Surface Display-Enabled Miniprotein Evolution for Brain-Targeted Therapeutics

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Introduction

Crossing the blood-brain barrier (BBB) remains a central challenge for CNS drug delivery. Tightly regulated endothelial barriers severely limit the brain penetration of peripherally-injected biologics like antibodies, lowering the therapeutic efficacy of current neurological disease treatments[1].

Tissue binding domains (TBDs) **offer a promising solution**. By facilitating passage across endothelial barriers, TBDs enable brain penetration of biologics[2] while potentially spatially restricting activity to target tissue. Many proteins can be TBD templates, with known structures enabling rapid, modular development.

Here, we present a directed evolution pipeline using surface display to discover novel, miniprotein-based CNS TBDs, demonstrating a **generalizable approach to tissue-targeted therapeutic development**.

Methods

Computational Library Creation *(Figure 1)*

1. Identified small (~80 a.a.), simple, well-characterized synthetic miniprotein scaffold.
2. Selected mutation residues computationally (py3Dmol, freeSASA) - filtered for hydrophilicity, centroid distance (≥20th percentile), and SASA (≥75th percentile).
3. Partitioned residues into combinatorial libraries by protein face, increasing combinatorial efficiency.

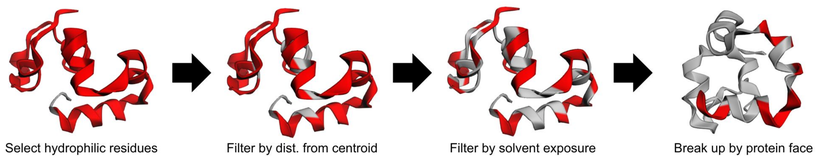

Figure 1. Example residue selection pipeline. Structure pictured is the villin headpiece[3].

Cloning

1. Created “stop template” genes based on combinatorial libraries.
2. Cloned wild-type scaffold into phage display vector via restriction enzyme cloning (REC).
3. Cloned stop templates into surface display vector via REC.
4. Verified constructs via plasmid sequencing.

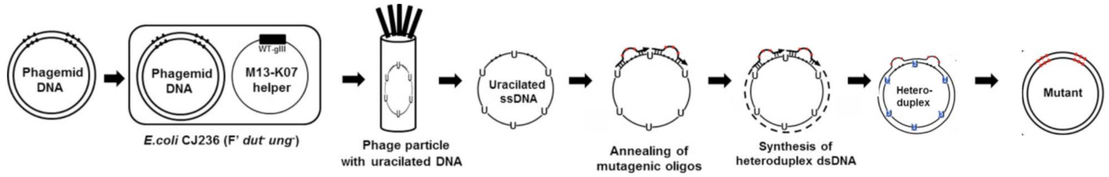

Figure 2. The Kunkel mutagenesis principle. Figure adapted from Huang et al., 2012[4].

Library Construction *(Figure 2)*

1. Designed two degenerate mutagenic primers per library for Kunkel mutagenesis (KM).
2. Ran pilot protocol to verify dU-ssDNA production and mock KM conversion via gel electrophoresis.
3. Created TBD libraries via KM, transformed library into host organism, and diluted cultures to standard OD.
4. Verified TBD library size via serial dilution.

Discovery via Phage Display & Liquid-Phase Panning *(Figure 3)*

1. Combined cultures containing TBD libraries to create initial screening pool.
2. Incubated screening pool with streptavidin beads and collected supernatant to remove non-target binders.
3. Screened pool against target-conjugated beads, washed, and recovered binders for subsequent rounds.
4. Conducted host organism-specific ELISA to confirm propagation of remaining binders.
5. Repeated steps 2-4 three additional times, decreasing target concentration from 100 nM to 12.5 nM.

Clone Screening *(Figure 3)*

1. Plated final screening pool and isolated individual clones in liquid culture.
2. Conducted supernatant ELISA for TBD clones to determine relative binding activity.

