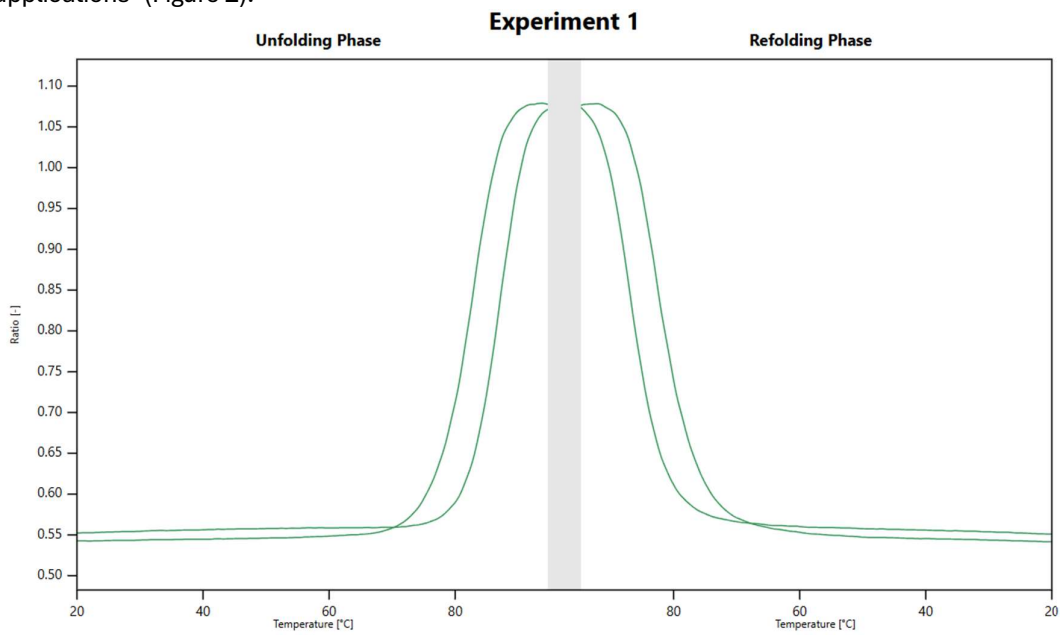

Figure 2. Constructs A and B show considerable thermostability in solution. The curves show change in tryptophan fluorescence as a function of temperature. Samples were heated from 20-95 °C and then cooled back to 20 °C with a temperature change rate of 1 °C/minute and Trp fluorescence monitored at 330 and 350 nm. Construct A, which contains the SH3 binding peptide, exhibited a melting temperature (T_m) of 82.8°C, while the unmodified scaffold Construct B showed a T_m of 87.1°C. These results are consistent with both constructs being properly folded and retaining thermally stability, and with the modification causing minimal impact on the stability of Construct A. Samples were prepared in buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM DTT (for cysteine-containing samples) at a concentration of 100 µM.

The interaction between the scaffold and the SH3 domain was characterised using Surface Plasmon Resonance (SPR). The constructs were covalently linked to a CM5 SPR chip through a C-terminal Cys tag, ensuring stable and site-specific covalent immobilisation. SPR analysis revealed a clear binding response, indicating a specific interaction between Construct A and the SH3 domain. Construct B showed no binding to the SH3 domain analyte. From the kinetic data, we calculated a dissociation constant (K_d) of 3.1 ± 0.2 µM, which is consistent with previously reported values from my host group. These results show that the scaffold can be immobilised onto a surface and bind its intended target with the anticipated affinity, which further demonstrates the potential of the scaffold for biosensing applications (Figure 3).

Previous analyses conducted in my host group have shown that single scaffold domains are highly stable and capable of refolding after exposure to denaturants in solution. Here, we sought to use SPR to determine whether the scaffold can be denatured and refolded on a surface and whether the refolded protein has the same properties.

