Chapter 1

Synthetic Antibody Analogues and their Applications

Antibodies are a form of biological aptamer that have been utilized in the development of therapeutics and diagnostics. However, antibodies are a large biomolecule that is not perfectly optimized for these applications. This fueled the rise of synthetic aptamer classes to replace antibodies including peptide-based aptamer classes. In this review we look at the development and application of the peptide-based synthetic aptamer classes affimers, free-peptides, protein-catalyzed capture agents, and peptoids.

Synthetic Antibody Analogues and their Applications

Section 1.1-Introduction

Biological aptamers such as antibodies form an integral component of immune systems and have a variety of jobs *in vivo* including distinguishing cell types, detecting foreign invaders, and triggering allergic reactions.[1] Antibodies have also been utilized in the development of cancer treatments and microarray diagnostic kits. For example, antibody drug conjugates (ADCs) link a cancer drug to an antibody raised against specific cell type in order to deliver the drug to cancer cells bound by the antibody.[2] Secondly, immunotherapy treatments employ antibodies such as anti-PD1 to block the interactions between a T-cell and a tumor cell that prevent the T-cell from killing the tumor cell.[3,4] Additionally, the evolution of a patient's proteomics over time can be tracked via pull-down of various proteins using antibody microarrays such as the DNA Encoded Antibody Library (DEAL) technology.[5–7]

Antibodies are valuable biological tools, but they possess characteristics that present a challenge for their utilization. For example, interspecies antibodies are immunogenic and cannot be used in humans. Secondly, antibodies are the result of injecting an animal, usually a mammal, and extracting the resulting antibodies from their bloodstream. The use of animals to generate antibodies makes the antibody quality dependent on the animal's health and which species of animals is being used. Antibodies are also large proteins with the average IgG antibody weighing ~150 kDa.[8] As a result, antibodies have poor cellular penetration and are susceptible to thermal and proteasome degradation. Furthermore, antibodies are poorly optimized for direct surface immobilization, as this can affect their structure, which limits their usefulness in directly-patterned microarrays.

Some disclosed workarounds to the above issues include humanizing antibodies,[9] producing known antibodies in transformed cells,[10] and substituting single-domain antibody fragments for full-antibodies.[11,12] However, an alternative approach would be to develop a synthetic replacement, which mimics the selectivity and binding affinity of an antibody, but is better suited for biomedical applications. Synthetic aptamers[13] designed as antibody surrogates can be prepared from various biomolecules including nucleic acids[14] and peptides.[15] The peptide-based aptamer class includes the subclasses of affimers or peptamers,[16] free peptides, protein-catalyzed capture (PCC) agents,[17] and peptoids[18] (for representative structures of each see Figure 1.1). The remainder of this review will focus on these classes of peptide-based aptamers.

Section 1.2-Affimers

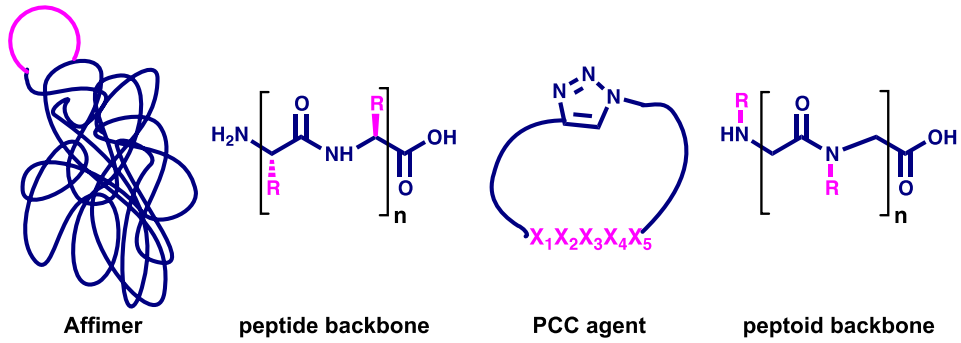


Figure 1.1. Representations of each affimer class. The parts colored blue are the backbone or scaffold of the aptamer and the part colored pink corresponds to the recognition region.

