

# Direct, Competitive Comparison of Linear, Monocyclic, and Bicyclic Libraries Using mRNA Display

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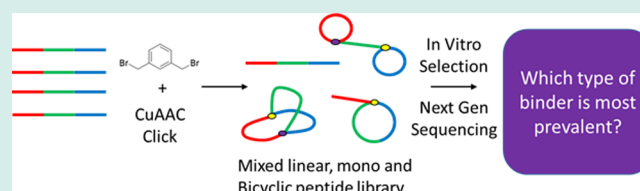
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**ABSTRACT:** Peptide macrocyclization is typically associated with the development of higher affinity and more protease stable protein ligands, and, as such, is an important tool in peptide drug discovery. Yet, within the context of a diverse library, does cyclization give inherent advantages over linear peptides? Here, we used mRNA display to create a peptide library of diverse ring sizes and topologies (monocyclic, bicyclic, and linear). Several rounds of in vitro selection against streptavidin were performed and the winning peptide sequences were analyzed for their binding affinities and overall topologies. The effect of adding a protease challenge on the enrichment of various peptides was also investigated. Taken together, the selection output yields insights about the relative abundance of binders of various topologies within a structurally diverse library.

**KEYWORDS:** mRNA display, peptide library, in vitro selection, peptide cyclization, binding affinity



A common variable that is considered in peptide library design experiments is conformational restriction through macrocyclization. Cyclization can endow peptides with a number of secondary benefits for drug discovery including resistance to proteolytic degradation and enhanced cell permeability.<sup>1–3</sup> But more fundamentally, it is argued that cyclization is likely to lead to higher affinity binders by “pre-paying” the entropic cost of binding; there are many examples of rational design which support this assertion.<sup>4–6</sup>

However, the consequences of cyclization for a given peptide are less clear within the context of a diverse peptide library. Cyclization of certain peptides will likely promote binding affinity through promoting optimal binding conformations, while cyclization of many others is likely to eliminate their ability to bind by locking them into conformations incompatible with the protein surface. One might expect, therefore, that the prevalence of cyclic binders within an unbiased library might be small in comparison to a linear library. Yet, very few screening or in vitro selection experiments have focused on this fundamental question.<sup>7</sup>

To get at these questions comprehensively, one needs significant sequence diversity and libraries with diverse cyclic structures.<sup>8,9</sup> mRNA display has an advantage in this context because it is able to create libraries containing over 10 trillion sequences.<sup>10–15</sup> Moreover, the compatibility of mRNA display with the introduction of noncanonical amino acids (ncAAs) allows many choices for cyclization chemistries.<sup>12,15–19</sup> We recently reported adding ncAAs for the development of two orthogonal cyclization reactions within mRNA display, copper-mediated azide–alkyne cycloaddition<sup>20</sup> and cysteine bis-

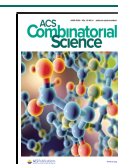
alkylation with *m*-dibromoxylene (DBX),<sup>16</sup> Figure 1A. Our new strategy allows controllable creation of peptide libraries with diverse topologies, as each of the four cyclization positions can be varied. In this Letter, we use this strategy to create a library that contains a mixture of linear, mono- and bicyclic peptides to see which type of binders are the most prevalent selection “winners.”

To generate our scaffold-diverse libraries, we designed three degenerate oligonucleotides with codons strategically placed to permit the chemistry necessary for cyclization but only in those peptides that contained the complementary cyclization residue (Figure 1A). For each library, we replaced methionine with  $\gamma$ -azido-*L*-homocysteine (Aha) and phenylalanine with *p*-ethynyl-*L*-phenylalanine (YnF). These residues, along with cysteine, allowed for cyclizable groups to be randomly encoded with degenerate codons. For two of the libraries (Lib 1 and Lib 2, Figure 1B–C), we used the NNY codon (Y = C or T) in the random region to increase the presence of cyclizable amino acids (Cys or YnF) because this codon increases the prevalence of Cys and Phe to 6.25% as compared to the standard NNS codon (3.13%) used in Lib 3. The trade-off for this choice is that five amino acids (Aha, K, E, Q, and W) were omitted from the random region of libraries 1 and 2. Each

