

Enhancing the Therapeutic Properties of a Cancer-Targeting Affibody by Soft Mutagenesis with Combinatorial Variant Libraries

ABSTRACT

Affibodies offer many advantages over monoclonal antibodies, including smaller sizes, enhanced thermal stability, and rapid refolding capability. This application note describes the optimization of pharmacologically relevant properties in an affibody already exhibiting strong Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) binding. The authors explored functional sequence space using multiple Combinatorial Variant Libraries (CVLs) designed *in silico* with a soft mutagenesis profile, and the Protein Repair One-Stop Shop (PROSS). Compared to the original binder, the engineered variants displayed improved thermal stability, refolding capacity, and biodistribution, while retaining the ability to bind onto their target.

INTRODUCTION

Affibodies are synthetic antigen-binding proteins originally derived from the combinatorial protein engineering of Protein A's Z domain. They mimic the function of monoclonal antibodies, possessing high affinity and highly specific binding properties for their cognate antigen. Affibodies are particularly exciting due to their small size. Whereas antibodies are roughly 150kDa in size, affibodies are much smaller at approximately 6kDa. Affibodies are also robust and can be engineered to withstand higher temperatures and larger pH ranges compared to monoclonal antibodies (Löfblom et al., 2010). These advantages make affibodies an attractive platform for the development of therapeutics, diagnostics, and bioimaging technology (Ståhl et al., 2017).

In a recent study published in Nature Scientific Reports, researchers at the KTH Royal Institute of Technology and Uppsala University sought to engineer previously identified affibody molecules targeting VEGFR2 (Güler et al., 2020). VEGFR2 is a key receptor in the angiogenesis pathway. Efficient VEGFR2 targeting is therefore valuable for both basic biological research into the vascularization process, as well as a potential target for the treatment of several diseases including cancer and macular degeneration.

The authors had previously generated a high-affinity heterodimeric protein consisting of two unique affibodies that together blocked the interaction between VEGFR2 and its signaling molecule,

VEGFA, in mouse models. This affibody-based system was shown to efficiently block angiogenesis *in vitro* and was successfully used as an imaging agent for the study of vascularization in mouse models. However, the authors also identified a number of issues with the molecule, including poor thermostability, a propensity to aggregate due to poor re-folding, and an increased uptake in the liver and spleen—problematic properties if the molecule was to be transferred into the clinic. Güler et al., 2020 sought to optimize these proteins without negatively impacting existing affibody function using a combination of directed evolution and rational design.

Directed evolution for optimizing a given protein property relies on introducing new diversity into the parental gene of interest, followed by the functional selection of improved protein variants. The screening of these variants can be optimized by ensuring the majority of variants screened still retain their original function. This process is typically referred to as tuning the “softness” of mutagenesis, which can be achieved through the careful design of synthetic variant libraries [box 1]. To generate the desired sequence space, the authors leveraged Twist Bioscience's Combinatorial Variant Libraries (CVLs).

CVLs are highly diverse variant libraries containing the complete combinatorial set of specified mutations across defined amino acid positions in a given wildtype sequence; up to 10^{10} variants. Traditional library fabrication methods such as error-prone PCR, NNK, and TRIM can introduce premature stop codons, suffer from skewed amino acid distributions, contain uneven or incomplete variant representation, and provide little control over unwanted DNA or amino acid motifs. The lack of tunability, missed variants, and inherent bias ultimately leads to a need for increased screening depth to thoroughly scan the available sequence space. To circumvent these issues, Twist Bioscience synthesizes each variant precisely on a proprietary, high-throughput silicon-based oligonucleotide synthesis platform. All 64 codons can be implemented at user-defined ratios without the incorporation of unwanted motifs, providing much-improved control over the location and frequency of each mutation.



TUNING THE SOFTNESS OF MUTAGENESIS

Protein optimization presents a trade-off between making amino acid changes that improve desired properties and making amino acid changes that negatively impact protein function. This trade-off can be effectively navigated by prioritizing wild-type sequences in the mutagenesis. In library design, this process is often referred to as tuning the softness of the mutagenesis.