Peptamers consist of a modified small protein (~15 kDa) that serves as a scaffold to one or more peptide loops introduced into its structure by the use of restriction enzymes. The first peptamers were generated on the modified Thioredoxin A (TrxA) protein scaffold,[19] with multiple scaffolds reported since (Table 1.1).[19–25] The inserted peptides range in length from roughly 8-20 residues, which mimics the size of the antibody recognition

region while minimizing the number of defective peptamers in a library as a result of a random stop codon present in the peptide sequence.[26]

Table 1.1. Scaffolds used to develop affimer reagents.

Scaffold	Ref.
TrxA	19
STM	20
GFP	21
Affibodies	22
Anticalins	23
Adhiron	24
SQM	25

Peptamers libraries are generated by the preparation of plasmids containing the protein scaffold with the inserted random peptide sequences. The use of two orthogonal restriction enzyme sites to add each peptide sequence into the scaffold yields libraries with the greatest number of functional affimers, as the peptide sequence can only be inserted in a single direction.[26] A typical peptamer library ranges in size from 10^6 to 10^{12}, with library sizes $>10^9$ favored. The resulting plasmids are then transformed into eukaryotic yeast or mammalian cells in preparation for the library screen. Screening in eukaryotic cells ensures proper folding of the target proteins and tests the peptamers in conditions that mimic conditions found in the human body. Scale up production of affimer hits can occur in either *E. coli* or eukaryotic cell-based expression systems.

There are several different methods to screen peptamer libraries. One of the most common screening methods is the yeast two-hybrid system (Figure 1.2).[26,27] This screen exploits the discovery that the discrete DNA-binding domain (BD) and activating domain (AD) of certain transcription factors can still induce expression if they are fused to different molecules, but still in close albeit with reduced expression efficiency.[27] In the yeast two-hybrid system the TF's BD is fused to the target protein to produce the "bait", and the "prey" consists of the TF's AD-domain fused to both a nuclear localizing sequence (NLS) and unique members of the peptamer library. The gene under the TF's control is chosen carefully such that the screen's readout is either the growth of cells in

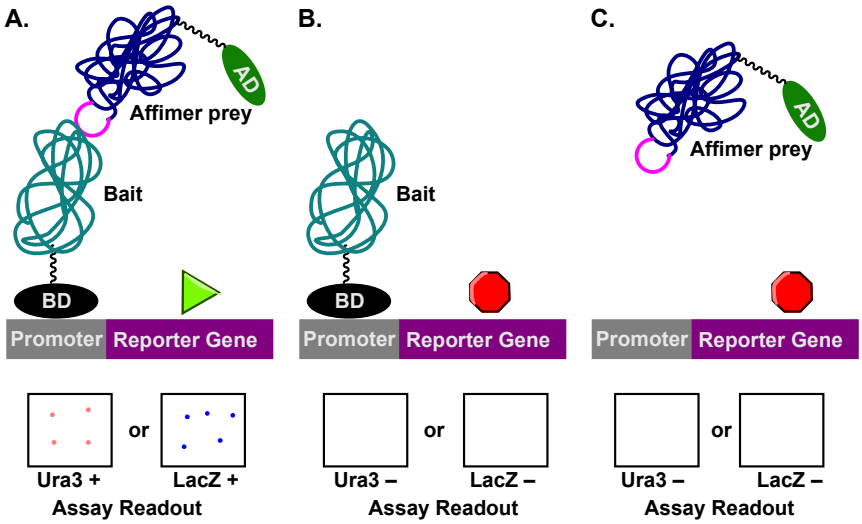


Figure 1.2. A representative schematic of the outcome of a yeast-two hybrid assay. A) The affimer prey binds successfully to the protein bait, which triggers activation of the reporter gene. Readout is either growth of the yeast cells in a nutrient deficient medium as seen in the Ura3+ box or a blue color as seen in the LacZ+ box. B) A lack of binding results in no activation of the reporter gene and no readout. C) Expressing the affimer library without the protein bait does not result in activation of the reporter gene and no readout.

media lacking essential nutrients (Ura3, Leu2, Ade2, His3, etc.) or the development of color due to enzymatic activity (LacZ).[28] Screens conducted in mammalian cells utilize a similar "bait and prey" interaction approach with different readouts: mammalian protein-protein interaction trap (MAPPIT, cytokine activation readout)[29,30] and cell array protein-protein interaction array (CAPPIA, fluorescent readout).[31] Mammalian-cell based screens are even better mimics of *in vivo* conditions than yeast cells, which can be important when developing affimers for *in vivo* applications. For libraries with the Adhiron scaffold, screening involves iterative bio-panning of phage-displayed peptamers.[24] After the library screen cells containing a "hit" are lysed, and the collected plasmids are sequenced before scale-up expression of the peptamers via standard protocols.