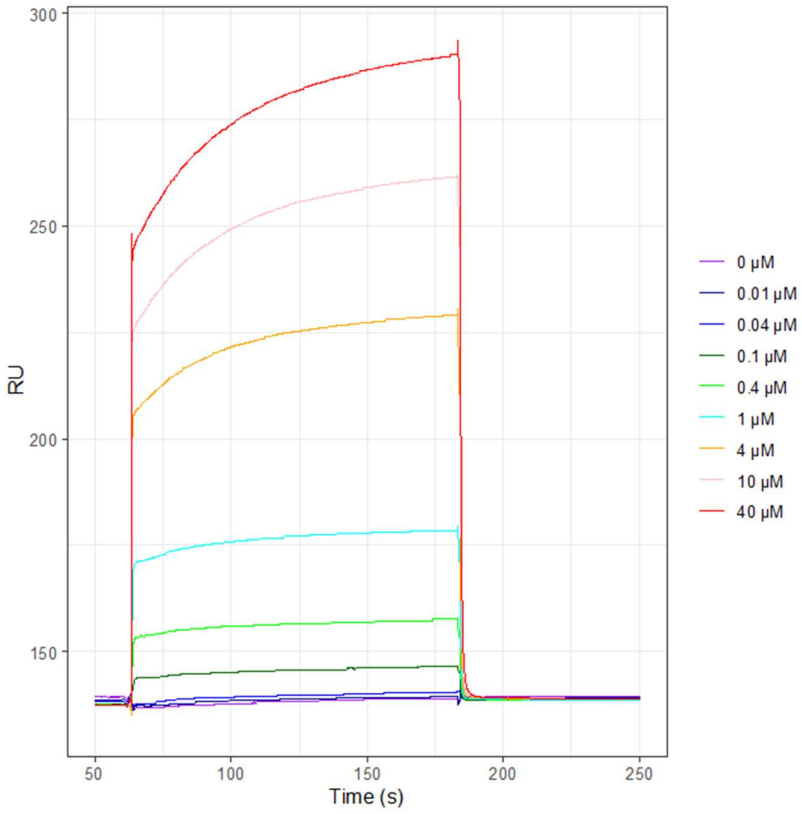

Figure 3 – Surface immobilised scaffold binds an SH3 domain target*. Surface immobilised Construct A or Construct B were titrated with an SH3 domain analyte at concentrations of 0.01, 0.04, 0.1, 0.4, 1, 4, 10 and 40 µM. Both constructs were covalently linked to the SPR chip via a terminal cysteine tag. Analysis of SPR sensorgrams yielded a dissociation constant (K_d) of 3.1 ± 0.2 µM. SPR was carried out using a Biacore T200. Data was processed with Biacore analysis software and visualised using R studio*[3]*. Experiments were conducted in phosphate buffer (20 mM sodium phosphate, pH 6.0, 100 mM NaCl) at 25° C.*

The SPR chip was washed with buffer to remove SH3 domain before treatment with 6 M guanidinium HCl (Gdn-HCl). The chip was then washed back into buffer to remove Gnd-HCl and SH3 domain reapplied. The SPR response showed that binding occurred, and analysis of the data yielded K_d of 1.7 ± 0.5 µM, similar to the affinity of 3.1 µM measured before treatment (Figure 3). These results suggest that the immobilised scaffold protein was refolded on the surface following chemical denaturation (Figure 4). The slight difference in binding affinity is likely due to experimental variation and the experiment will need to be repeated to determine the precision of the binding affinities calculated. The maximum change in response units (RU) observed before Gdn-HCl treatment was 142 RU, which is significantly higher than the 27.5 RU recorded after treatment. Since construct A was covalently immobilized, it is unlikely that the reduction in RU is due to dissociation. Instead, the lower maximum response is likely due to a proportion of construct A failing to refold, reducing the surface's capacity to bind the SH3 domain. Given that only about 20% of the original RU was recovered, optimizing the regeneration protocol (e.g., using a stepped reduction in Gdn-HCl) may improve the recovery percentage, and repeating the experiment with N = 3 would be necessary to confirm this result.

