Chapter 3

Affinities, Specificities, and Implementation of Multi-ligand Capture Agents in Standard Assays of Protein Detection

3.1 INTRODUCTION

In Chapter 2, the screening methodology for discovery of a triligand capture agent for a specific target, namely human and bovine carbonic anhydrase II (hCAII and bCAII, respectively), was explored as a proof of concept. During the course of multi-ligand development, measurements of binding affinity, specificity, and other physicochemical properties for the isolated ligands were performed. Characterization of hit-derived compounds provided guidance on selecting the most suitable anchor ligand(s), evaluating the quality of the screen, and deciding how many screens to perform. The resultant triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl was further studied for efficacy as a capture and detection reagent in standard assays including dot blot, Western blot, and sandwich (ELISA-like) assay. Through their potential to remove reliance on antibodies, multi-ligand capture agents may directly impact quantitative biology through such implementation in standard assays for protein detection.

The binding affinities describing the interaction between b(h)CAII and the anchor ligands, biligands, and triligands have been characterized via several techniques, including fluorescence polarization and surface plasmon resonance (SPR). The terms “binding affinity” or “affinity” as used herein indicate the strength of the binding between a ligand and protein target (CA II), and is expressed as an equilibrium dissociation constant (K_D). Binding affinities are influenced by non-covalent intermolecular interactions between the two molecules such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and van der Waals forces. The smaller the dissociation constant, the more tightly bound is the ligand, or the better the binding affinity between the two molecules.

Specificities of multi-ligands have been demonstrated and optimized in one case (dot blot). The term "specificity," with reference to the binding of a ligand to a protein target (CA II), refers to the recognition, contact, and formation of a stable complex between the first molecule and the second molecule, together with substantially less to no binding interaction with other molecules that may be present. With the protein target spiked in serum, dot blots, Western blots, and sandwich (ELISA-like) assays were employed to compare specificities of antibody vs. multi-ligand. Detection sensitivities of triligand vs. biligand vs. anchor ligand were also studied. As anticipated, the triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl was the most sensitive, detecting CA II at the ≥20 ng level from 10% porcine serum.

Physicochemical properties of multi-ligands provide additional information on utility of capture agents in various biological assays. Circular dichroism (CD) measurements indicated that the 1,2,3-triazole linker (Tz1 and Tz2) in a multi-ligand induces formation of a random coil structure, which is likely to influence the mechanism of binding to the protein target. On the other hand, an activity assay of bCAII was utilized to assess capacity for active site binding by multi-ligands. In addition, non-natural amino acids in the form of D-stereoisomers were found to be useful ligand building blocks because they are not susceptible to enzymatic degradation. Because the multi-ligands can be chemically synthesized and stored as a lyophilized powder, they have long shelf lives (>1 yr). Since we have highly modular chemical control over capture agent synthesis, additional molecules or functional groups (e.g., fluorophores, small molecules, oligonucleotides, haptens, and other proteins) can be installed in desired locations to provide desired chemical or biological activity. Similarly, if ultra-

high affinity (e.g., $K_D \approx$ pM) is a desired goal, the triligand can potentially be matured into a tetraligand capture agent via another iteration of the in situ click/OBOC screen.

3.2 MATERIALS AND EXPERIMENTAL METHODS

3.2.1 Chemicals

For bulk biligand and triligand synthesis (see Chapter 2), acetylation reagents (acetic anhydride, 2,6-lutidine, and *N*,*N*-dimethylformamide) were purchased from Sigma-Aldrich (St. Louis, MO). For the on-bead Cu(I)-catalyzed click reaction, copper(I) iodide, L-ascorbic acid, and sodium diethyldithiocarbamate trihydrate were purchased from Sigma-Aldrich (St. Louis, MO).

Fluorescein isothiocyanate (FITC) was obtained from AnaSpec. D-biotin and 4-nitrophenyl acetate were obtained from Sigma-Aldrich (St. Louis, MO).

3.2.2 Characterization of Affinity by Fluorescence Polarization

The N-terminus of the anchor ligand was labeled with FITC following published protocols.[1] After resin cleavage, the crude fluoresceinated anchor ligand was precipitated with ether and then purified to >95% by C_{18} reversed phase HPLC.

Luminescence spectra were recorded by Fluorolog2 spectrofluorimeter (Jobin Yvon, Longjumeau, France) in the Beckman Institute Laser Resource Center (Pasadena, CA). All samples contained 6 μM fluoresceinated anchor ligand and a concentration gradient of bCAII (0.2 to 800 μM) in PBS (pH 7.4) + 3% (v/v) DMSO. Stock protein and anchor ligand concentrations were verified by UV-Vis using ε_{280} (bCAII) = 57,000 $M^{-1}cm^{-1}$ or ε_{494} (FITC, 0.1 N NaOH) = 68,000 $M^{-1}cm^{-1}$ for fluoresceinated anchor ligand. After incubation for 1 h at 25 °C in the dark, samples were excited at

488 nm (2-nm band-pass), and luminescence spectra were obtained between 500 and 700 nm (4-nm band-pass). All measurements were taken at 2-nm intervals with 0.5 s integration times at 25 °C. All luminescence spectra were subjected to background subtraction.

The ratio of sensitivities (G) for the vertically and horizontally plane-polarized light in the system was calculated by the equation $G=I_{HH}/I_{HV}$ using the I_{HH} and I_{HV} luminescence spectra obtained from a peptide-only sample. The luminescence spectra I_{VV} and I_{VH} were integrated, and the fluorescence polarization value (P) was calculated by applying Equation (1). The polarization value, P, being a ratio of light intensities, is dimensionless, and is sometimes expressed in millipolarization units (1 polarization unit = 1000 mP Units).

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \tag{1}$$

The polarization values were fitted with a sigmoidal dose-response curve using Origin 6.1 (Northampton, MA).

3.2.3 Characterization of Affinity by Surface Plasmon Resonance

Affinity measurements were performed using a Biacore T100 SPR (Caltech Protein Expression Center, Pasadena, CA) and research grade CM5 sensor chips (GE Heathcare). The instrument was first primed with HBS-P^+ [10 mM HEPES, 150 mM NaCl, 0.05% Tween20 (pH 7.4)] + 3% DMSO. Flow cell 1 was used as a reference to subtract nonspecific binding, drift, and the bulk refractive index, while flow cell 2 (or 3) was immobilized with bCAII (or hCAII) following standard procedures. A 1:1 mixture of 0.4 M EDC and 0.1 M NHS was used to activate flow cell 2, and 0.25 mg/mL bCAII

solution [prepared in 10 mM sodium acetate (pH 5.0)] was injected.[2] Similarly, flow cell 3 was immobilized with hCAII following standard procedures using 0.25 mg/mL hCAII prepared in 10 mM sodium acetate (pH 5.5) buffer.[3] Immobilization levels of ~4000 RU were achieved using a flow rate of 100 μL/min over 420 s. The instrument was then primed using running buffer (HBS-P^+ + 3% DMSO). Prior to the peptide analyte experiment, 8 buffer-alone cycles were completed to establish baseline stabilization.

Triligands were dissolved in HBS-P^+ + 3% DMSO buffer to produce 2 μM peptide stock solutions for each peptide, which were serially diluted by a factor of 2 to produce a concentration series down to 0.1 nM. Biligands were dissolved in HBS-P^+ + 3% DMSO buffer to produce 5 μM peptide stock solutions for each peptide, which were serially diluted by a factor of 2 to produce a concentration series down to 2 nM. Anchor (1°) ligands were dissolved in HBS-P^+ + 3% DMSO buffer to produce ~10 μM peptide stock solutions for each peptide, which were serially diluted by a factor of 2 to produce a concentration series down to 300 nM. For a given affinity measurement, these series of peptide solutions successively were injected into flow cell 2 (or 3) for 120 to 180 s of contact time, 300 s of dissociation time, and 200 s of stabilization time using a flow rate of 100 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). Equilibrium dissociation constant (K_D) values for 1:1 binding were extracted by nonlinear regression fitting of the data to Equation (2).

$$RU_{eq} = RU_{max}[peptide]/(K_D + [peptide]), \quad (2)$$

where RU_{eq} is the measured response unit at a certain peptide analyte concentration and RU_{max} is the maximum response unit.