The last twenty-two years has seen affimers utilized as functional effectors on proteins[26,32] with the biotech company Avacta working to commercialize the affimer technology. Affimers, especially affimers based on the STM or Adhiron scaffolds, have

also been engineered such that they can be attached to microelectrodes to form apatasensors.[33–39] The aptasensors can form a microarray for the detection of cancer biomarkers or for proteomic analysis of a serum sample. Peptasensor microarrays can be readily produced using dot-printing production and other microfabrication protocols.

Section 1.3-Peptides

Peptides consist of a short polypeptide chain (<30 residues) and can be prepared as either a linear polypeptide or as a stapled (cyclic) macrocyclic peptide, which will be focused on in this review. Stapled peptides have increased affinity and proteolytic stability relative to linear peptides likely as a result of their constrained structure. Cyclization reactions include head-to-tail amidation, disulfide bond formation, thioether formation,[40] side-chain-to-tail amidation/N-alkylation, copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC),[41–44] and ring-closing metathesis[45–47] of unnatural, alkenyl side-chains in an *i, i+4* or *i, i+7* relationship.[48] Peptides can be prepared either via expression in transformed cells, through the use of purified ribosomes[49] or synthetically through solution phase or on-bead solid phase peptide synthesis (SPPS).[50–52] The heterogeneous synthesis environment of SPPS enables the high-throughput, automated synthesis of high-purity peptides that can contain amino acids not present in the original chiral pool.

Synthetic libraries of cyclic peptides can be prepared through a variety of different methods. Libraries prepared *in vivo* involve transforming cells to generate the library fused to some sort of biomolecule such as phages,[53] ribosomes,[54] mRNA,[55] and DNA. [56] Phage-displayed libraries typically undergo an iterative method of screening called biopanning.[57–59] In this recursive method phages that bind to the desired target are used as the starting point for successively smaller, focused libraries. These libraries are screened

under increasingly more selective conditions until peptides with high binding affinity for the target protein are isolated. After a library screen the plasmids corresponding to library hits are sequenced and the peptides are scaled up for further characterization. For peptides that are prepared in a purely synthetic fashion, the best method for generating a large combinatorial one-bead-one-compound library is the split-and-mix method independently discovered by researchers Furka[60] and Lam[61] (Figure 1.3).

