Chapter 4

Assays for Quantifying Protein-Catalyzed Multi-ligands and Extensions to Other Proteins

4.1 INTRODUCTION

Protein-templated in situ click chemistry is a low-yielding reaction, as it requires precise alignment of the azide and alkyne with respect to each other and the protein. Therefore, only a small fraction (<<1%) of the peptides on a particular bead will be converted to multi-ligands. Previously in Chapters 2 and 3, I discussed the discovery of a triligand capture agent, possessing antibody-like attributes, for the model protein, bCAII. We initially validated the in situ click assembly by analysis of sequence homology and binary component screens (monitored by MALDI-MS). However, challenges remained in developing direct, quantitative assays to assess the yield of multi-ligand capture agent following the in situ click/OBOC screen. Such quantitative assays define the signal-to-noise ratio for the in situ click/OBOC selection, since background chemical processes can also contribute to "false" hits. In this chapter, we will explore different assays for detecting on-bead, protein-templated triligand, such as colorimetric and quantitative polymerase chain reaction (QPCR) assays. The low but detectable yield per protein-catalyzed in situ click reaction—approximately 0.000005% for bCAII—confirms the exquisite demands of the process, and also provides guidance for the types of methods that can improve the signal-to-noise ratio for the in situ click/OBOC screening process.

This result encouraged us to develop more sophisticated screening strategies for improving signal-to-noise ratio during in situ click/OBOC screens. Such strategies incorporated anti-selections (following the selections) so as to remove hits that resulted from potential side reactions. The strategies also included the direct detection of the bead-bound products of the protein-catalyzed click reaction. Such product-based screens provide information that is highly complementary to that obtained from screens in which

hits are identified according to the presence of the target (i.e., the fluorescently labeled protein) on bead. These strategies were able to take advantage of the modular construction of the multi-ligand capture agents. As one example, by site-specific labeling the anchor (1°) ligand with biotin, we have a label that permits the direct monitoring of the in situ click reaction between 1° ligand and bead-bound 2° ligands. The use of this label is described in some detail within this chapter. In a second example, through the use of a labeled antibody, we can probe for bead-bound proteins during an in situ click/OBOC screen. These new screening strategies were applied toward the in situ click/OBOC selection of a biligand capture agent ($K_D \approx 140$ nM) against the blood-based protein biomarker prostate-specific antigen (PSA). The rapid assembly of the biligand capture agent by the protein-catalyzed process was expedited to two weeks by utilization of a previously reported anchor ligand[1] and the new selection/anti-selection strategies, and demonstrates the feasibility of a high-throughput route toward production of high-affinity, high-specificity protein capture agents.

4.2 MATERIALS AND EXPERIMENTAL METHODS

4.2.1 Materials

Proteins. Bovine and human carbonic anhydrase II (bCAII, C2522; hCAII, C6165), from erythrocytes, lyophilized powder, were obtained (Sigma-Aldrich; St. Louis, MO) and used as received. Human transferrin (Tf) and bovine serum albumin (BSA, ≥98%) were also purchased from Sigma-Aldrich as lyophilized powders. Prostate-specific antigen (PSA) was isolated by Scripps Laboratories (San Diego, CA) and shipped as a lyophilized powder. PSA activity was confirmed by an optical assay

employing the chymotrypsin substrate Suc-Arg-Pro-Tyr-pNA (AnaSpec, San Jose, CA; pNA = p-nitroaniline) as a chromogenic substrate.

4.2.2 On-Bead Detection of In Situ Triazole Formation

A biotin conjugate of the biligand anchor was prepared by modifying the N-terminus with an ethylene glycol linker (Fmoc-NH-$(PEG)_5$-COOH, EMD Biosciences) followed by biotin, by standard SPPS. A stock solution of this biotinylated biligand anchor Biotin-$(EG)_5$-(D-Pra)-kwlwGl-Tz1-kfwlkl (1.25 mM, alkyne) was prepared in DMSO (EG = ethylene glycol). Stock solutions of bCAII (30 μM) and hCAII (30 μM) were prepared in 50 mM Tris-Cl buffer (pH 7.2). For control experiments, stock solutions of human transferrin (Tf, 30 μM) and bovine serum albumin (BSA, 30 μM) were prepared in 50 mM Tris-Cl buffer (pH 7.2), and Biotin-RPRAAA-Pra (1.25 mM, alkyne with no documented affinity for CA II) was prepared in DMSO. The consensus 3° ligand Az4-nlivfr (azide) was synthesized in bulk on TentaGel S-NH_2 beads. Each in situ click reaction contained 0.5 mg beads appended with 3° ligand, 30 μM biotinylated peptide-alkyne, and 15 μM protein in a final volume of 50 μL 50 mM Tris-Cl buffer (pH 7.2) + 2.5% DMSO (v/v). In situ click reactions proceeded for 24 h at 25 °C with shaking. Reactions were quenched with 50 μL 7.5 M guanidine hydrochloride (GuHCl, pH 2.0). Following incubation with GuHCl (pH 2.0) for 1 h, the beads were washed with 10 × 200 μL water, leaving only covalently bound peptides (3° ligand and biotinylated in situ triligand) on the bead.

To prepare for the enzyme-linked, colorimetric assay,[2] beads were washed with 3 × 100 μL Blocking Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 150 mM NaCl, 14 mM 2-mercaptoethanol, 0.1% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.5). Beads were then

incubated in Blocking Buffer for 1 h with shaking. Alkaline phosphatase-streptavidin (AP-SA, Promega) was introduced at 1:300 dilution in Blocking Buffer to bind to any potential bead-bound biotinylated triligand. This AP-SA solution was incubated for 1 h with shaking. Excess AP-SA was then removed by washing the beads with 3 × 300 μL Wash 1 Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 150 mM NaCl, 14 mM 2-mercaptoethanol, pH 7.5), followed by 2 × 250 μL Wash 2 Buffer (25 mM Tris-Cl, 14 mM 2-mercaptoethanol, pH 7.5). Beads were developed for 2 h in 50 μL of the chromogenic substrate BCIP (5-bromo-4-chloro-3-indoyl phosphate, Promega).

4.2.3 QPCR Assay for the Detection and Quantitation of the Formation of On-Bead, Protein-Catalyzed Triligand Capture Agent

The Streptavidin-oligo reagent was prepared as described below: SAC expression was performed according to previously published protocols.[3] Prior to use, stock SAC (streptavidin-cysteine) was buffer exchanged to Tris buffered saline (TBS) containing 5 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) using desalting columns (Pierce). MHPH (3-N-Maleimido-6-hydraziniumpyridine hydrochloride, Solulink) in DMF was added to SAC at a molar excess of 300:1. In parallel, SFB in DMF (succinimidyl 4-formylbenzoate, Solulink) was added in a 40:1 molar excess to the 5' aminated oligo. The mixtures were allowed to react at room temperature for 3 to 4 h. Excess MHPH and SFB were removed and samples were buffer exchanged to citrate buffer (50 mM sodium citrate, 150 mM NaCl, pH 6.0) using Zeba desalting spin columns (Pierce). The SFB-labeled oligo was then combined in a 20:1 molar excess with the derivatized SAC and allowed to react for 2 to 3 h at room temperature before transferring to overnight incubation at 4 ^{o}C. Unreacted oligos were removed using a

Pharmacia Superdex 200 gel filtration column at 0.5 mL/min isocratic flow of PBS. Fractions containing the SAC-oligo conjugates were concentrated using 10K MWCO concentration filters (Millipore). The synthesis of SAC-oligo constructs was verified by non-reducing 8% Tris-HCl SDS-PAGE.