A library with a soft mutagenesis profile will contain all of the desired variants, but the majority of targeted positions will present as wild-type when screening a single clone. In theory, by not deviating too far from the wild type sequence, the maintenance of primary functionality is maximized, while other beneficial properties can be panned and identified. On the other hand, a library with a complete mutagenesis profile will contain very few or no wild-type sequences in the design. Utilizing Twist Bioscience’s CVL synthesis technology, libraries can be designed *in silico* with pre-defined ratios of wild-type mutants. Each unique variant is generated independently during oligo synthesis, and these variants are combined in the required ratios to generate the final highly uniform, evenly represented, true-to-design library.

This application note describes the design, synthesis and use of a CVL library with a soft mutagenesis profile to optimize the clinical properties of an anti-VEGFR2 affibody. Combinatorial mutagenesis was combined with rational mutagenesis directed by the online tool PROSS. Engineered anti-VEGFR2 affibodies demonstrated improved thermal stability, refolding, and biodistribution compared to the original binders. These affibodies were intentionally designed to reduce hydrophobicity while retaining high affinity for the receptor. Taken together, the results provide a model for the optimization of functional proteins, and show VEGFR2-binding affibody molecules are promising candidates for further development in molecular imaging and therapeutic applications.

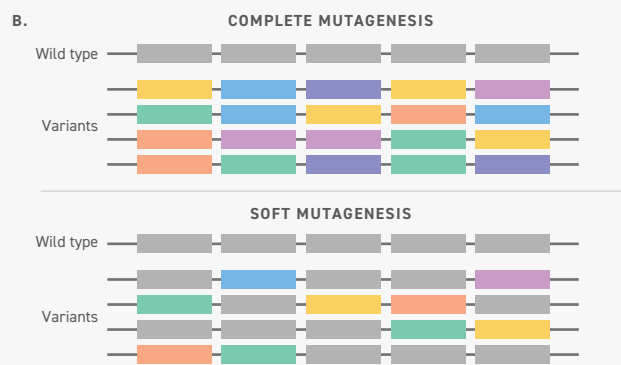
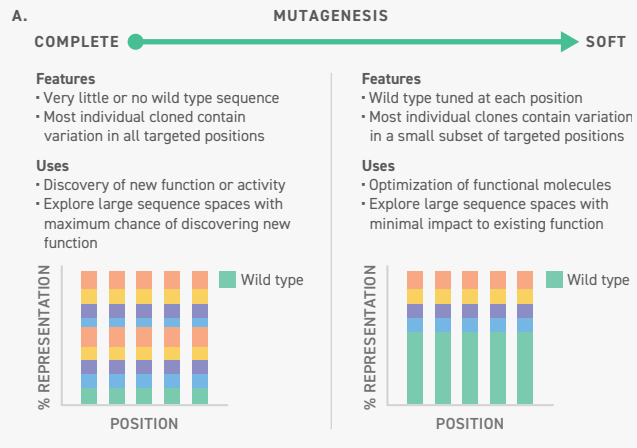


Figure 1. A comparison of a complete mutagenesis profile and a soft mutagenesis profile for combinatorial variant libraries. A) The features and common use cases of a complete mutagenesis profile vs. a soft mutagenesis profile in combinatorial variant library design. B) A representation of the amino acid makeup of individual variants selected from a library with a complete mutagenesis profile vs a library with a soft mutagenesis profile. Clones selected from a library with a complete mutagenesis profile typically contain variation at every position. Soft mutagenesis allows the user to control the prioritization of wild type at each position, tuning the number of variant positions expected in each individual clone.