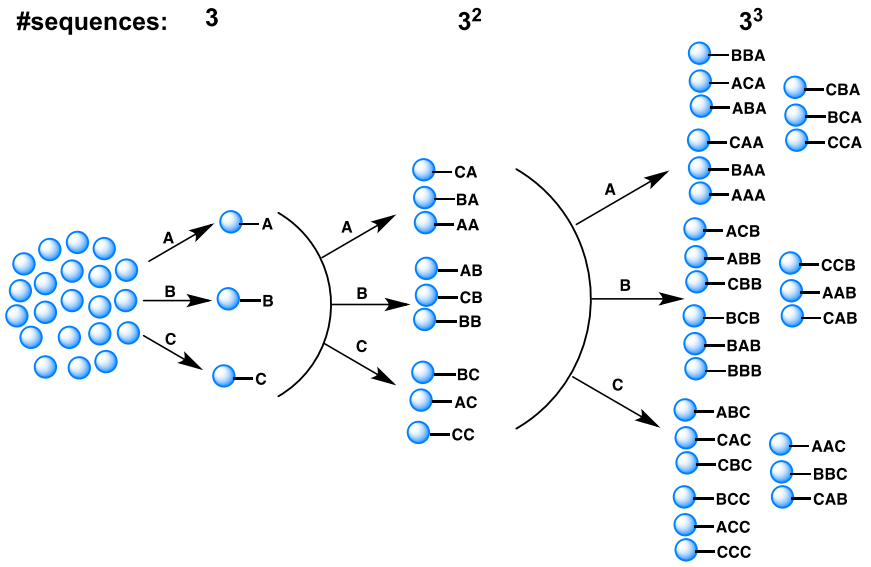


Figure 1.3. A demonstration of a combinatorial split-and-mix protocol. For each round of the split-and-mix protocol the beads are split into n pools and coupled with one of the n amino acids from the pool. After combining the separate batches the beads are split again for the following round. In the above figure the beads are split into three batches and undergo three rounds of coupling to generate 3^3 sequences.

In all peptide libraries the theoretical sequence diversity is roughly X^n, where X is the number of amino acids in the residue pool and n is the length of the peptide chain. As a result peptides six residues long can result in libraries that contain $>10^7$ unique sequences with peptide libraries of 20mers reaching theoretical sizes of $>10^{25}$, which is an incredibly large sequence space. Library screens can occur on-bead with the resulting peptide hits sequenced using Edman degradation[62] on-bead or the peptides are linearized

and cleaved from bead for mass spectrometry analysis methods.[63] The known sequences are then synthesized using standard SPPS protocols[64] and characterized further.

Cyclic peptides have been developed against a variety of protein targets for both *in vivo* and *in vitro* applications.[54,59,65–69]

Section 1.4-Protein-Catalyzed Capture Agents

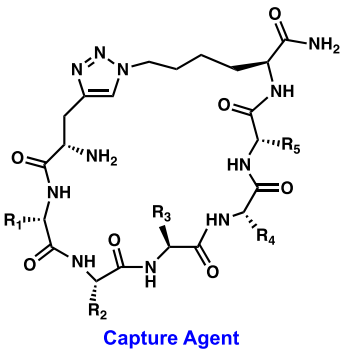
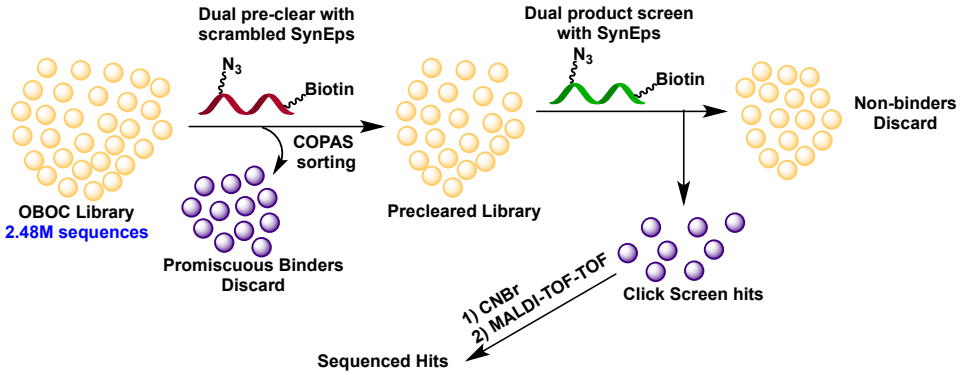


Figure 1.4. Example of a cyclic PCC ligand closed by a (1,4)-triazole linkage.

Protein-catalyzed capture (PCC) agents are a specialized class of peptides that were first disclosed by Heath and coworkers in 2009. PCC agents differ from other cyclic peptides in that the library screening process involves a protein/oligopeptide-catalyzed reaction. The PCC technology initially was composed of purely linear ligands, but now consists of cyclic ligands as the cyclic PCCs exhibit greater stability and binding affinity at the 1° ligand stage (Figure 1.4). The cyclic PCC agents have a variable 5-mer recognition region that is held in place by either a 1,4-triazole, resulting from a copper catalyzed (1,3)-dipolar cycloaddition, or an alkenyl ring closure, resulting from a ring-closing metathesis (RCM).