The triligand-containing beads were prepared as described in Section 4.2.2. After dissociation of the target, 0.5 mg beads were washed 10 times in water and resuspended in Blocking Buffer (0.15% BSA (w/v), 0.1% Tween-20, 150 μg/mL sheared salmon sperm DNA, in PBS pH 7.4). The beads were washed with 3 × 100 μL Blocking Buffer and incubated for 1 h at 25 °C in 100 μL Blocking Buffer. The beads were then filtered and washed twice more in 100 μL Blocking Buffer. Streptavidin-oligo (100 μL of 170 ng/mL dilution prepared in Blocking Buffer) was added and the beads were incubated for 1 h at 25 °C. The beads were washed 5 times in 250 μL Blocking Buffer followed by 3 washes in 250 μL PBS. The beads were resuspended in dH_2O and spotted on a glass slide. After evaporation, the beads were manually picked and placed in thin-walled PCR tubes.

Quantitative PCR (QPCR) was carried out on a Bio-Rad Real-Time PCR system. To each tube containing 1 to 5 individual beads was added 12.5 μL iQ SYBR Green Supermix (Bio-Rad), 11.5 μL dH_2O, 100 nM Forward Primer (5'…TAATACGACTCACTATAGGGACAATTACTATTTACAATTACA…3' –SEQ ID NO: 2), and 100 nM Reverse Primer (5'…ACCGCTGCCAGACCCCGATT TGGCCTGGGAGACGAACTCG…3' –SEQ ID NO: 3). Real-time PCR was carried out for 30 cycles with the following thermal profile: 94 ºC, 30 s, 50 ºC, 45 s, 72 ºC, 60 s. A standard curve was generated using known template concentrations ranging from 0.01 nM to 0.01 pM. The Ct values for each of the known concentrations were plotted

against the log of the template concentration to generate a linear standard curve which was then used to determine the concentration of oligo in each of the sample tubes. This was adjusted based on the number of oligonucleotide templates present per streptavidin tetramer as estimated by SDS-PAGE.

4.2.4 Selection of Biligand Capture Agent for Prostate-Specific Antigen

Two-stage in situ click/OBOC screen with biotinylated cyclic anchor. The comprehensive 5-mer Library X, displaying an N-terminal azidoalkyl amino acid, is first blocked overnight (0.2 g TentaGel scale) at 25 °C in Blocking Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 150 mM NaCl, 0.1% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.5). Then, 5 to 40 nM PSA is prepared with 2.5 μM biotin-labeled cyclic anchor (Biotin-$(EG)_5$-Pra-cy(CVFAHNYDYLVC), Figure 4.7A) in 1 to 3 mL Blocking Buffer. The protein and cyclic anchor were allowed to incubate for 1 h at 25 °C. This solution was subsequently combined with the blocked portion of Library X. After screening for 1 h at 25 °C, the library/PSA complex was washed with 5 × 3 mL Blocking Buffer to remove excess target and then incubated with a primary antibody for 1 h at 25 °C [mouse monoclonal anti-PSA, clone PS2 or PS6 (#M86433M or #M86111M, Meridian Life Science, Saco, ME)]. Primary antibodies were prepared at 1:5000 to 1:50,000 dilution in Blocking Buffer. Beads were then washed with 5 × 3 mL Blocking Buffer to remove excess primary antibody, and then incubated with a secondary antibody [anti-mouse IgG, alkaline phosphatase (AP)-conjugated, at 1:5000 dilution in Blocking Buffer; Cell Signaling] in Blocking Buffer for 30 min at 25 °C. Excess secondary antibody was removed by washing the beads with 5 × 3 mL Blocking Buffer, followed by 5 × 3 mL Wash 1 Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 700 mM NaCl, pH 7.5), and last by

5 × 3 mL Wash 2 Buffer (25 mM Tris-Cl, pH 7.5). Beads were developed for 30 to 90 min in the chromogenic substrate BCIP:NBT (Promega, #S3771), freshly prepared in Alkaline Phosphatase Buffer [100 mM Tris-HCl (pH 9.0), 150 mM NaCl, 1 mM $MgCl_2$], as recommended by the vendor. The darkest purple beads ("initial hits") are selected by micropipette, washed with 7.5 M guanidine hydrochloride (GuHCl pH 2.0) for 1 h to remove bound protein, then with 10 × 500 μL water. Beads are then decolorized overnight by incubation in DMF. After the purple dye has completely dissociated from the beads, the initial hits are re-swollen for at least 12 h in Blocking Buffer before moving on to the next step.

The second screen for direct detection of on-bead protein-templated biligand is achieved by incubating the initial hits with AP-SA (Promega) at 1:300 dilution in Blocking Buffer for 45 min at 25 °C. Excess AP-SA is removed by washing the beads with 5 × 3 mL Blocking Buffer, followed by 5 × 3 mL Wash 1 Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 700 mM NaCl, pH 7.5), and last by 5 × 3 mL Wash 2 Buffer (25 mM Tris-Cl, pH 7.5). Beads are developed for 30 to 90 min in the chromogenic substrate BCIP:NBT as described above. The darkest purple beads ("true hits") are selected by micropipette, washed with 7.5 M GuHCl (pH 2.0) for 1 h to remove bound protein, then with 10 × 500 μL water. Following this wash, the purple hit beads are analyzed directly by Edman degradation, and the sequences of the candidate 2° ligands are determined.

Synthesis of cyclic biligand candidates. Click reactions were performed in solution between cyclic anchor and 2° ligand. To begin, HPLC-purified cyclic anchor Biotin-$(EG)_5$-Pra-cy(CVFAHNYDYLVC) (EG = ethylene glycol, cy = denotes cyclized sequence) was dissolved in DMF to make a stock of 15 to 30 mM. Similarly, HPLC-

purified 2° ligands (Az8-iyydt, Az8-kyydt, and Az8-iyiet) were each dissolved in DMF to make a stock of 15 to 30 mM. In 200 μL of 4:1 DMF:H_2O, the in-solution reaction was set up with the following final concentrations: 2 mM Cyclic anchor, 3 mM 2° ligand, 3 mM CuI, 10 mM Ascorbic acid, 10 mM TBTA [Tris-(benzyltriazolylmethyl)amine]. TBTA is a ligand which accelerates catalysis while simultaneously protecting and stabilizing the copper(I) from oxidation, thus further improving the efficiency of the CuAAC.[4] After overnight reaction at 25 °C, the entire crude mixture was loaded onto the HPLC, and cyclic biligands were isolated at approximately 30% B (where A = H_2O/0.1% TFA and B = ACN/0.1% TFA). Non-optimized yield was >25%.

It should be noted that the protein-templated in situ click reaction may yield product regioisomers that are either *anti* (1,4), *syn* (1,5), or a mixture of the two geometries. Although we have not yet determined which regioisomers of the in situ click products were formed, the authentic multi-ligands synthesized by CuAAC were definitely the 1,4-triazole [Tz1 = triazole formed between Pra (appended from the cyclic anchor) and Az8 (on the 2° ligand)]. After bulk synthesis, cyclic biligands were purified by HPLC and analyzed by MS prior to use. Their characterization is as follows:

Biotin-$(EG)_5$-Tz1-cy(CVFAHNYDYLVC)-iyydt. MALDI-MS of the purified biotinylated cyclic biligand gave a peak at *m/z* 2982.9 for $[M + H]^+$.