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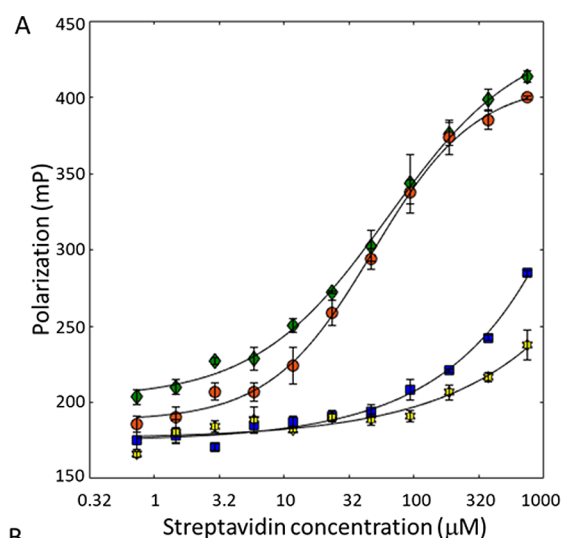
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deeply into the sequence winners, none of the top 1000 sequences were from libraries 1 or 2.

We note that many of the most abundant sequences have the HPQ motif that is common among streptavidin binders (Figure 2B and C).<sup>27</sup> To make sure that our abundant sequences bound to streptavidin, we selected three of the winning sequences that represented different families (Figure 2C, 8.1, 8.2, and 8.3, percentages show library abundance) and prepared them by solid-phase peptide synthesis, adding a 5(6)-carboxyfluorescein group and aminohexyl spacer to the N-terminus (analytical data for each peptide is found in Figures S1–S4). Each peptide was analyzed for binding against streptavidin using fluorescence polarization. Strep-Tag II<sup>28</sup> served as a control (Figure 3). Peptides 8.2 and 8.3 were mid-



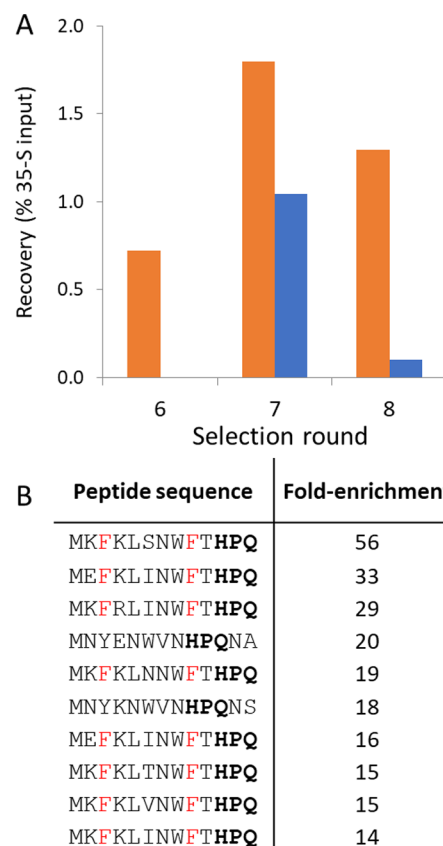
Name	Sequence	Kd (μM)
8.1	FAM-Ahx-N <sub>3</sub> NYEYELLPSHPQ	>500
8.2	FAM-Ahx-N <sub>3</sub> NYKNWVNHPQNY	48 ± 4
8.3	FAM-Ahx-N <sub>3</sub> TRYQWNTHPQNV	63 ± 8
StrepTag II	FAM-Ahx-WSHPQFEK	>500

**Figure 3.** In vitro binding affinity analysis of selected winner peptides. (A) Fluorescence polarization experiments demonstrating binding with each peptide: 8.1, yellow hexagons; 8.2, red circles; 8.3, green diamonds; Strep Tag II, blue squares. Measurements were performed after overnight incubation at 4 °C of the peptide (40 nM) with increasing concentrations of streptavidin. Error bars denote the standard deviation from three independent experiments. The streptavidin concentrations listed on the X-axis are the concentration of monomeric subunits. (B) Table of binding affinities. N<sub>3</sub> =  $\gamma$ -azidohomo-L-alanine, Ahx = 6-aminohexanoic acid, and FAM = 5(6)-carboxyfluorescein. We note also that we prepared a mutant of 8.1 for these studies where the N-terminal His was converted to an Asn.

micromolar binders in line with many other linear streptavidin binding peptides,<sup>27</sup> yet each bound better than Strep-Tag II which was a very weak binder under these conditions.