3.2.4 Enzymatic Activity Assay of Carbonic Anhydrase II

Following previous methods,[4] solution assays for esterase activity were conducted with 1.4 μM bCAII, 5 μM triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl, and 50 μM 4-nitrophenyl acetate (4-NPA) in Tris buffer composed of 9 mM Tris-HCl and 81 mM NaCl + 9% acetonitrile (v/v) + 1% DMSO (v/v). Control assays were conducted in the absence of triligand, and in the absence of protein. The hydrolysis of 4-NPA was monitored over a time course of 60 min, with absorbance measurements taken every 6 min.

3.2.5 Circular Dichroism of Triligand

Circular dichroism spectra were measured by Aviv 62AD Spectropolarimeter (Aviv Associates, Lakewood, NJ) in a 1 mm cuvette at 25 °C. Measurements of 15 μM triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl in 100 mM Tris-HCl (pH 7.5) were recorded between 200 and 260 nm with a band-pass of 1.5 nm.

3.2.6 Dot Blot Specificity/Sensitivity Assays of Biligand and Triligand in Serum

For these tests, Biotin-PEG-NovaTag resin (0.48 mmol/g; Novabiochem) was utilized for bulk synthesis of C-terminal biotin-labeled multi-ligands (Figure 3.7). After resin cleavage, the crude biotinylated multi-ligand was precipitated with ether and then purified to >95% by C_{18} reversed phase HPLC.

The b(h)CAII antigens were prepared as 1 mg/mL stocks in PBS (pH 7.4). A dilution series of antigen was applied to a nitrocellulose membrane, typically ranging from 2 μg to 0.5 ng per spot. The membrane was blocked at 4 °C overnight in 5% milk in Tris-buffered saline (TBS) [25 mM Tris, 150 mM NaCl, 2 mM KCl (pH 8.0)]. The

membrane was then washed with TBS. The biotinylated multi-ligand was prepared at 1 μM in 10% porcine serum in TBS + 0.1% DMSO (v/v) and incubated over the membrane at 4 °C overnight. After washing with TBS for 1 h, 1:3000 Streptavidin-HRP (Abcam, Cambridge, MA) prepared in 0.5% milk/TBS was added to the membrane and incubated for 1 h. After washing with TBS for 1 h, the membrane was developed with SuperSignal West Pico Chemiluminescent Enhancer and Substrate Solutions (Pierce, Rockford, IL) and then immediately exposed to HyBlot CL AR film.

3.2.7 Western Blot Analysis Using Triligand

For denaturing Western blot analysis, bCAII-spiked porcine serum was electrophoresed on a 12% Tris-HCl PAGE gel (Bio-Rad; Hercules, CA) in 1 × TGS [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3]. Samples were prepared in Laemmli Sample Buffer (Bio-Rad) containing 0.05% (v/v) 2-mercaptoethanol, and boiled before electrophoresis. Gels were transferred to nitrocellulose in 1 × TGS containing 20% methanol, over 1 h at 100 V.

For native Western blot analysis, bCAII-spiked porcine serum was electrophoresed on a 12% Tris-HCl PAGE gel (Bio-Rad; Hercules, CA) in 1 × TG [25 mM Tris, 192 mM glycine, pH 8.3]. Samples were prepared in Native Sample Buffer (Bio-Rad) for electrophoresis. Gels were transferred to nitrocellulose in 1 × TG containing 20% methanol, over 3 h at 100 V.

After transfer, the nitrocellulose membrane was blocked at 4 °C overnight in 5% milk/TBS. The membrane was then washed with TBS. The biotinylated triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl-$(EG)_3$-Biotin was prepared at 1 μM in 0.5% milk/TBS + 0.1% (v/v) DMSO and incubated over the membrane overnight at 4 °C. Alternately, a

separate membrane was probed with 1:4000 primary antibody (biotinylated anti-bCAII; Rockland Immunochemicals, PA) for 1 h at 4 °C. After washing with TBS for 1 h, 1:3000 Streptavidin-HRP prepared in 0.5% milk/TBS was added to the membranes and incubated for 1 h. After washing with TBS for 1 h, the membranes were developed with SuperSignal West Pico Chemiluminescent Enhancer and Substrate Solutions (Pierce; Rockford, IL) and then immediately exposed to HyBlot CL AR film.

3.2.8 Sandwich (ELISA-like) Assays Using Triligand

Reacti-Bind Streptavidin high binding capacity coated 96-well plates (~125 pmol biotin/well; Pierce, Rockford, IL) were utilized for this experiment. The biotinylated multi-ligand was prepared at 3 μM in 0.5% milk/TBS, and incubated for 1 h at 25 °C. After washing each well with 5% milk/TBS (3 ×), the plate was filled with 5% milk/TBS and blocked for 1 h at 25 °C. A serial dilution of bCAII antigen was prepared in 10% porcine serum, ranging from 1 mM to 1 pM, and incubated for 1 h at 25 °C. After washing each well with 5% milk/TBS (3 ×), 1:1000 polyclonal anti-bCAII, HRP conjugate (Abcam, Cambridge, MA) was added to each well in blocking buffer and incubated for 30 min at 25 °C. After washing each well with 5% milk/TBS (3 ×), chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine) was supplied to each well. After 20 min, the reaction was quenched with 1 M H_2SO_4 and analyzed by absorbance at 450 nm on a plate reader.

3.3 RESULTS AND DISCUSSION

With the addition of each ligand to the capture agent, the affinity and the specificity of that capture agent for its cognate protein rapidly increase. The screen

illustrated by Figure 2.1 was used to identify lklwfk-(D-Pra) as the anchor ligand and kwlwGl-Tz1-kfwlkl as the biligand, and ultimately implemented (D-Pra)-kwlwGl-Tz1-kfwlkl as the new anchor ligand for identification of a triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl against bCAII, according to the methods described in Chapter 2.

3.3.1 Characterization of Anchor (1°) Ligand Affinities

Fluorescence polarization. To determine the binding affinity of the anchor (1°) ligands lklwfk-(D-Pra) and Az4-kfwlkl, fluorescence polarization was employed. Fluorescence polarization is a measure of the extent of molecular rotation by a fluorescent ligand during the period between excitation and emission with plane polarized light.[5] Free ligands rotate quickly and tumble in and out of plane during their excited states. Therefore, they have low polarization values upon excitation. When a ligand is bound to a receptor (i.e., protein), the molecule remains largely stationary, and so the rotation of the ligand is smaller in its excited state, and hence high polarization values are observed. In these experiments, ligands are typically labeled with a fluorescent dye of a high quantum yield, such as FITC (~4 ns excited lifetime).