Biotin-$(EG)_5$-Tz1-cy(CVFAHNYDYLVC)-kyydt (Figure 4.7B). MALDI-MS of the purified biotinylated cyclic biligand gave a peak at *m/z* 2998.9 for $[M + H]^+$.

Biotin-$(EG)_5$-Tz1-cy(CVFAHNYDYLVC)-iyiet. MALDI-MS of the purified biotinylated cyclic biligand gave a peak at *m/z* 2946.0 for $[M + H]^+$.

Characterization of affinity by surface plasmon resonance. SPR experiments were performed as described in Chapter 3, with a few minor modifications. Here, PSA [30 to 60 μg/mL in 10 mM sodium acetate (pH 5.5)] was immobilized to ~3000 RU on the CM5 chip using a running buffer of HBS-P^+ [10 mM HEPES, 150 mM NaCl, 0.05% Tween20 (pH 7.4)]. Cyclic biligands were dissolved in HBS-P^+ buffer to produce 2.5 μM stock solutions for each peptide, which were then serially diluted by a factor of 2 to produce a concentration series down to 0.3 nM. Cyclic anchor ligands were dissolved in HBS-P^+ buffer to produce 10 μM stock solutions for each peptide, which were then serially diluted by a factor of 2 to produce a concentration series down to 1 nM. For a given affinity measurement, these series of peptide solutions successively were injected into flow cell 2 (or 3) for 360 s of contact time, 300 s of dissociation time, and 200 s of stabilization time using a flow rate of 50 μL/min at 25 °C. Data processing and affinity analysis, including background subtraction, was performed using Biacore T100 evaluation software (Version 2.0.1, Biacore) as before.

4.3 RESULTS AND DISCUSSION

4.3.1 Initial Validation of Protein-Catalyzed Multi-ligand Product

Protein catalyzed, multi-ligand capture agents were prepared according to the scheme of Figure 2.1. When an in situ multi-ligand screen was carried out as illustrated in Figure 2.1, only a very small fraction of the on-bead n-order ligands were covalently coupled to the solution-phase 1° ligand by the protein. Analysis of the n-order ligands on the bead using standard methods yields information largely about the sequences of the n-order ligands themselves, since they comprise >99% of the molecules bound to the bead, and not the complete multi-ligand. For previously published in situ click

chemistry screens, the triazole product was identified using chromatographic separation followed by mass spectrometry.[5–9] For the case of the in situ click/OBOC biligand screens (Figure 2.1), the binary component screen was adopted. This was not a broadly applicable method, but showed efficacy in one exemplary case, which was discussed in Chapter 2 (Figure 2.9). Thus, alternative strategies were developed for demonstrating that the protein-catalyzed multi-ligand capture agent selections are indeed successful.

Two alternative strategies include: sequence homology analysis, and assays involving amplification of one or more labeled ligands. For both the first-generation biligand and triligand screens, a striking result was the extremely high sequence homology that was observed for the hit beads. For example, for the first 17 hit beads sequenced from screen **Bi1**, two peptides were identical, and a third peptide varied by only a single amino acid (see Appendix B). For screen **Tri1** (against the same library), the most commonly observed amino acids by position almost exactly reflect the consensus sequence identified in the second generation (focused) screen **Tri3** (see Appendix B). Such sequence homology was unique to in situ click/OBOC screens, and argues that these screens generate highly selective hits.

4.3.2 Direct Detection of Protein-Catalyzed In Situ Multi-ligand

Assays with labeled ligands. An enzyme-linked, colorimetric assay was developed for detecting on-bead, protein-templated multi-ligand (Figure 4.1). This approach relies upon appending a small molecule, such as biotin, to the solution-phase anchor (1°) ligand that is used in the screen. Once the screen has been completed, the small molecule will be covalently functionalized on only those beads that contain the protein-catalyzed multi-ligand. That small molecule can then provide a handle for

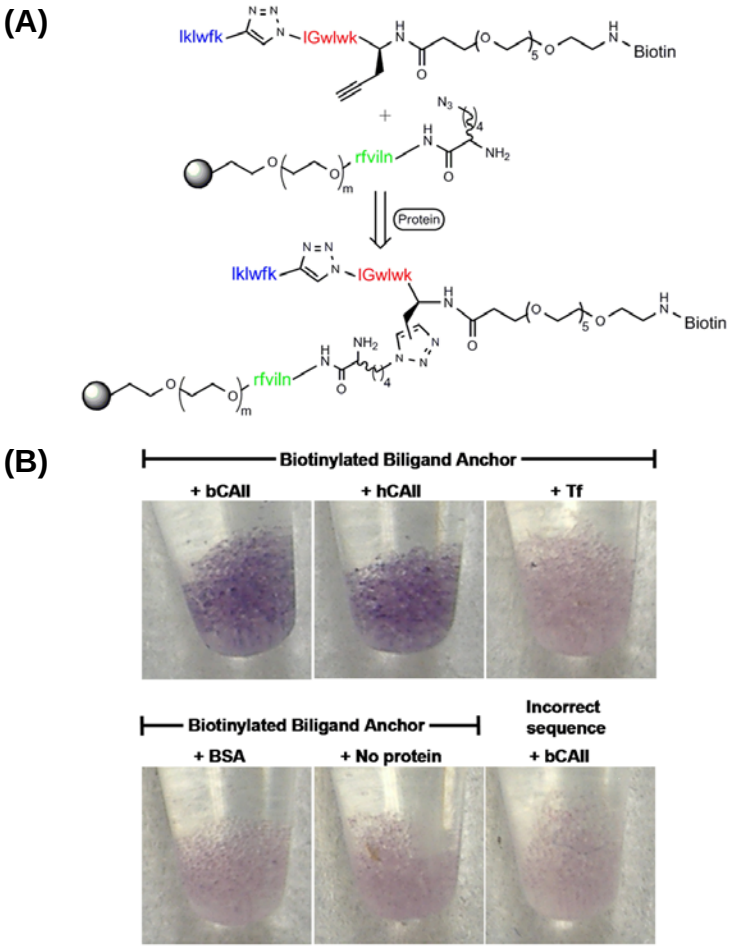


Figure 4.1. **(A)** Schematic of in situ click assay for on-bead triazole formation, using a biotinylated biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl. **(B)** Purple beads are visualized as a positive indicator of triazole formation.

building up a chemical construct that can generate some detectable signal. The most successful approaches will rely on signals that can be amplified. For example, if an enzyme is appended to the small molecule, and then that enzyme can be utilized to catalyze some chemical process, which in turn represents an amplified signature of the on-bead protein-catalyzed multi-ligand. The product molecules from the enzymatic reaction can be uniquely colored, fluoresce or have some other unusual chemical or physical property that can be detected, thus providing evidence for the formation of the on-bead multi-ligand product. Results of such an assay, utilized to detect the on-bead formation of the triligand shown as the product of the 3° ligand screen of Figure 2.1, are presented in Figure 4.1.

