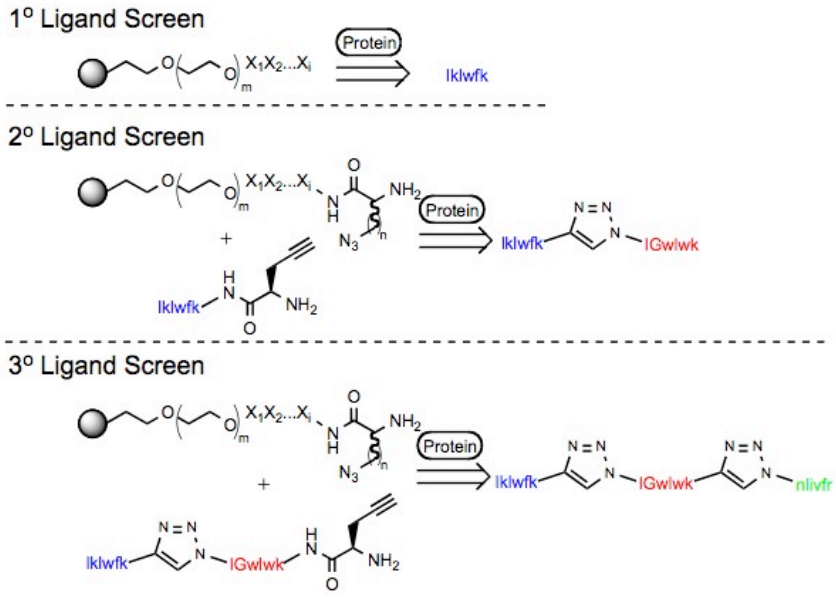
Chapter 3: Generating a High-Quality Triligand Capture Agent for bCAII

3.1 Introduction

Chapter 3 describes an effective and generalized *in situ* click chemistry catalyzed approach using peptide ligands towards a model protein biomarker, bovine carbonic anhydrase II (bCAII). Carbonic anhydrase II is from a family of metalloenzymes that catalyze the reversible hydration of carbon dioxide. This protein has been used for studying protein-ligand interactions and it is a demonstrated receptor for bivalent ligands.[1–5] In addition, expression of human carbonic anhydrase II (hCAII) is induced in the endothelium of neovessels in melanoma and renal and other cancers.[6] Furthermore, CA II represents a major target antigen stimulating an autoantibody response in melanoma patients,[7] and it is also potentially a therapy target for glial tumors.[8] In this work, a triligand peptide capture agent was developed for bCAII and hCAII, which exhibited 64 and 45 nM binding affinities, respectively, by the sequential *in situ* click-catalyzed assembly of three 7-mer peptide ligands (Scheme 3.1).



Scheme 3.1 Preparation of protein catalyzed, multi-ligand capture agents

A completely random OBOC library of 22-mer peptides would be impractical for constructing and identifying the protein capture agent. We further report that this triligand capture agent can detect bCAII and the hCAII from a serum consisting of multiple naturally occurring proteins. These data suggest that triligands (and higher-order multi-ligands) assembled by *in situ* click chemistry will be a great source of robust and specific capture agents for *in vitro* diagnostics. Our generalized methods should serve as the basis for inexpensive capture agent development for any protein. This is of particular interest for proteins that do not respond well to traditional capture agents.

Herein, I describe, step-by-step, the screens and results associated with developing an anchor ligand, biligand, and triligand. Binding affinity and selectivity measurements are also covered in this chapter. Appendix B gives the complete structures of biligands and triligands, along with mass spectrum analysis. Attached to Appendix C is our recent literature paper: "Iterative *In Situ* Click Chemistry Creates Antibody-Like Protein-Capture Agents," that can be further reviewed.

3.2 Peptide Library Construction

One-bead one-compound (OBOC) peptide libraries were synthetically constructed and discussed in Section 2.3. Table 3.1 shows the specific libraries that were fabricated and used for screening against bCAII.