The results of a fluorescence polarization experiment to characterize the interaction between bCAII and a fluoresceinated lklwfk-(D-Pra) are shown in Figure 3.1. The fluoresceinated anchor ligand was titrated with increasing concentrations of the protein target (0.2 to 800 μM). In high bCAII concentration, most fluoresceinated anchor ligands are bound to the protein. This fluorescent ligand-protein complex will exhibit high fluorescence polarization. However, as less bCAII is titrated, increasing amounts of free fluoresceinated anchor ligand will exist in the solution. These unbound anchor ligands will contribute to a low fluorescence polarization reading. Therefore, by

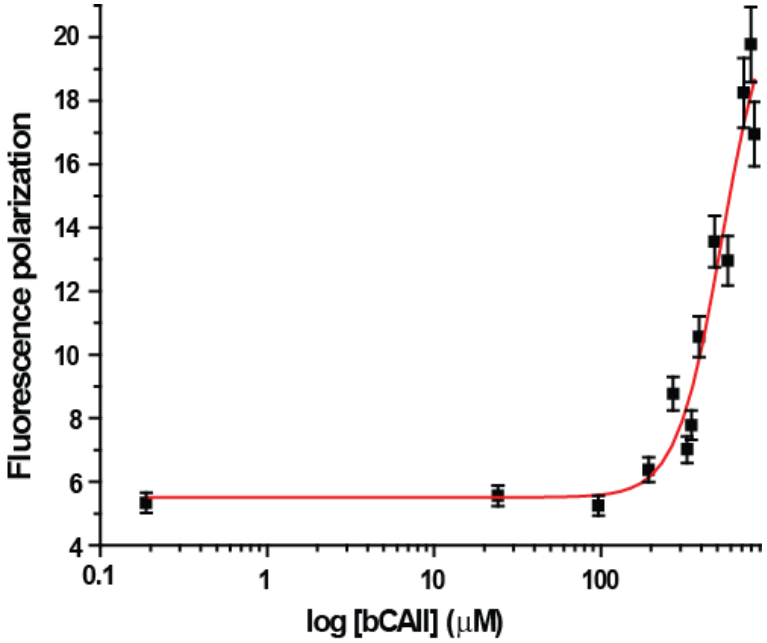


Figure 3.1. Fluorescence polarization binding isotherm for the anchor ligand lklwfk-(D-Pra), showing $K_D \approx 500$ μM. For fluorescence polarization experiments, the anchor ligand was labeled with FITC at the N-terminus. All samples contained 6 μM FITC-anchor ligand and varying concentrations of bCAII (0.2 to 800 μM).

fitting the fluorescence polarization against the protein concentration, a value of $K_D \approx$ 500 μM was extrapolated to describe the strength of the bCAII/anchor ligand interaction.

Surface plasmon resonance. The binding affinities of 1° ligands lklwfk-(D-Pra) and Az4-kfwlkl were also determined by SPR, and confirm the previous fluorescence polarization result. In SPR, real-time, label-free optical sensing of biomolecular binding events may be achieved through measurements of thickness (and refractive index) of films adsorbed on gold substrates.[6] A transducing medium is usually formed on the gold substrate film through surface-immobilized biomolecules (e.g., receptors). Changes in the refractive index of this transducing layer are induced by the binding of analyte to the biomolecule. Measurement in binding response over time yields sensorgrams which can be fitted for K_D and kinetics following a Langmuir binding isotherm.

In Figure 3.2, sensorgrams depict the interaction of surface-immobilized bCAII with increasing concentration (300 nM to ~10 μM) of 1° ligands (A) lklwfk-(D-Pra) and (B) Az4-kfwlkl. The analyte responses were quite weak, demonstrating $K_D > 10^{-5}$ μM binding affinities for both 1° ligands, and represent a limit for Biacore analysis. Since weak affinities are hard to quantify, this value is only an estimate.

3.3.2 Characterization of Biligand Affinities

Three candidate biligands were obtained by screening bCAII. One biligand (lklwfk-Tz1-kiwiG) is the result of an in situ click/OBOC screen between a comprehensive bead library of azides, anchor ligand lklwfk-(D-Pra), and bCAII. Two biligands (kwlwGl-Tz1-kfwlkl and kwiwGw-Tz1-kfwlkl) are the result of an on-bead

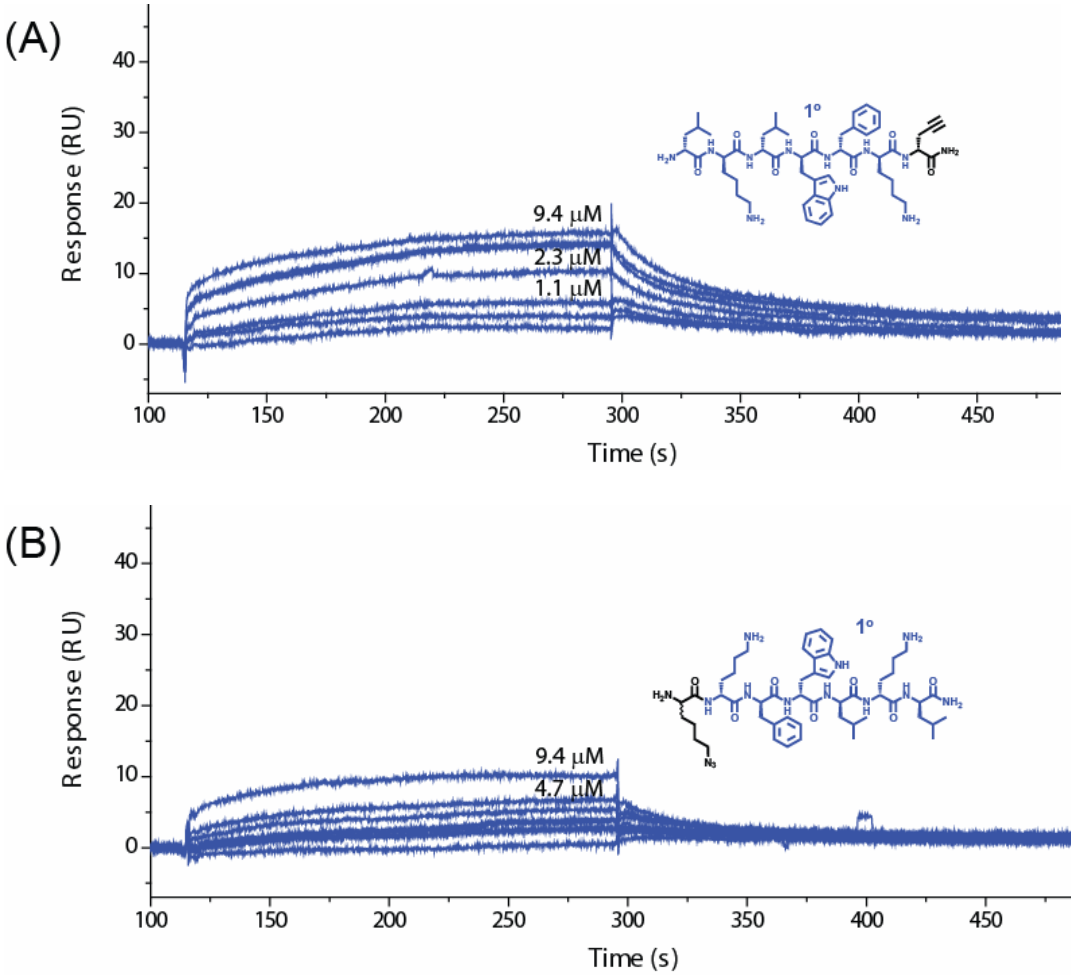


Figure 3.2. SPR response sensorgrams obtained with increasing concentration (300 nM to ~10 µM) of 1° ligands **(A)** lklwfk-(D-Pra) and **(B)** Az4-kfwlkl demonstrate K_D >10-µM binding affinities to immobilized bCAII.

CuAAC biligand library screen. These three biligands were synthesized in bulk, and their binding affinities for bCAII were measured using SPR.

The binding responses (Figure 3.3A-B) reveal $K_D \approx 10^{-6}$ M affinity of two biligands toward bCAII. In particular, sensorgrams obtained with increasing concentration (2 nM to 5 μM) of the biligands (A) kwlwGl-Tz1-kfwlkl and (B) lklwfk-Tz1-kiwiG demonstrate 3-μM and 11-μM binding affinities, respectively. This proves that the in situ click/OBOC screen, whose selected biligand is depicted in Figure 3.3B, and the on bead CuAAC biligand library screen, whose selected biligand is depicted in Figure 3.3A, converge on similar biligand sequences with similar affinities, further validating our selection approach. Furthermore, the SPR data for the best-binding biligand kwlwGl-Tz2-kfwlkl (Figure 3.3A), with an extrapolated affinity of $K_D \approx 3$ μM, represents a ~100-fold improvement over the binding affinity for 1^o ligand interaction with the same protein.