A PCC library is prepared using SPPS protocols on Tentagel resin using a pool of roughly 18-20 amino acids in a split-and-mix protocol using a pool of roughly 18-20 amino acids to yield a theoretical library size of 1.78 M-3.20 M sequences. Following ring-closure an amino acid containing a click handle is added to the N-terminus amino group and all acid-labile side chain protecting groups are removed.[70] While either an azido-presenting $^{4\text{-N3}}$K (Az4) or alkyne-presenting $^{\alpha\text{-propargyl}}$Gly (Pra) residue can be added, the Az4 group is prone to reduction over time to form Lys plus nitrogen gas, and

consequently Pra-presenting libraries are preferred. Screening a library against a particular target protein involves first selecting a solvent-exposed region of the protein that corresponds to a unique signature for the protein e.g. a point mutation for oncogenic



Scheme 1.1. A graphical flow-chart of the *in-situ* library click screen to identify PCC agents against a SynEp.

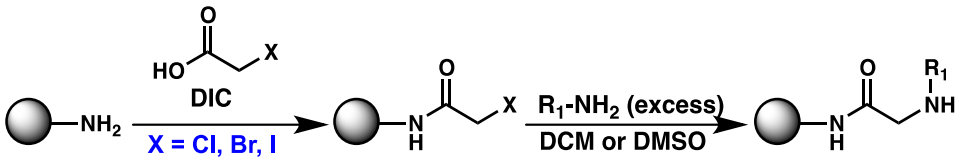
proteins or sequence divergence for polymorphic proteins. This epitope of 9-30 amino acids gets synthesized with both a biotin handle for detection and with one of its amino acids substituted for a structurally similar complementary click handle to prepare a synthetic epitope (SynEp) for screening.[70] Common substitutions are: I, K, L→Az4 and Pro→ $^{4\text{-N3}}$Pro or G → Pra.

The *in-situ* PCC library screening process involves two separate screening rounds in order to minimize the amount of "sticky" sequences isolated (Scheme 1.1). The *in-situ* click screen utilized is a very low-yielding reaction, which results in the routine isolation of cyclic PCCs with low nM binding affinity for the target protein.[71] First, the library undergoes a pre-clear typically with a scrambled SynEp to identify beads that bind non-selectively. The resulting colored beads are removed either by hand or through an automated complex object parametric analysis and sorting (COPAS) protocol,[72] and the remaining library is incubated with the SynEp to identify hits. The latest version of this screening protocol typically yields 5-10 colored beads after the product screen, which are

then sequenced either using Edman degradation[62] or the more accurate matrix-assisted laser desorption ionization time-of-flight time-of-flight (MALDI-TOF-TOF).[73] Hit sequences are scaled up using standard Fmoc/^{t}Bu SPPS protocols[74] with a conjugated biotin assay handle before testing the hits against the full-protein. This evaluation typically involves measuring the binding affinity of the ligands through an immunoassay either through a bulk 96-well plate setup or on the recently disclosed barcoded rapid-assay platform.[75] The best ligands identified by the best binding affinity (EC_{50} value) are subjected to further characterization/medicinal chemistry optimization in order to arrive at fully optimized ligands for the desired application. Biligands can be formed by raising PCC ligands against multiple regions of the same protein and linking the best ligands through a flexible PEG_n linker.

Protein catalyzed capture agents have been utilized for both the detection of proteins that function as disease biomarkers[70,76–79] and for exerting a functional effect on a particular protein.[17,75,80–86] A few PCCs have exerted this effect in cellular assays, which suggests that PCC agents could be used *in vivo* as therapeutics particularly as cancer drugs.