We were somewhat surprised that all of the peptide winners, even under low stringency conditions, were linear peptides, and we wondered if this outcome would change if we introduced a protease treatment step<sup>29</sup> during the selection. Starting with the output of round 6, we then repeated rounds 7 and 8, but prior to capture on streptavidin, we treated the

library with immobilized chymotrypsin. The fractional recovery of each round and the most highly enriched peptides are shown in Figure 4 and Table S4. Notably, 8 of the 10 most



**Figure 4.** Results of the second in vitro selection containing an added chymotrypsin digestion step. (A) In vitro selection rounds 6–8 for both selections. Orange bars denote the recovery of <sup>35</sup>S-containing peptides from streptavidin magnetic beads upon addition of biotin (this data is repeated from Figure 2). Blue bars are the parallel experiments done with a chymotrypsin challenge. (B) Peptide sequences showing the highest enrichment rates from rounds 6–8 in the selection that included chymotrypsin digestion. YnF residues are shown in red and the HPQ motif is highlighted in bold.

highly enriched sequences contain two YnF residues, which are sites for click cyclization with the N-terminal Aha residue. Presumably these peptides survived the protease challenge due to their cyclic nature.

Thousands of peptide binders have been uncovered against streptavidin,<sup>27</sup> however to our knowledge this is the first example where libraries of linear, mono, and bicyclic peptides were placed in competition with one another. As a whole, cyclic peptides that have been uncovered against streptavidin have higher binding affinity, so an interesting question raised by our work is, “Why were the selection winners dominated by linear peptides?” Here we offer some potential explanations for this surprising result. First, to enhance the proportion of cyclic peptides in libraries 1 and 2, we used the NNY codon, which eliminates 5 of the 20 amino acids, including glutamine, which is present in the ubiquitous HPQ motif.<sup>27</sup> Still, there are some peptides that bind to streptavidin that lack this motif,<sup>21,27,30</sup> including cyclic peptides we have uncovered using mRNA display.<sup>16</sup> Perhaps the more important feature is that our three libraries, while matched in peptide length, were not matched in

diversity due to the presence of a fixed codon at the C-terminal position in libraries 1 and 2 and the use of a less degenerate library codon in these libraries. Libraries 1 and 2 contain  $15^{11}$  potential sequences ( $8.6 \times 10^{12}$ ), while library 3 contains  $20^{12}$  potential sequences ( $4.1 \times 10^{15}$ ), nearly 3 orders of magnitude more diverse. While the scale of our mRNA experiments (24 pmol,  $1.4 \times 10^{13}$  molecules in round 1) means that we sampled only a fraction of these sequences, the proportion of similar sequences in libraries 1 and 2 would still be much higher than in library 3. Still, even within library 3, almost 35% of the sequences are expected to be mono or bicyclic (Figure 1C). Why did these not win out over the linear peptides? Perhaps cyclic peptide solutions to this target are more rare than linear ones, and under lower stringency conditions where higher affinity capture is not prioritized, the more common linear peptides win. Further experiments are underway to answer this interesting question. Finally, our last set of experiments (Figure 4) demonstrates that adding a protease digestion step during the selection process does, indeed, lead to selective enrichment of cyclic peptides. This result correlates with the known protease resistance of other classes of cyclic peptides.<sup>2,3</sup>

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscombsci.0c00016>.

Experimental methods, calculations of peptide shapes within the library, HPLC traces and MALDI-MS spectra for each peptide synthesized, and tables of the oligonucleotides used and high-throughput sequencing data (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ DEDICATION

Dedicated to the memory of David E. Hacker, who passed away during the review process.

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