In Figure 3.3C, SPR response sensorgrams for biligand kwiwGw-Tz1-kfwlkl are represented. These data were irregular and illustrated a significant amount of non-specific binding at high analyte concentrations (i.e., evidenced by RU exceeding R_{max} and high background binding on flow cell 1, data not shown). As this biligand sequence differs from the best-binding biligand of Figure 3.3A by only two residues (Res3: l→i and Res6: l→w), we have indirect evidence of the apparently high binding specificity of bCAII for only certain sequences.

In view of the above considerations, the biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl was synthesized. The D-propargylglycine linker was installed at the N-terminus of the peptide, to minimize perturbation to the linear biligand sequence. In the presence

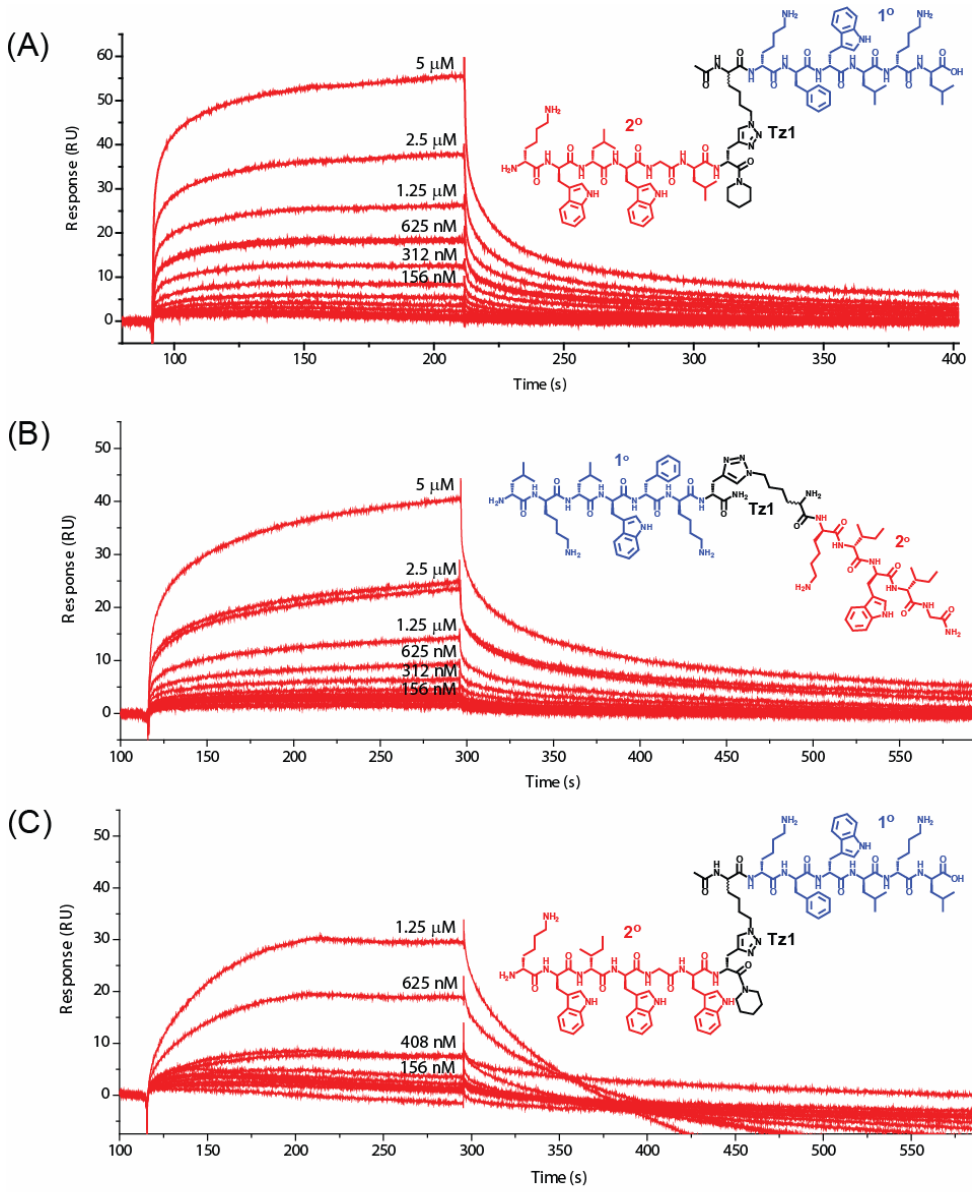


Figure 3.3. SPR response sensorgrams obtained with increasing concentration (2 nM to 5 µM) of the biligands **(A)** kwlwGl-Tz1-kfwlkl and **(B)** lklwfk-Tz1-kiwiG demonstrate 3-µM and 11-µM binding affinities, respectively, to immobilized bCAII. **(C)** Sensorgrams for biligand kwiwGw-Tz1-kfwlkl were irregular and illustrated a significant amount of non-specific binding.

of this new anchor unit, an in situ click/OBOC screen between bCAII and the same bead library of azides was performed to identify triligand candidates.

3.3.3 Characterization of Triligand Affinities

Only one candidate triligand was obtained by screening bCAII, because the sequence rfviln-Tz2-kwlwGl-Tz1-kfwlkl was repeated several times in both generations of in situ click/OBOC screen. This consensus triligand (Figure 3.4A) was synthesized in bulk and its binding affinity for both bCAII and hCAII was measured using SPR. The binding responses (Figure 3.4B-C) reveal $K_D \approx 45$ nM (for hCAII) and $K_D \approx 64$ nM (for bCAII). These equilibrium dissociation constants represent a 50-fold affinity enhancement compared to the interaction between biligand and target, and $>10^3$-fold affinity enhancement compared to the binding of 1° ligand and target (see Figures 3.1-3.3, for comparison).

3.3.4 Enzymatic Activity Assay of Carbonic Anhydrase II

Nature of triligand binding to bCAII. The active site of bCAII possesses an intrinsic esterase activity which can be monitored spectrophotometrically.[4] Specifically, bCAII catalyzes the hydrolysis of 4-nitrophenyl acetate (4-NPA) to 4-nitrophenol (4-NP), whose absorption can be monitored at 400 nm. The enzyme-catalyzed hydrolysis proceeds at a range of pH and serves as a test for active site binding by common inhibitors (Scheme 3.1). We utilized this assay to study the functional activity of bCAII as an esterase in the presence and absence of the triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl. The activity assay was performed to qualitatively assess the possibility of active site binding by the triligand.