In particular, the illustration of Figure 4.1A shows the schematic of in situ click assay for on-bead triazole formation, using a biotinylated biligand anchor Biotin-$(EG)_5$-(D-Pra)-kwlwGl-Tz1-kfwlkl. After dissociation of the protein target, Figure 4.1B shows that treatment with alkaline phosphatase-streptavidin (AP-SA) then BCIP (5-bromo-4-chloro-3-indoyl phosphate) yields purple beads as a positive indicator of multi-ligand formation. In situ triligand was only formed in the presence of b(h)CAII protein, and not when the protein was human transferrin (Tf), BSA, or absent. Also, triligand is not observed when the biligand anchor sequence is incorrect.

QPCR assay for the detection and quantitation of the formation of on-bead, protein-catalyzed multi-ligand protein capture agent. Quantitative polymerase chain reaction (QPCR) enables both detection and quantification of oligonucleotide templates (as an absolute or relative copy number) through real-time monitoring of the intercalation of double-stranded DNA-binding fluorescent dyes during template

amplification. Fluorescence emission during the elongation step of each cycle is proportional to the amount of PCR product and enables direct monitoring of the PCR reaction. The resulting PCR curve is used to define the exponential phase of the reaction, which is a prerequisite for accurate calculation of the initial copy number at the beginning of the reaction.[10] Real-time PCR assays are characterized by a wide dynamic range of quantification, a high technical sensitivity (< 5 copies of template oligo) and a high precision (< 2% standard deviation).[11,12]

To quantify the formation of on-bead, protein-catalyzed triligand obtained by the assay of Figure 4.1A, it was necessary to transform the biotin label into an oligonucleotide label. The PCR-based assay shown in Figure 4.2 is a variation of the enzymatic assay where AP-SA is replaced with streptavidin conjugated to a small template oligonucleotide (5'…NH_2–$(CH_2)_6$–GGGACAATTACTATTTACAATTAC AATGCTCACGTGGTACGAGTTCGTCTCCCAGG…3' –SEQ ID NO: 1). Binding of this reagent to biotinylated triligand results in the recruitment of the template oligonucleotide to the bead surface where it can be amplified by PCR. The extent of amplification (i.e., number of PCR cycles required to produce a band) is directly proportional to the amount of oligonucleotide at the bead surface, providing a quantitative readout of the assembled triligand and hence the efficiency of the in situ click reaction.

The results shown in Figure 4.2 are roughly in line with the colorimetric AP-SA assays. The percent yield for the bCAII-catalyzed click reaction between biotinylated biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl and 3° ligand Az4-nlivfr may be estimated as 0.000005% from the QPCR assay of Figure 4.2. This takes into account a stoichiometry of 4 oligos per streptavidin tetramer, and estimates that single beads

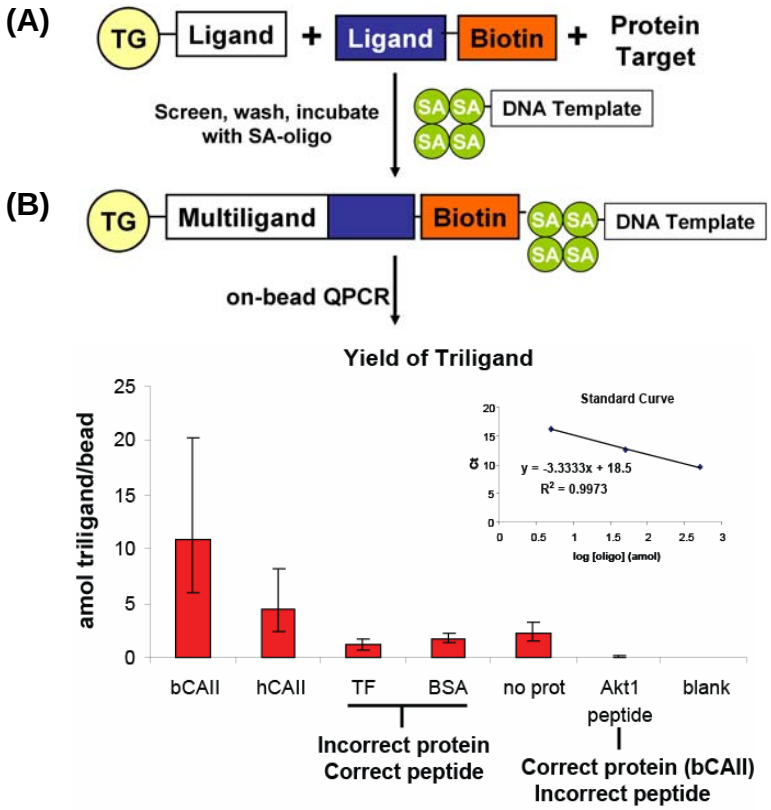


Figure 4.2. **(A)** General method for detecting on-bead multi-ligand by QPCR. **(B)** In a specific example, the results of a QPCR assay quantifying the yield of biotinylated triligand from the protein-catalyzed in situ click reactions of Figure 4.1 are illustrated. Results are expressed by bar graph in units of mean amol triligand/bead for 5-bead samples (N = 3). The Ct values for a series of known template concentrations were used to generate a linear standard curve (inset), from which the concentration of streptavidin-oligo reagent in each 5-bead sample was extrapolated.

display a uniform loading of 100 pmol/bead. It is interesting that while bCAII apparently makes 2 times more triligand product per QPCR assay than hCAII, the triligand displays a slightly higher binding affinity for hCAII (see Chapter 3). Out of the controls, the "no protein" control displays the consistently higher background reaction than the BSA and Tf controls. It is possible that BSA and Tf are blocking reactive azides on the bead and attenuating the background click reaction. It is also interesting that the Akt1 peptide control displayed the least background, showing nearly no triligand formation, and is comparable with blank beads. Here, bCAII is evidently binding to the bead but not to the Akt1 peptide, and azides on these beads are blocked more effectively with bCAII than with BSA or Tf.

4.3.3 Strategies for Improving Signal-to-Noise Ratio during In Situ Click/OBOC Screens

Based on the success of the colorimetric assay in Figure 4.1, a new method for visualization of hits from the in situ click/OBOC screens emerged. Rather than stratifying hit beads based on fluorescence (via binding of a fluorescently labeled protein target, see Chapter 2), it became apparent that the assay of Figure 4.1 could be easily modified to accommodate screening of an entire bead library rather than a single sequence. This colorimetric approach removed dependence on a fluorescence microscope (or array scanner) for identification of hits, and allowed the researcher to pick the hits in real time while monitoring the BCIP development with a standard light microscope. Furthermore, the intrinsic autofluorescence[13,14] of TentaGel S-NH_2 beads was no longer a potential challenge to the signal-to-noise ratio. It also should be noted that the more delicate of protein targets (such as phospho-Akt), which were previously

intolerant to covalent modification with fluorophores, now became candidates for multi-ligand capture agent development.

We were surprised by the extremely low yield of the protein-catalyzed click reaction. Such a low-frequency event may potentially be surrounded by a high level of background. A major source of background would arise from the binding of protein target to the bead, but without any click reaction occurring. Since our methods of Chapter 2 relied on protein detection (by way of the fluorophore), "background hits" would not have been distinguished from "true hits." Thus, the general concept of multi-stage screening was explored (see Figures 4.3-4.4).