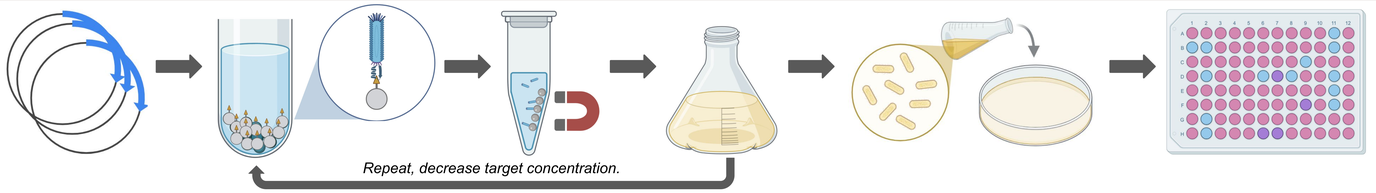

Figure 3. Liquid-phase panning workflow for binder discovery via phage display. Mutant library is added, binders are separated and re-cultured. After multiple repetitions with decreasing amounts of target, remaining mutants are plated, and individual colonies screened for relative activity.

Results

Three libraries were created with 14 mutated residues each. Sequencing **confirmed** scaffold & stop template cloning. Pilot KM steps were confirmed via ELISA and gel electrophoresis. For library KM reactions, propagation of host organism confirmed success. Total number of mutants was **determined to be 10^5 transformants** via serial dilution of each library (Fig. 4).

Phage recovery was confirmed via phage ELISA after each round of screening (Fig. 5). The pool remaining after the final screen was plated to isolate individual clones. ~75 tissue-binding domain clones were isolated from individual host bacterial colonies. **Clones analyzed showed binding activity,** with highest performers showing signal +1SD higher than mean (Fig. 6).

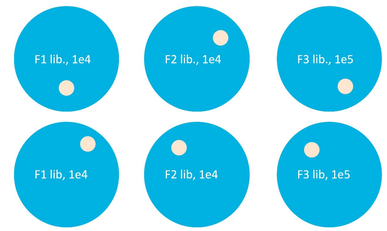

*Figure 4 (above). Mockup of post-transformation library plates. Final library size is 2M * 10^N, where M is avg. colony number counted on plate diluted 10^N.*

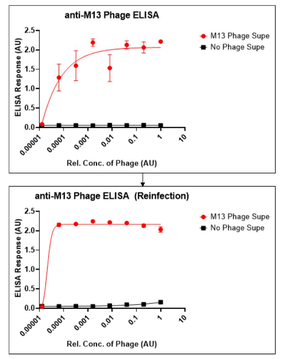

Figure 5 (right). M13 ELISA pre- and post-library recovery.

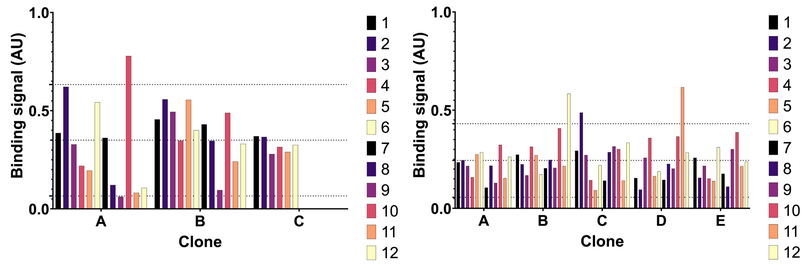

Figure 6. Results of individual clone screening on 96-well plates, labeled by well index.

Discussion

Broadly, our results **confirm the pilot discovery of a unique set of miniprotein-derived binders** for our target, validating a pipeline for isolation and further characterization of brain targeting agents.

We encountered multiple challenges during the workflow execution. Using a multi-site RE led to cloning issues; conducting titration initially would have offset the resulting time loss. Additionally, transformation inefficiency may have led to a smaller initial pool of mutants. To validate the workflow, we decided to proceed after identifying potential changes for subsequent trials.

However, **our pipeline is versatile**. Our scaffold’s small size, simplicity, and high number of variants (5×10^{17} potential) suggests broad cross-target reusability. Our plasmid-based library allows for translation across display techniques, further increasing flexibility. We executed 4 panning rounds by hand in 2 weeks, demonstrating the potential for rapid discovery.

Future Work

Titrating high-performing mutants against literature binders and the wild-type scaffold would provide binding affinity data. **Further characterization via cell binding and transport assays** or a murine study would follow after benchmarking and selection of best binder candidates. NGS of each post-pan pool combined with high-throughput functional screening could inform rational design approaches and identify mutation-tolerant residues.

Integration of PFunkel[5] and KM+RCA[6] into a unified protocol could **increase mutagenic efficiency and library complexity**. Panning automation could decrease time to discovery. Finally, comparing experimentally generated binders to computationally designed variants via ProteinMPNN and Rosetta could supplement our approach and add insight to candidate selection.

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Some figures and data have been modified or omitted in accordance with a confidentiality agreement with NextCure, Inc.