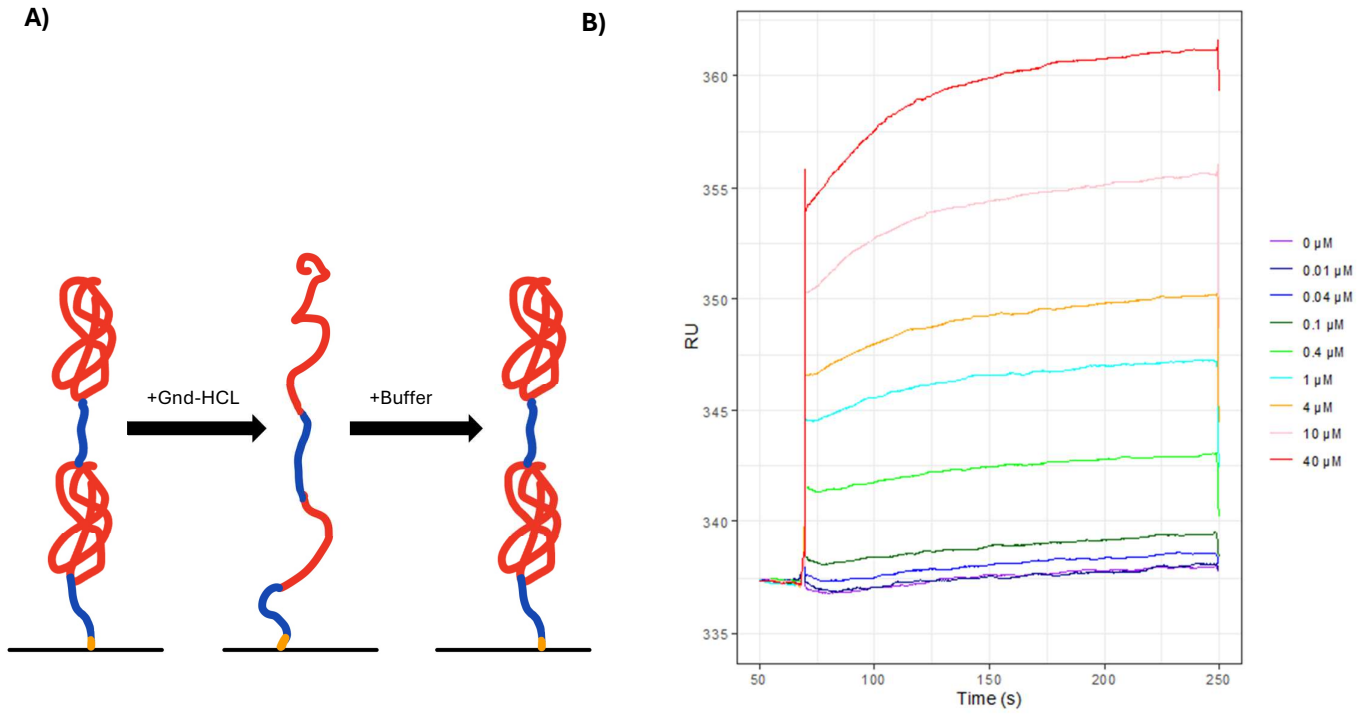

Figure 4. The scaffold can refold on a surface following chemical denaturation. *A) Schematic of the experiment. The scaffold domains are expected to unfold in the presence of Gnd-HCl and then refold in buffer, restoring structure and function. B) SPR sensorgram showing SH3 binding to refolded scaffold after buffer rewash. Following immobilisation and denaturation with 6M Gnd-HCl, the SPR chip was rewashed with buffer and titrated with SH3 domain analyte at concentrations of 0.01, 0.04, 0.1, 0.4, 1, 4, 10 and 40 µM. The resulting sensorgrams show that the refolded scaffold retained SH3 binding properties with a K_d of 1.7 ± 0.5 µM, similar to the pre-denaturation affinity. SPR was carried out using a Biacore T200. Data was processed with Biacore analysis software and visualised using R studio[3]. Experiments were conducted in phosphate buffer (20 mM sodium phosphate, pH 6.0, 100 mM NaCl) at 25° C.*

Although the SPR data supports the hypothesis that our protein is highly stable and may be capable of refolding after exposure to denaturing conditions, it does not provide conclusive evidence to confirm this. For example, the SH3 binding peptide motif inserted into Construct A could still bind its target if the scaffold remained unfolded after denaturation. A technique called quartz crystal microbalance with dissipation (QCM-D) has the capability to probe both analyte binding and folding and unfolding of proteins on surfaces and therefore could be used to evaluate the scaffold system in more detail.

The long-term vision of my host research group is to create proteins that can function as receptors in biosensors. The group are interested in proteins that can be used to detect a wide variety of targets, ranging from proteins to small molecules and other analytes. The ability of the scaffold to be engineered as a tandem repeat and its tolerance of peptide insertions, as demonstrated in this study, opens avenues for developing biosensors capable of targeting multiple analytes simultaneously or responding to specific molecular cues. The results presented here also suggest that the scaffold may refold after chemical denaturation, which would be valuable for producing regeneratable biosensors.

In conclusion, this project demonstrates the potential of a novel protein scaffold for biosensing, especially in detecting protein-protein interactions. Its natural stability, resilience to harsh conditions, and ability to accommodate modifications without losing structural integrity make it ideal for biosensors. The scaffold's stability, binding affinity, and refolding after denaturation further highlight its robustness for analytical applications, paving the way for future biosensor developments.

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