For the most important cancer-specific protein biomarkers, antibodies are available. Therefore, primary and AP-labeled secondary antibodies initially could serve as reagents to detect bead-bound protein from a simple OBOC screen, as shown in Figure 4.3A and Figure 4.4A, all the way to the in situ click/OBOC screen (see Figure 4.5A for a specific example). This antibody-based screening approach is essentially a sandwich (ELISA-like) assay, but with the solid support being a bead rather than a microwell of a 96-well plate. As an added bonus, this approach also selects for those hit beads and peptides that can eventually form a multi-ligand capture agent that, together with the antibody, form an ELISA pair.

The antibody-based screening approach also allowed for improvement of the signal-to-noise ratio, through pairing each screen with an anti-screen. Figure 4.3B illustrates an anti-screen which would be performed following the 1° ligand screen of Figure 4.3A. This screen eliminates "background hits" which would represent natural antibody-binding epitopes. Figure 4.4B illustrates an anti-screen which would be performed following the *epitope-targeted* 1° ligand screen of Figure 4.4A. This screen

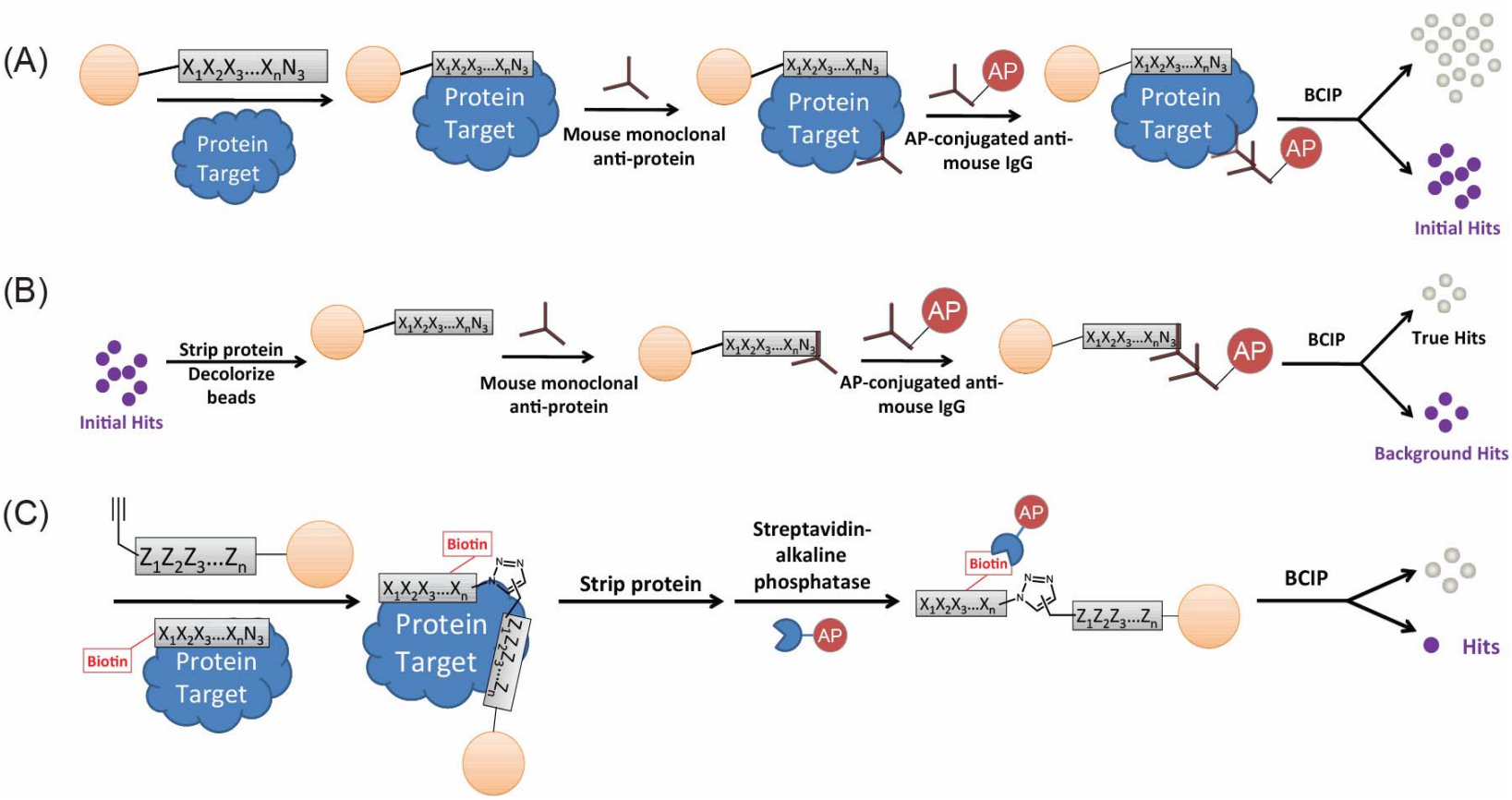


Figure 4.3. General screening strategies to improve signal-to-noise ratio and reduce number of false positives in OBOC selections. **(A)** Colorimetric antibody screen for initial hits. This screen can be used at any level of multi-ligand discovery (i.e., anchor, biligand, etc.), as only bound protein is detected. **(B)** Anti-screen for removing background hits. **(C)** Direct detection of on-bead biligand.

