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Review

Protein Engineering by Combined Computational and *In Vitro* Evolution Approaches

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Two alternative strategies are commonly used to study protein–protein interactions (PPIs) and to engineer protein-based inhibitors. In one approach, binders are selected experimentally from combinatorial libraries of protein mutants that are displayed on a cell surface. In the other approach, computational modeling is used to explore an astronomically large number of protein sequences to select a small number of sequences for experimental testing. While both approaches have some limitations, their combination produces superior results in various protein engineering applications. Such applications include the design of novel binders and inhibitors, the enhancement of affinity and specificity, and the mapping of binding epitopes. The combination of these approaches also aids in the understanding of the specificity profiles of various PPIs.

Trends

Novel protein binders to various targets can be engineered by first applying computational approaches and then optimizing the binder with yeast surface display (YSD). Computational methods can narrow down the choices of possible mutations and combinations of mutations, thereby enabling the construction of smaller, more focused libraries.

Together, combinatorial and computational techniques can be used to map binding epitopes of poorly characterized PPIs, thus identifying binding hot-spots and affinity-enhancing mutations.

Binding-specificity profiles can be mapped with a combination of combinatorial approaches and next-generation sequencing (NGS). Computational methods contribute to understanding the nature of specific PPIs, determining their binding specificity, and predicting ligands for homologous targets.

Engineering Protein–Protein Interactions

PPIs are crucial for all essential processes in the cell, including transcription, translation, replication, intra- and intercellular signaling, and molecular transport. Thus, it is not surprising that aberrant PPIs have been implicated in several types of disease, including neurodegenerative diseases and cancers. Studies from many different laboratories have shown that it is possible to modify various characteristics of PPIs through mutations and even to 'create' novel PPIs. Therefore, PPI engineering presents an attractive strategy in synthetic biology and in the design of biosensors, imaging agents, and novel therapeutics.

Two different approaches are commonly used in PPI engineering: the combinatorial approach and computational protein design (CPD). In the first approach, also known as directed evolution, large libraries of protein mutants are constructed, proteins with certain binding characteristics are selected, and the sequences of the selected proteins are determined. This 'irrational' approach is used for affinity maturation [1], for identifying target-specificity profiles [2,3], and for producing high-affinity and high-specificity PPI inhibitors from antibodies [4], natural protein effectors [5,6], and unrelated protein scaffolds [7–9]. The main advantage of combinatorial methods is that they require only minimal knowledge of the PPI under study. However, combinatorial approaches do not provide much information about the nature of the created intermolecular contacts, which often hampers an understanding of the obtained results. An additional drawback is that the number of sequences that can be explored by such methods is limited to several million, thus allowing the exploration of only a small fraction of the protein sequence space.

The second approach, CPD, is a 'rational' methodology that relies on our understanding of the biophysical forces that govern protein binding. This method requires a detailed knowledge of the

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structure and function of the PPI under study. As for the combinatorial approach, CPD has been successfully applied to manipulating PPI binding specificity [10–14] and binding affinity [15–20]. More recently, it has also been used to create novel binding interactions [21–23]. The advantage of CPD lies in its ability to explore a huge sequence space *in silico* and to select a few tens of protein sequences for experimental verification. Yet, CPD is impeded by the inaccuracy of the energy functions for calculating binding energetics and by current sampling methods that sometimes 'miss' the correct conformations of the binding interface residues.

Thus, each method has its particular advantages and disadvantages. Recent studies have shown that combining the two approaches could overcome the above limitations and produce superior results in PPI design. In this review, we describe the application of combined computational and combinatorial methodologies to problems in PPI characterization and engineering.

Increasing the Affinity and Specificity of Binding Interactions

Combinatorial and computational methods, separately or in combination, can be used to alter the binding characteristics of natural PPIs for various biomedical and synthetic biology applications [5,6,24–28]. All combinatorial approaches are limited in the size of the libraries that they can explore (a maximum of 10^{10} mutants; Box 1), which means that, in binding selection experiments, eight positions at most in a protein can be randomized to all 20 amino acids. Yet, the number of protein residues that can affect binding affinity and specificity, either through direct contacts or through allosteric effects, is usually larger than eight [29]. To overcome this limitation and to better exploit the randomization strategy, CPD can be used to design focused libraries of protein binders by identifying positions on the protein–protein interface at which mutations have the highest potential for affinity and specificity improvement and the lowest potential to compromise the protein structure [30].

Guntas *et al.* [31] used such an approach to generate a photoswitchable binding protein based on a naturally occurring photoswitch, the light-oxygen-voltage 2 (LOV2) domain, which partially unfolds upon exposure to light. The authors embedded the SsrA peptide into the LOV2 domain and engineered a light-sensitive binder for the natural ligand of SsrA, SspB. CPD was used to

Box 1. Principles of Combinatorial Approaches

The most commonly used combinatorial approaches for PPI engineering are phage display (PD), yeast surface display (YSD), and human surface display (HSD). In all these techniques, large combinatorial libraries of proteins are displayed on a cell surface, and a receptor protein is used as a 'bait' to select for binders. Since each cell contains the DNA for the displayed protein mutant, the sequence of the selected protein binders can easily be recovered.

PD

In PD, the library of interest is fused to a bacteriophage coat protein and displayed on the phage surface. Thereafter, to isolate specific binders, the pool of phages is mixed with a target protein that has been immobilized on either paramagnetic beads or microtiter plates [77]. The binding of the selected clones is verified by phage ELISA, in which the phage-displayed protein mutant is added to the plate-immobilized target and binding is detected via colorimetric output. The library size for PD, the largest of the cell display methods, can reach 10^{10} mutants. The limitation of this technique is that large proteins, proteins containing disulfide bonds, and proteins with post-translational modifications (PTMs) are frequently not compatible with the technology.

YSD

In YSD, combinatorial protein libraries have a maximal size of 10^8 mutants and are displayed on the surface of *Saccharomyces cerevisiae* cells [78,79] (Figure IA). The large size of yeast cells enables the selection of antigen-binding cells by flow cytometry (fluorescence-activated cell sorting; FACS) (Figure IB), thereby conferring a major advantage over the PD technology. YSD utilizes a two-color fluorophore labeling system (Figure IC), with one fluorophore detecting expression and the other detecting antigen binding. Thus, stability and affinity screenings are accomplished simultaneously. Once the selection process is complete, the binding affinity of the individual protein mutants can be estimated while the protein is displayed on the yeast surface, thereby enabling rapid screening of the clones without the need for lengthy expression and purification processes.

HSD

In HSD, a protein library (with a maximal size of 10^6 mutants) is expressed on the surface of human cells, and the cells carrying binding mutants are selected by FACS (Figure ID). The particular advantage of HSD is that it facilitates correct protein folding and the display of human proteins with PTMs [80–83]. The use of HCD greatly enhances the probability of selecting protein variants that function as agonists or antagonists of human proteins and could thus serve as future therapeutics.

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