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DIRECTED EVOLUTION: Overcoming biology's limitations

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Abstract

A process in which peptide nucleic acids may be used for *in vitro* evolution has been developed. This method can offer enormous opportunities to evolve stable, non-natural molecules for therapeutic applications.

The field of directed, *in vitro* evolution utilizes biological machinery to develop nucleic acid and peptide oligomers that bind to molecular targets, some of which are important for therapeutic applications.^{1,2} The advantage of this approach over traditional small molecule-based medicinal chemistry is based in part on the number of molecules that may be screened: *in vitro* evolution can screen upwards of 10¹⁵ different molecules for binding to a target while the best combinatorial libraries of small molecules are in the more modest range of 10² to 10⁷ molecules.^{3,4} The utility of this approach is nicely illustrated by the drug Macugen, which was approved by the FDA in 2004 for treatment of macular degeneration.⁵ The initial lead for this drug was identified by *in vitro* evolution screening of an oligonucleotide library for binding to vascular endothelial growth factor (VEGF). The main weakness of evolution-based techniques, however, has been the susceptibility to degradation of the oligonucleotides and peptides obtained from the process. Macugen, for example, is a highly modified version of the original oligonucleotide in which numerous chemical groups had to be incorporated to slow degradation. Furthermore, the reliance on enzymes for *in vitro* evolution has largely constrained the field to using natural biopolymers, although excellent advances have been made in identifying non-natural nucleotides and amino acids that are tolerated by enzymes in the process.⁶ A new study in this issue paves the way to use a class of non-natural polymers in directed evolution.⁷

Re-engineering *in vitro* evolution to use a completely non-natural backbone would eliminate concerns about degradation. In this regard, a class of molecules called Peptide Nucleic Acids (PNAs) would appear to be ideal. First described by Nielsen, Buchardt, and coworkers in 1991,⁸ the aminoethylglycyl PNA (aegPNA) has become one of the gold standards among non-natural nucleic acid mimics that bind strongly and in a highly sequence-dependent manner to complementary DNA or RNA sequences.^{9,10} PNAs are completely resistant to degradation by nucleases and proteases;¹¹ however, polymerases that are essential for the process of *in vitro* evolution do not recognize PNA either.¹²

In the manuscript by Brudno *et al.*, the authors overcome two significant obstacles to using PNA in the process of *in vitro* evolution. The first key to this system was to eliminate the need for a polymerase to generate a library of PNA oligomers. In this study, the authors cleverly and carefully optimized conditions for a DNA-templated synthesis of PNA that does not require enzymes and still has fidelity similar to that seen with polymerase replication (Fig. 1a). In this paper, DNA templates that are 40–60 nucleotides long are used to condense a mixture of 12 PNA pentamer building blocks. Once the PNA library has been made (Fig. 1b), liberating the PNAs from the DNA templates to which they are bound is a

challenge as PNA binds very strongly to complementary DNA. After extensive screening, the Herculanase II DNA polymerase was found to efficiently displace PNA from the DNA template at the 40 nucleotide length (Fig. 1c). Moving forward, the authors next demonstrate that the DNA-coding template strand that was used to make the PNA oligomer can be isolated and amplified in a PCR protocol (Fig. 1d). While these three steps (translation, displacement, and amplification) constitute most of the basic requirements of *in vitro* evolution, the process still needs to be iterated for several rounds of selection. Subjecting this system to such a test, the authors generated a mock selection system in which one PNA pentamer was labeled with biotin. The labeled-PNA was uniquely incorporated into a DNA template that was spiked at varying levels into a 10^8 member PNA library. Next, six rounds of selection for streptavidin binding were performed, which included amplification by PCR and retranslation into PNA. Beginning with a library that contained a 1:10⁶ ratio of the biotin-labeled PNA to non-labeled PNA members, the authors show that positive control templates are the primary sequences identified at the end of the selection process.

The next step for PNA-based evolution will be to identify a sequence that binds to a biologically relevant target. Presumably, PNA, like DNA and RNA, can fold into three-dimensional structures to create binding pockets with good molecular recognition properties, but this is only speculative and proving that PNA can fold in such a complex manner remains to be demonstrated. In addition, the requirement to use carefully designed pentamers as the building blocks for the translation part of PNA-based evolution will limit the sequence diversity that can be explored compared to oligonucleotide-based *in vitro* evolution. At the same time, there are numerous methods to chemically modify the PNA backbone with diverse types of sidechains that could broaden the chemical diversity of libraries generated using PNA-based evolution.¹⁰ Clearly there is more work to be done before *in vitro* evolution using PNAs can compete with the oligonucleotide versions, but the report of Brudno *et al.* lays the foundation to explore whether PNA can indeed be evolved to target proteins. Accomplishing this feat will certainly jumpstart the next generation of *in vitro* evolution research, and the system to start this process is now in place.

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Figure 1.

Scheme for *in vitro* evolution using peptide nucleic acids (PNAs). For the translation step (a), PNA pentamers with reactive aldehydes and amines on the termini are condensed in a sequence-dependent manner on a DNA template using reductive amination. This step uses a mixture of DNA templates to generate a library of PNAs bound to their complementary DNA sequences. For displacement (b), Herculanase II polymerase is used to make DNA duplexes and dislocate the PNAs from the DNA templates. Selection (c) is then performed followed by isolation and amplification (d). The process can be iterated several times to achieve enrichment for streptavidin binding of a biotin-labeled PNA present in the library at a 1:10⁶ ratio. While the current process is a proof-of-concept, this scheme lays the foundation to explore PNA-based evolution for biologically important protein targets.