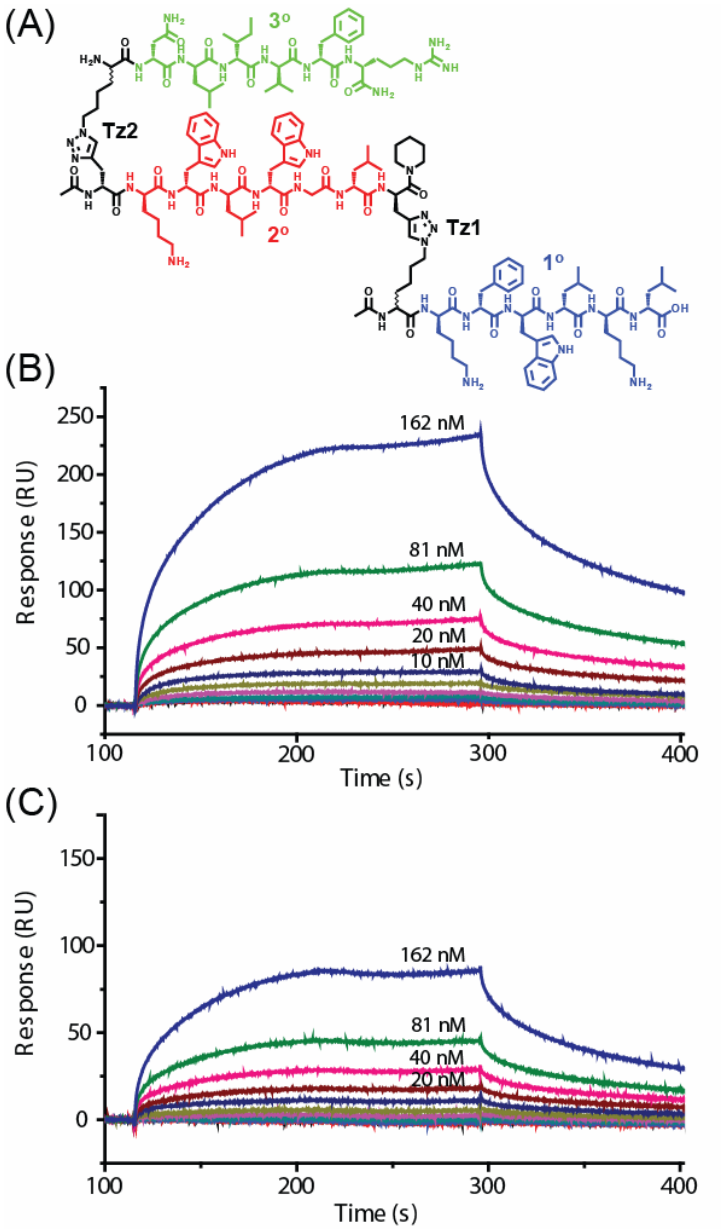
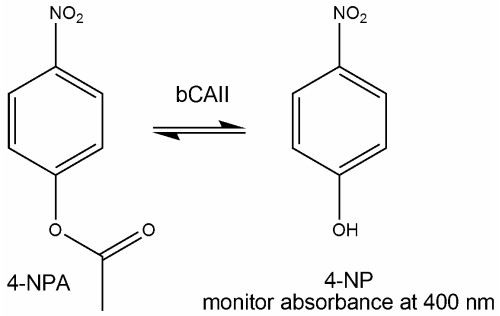


Figure 3.4. (**A**) Triligand capture agent, rfviln-Tz2-kwlwGl-Tz1-kfwlkl. SPR response sensorgrams with increasing peptide concentration (0.1 to 162 nM) characterize triligand binding to immobilized human **(B)** and bovine **(C)** CA II targets, respectively. Data analysis of this biomolecular interaction provided values of $K_D \approx 45$ nM (hCAII) and $K_D \approx 64$ nM (bCAII).



Scheme 3.1. Esterase activity of bCAII, using 4-NPA as the hydrolytic substrate.

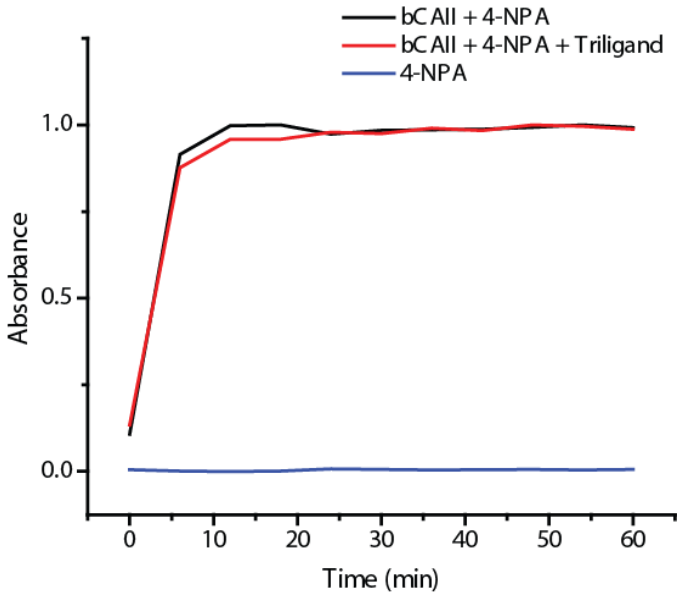


Figure 3.5. Enzymatic activity of bCAII in the presence of the triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl. Absorbance data monitor the bCAII-catalyzed hydrolysis of 4-NPA to 4-NP (ε = 18,400 $M^{-1}cm^{-1}$ at 400 nm) at the protein active site. Experiments were performed with (red) and without (black) capture agent. Additionally, an assay was performed in the presence of 4-NPA alone (blue) to determine the slow background hydrolysis of the ester in aqueous solution. [bCAII] = 1.4 μM, [Triligand] = 5 μM, and [4-NPA] = 50 μM in Tris buffer [9 mM Tris-HCl, 81 mM NaCl, 9% acetonitrile (v/v), 1% DMSO (v/v)].

The experimental results are presented in Figure 3.5. Regardless of whether the assay contained triligand, there was an initial "burst" in 4-NP formation, followed by a slow increase in the product formation over the 60 min. Because there were no appreciable changes in the bCAII esterase activity when the triligand capture agent was included in the assay, apparently this peptide binds to an epitope distinct from the bCAII active site.

3.3.5 Circular Dichroism of Triligand

Circular dichroism (CD) measures the differential absorption of left- and right-handed circularly polarized light in solutions of optically active molecules such as peptides, proteins, and nucleic acids. For peptides and proteins, secondary structures such as α-helix and β-sheet are easily resolved by CD. The signature peaks for an α-helix and β-sheet can be found at 222 and 208 nm, respectively.[7]

The triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl was characterized as a random coil by CD (Figure 3.6). The unfolded random coil structure may be a reflection that this oligopeptide was assembled linearly through successive protein-templated in situ click screens. Since the random coil is not one specific shape, but a statistical distribution of shapes, this conformation suggests the idea that, in the absence of specific, stabilizing interactions with the protein target, the oligopeptide will "sample" all possible conformations randomly.[8]

3.3.6 Dot Blot Specificity/Sensitivity Assays of Biligand and Triligand in Serum

Dot blots are a common method for detecting proteins. The sensitivity and

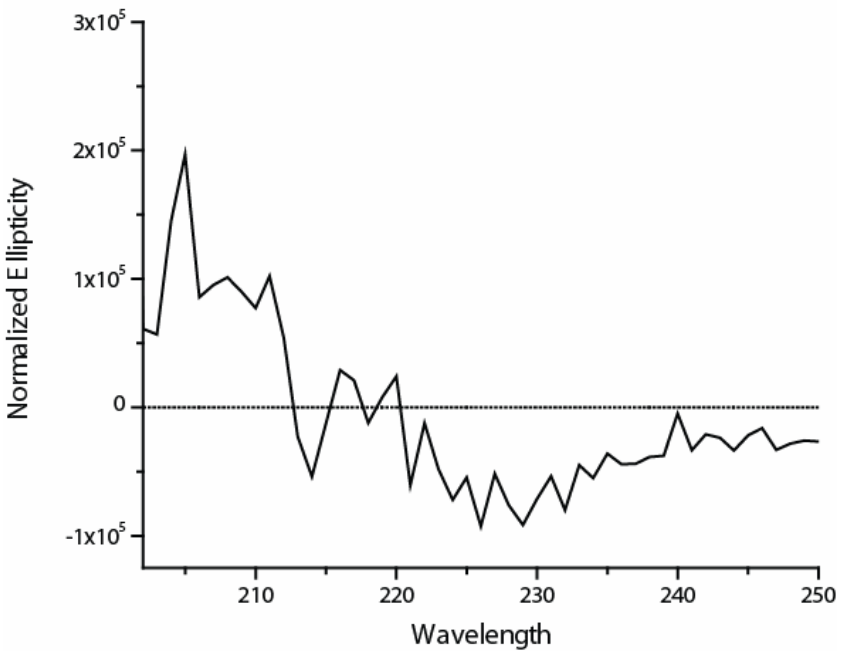


Figure 3.6. CD spectrum for triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl, acquired at 15 μM in 100 mM Tris-HCl (pH 7.5). Lack of signature peaks at 222 nm (for α-helix) and 208 nm (for β-sheet) indicates that the peptide structure is that of a random coil.

specificity of multi-ligand capture agents for detecting b(h)CAII in complex environments were demonstrated through the use of dot blot experiments in 10% porcine serum. For a dot blot, the solution containing the protein of interest is simply deposited onto an absorbent membrane material (typically nitrocellulose). The capture agent (typically an antibody, or one of the multi-ligands of Figure 3.7) is labeled with biotin, and then exposed to the entire nitrocellulose membrane. The membrane is washed to remove unbound material, and then horseradish peroxidase (HRP)-labeled streptavidin is added, attaching to the protein-bound biotin. Optical methods are typically utilized to detect this binding. Because we conducted dot blots experiments with the multi-ligand capture agent in dilute serum, both sensitivity and specificity may be addressed in a single assay.