WORKFLOW AND RESULTS

CVL library generation

The author’s target molecule consists of a heterodimeric pair of affibodies. A Twist CVL was designed and ordered for each affibody in the system targeting 13 positions (Figure 2). To remove potentially destabilizing mutations, hydrophobic, cysteine, and proline residues were excluded from the randomized positions. Soft mutagenesis was simulated in both libraries by synthesizing the complete sequence space such that each mutant would contain 1, 2, or 3 mutations per affibody. The resulting libraries had a final complexity of approximately 2.5×10^5 variants. Each library was PCR-amplified, purified, enzymatically digested, and ligated into surface expression vectors.

Selected affibody monomer variants from FACS screening display high thermal stability and binding affinity

Surface expression vectors were transformed using electroporation into *Staphylococcus carnosus* for surface display.

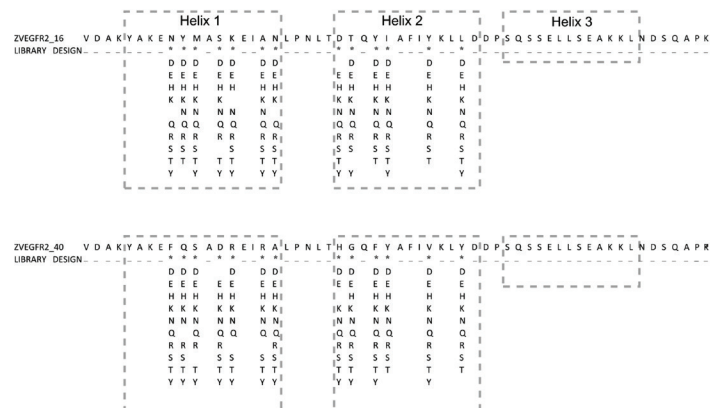


Figure 2. Targeted positions for mutagenesis, and selected variants in both affibody domains. The library was synthesized such that each clone contained one, two or three variant residues.

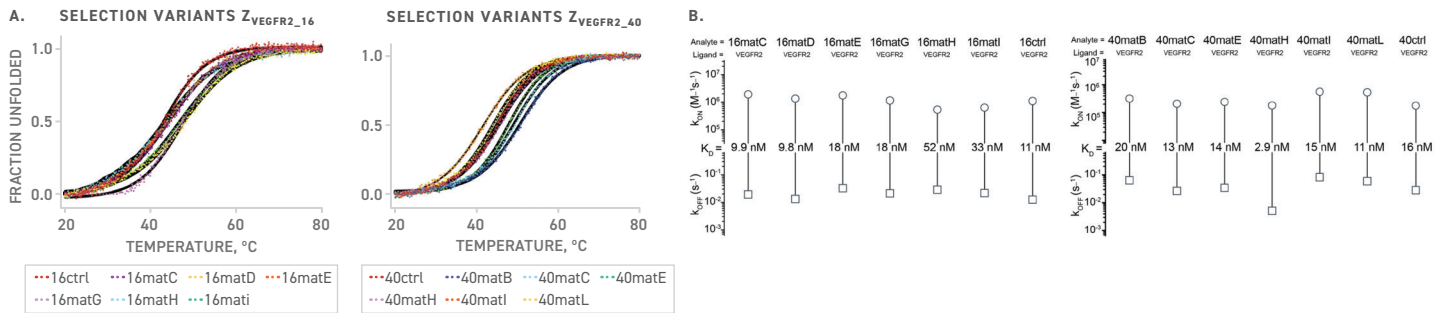


Figure 3. Thermal stability and affinity of monomer variants identified by selection with FACS. A) Thermal stability of each variant identified by observing the fraction of unfolded protein measured by variable temperature CD spectroscopy B) Rate scale plots of variants from selection, showing binding affinity.

The combinatorial libraries were incubated with fluorescently labeled targets and sorted for binding variants using Fluorescent Activated Cell Sorting (FACS). Sorting was repeated for several rounds with amplification by growth in between cycles until the required enrichment level was reached. After sorting, the isolated recombinants were DNA sequenced and affinity and specificity were determined using flow cytometry.