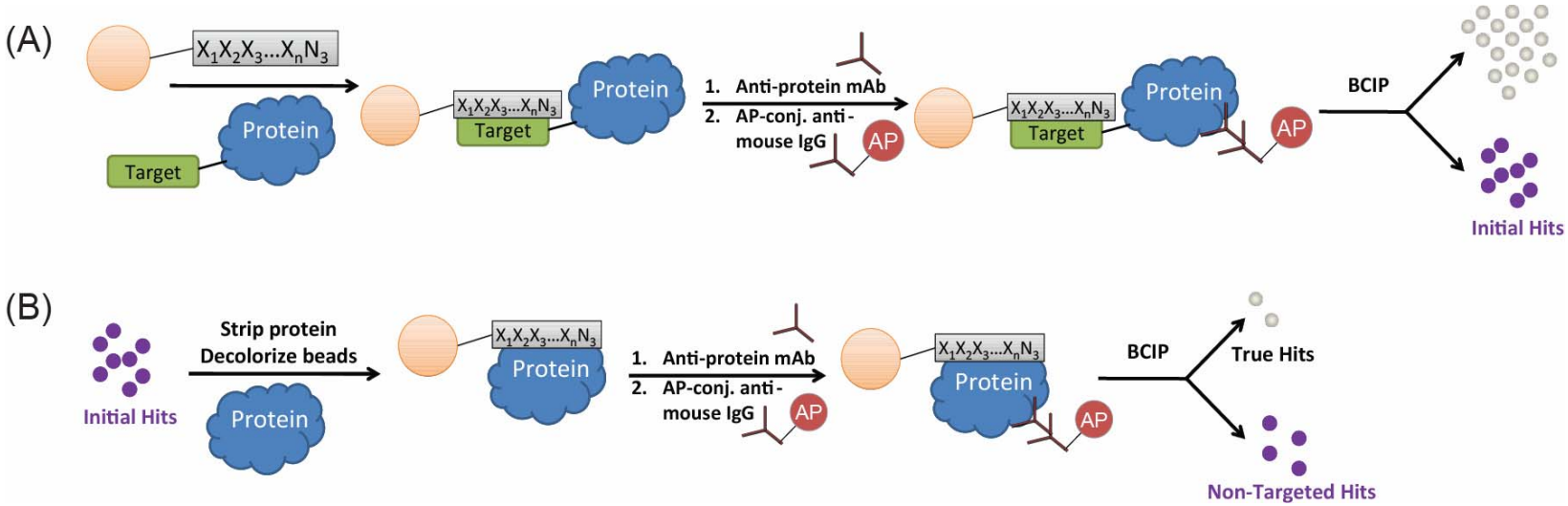
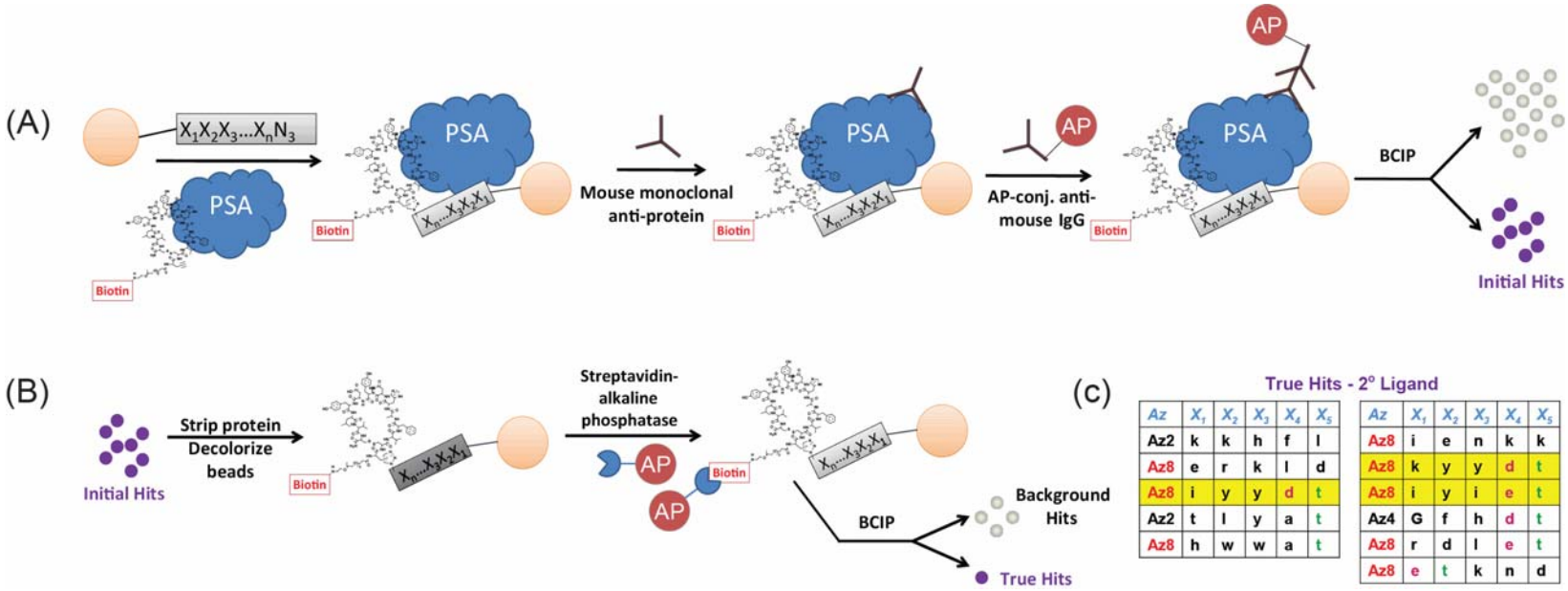


Figure 4.4. **(A)** Screening and **(B)** anti-screening strategies to target a particular protein epitope or modification.



Az	X_1	X_2	X_3	X_4	X_5
Az2	k	k	h	f	l
Az8	e	r	k	l	d
Az8	i	y	y	d	t
Az2	t	l	y	a	t
Az8	h	w	w	a	t

Az	X_1	X_2	X_3	X_4	X_5
Az8	i	e	n	k	k
Az8	k	y	y	d	t
Az8	i	y	i	e	t
Az4	G	f	h	d	t
Az8	r	d	l	e	t
Az8	e	t	k	n	d

Figure 4.5. Two-stage in situ/click OBOC screening strategy to identify a biligand capture agent for PSA using a previously identified cyclic peptide Biotin-$(EG)_5$-Pra-cy(CVFAHNYDYLVC) as the anchoring unit. It should be noted that this strategy is a specific application of Figures 4.3A,C. **(A)** Probing for bound PSA. **(B)** Probing for on-bead, protein-catalyzed biligand. **(C)** True hits displaying a high degree of sequence homology.

eliminates "background hits" which would represent non-targeted peptides. Figure 4.5B illustrates the specific case of an anti-screen that results in direct detection of the bead-bound biligand products of the protein-catalyzed click reaction. Through detection of the biotin label, we can parse out the false-positive beads which bind to PSA (in the Figure 4.5A screen) but do not undergo in situ click reaction with the biotinylated anchor ligand. A general screen for direct detection of protein-catalyzed in situ hits is shown in Figure 4.3C, and this is simply the whole-library extension of the Figure 4.1 assay.

4.3.4 Selection of Biligand Capture Agent for Prostate-Specific Antigen

The in situ click/OBOC selection of an epitope-targeted biligand capture agent against prostate-specific antigen was explored as a feasibility demonstration for the antibody- and biotin-based multi-stage screening strategies.

Prostate-specific antigen. PSA is a 30-kDa serum glycoprotein and protein biomarker for detection and management of prostate cancer. This protein is present in normal prostatic tissue, but increased levels of PSA are a reliable indicator of prostate cancer and are widely used as a marker of potential cancerous growths or disease status.[15] Differences in concentration between the active form of PSA and enzymatically inactive versions (e.g., proPSA, nicked inactive PSA, ACT-PSA complex) may provide distinguishing information between cancer and benign prostatic hyperplasia (BPH), which is a common misdiagnosis. Additionally, PSA has a single N-oligosaccharide chain attached to Asn-45, and it has been reported that one can distinguish PSA origin (healthy vs. tumor) through differences in glycosylation patterns.[16] Creating specific

multi-ligands that target these minor variants of PSA may potentially facilitate more accurate diagnosis of prostatic diseases.

Preparation of anchor (1°) ligand. Due to its relevance in the diagnosis and monitoring of human prostate cancer, PSA is a well studied protein target for ligand development. Phage display,[1,17] polysome selection,[18] and *in silico* structure-guided design[19,20] have all been used to isolate peptide ligands of μM to nM binding affinity against PSA. It was our idea that the multi-ligand discovery process can be expedited through implementation of one of these peptides as the anchor (1°) ligand. Indeed, the 1° ligand OBOC screen is the most challenging step of Figure 2.1 since only weak binding interactions are probed. After evaluating several of these reported peptides, we chose the optimized phage-derived cyclic sequence cy(CVFAHNYDYLVC) as the 1° ligand for the rapid selection of a multi-ligand capture agent against PSA.[1] SPR measurement determined that this peptide displays a binding affinity of $K_D \approx 2.4$ μM for its interaction with PSA. As this K_D value is approximately the same affinity as our *biligand* for bCAII, we concluded that cy(CVFAHNYDYLVC) was an excellent starting point for building a multi-ligand capture agent that specifically recognizes the active site of PSA.

Cyclic peptides, due to their conformational rigidity, lose less entropy and free energy upon binding to targets than their linear counterparts.[21] This translates to enhancements in receptor-binding affinity, specificity, and stability. To illustrate this point, we determined that cy(CVFAHNYDYLVC) binds to the active site of PSA when cyclized, but does not bind as a linear sequence (SPR, data not shown).