Results for the dot blot to use the triligand (Figure 3.7A) and the biligand anchor (Figure 3.7B) to detect hCAII and bCAII from dilute porcine serum are shown in Figure 3.8. It is noted that bCAII and hCAII are >80% identical by sequence (PDB ID: 1CA2, 1V9E), and so both proteins were expected to be captured in this assay. The results of this assay illustrate ~20 ng b(h)CAII detection sensitivity by the triligand in 10% porcine serum, while ~0.2 μg hCAII detection sensitivity is attained by the biligand anchor when the assay is performed under similar conditions. We reason that the sensitivity correlates with overall affinity of the capture agent, and so it is no surprise that the triligand is the more sensitive binder. Similarly, these results suggest that through the in situ click/OBOC screening method, we build specificity into our multi-ligands with each screening iteration.

We also wanted to directly compare binding specificity of the triligand (Figure 3.7A) against a commercially available antibody. Figure 3.9 shows the results of dot

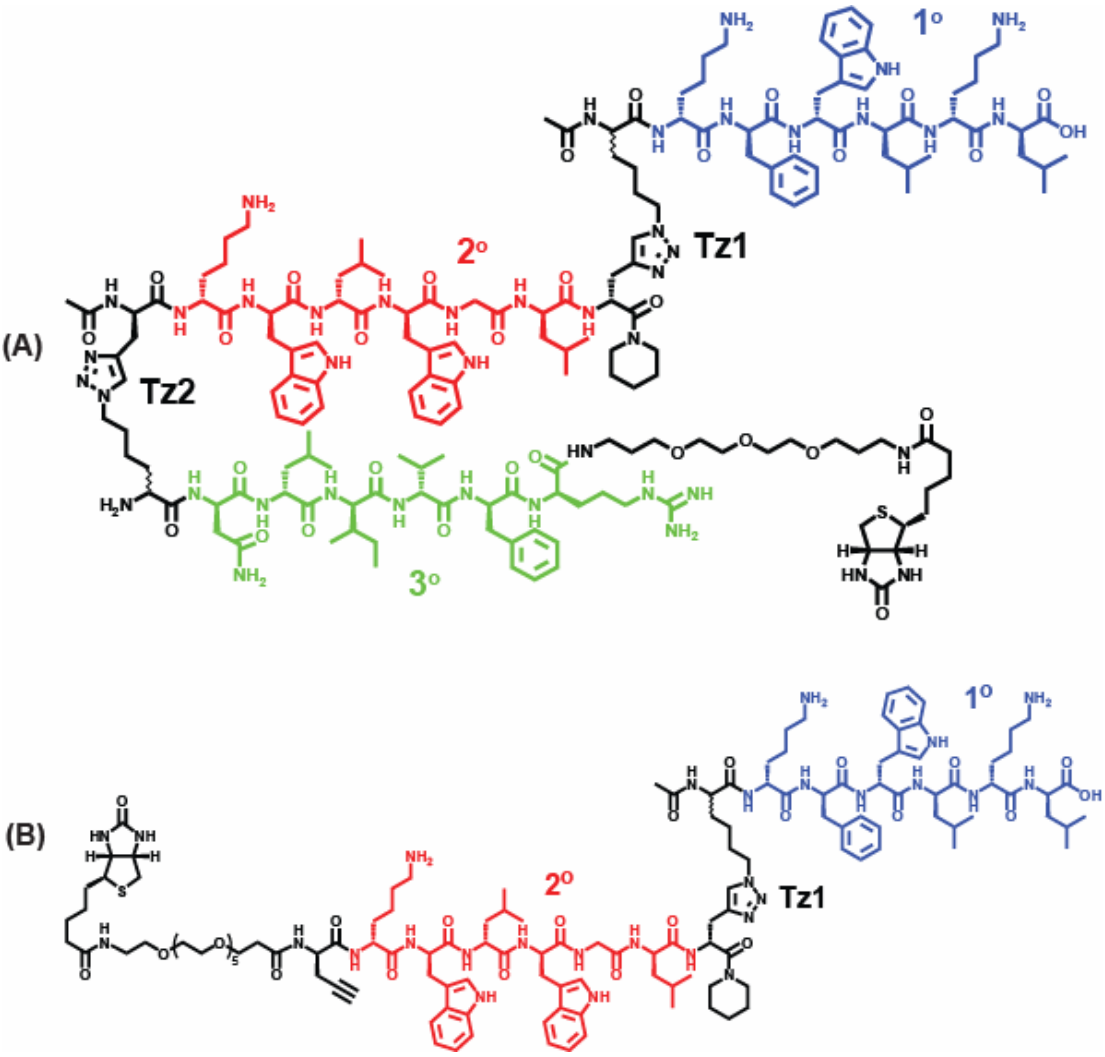


Figure 3.7. Biotin conjugates of the **(A)** triligand rfviln-Tz2-kwlwGl-Tz1-kfwlkl and **(B)** biligand anchor (D-Pra)-kwlwGl-Tz1-kfwlkl. These capture agents were implemented in dot blots, Western blots, and sandwich (ELISA-like) assays of bCAII.

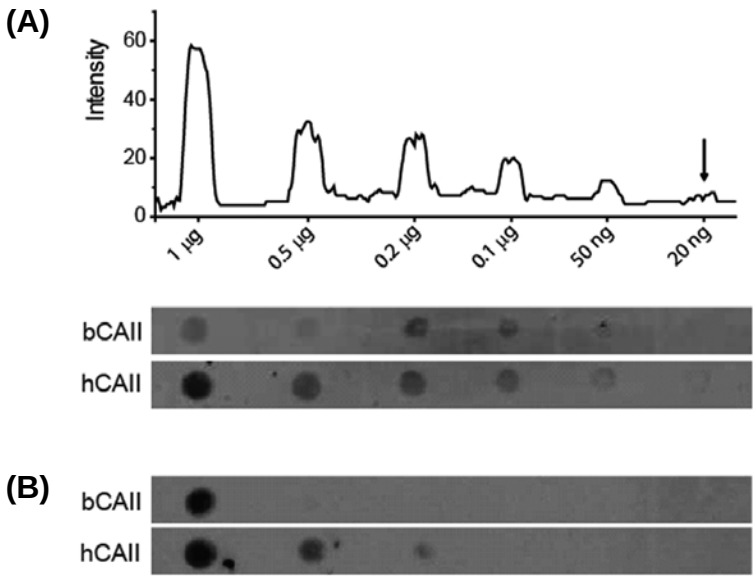


Figure 3.8. **(A)** Dot blot illustrating ~20 ng b(h)CAII detection sensitivity by the triligand of Figure 3.7A in 10% porcine serum. When the biligand anchor of Figure 3.7B is used as the primary capture agent in 0.1% serum **(B)**, the sensitivity is reduced by more than 10-fold.