Section 1.5-Peptoids

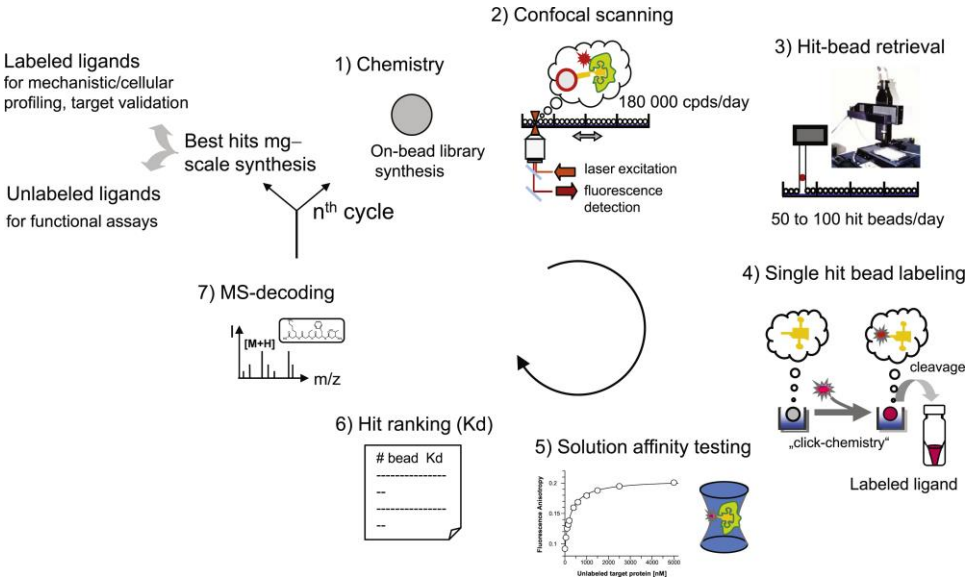


Scheme 1.2. The submonomer synthesis method for the preparation of peptoids. The submonomer pathway begins by coupling a 2-halo acid, typically 2-bromoacetic acid through a DIC active-ester intermediate. Next, a nucleophilic S_N2 displacement with an excess of a primary amine installs the N-alkyl group ready for another round of coupling.

Peptoids, or N-substituted polyglycine polymers, are a class of synthetic polymer that was first disclosed in 1992.[18,87] While similar to peptides in terms of having a polyamide backbone, the side chains migrated from the α-carbon to the amide nitrogen. As a result, the biomimetic polymer has an achiral backbone with tertiary amides that are completely resistant to proteolytic degradation. Peptoids are prepared via a modified solid-phase synthesis method called the submonomer method, which has been adapted to automated synthesis (Scheme 1.2).[88] This iterative two-step process involves acetylation of the free amine group with a 2-halo acetic acid, typically 2-bromo acetic acid, followed by S_N2 displacement of the halogen with a primary amine. The use of a primary amine to introduce functionality greatly expands the pool of potential side chains beyond the original twenty side chains found in α-amino acids. As a result, non-canonical side chains can be readily introduced. Most early reports of peptoids were linear, but recently cyclization methods have been developed for both solution phase and on-bead cyclization[89] including head-to-tail amide formation,[90–93] side-chain-tail cyclization,[94] side-chain click reactions,[95,96] side-chain ring-closing metathesis (RCM),[47,97] and triazine thioether formation.[98,99] Peptoid libraries are prepared using the aforementioned split-and-mix protocol to prepare OBOC libraries ranging in size from modest (~10^{3-4}) to large (~10^{5-6}) as the pool of viable amines increases.

Peptoid libraries can be screened by a variety of processes. For example, a classic OBOC library screen protocol for peptoid libraries was reported by Kodadek *et al.* [100] The main difference between this screening process and related ones reported for peptide libraries is the rescreening of hit peptoid beads to distinguish true hits from sticky false positives. Kodadek and coworkers also reported on a two-color cell-based procedure for

identifying peptoids that bind to membrane proteins.[101] In this screening process hits that bind cells with the overexpressed protein, but not the control cells, were taken as hits. Another screening protocol incubates the peptoid library with a His_6-tagged target protein and captures hits with magnetic anti-His_6 beads. The hits from a library screen can be sequenced in a manner similar to peptides and PCCS: Edman degradation on-bead or tandem mass-spectrometry after cyanogen bromide cleavage from bead.[102] In the case of cyclic peptoids a methionine residue is be incorporated both into the macrocycle and



Scheme 1.3. A protocol to directly analyze the binding affinity of library screen hits directly. From reference 97.

adjacent to the resin linker in order to linearize the hits and cleave from the bead in a single step.[100] Similar to the aforementioned aptamer classes, validation of the scaled-up hits involves measuring their desired performance off-bead and can occur after spotting the hits onto a spin-coated glass slide in a microarray format. However, Weidemann *et al*. disclosed a strategy where the library hits can be analyzed for binding affinity *before* scale-up occurs (Scheme 1.3).[103] After resin cleavage a fluorescent tag gets clicked onto the peptoid hit for measurement of the binding affinity with fluorescence polarization

(FP) measurement. As a result only the most promising hits are subject to scale-up and further investigation.