In order to prepare the peptide cy(CVFAHNYDYLVC) for in situ click chemistry, the artifical amino acid L-propargylglycine was installed on the N-terminus during solid-phase peptide synthesis of the linear sequence. Following the coupling of L-propargylglycine, two other chemical modifications were made. First, Fmoc-NH-$(PEG)_5$-COOH (22 atoms) (Novabiochem) was installed to impart better water solubility to the cyclic anchor ligand. Second, biotin was added to cap the N-terminus, thus providing the label for performing the two-stage in situ click/OBOC screen with direct detection of on-bead protein-catalyzed biligand. Peptide cyclization by formation of a disulfide bond was achieved by an oxygen/Cu(II)(1,10-phenanthroline)$_3$ system.[22] The final structure of cyclic anchor ligand Biotin-$(EG)_5$-Pra-cy(CVFAHNYDYLVC) is shown in Figure 4.7A. Results from SPR determined that the addition of these linker moieties did not affect the overall binding affinity of the anchor to PSA. It was found that this modified cyclic anchor ligand had an affinity of 2.1 μM (Figure 4.7C). A kinetic fit of the data yielded $k_d = 0.09\ s^{-1}$ and $k_a = 4.5 \times 10^4\ M^{-1}s^{-1}$ (for 1:1 binding interaction).

Binding specificity for the cyclic anchor ligand of Figure 4.7A was characterized by sandwich (ELISA-like) assays on streptavidin-functionalized microtiter plates. Similar to Chapter 3, the cyclic anchor (1° capture agent) was paired with a commercial mouse monoclonal anti-PSA antibody (2° capture agent) for quantification of captured PSA. For a sandwich assay performed with PSA presented in 10% porcine serum, the analytical sensitivity was ~2 μM (~60 μg/mL), further confirming the SPR result of Figure 4.7C even in a high protein background.

Cyclic peptides were prepared in bulk by solid-phase synthesis, purified by HPLC, and analyzed by MS prior to use. Their characterization is as follows:

cy(CVFAHNYDYLVC). MALDI-MS of the purified cyclic peptide gave a peak at *m/z* 1443.2 for $[M + H]^+$.

Biotin-$(EG)_5$-Pra-cy(CVFAHNYDYLVC) (Figure 4.7A). MALDI-MS of the purified biotinylated cyclic anchor gave a peak at *m/z* 2100.0 for $[M + H]^+$.

Two-stage in situ click/OBOC screen for biligand capture agent using a biotinylated anchor ligand. The two-stage in situ click/OBOC screening approach for selection of a biligand capture agent against PSA is shown in detail in Figures 4.5A-B. Note that this screening procedure is an application of the general methods in Figure 4.3. For these screens, a single comprehensive library of 3×18^5 hexamers was used (Library X): Az-$X_1X_2X_3X_4X_5$-TentaGel, where Az = azidoalkyl amino acids Az2,[23] Az4, or Az8, and X = all D-amino acids except D-Cys and D-Met. In the first stage (Figure 4.5A), two antibodies were applied to detect and amplify the binding of PSA to the bead library during an in situ/click OBOC screen. Binding of monoclonal anti-PSA antibody to the PSA-bound bead was visualized by probing with an AP-labeled secondary anti-IgG, followed by treatment with BCIP. The percentage of hits (purple beads) following this first screen was 10%, a value which indicated that a significant number of "background hits" were likely selected along with the "true hits." This first screen may be optimized to achieve a more manageable number of hits, through modulating antibody and/or PSA concentrations, or by testing different blocking buffers. Instead, we chose to perform a second screen, against only the initial hits from the first screen (Figure 4.5B). Because a biotinylated cyclic anchor ligand was applied in the first screen, we have a label for direct monitoring of the in situ click hits, representing protein-catalyzed conjugation of 1°

ligand to bead-bound 2° ligands. In the second screen, AP-SA followed by BCIP treatment allowed visualization of only the in situ click biligand hits. Surprisingly, only 10% of the initial hits were "true hits" in this assay. This result confirms the practical importance of the multi-stage screening method to enrich for the best hits.

A representative image of the beads in this second screen is depicted in Figure 4.6, and it illustrates the high signal-to-noise ratio that may be achieved by colorimetric detection. Edman sequencing of the true hits (i.e., in situ click biligands) yielded the table of results shown in Figure 4.5C. There is an incredible sequence homology displayed by these biligand hits. All hits demonstrate an extremely high preference for X_5 = t and significant propensity for Az = Az8. Also, two sequences show the homology of "yy" in positions X_2 and X_3. This motif was reinforced by two additional occurrences of y at these positions. The 2° ligand motif Az8-X_1yydt was observed twice (X_1 = k, i).

Validation of PSA-binding cyclic biligands. Based on the Edman sequencing results (Figure 4.5C), the cyclic biligands comprised of Az8-iyydt, Az8-kyydt, and Az8-iyiet were chosen as candidates to test for binding affinity by SPR. The best-binding cyclic biligand was Biotin-$(EG)_5$-Tz1-cy(CVFAHNYDYLVC)-kyydt, whose structure is shown in Figure 4.7B. SPR measurement determined that this peptide displays a binding affinity of $K_D \approx 140$ nM for its interaction with PSA, which is a factor of ~15 improvement from the cyclic anchor (Figure 4.7D). Biligands Biotin-$(EG)_5$-Tz1-cy(CVFAHNYDYLVC)-iyydt and Biotin-$(EG)_5$-Tz1-cy(CVFAHNYDYLVC)-iyiet displayed affinities of $K_D \approx 480$ nM and $K_D \approx 5$ μM, respectively (data not shown). Thus,

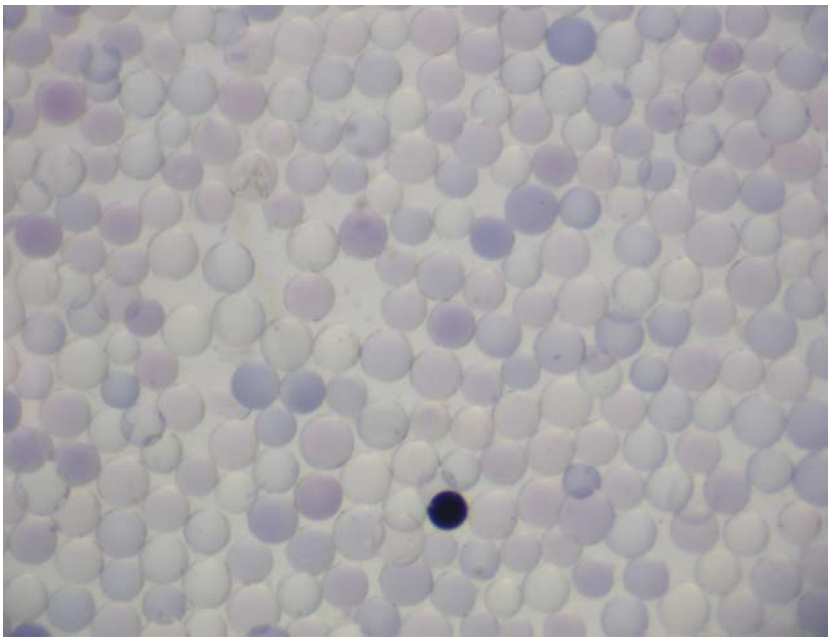


Figure 4.6. Representative image of an in situ click/OBOC screen with enzymatic amplification. In a single assay, $>10^6$ TentaGel beads (90-μm diameter) present individual 2° ligands to a solution of PSA and the biotinylated cyclic anchor (of Figure 4.7A). Specific binding by PSA and formation of in situ click product (purple color) is visualized by treatment with AP-SA and the chromogenic substrate BCIP.