The top 12 candidate proteins isolated from FACS screening were analyzed for thermal stability and secondary structure using Circular Dichroism (CD) spectroscopy, as well as binding affinity using Surface Plasmon Resonance (SPR). The majority of the isolated clones demonstrated higher melting temperatures ranging from 42–49°C and more complete refolding capabilities compared with the original binders (Figure 3a). In addition, the affibody variants retained their ability to bind to the receptor, with binding affinities ranging between 3–50nM (Figure 3b). Based on thermal stability, refolding capability, and binding affinity, the two top candidates for each monomer were chosen for dimer construction and further analysis.

Engineered dimeric proteins demonstrate complete refolding

In parallel to CVL screening, homology-modeled protein structures of both affibodies in the heterodimeric system were generated and submitted to the Protein Repair One Stop Shop (PROSS) according to the web server instructions (<https://pross.weizmann.ac.il/>). All amino acids aside from the 13 that are a part of the binding interface on helices 1 and 2 of the affibody molecule were subject to mutations. Variant genes generated by PROSS were synthesized and resultant proteins were characterized as before in parallel to the CVL library. A total of four additional mutations were identified that additively increased the stability of the original affibody molecules by up to 10°C (data available in Güler et al., 2020).

The four additional mutations identified by PROSS were grafted into the four most promising candidates from the FACS screening of CVLs. Dimers of the candidates were then synthesized separated by an S4G linker, with an engineered Albumin Binding Domain (ABD) added to the C-terminus for affinity capture. The refolding capability and stability of the most promising dimeric mutant candidate (Z_{VEGFR2_3gen}) was characterized using CD spectroscopy. Engineered dimers demonstrated an increase in stability of 15°C compared to the original binder (Figure 4a), as well as complete refolding (Figure 4b).

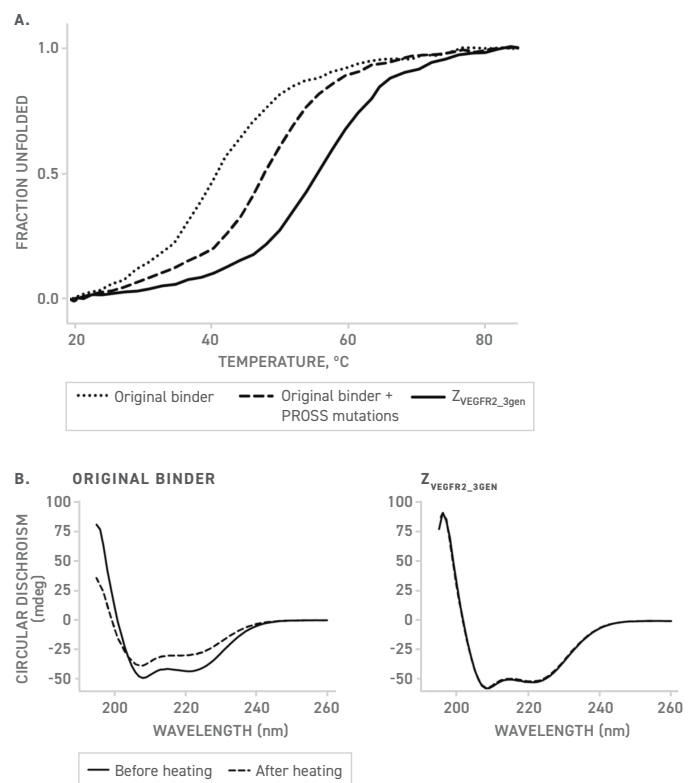


Figure 4. Stability and refolding capacity of dimeric engineered proteins identified from CVL screening + PROSS mutations. A) Thermal stability of the original binder, the original binder and rationally designed PROSS mutations, and the thermal stability of Z_{VEGFR2_3gen} determined by observing the fraction of unfolded protein measured by variable temperature CD spectroscopy. B) Refolding capability of original dimer (left) and Z_{VEGFR2_3gen} after heating and cooling, measured by CD spectroscopy.