Peptoids have been raised against a variety of proteins both for detection of proteins in a microarray[104] and for functionally affecting the target protein in a manner that makes them potential therapeutics.[101,105–109] While the above examples highlight the ability of peptoids to target a wide variety of proteins, the affinity is modest likely due to the linear nature of the peptoids. Cyclic peptoids would be expected to have enhanced affinity/functional effects as a result of their more constrained nature.

Section 1.6-Analysis of the Synthetic Aptamer Classes and Future Outlook

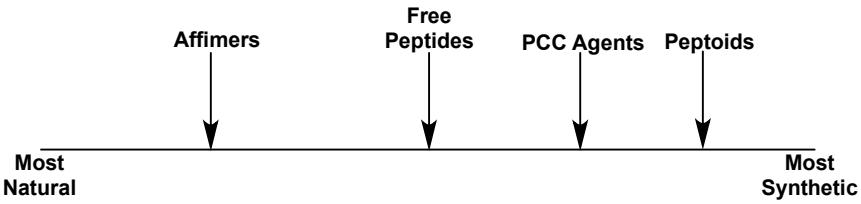


Figure 1.5. Ranking the different synthetic aptamer classes in terms of their similarities to naturally occuring biological motifs.

The different aptamer classes can be ranked in terms of their "naturalness" (Figure 1.5). Affimers are the most natural as they contain a protein scaffold similar to antibodies and are typically prepared using cell-based expression systems. Peptides are a transitional aptamer sub-class as peptides can be inspired by naturally occurring peptide sequences and generated via cellular expression, but a growing number of reported peptides are the result of random sequence generation and synthetic SPPS generation. PCCs are a more synthetic sub-class of aptamers relative to free-peptides as the stapling methods used are purely synthetic-based and the ligands are derived solely from combinatorial libraries. However, PCC agents still utilize the chiral pool of amino acids as a starting basis set

even if unnatural amino acids are included. Peptoids represent a purely synthetic class that cannot be traced back to any naturally occurring biomolecules or biopolymers, and a method of synthesis that is purely synthetic although there are limited reports of ribosomes preparing peptoids. While the synthetic aptamer classes are easier to prepare then antibodies, as they do not require the use of animals, only the more synthetic classes are able to incorporate unnatural motifs or easily modify the aptamer through modular substitution of "residues" (Table 1.2).

Table 1.2. Comparison of structure and preparation of antibodies and synthetic aptamers.

Property	Peptamers	Peptides	PCC Agents	Peptoids	Antibodies
Typical Size	~15 kDa	0.5-3 kDa	1-3 kDa	0.5-3 kDa	>150 kDa
Preparation	Expression	SPS/Expression	SPS	SPS	Expression
Modular Synthesis?	No	Yes	Yes	Yes	No
Introduction of Unnatural Motifs	Difficult	Easy for SPS preparation	Easy	Very Easy	Difficult
Scalable Synthesis	No	Yes if SPS	Yes	Yes	No
Multimers?	No	Yes	Yes	Yes	Yes

In fact, the automated synthesis methods available to prepare peptides, PCCs, and peptoids, respectively means that is easy to generate the large number of derivative sequences needed to interrogate the structure-activity relationship (SAR) between the recognition regions and desired functionality in a medicinal chemistry optimization approach.

Each aptamer class has physical properties superior to antibodies (Table 1.3) with peptamers and free-peptides the best characterized. The lack of information on immunogenicity for PCC agents and peptoids likely results from their limited use *in vivo*.

The aptamers wherein screening can occur on-bead have screening and sequencing procedures that enable rapid identification of hits without intensive plasmid isolation and screening. However, the use of cell-based screening assays with affimers yields information about functional performance in a complex environment.

Table 1.3. Comparison of physical properties of antibodies and synthetic aptamers.