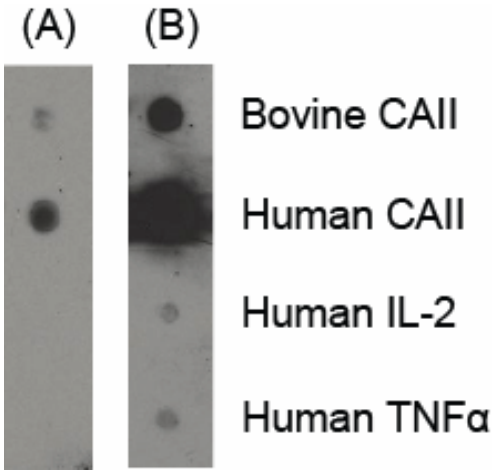


Figure 3.9. Results of dot blots performed in 0.5% milk/TBS where the **(A)** triligand of Figure 3.7A or **(B)** polyclonal anti-bCAII were utilized as the primary capture agent. **(A)** The triligand appears to be specific for CA II. **(B)** The polyclonal antibody displays an apparent cross-reactivity with unrelated proteins. Proteins = 2 μg per spot.

blots performed in 0.5% milk/TBS where the (A) triligand (Figure 3.7A) or (B) polyclonal anti-bCAII were utilized as the primary capture agent. Besides bCAII and hCAII, two human secreted proteins interleukin-2 (IL-2) and TNFα were included in the protein panel. We also tested bovine serum albumin (BSA) as the spotted antigen in a separate blot, and neither triligand nor antibody displayed detectable cross-reactivity (data not shown). While the triligand displayed a high degree of specificity for CA II in the blot of Figure 3.9, the antibody showed an apparent cross-reactivity for the unrelated human proteins. This result is not surprising, as polyclonal antibodies generally sample diffuse epitopes. However, qualitative analysis of spot intensity suggests that the antibody is the more sensitive capture agent. From the results of Figures 3.8-3.9, we conclude that the triligand capture agent displays a comparable, or even better, specificity for b(h)CAII than the antibody, but the sensitivity remains to be optimized.

3.3.7 Western Blot Analysis Using Triligand

The Western blot is another common method for detecting proteins. For the standard Western blot, proteins are subjected to denaturing gel electrophoresis and transfer to nitrocellulose. For the native Western blot, proteins are exposed to non-denaturing conditions for both electrophoresis and transfer. Antibody or multi-ligand capture agents are then used to interrogate the proteins on the nitrocellulose membrane. After specific binding of the capture agent to the target, a secondary detection agent is added to specifically bind to the capture agent. The secondary detection agent (e.g., streptavidin-HRP) often exhibits chemiluminescence which allows visualization of the results on film.

Demonstrations of Western blots to detect bCAII, with direct comparisons between the triligand (Figure 3.7A) and a commercial antibody, are shown in Figure 3.10. The denaturing Western blot of Figure 3.10A, utilizing polyclonal anti-bCAII as the primary capture agent, shows ~50 ng bCAII detection sensitivity. Curiously, on the same gel (Figure 3.10B), bCAII was not detected by the triligand. This result suggests that the triligand capture agent recognizes a 3-D protein epitope that is destroyed when the protein is subjected to denaturing conditions.

To test this hypothesis, native Western blots were performed under similar, but non-denaturing, conditions. We also took this opportunity to interrogate specificity by utilizing the antibody and triligand capture agents as probes against bCAII spiked in dilute serum. The native gel of Figure 3.10C details the electrophoresed bCAII and serum proteins. When this native gel was transferred and probed with polyclonal anti-bCAII (Figure 3.10D), bCAII and a serum protein (MW ≈ 30-35 kDa) are detected. We hypothesize that this upper band may be one of the related isozymes CA I or CA III, which show 58%-60% identity with each other and with CA II in amino acids at similar positions.[9] Furthermore, CA I is five to six times as abundant as CA II in erythrocytes.[9] When the same native blot is probed with the triligand of Figure 3.7A (Figure 3.10E), only bCAII is detected, illustrating triligand specificity for native bCAII epitopes. Even in the presence of serum, native Western analysis suggests that the triligand is potentially more specific than the commercial anti-bCAII antibody, and this result confirms our previous dot blot analysis.

It should be noted that the detection sensitivity for the triligand in the native Western blot is not as high as in the dot blot (1 μg vs. 20 ng bCAII). Under non-denaturing conditions, the gel transfer step requires high voltage and is still inefficient,

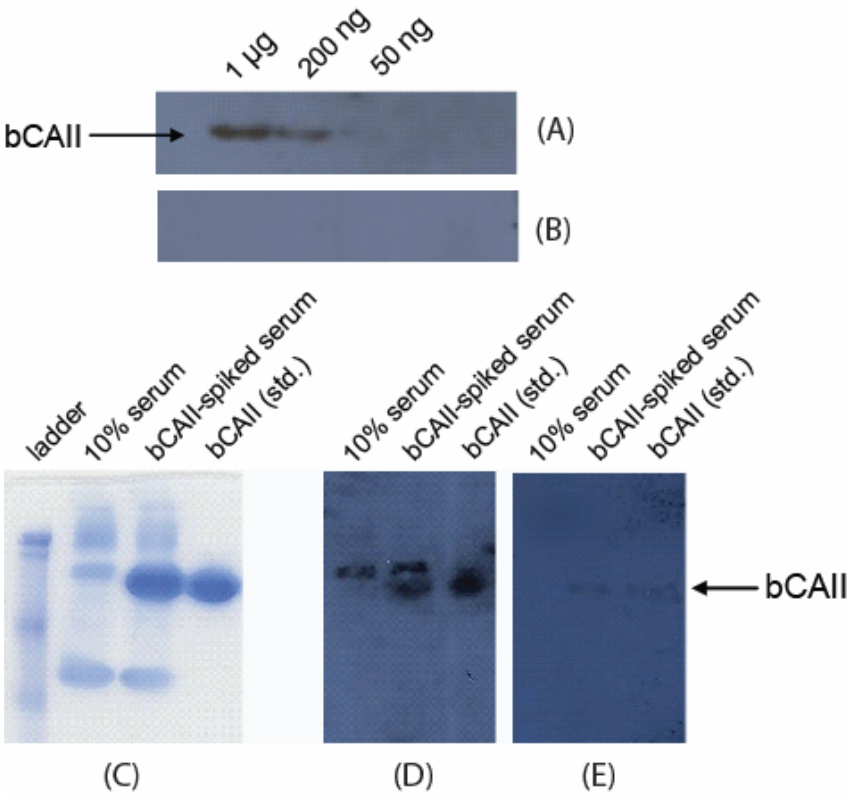


Figure 3.10. Results of Western blots performed under denaturing **(A, B)** and non-denaturing conditions **(C, D, E)**. **(A)** The denaturing Western blot, utilizing polyclonal anti-bCAII as the primary capture agent, shows ~50 ng bCAII detection sensitivity. **(B)** On the same gel, bCAII was not detected by the triligand of Figure 3.7A. **(C)** The native PAGE gel was stained with Coomassie, and details total protein content. **(D)** When this native gel is transferred and probed with polyclonal anti-bCAII, bCAII and a serum protein (MW ≈ 30-35 kDa) are detected. **(E)** When the same native blot is probed with the triligand of Figure 3.7A, only bCAII is detected, illustrating triligand specificity for native bCAII epitopes. bCAII loading **(C, D, E)** = 1 µg per lane.

as the proteins are only natively charged. The poor transfer leads to the perceived reduction in sensitivity by the triligand in the native Western blot.