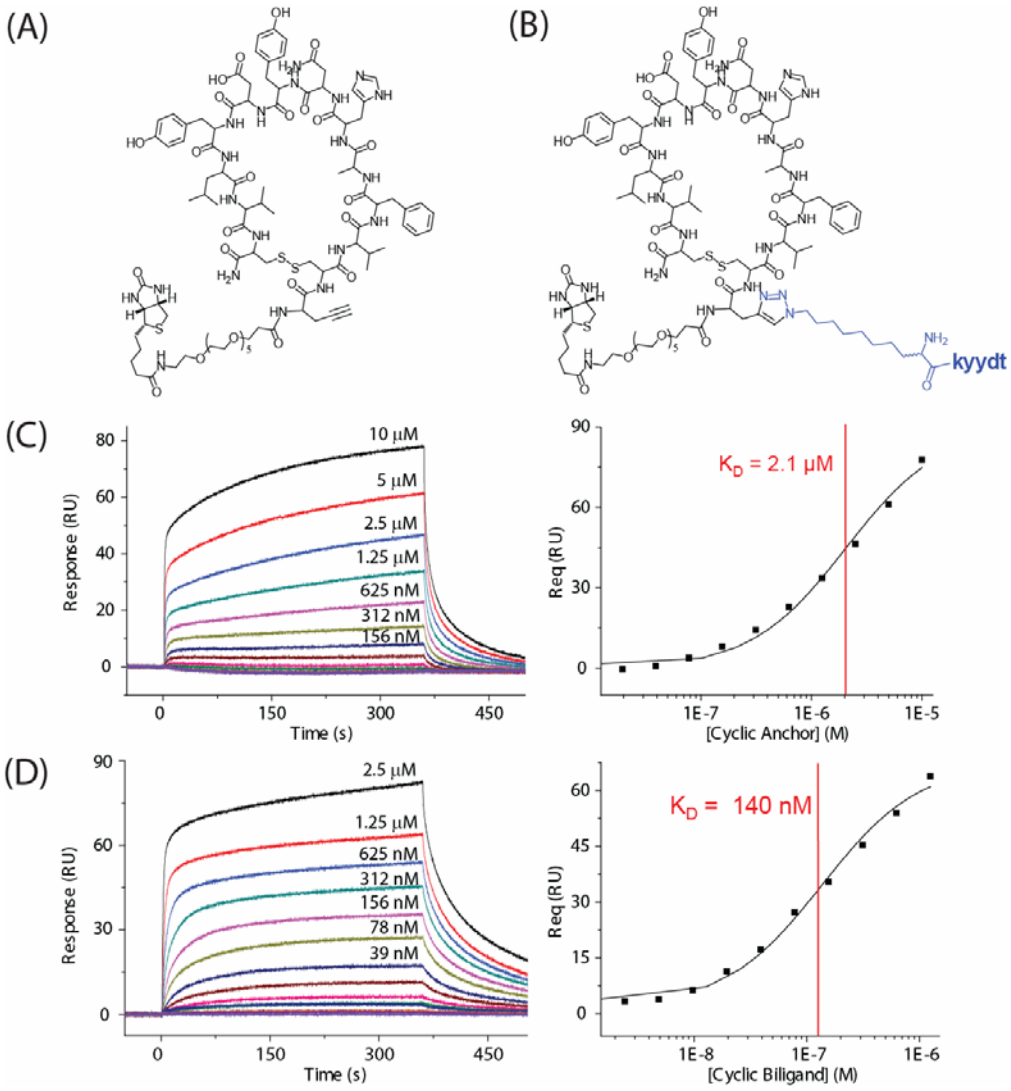


Figure 4.7. Structures of active site targeted cyclic anchor **(A)** and cyclic biligand **(B)** against PSA. Note that the anchor (black) is comprised of L-amino acids, while the 2° ligand (of the biligand) is composed of D-stereoisomers. **(C)** SPR response sensorgrams obtained with increasing concentration (1 nM to 10 µM) of cyclic anchor demonstrate K_D ≈ 2.1 µM binding affinity to immobilized PSA. **(D)** SPR response sensorgrams obtained with increasing concentration (0.3 nM to 2.5 µM) of cyclic biligand display K_D ≈ 140 nM binding affinity to immobilized PSA.

it appears that the area proximal to the active site of PSA is negatively affected by binding of isoleucine.

To further evaluate the cyclic biligand of Figure 4.7B as a suitable capture agent for PSA, a sandwich (ELISA-like) assay will be performed in parallel with the standard commercial 2-antibody ELISA kit for PSA capture and detection. The analytical sensitivities will also be compared for cyclic anchor vs. cyclic biligand as 1° capture agents. Based on the high specificity of the cyclic anchor, we expected that its corresponding biligand will have sufficient affinity and specificity to capture the PSA from even undiluted serum.

4.4 CONCLUSIONS

To both qualitatively and quantitatively assess the formation of protein-templated multi-ligand products from in situ click/OBOC screens, two complementary assays were developed. First, the colorimetric assay employing AP-SA and the chromogenic substrate BCIP allowed detection of on-bead multi-ligands by simple visual inspection. Second, the QPCR assay employing a novel streptavidin-oligo reagent allowed accurate and direct determination of the on-bead in situ click products. The low-yielding, but detectable, products of in situ click chemistry inspired us to develop next-generation, multi-stage screening strategies to improve the signal-to-noise ratio and reduce the number of false positives in our screens. Next-generation screening formats included colorimetric antibody-based screens for initial hits, anti-screens for removing background hits, and direct screening of on-bead biligand. These methods dramatically improved the efficiency of the in situ click/OBOC multi-ligand discovery process. Furthermore, these

new colorimetric methods were easier to perform as they did not require sophisticated instrumentation (e.g., fluorescent microscopes or array scanners).

As a specific application of the multi-stage screening strategies, the rapid selection of a biligand capture agent for PSA was demonstrated. The biligand selection process was expedited through both use of a previously reported, phage-derived cyclic anchor ligand and a two-stage in situ click/OBOC screening method. The cyclic anchor was shown by SPR to have an affinity of $K_D \approx 2.1$ μM, and it was a viable capture agent in sandwich (ELISA-like) assays, pulling down 2 μM (~60 μg/mL) PSA from dilute serum. After two screens, the initial pool of in situ click biligand hits was reduced to 10% true hits, and we obtained an extremely high sequence homology in these cyclic biligand sequences. The best cyclic biligand was shown by SPR to have an affinity of 140 nM. The sequence of this capture agent is unique in that it is a mixture of cyclic, L-chirality, and D-chirality components. Also, one can feasibly only obtain the resultant biligand from the in situ click/OBOC screening methodology described in this thesis. Using the cyclic biligand as a starting point, we next intend to synthesize a triligand capture agent of even higher affinity (e.g., $K_D \approx 1$ nM) and specificity for PSA.

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4.6 REFERENCES

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