Property	Peptamers	Peptides	PCC Agents	Peptoids	Antibodies
Proteolytic Stability	Low	Medium	Medium	High	Low
Temperature Stability	High	Medium	High	High	Low
Immunogenic?	Unknown	Medium	Unknown	Unknown	High
Cross reactivity	Low	Medium	Low	Unknown	Medium
Cellular Penetration	Yes	Yes	Yes	Yes	No
Nuclear Penetration	Yes	Yes	Unknown	Unknown	No
Typical EC_{50}/K_D values	nM	μM→nM	nM	μM→nM	nM→pM

At first glance the peptoid aptamer subclass appears to be the best suited for *in vivo* applications. In fact, the backbone of peptoids lends superior proteolytic stability as the backbone contains 3° amides. Additionally, the hydrophobic, floppy backbone mimics the structure of small molecules and consequently nearly all peptoids are cellular-penetrant although cyclic peptoids outperform linear peptoids. However, the affinity of peptoids hovers in the low μM to high nM range. The weaker affinity levels can stem from several causes. First, the polyglycine backbone is much more conformationally flexible and binding likely involves a larger entropic penalty than required with the more constrained peptide backbone. Conformational rigidity can be built into peptoids by cyclizing, incorporating bulky, chiral side chains on the amines, and substituting the 2-bromo acetic acid precursor for chiral 2-bromo propionic acids or rigid halogenated N-

heterocyclic acids. However, with the size of peptoid libraries typically in the 10^4 to 10^6 range, varying the composition of the backbone sharply limits the number of amines can be used to generate a library. Increasing the size of peptoid libraries further would dramatically increase the number of false positives/hits generated in a screen, which would require more resources to validate and identify lead compounds for further characterization. Additionally, most peptoids reported are linear peptoids, which are even more "floppy", with the number of cyclic peptoid disclosures expected to increase in the near future as most peptoid cyclization methods were reported in the last five years. Both peptides and PCCs have been regularly reported with nM affinity, and they can be engineered to penetrate cells either by making their backbones more rigid/hydrophobic with N-methyl amides or by attaching a cell-penetrating peptide sequence (CPP). However, the library screen of PCC agents generates the fewest hits, and, when Edman degradation artifacts are ignored, the lowest number of false positives of the discussed aptamer subclasses. In terms of overall stability and functionality PCC agents represent a "Goldilocks" solution between free-peptides and peptoids for *in vivo* applications.

For microarray development, while each aptamer class can be immobilized in an array format, the affimer class has a growing body of literature on their use as aptasensors, which makes affimers attractive for the development of proteomic microarrays. However, biotinylated PCC agents can be complexed with streptavidin-DNA conjugates for *indirect* assembly onto a microarray, which means that PCC agents can be independently prepared and modularly immobilized to the platform right before running an assay. This rapid assembly of separately generated components was demonstrated with the B-RAP technology and could be adapted to prepare highly modular PCC-based proteomic

analysis kits that can be rapidly adapted to the needs of the particular proteomic panel. To our knowledge the other aptamer classes are directly assembled onto the microarray platform, which requires the preparation of an entirely new microarray if a new protein panel is desired. As a result, the use of PCC agents in diagnostic microarrays and the advancement of PCCs into therapeutic clinical trials is expected to increase dramatically in the next 10-15 years.

Section 1.7-The Theme of the Thesis

The work summarized in the remainder of this thesis represents advances to the PCC technology. Chapter 2 summarizes contributions to an ongoing effort to develop a high-throughput production pipeline for PCC ligands with the development of a barcoded rapid assay platform (B-RAP) technology for the simultaneous evaluation of the binding affinity of up to fifteen different PCC agents in a single day. This chapter also discloses progress towards developing PCC agents against *difficult* proteins with the identification of PCC agents that bind to and inhibit the enzymatic activity of Kristen rat sarcoma (KRas) protein, whose oncogenic variants drive 20-25% of all cancers, but lack FDA-approved drug treatments. Chapter 3 summarizes another effort to target *challenging* protein targets with the development of PCC agents against the sticky *unstructured*, highly polymorphic protein Histidine rich protein II (HRP2).

Section 1.8-References

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