3.3.8 Sandwich (ELISA-like) Assays Using Triligand

The sandwich assay is a third common method for detecting proteins. Sandwich assays typically rely on two antibodies, a primary capture antibody (1°) and a labeled detection antibody (2° antibody), for detecting the protein of interest. In a typical ELISA sandwich assay, the 1° antibody is typically coated onto a surface, such as the surface of a well within a 96-well plate. A solution (e.g., serum, urine, etc.) expected to contain a particular target protein is added to the well. The target protein is then allowed to diffuse to the surface where it is captured by the 1° antibody. The 2° antibody is then added to the same well. This antibody is designed to bind to an orthogonal binding site, or epitope, of the target protein. Furthermore, this 2° antibody is labeled in a way that allows for the antibody/protein/antibody sandwich to be detected optically or by some other means.

For optical detection, the label is often an optically absorbent chromophore or a fluorescent dye molecule, and that label is often attached to the 2° antibody directly. The label is then detected by absorbance or fluorescence, and the signal intensity is proportional to the amount of protein captured in the assay. Alternatively, the 2° antibody may be conjugated to biotin, and in that case, a labeled protein (e.g., streptavidin-HRP) is added subsequently to visualize the biotin. Other methods are possible, such utilizing a gold nanoparticle as a label instead of the fluorescent or optically absorbent molecule, or using a radioactive molecule as the label, where the final detection is completed using a scintillation counter or appropriately sensitized film.

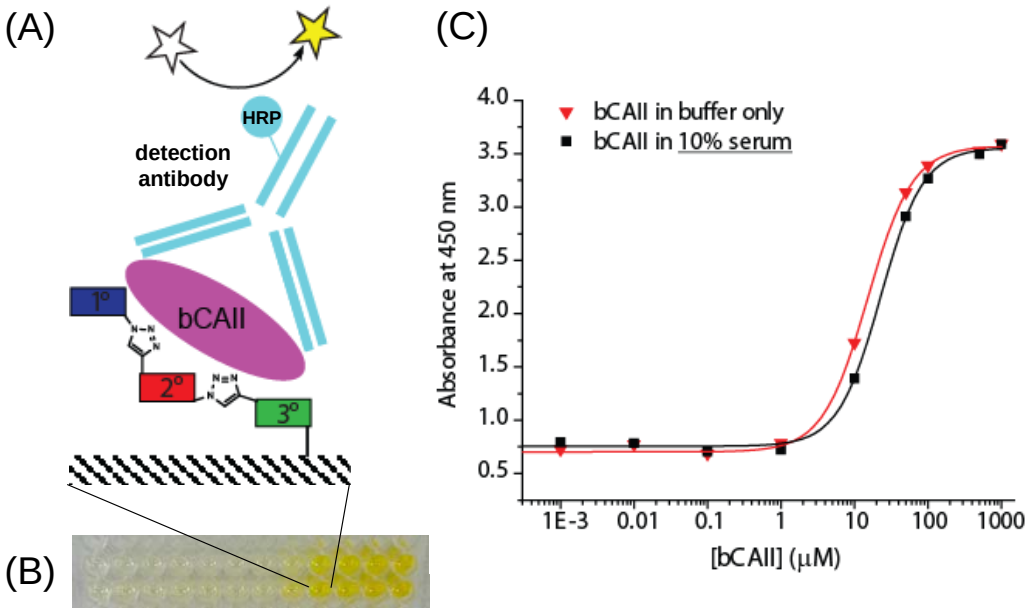


Figure 3.11. **(A)** Schematic illustration of the structure of fully assembled ELISA-like sandwich absorbance assays using the triligand of Figure 3.7A to detect bCAII protein. **(B)** Experimental data of ELISA assays at varying concentrations of bCAII as performed in the wells of a 96-well plate. Increasing bCAII concentration is detected as an increasing yellow color. **(C)** Diagrams illustrating two assay conditions. The target is presented in 0.5% milk/TBS (red curve) or in 10% porcine serum (black curve) to yield a sandwich assay with an analytical sensitivity of ~10 μM (~300 μg/mL).

Demonstration of sandwich-type ELISA assays on streptavidin-functionalized microtiter plates to detect bCAII using a combined commercial antibody (2° capture agent) and triligand of Figure 3.7A (as the 1° capture agent) is shown in Figure 3.11. Two assay conditions were used to compare the detection sensitivity for bCAII in buffered solution vs. a background of dilute serum (Figure 3.11C). For the sandwich assay performed with bCAII presented in 0.5% milk/TBS (red curve), the analytical sensitivity is ~10 μM (~300 μg/mL). This result is similar to the sandwich assay performed with bCAII presented in dilute serum (black curve), which further illustrates the utility of multi-ligand capture agents in standard assays of protein detection.

Our triligand sandwich (ELISA-like) assay, however, does not yet approach the analytical sensitivity expected for most commercial sandwich assays (~1 pg/mL). There are several areas for optimization of the Figure 3.11 assay. First, it is possible that the triligand of Figure 3.7A and the commercial polyclonal anti-bCAII (HRP conjugate) are not an optimized reagent pair. We did not test whether these two capture agents compete for similar (or the same) binding epitopes on bCAII before performing the sandwich assay. Competition of this kind would translate to reduced sensitivity. Second, our sandwich assay was an absorbance assay using TMB (3,3',5,5'-tetramethylbenzidine) as the chromophore to visualize bound proteins. Absorbance ELISAs are not nearly as sensitive as fluorescence ELISAs. It has been reported that a five- to six-fold enhancement in signal-to-noise ratio at a given analyte concentration and a two- to five-fold enhancement in sensitivity, as reflected by relative limits of detection, may be achieved with fluorogenic substrates.[10] Third, the background absorbance is high (0.6 Abs units), potentially masking sensitivity for the lower bCAII concentrations. This background may be caused by insufficient washing during the assay, or possibly use of

too much polyclonal anti-bCAII (HRP conjugate). Polyclonal antibodies display a higher risk of cross-reactivity since their epitopes are less precisely defined, and so there may have been some background binding to the serum- or milk-based proteins present in our assay.

3.4 CONCLUSIONS

As a companion to Chapter 2, this chapter focused on the properties and results of using multi-ligand capture agents in standard assays of protein detection. Measurements by fluorescence polarization and SPR served as direct evidence of the kind of affinity enhancement that one can achieve through multivalent binding interactions. Starting from lklwfk-(D-Pra) ($K_D \approx 500$ μM) as the anchor (1°) ligand, moderate affinity biligands such as kwlwGl-Tz1-kfwlkl ($K_D \approx 3$ μM) were assembled by in situ click chemistry and represent a ~100-fold affinity improvement. Using biligand (D-Pra)-kwlwGl-Tz1-kfwlkl as the new anchor unit, a triligand capture agent (rfviln-Tz2-kwlwGl-Tz1-kfwlkl, $K_D \approx 50$ nM) was isolated by in situ click/OBOC selection and represents a 50-fold affinity enhancement compared to the interaction between biligand and target, and a $>10^3$-fold overall affinity enhancement compared to the binding of 1° ligand and target. Interestingly, this triligand does not bind the active site of bCAII, but rather to a separate generalized epitope, and apparently the random coil structure of this peptide may become stabilized by specific binding with the target.

Protein capture agents should exhibit both an affinity for their cognate protein, as well as a specificity for detecting that protein in complex environments. Multi-ligands show initial efficacy as capture agents in standard assays including dot blot, Western blot, and sandwich (ELISA-like) assay. The triligand was found to detect ≥ 20 ng CA II

in porcine serum as illustrated by dot blot. The triligand was also found to detect ≥ 1 μg CA II from porcine serum in a non-optimized native Western blot. Curiously, the triligand only recognizes a 3-D (native) protein epitope which argues for the exquisite nature of the in situ click/OBOC discovery process. Non-optimized sandwich (ELISA-like) absorbance assays using the triligand for bCAII capture and a polyclonal anti-bCAII for detection yield an analytical sensitivity of ~10 μM (~300 μg/mL). These feasibility demonstrations show great promise toward the routine implementation of protein capture agents in basic research and as medical diagnostic tools.

3.5 ACKNOWLEDGEMENTS

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