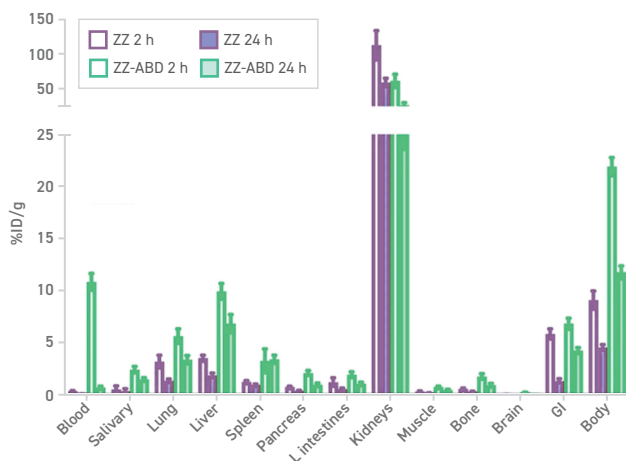


Figure 5. Biodistribution of 0.3 nmol radiolabeled ABD-conjugated and non-conjugated variants of VEGFR2_3gen in a mouse model, measured by Magnetic Resonance Imaging.

ABD-conjugated engineered dimeric proteins display prolonged *in vivo* biodistribution

It was subsequently investigated whether the ABD domain increases blood circulation in a mouse model. ABD unbound ($Z_{\text{VEGFR2_3gen}}$; ZZ) and bound ($Z_{\text{VEGFR2_3gen}}\text{-ABD}$; ZZ-ABD) variants of the protein were radiolabeled and analyzed for biodistribution two or 24 hours post-intraperitoneal injection. Overall, dimers without ABD conjugation exhibited rapid blood clearance compared with dimers conjugated with ABD, which circulated in blood 20-fold longer and thus had higher activity uptake in all tested tissue except for the kidneys (Figure 5). Importantly, compared to the original binder, $Z_{\text{VEGFR2_3gen}}\text{-ABD}$ showed 13-fold lower accumulation in the liver and the spleen, consistent with the improvement of protein stability and re-folding (data not shown).

CONCLUSIONS

Affibodies are an exciting alternative to monoclonal antibodies with applications in therapeutics, diagnostics, and biotechnology. Their small size, thermal stability, and intracellular refolding capacity make them attractive candidates for receptor targeting and bioimaging. However, their small size means optimizing pharmacokinetic properties without impairing function is a challenge. This study demonstrates how soft mutagenesis using Twist Bioscience's highly customizable CVLs enabled the engineering of a dimeric VEGFR2 binding affibody for improved stability, re-folding capability and bioaccumulation without negatively impacting its antigen binding properties. By prioritizing wild-type sequence in the library design, and leveraging Twist Bioscience's precise library synthesis technology, the number of mutants-per-clone could be precisely tuned for the experiment at hand. This screening principle can easily be applied to other proteins, including enzymes or pharmacologically relevant binding proteins to ensure the efficient navigation of sequence space.

REFERENCES

- Ferrara, N. & Kerbel, R. S. Angiogenesis as a therapeutic target. *Nature* 438, 967–974 (2005).
- Feldwisch, J. & Tolmachev, V. Engineering of affibody molecules for therapy and diagnostics. In: Voynov V., Caravella J. (eds) *Therapeutic Proteins. Methods in Molecular Biology (Methods and Protocols)*, vol 899. Humana Press, Totowa, NJ.
- Güler, R., et al. Increasing thermal stability and improving biodistribution of VEGFR2-binding affibody molecules by a combination of *in silico* and directed evolution approaches. *Sci Rep.* 10, 18148 (2020).
- Löfblom, J. et al. Affibody molecules engineered proteins for therapeutic, diagnostic and biotechnological applications. *FEBS Lett.* 584, 2670–2680 (2010).
- Ståhl, S. et al. Affibody molecules in biotechnological and medical applications. *Trends Biotechnol.* 35, 691–712 (2017).

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