Table 3.1 Libraries used in this study[†]

	Formula	Components	# of unique sequences
A	$x_1x_2x_3x_4x_5$	x_i = 19 D-amino acids (no D-Cys)	2,476,099
B	$x_1x_2x_3x_4x_5x_6$	x_i = r, k, l, w, f, h, y	117,649
C	**Az$_n$**-$x_2x_3x_4x_5x_6$-**Az$_n$**	x_i = 19 D-amino acids (no D-Cys) **Az$_n$** = 1/3 **Az4**, 1/3 **Az8**, 1/3 nothing	22,284,891
D	$x_1x_2x_3x_4x_5x_6$-**Tz1**-kfwlkl	x_i = k, l, w, f, i, g, v	117,649
Tz1 = triazole formed between **Az4** (on terminal k) and **D-Pra** (on x_6)			
E	$x_7x_6x_5x_4x_3x_2$-**Tz2**-kwlwGl-**Tz1**-kfwlkl	x_i = d, r, s, w, G, f, l	117,649
Tz1 = triazole formed between **Az4** (on terminal k) and **D-Pra** (on l) **Tz2** = triazole formed between **Az4** (on terminal x_2) and **D-Pra** (on k)			
F	**Az4**-$x_2x_3x_4x_5x_6x_7$	x_2 = r, n, l, i; x_3 = w, f, l, i; x_4 = r, w, f, l, i; x_5 = w, f, v, l; x_6 = r, w, f, l, k; x_7 = f, r	3200
G	$x_7x_6x_5x_4x_3x_2$-**Tz2**-kwlwGl-**Tz1**-kfwlkl		3200

[†] Randomized positions are denoted by x (for D-amino acids) and **Az$_n$** (for azide-containing artificial amino acids).

3.3 Screening and Results for Anchor Ligand

The first peptide, or anchor ligand, was selected from a 2-generation library screen (**An1, An2, An2b, Table 3.1**). Using the OBOC combinatorial library methodology, a randomized library of pentapeptides consisting of 2,476,099 D-peptide compounds was synthesized manually by a split-and-mix synthesis approach as previously discussed in Chapter 2. An approximately ten fold excess of beads was utilized to fabricate the peptide library. This pentapeptide library was expected to afford enough diversity to discriminate between weak and strong binders, and the sample size was large enough to ensure that

there will be some lead compounds. The library was also simple enough to manually prepare on the bench-top, and was readily modified with azide-containing amino acids.

3.3.1 First-Generation Screen for Anchor Ligand. For the first screen (**An1**), **Library A** was screened with 100 nM bCAII protein, labeled with Alexa Fluor 647 succinimidyl ester (Figure 3.1).

Figure 3.1 Screening for anchor ligand

The red Alexa Fluor 647 dye was chosen for protein labeling due to the low autofluorescence of the TentaGel bead at these wavelengths. The dye was conjugated to the bCAII protein at low level (< 3 fluorophores/protein) to minimize interference of the fluorophore with real binding interactions. Thus, the "hit" beads were identified by their fluorescence. The brightest fluorescent beads (< 0.02% of library) were manually separated from the non-hit beads. After removing the protein from the beads using 7.5 M guanidine hydrochloride (pH 2.0), the hit peptide-beads were sequenced by Edman degradation[9] (Table 3.2) and analyzed by a histogram correlating the frequency of amino acid occurrence vs amino acid identity (Figure 3.2). The results suggest that basic/charged (k, r) and aromatic residues (y, f, w) are important amino acids in an anchor ligand for bCAII.

Table 3.2 First-generation anchor ligand screen **An1** (100 nM) results

	X_1	x_2	x_3	x_4	x_5		x_1	x_2	x_3	x_4	x_5
hit1	r	r	y	h	r	**hit27**	w	r	--	y	r
hit2	m/v	r	w	k	r	**hit28**	h	r	w	r	r
hit3	k	r	w	y	y	**hit29**	w	y	r	k	r
hit4	w	k	k	k	w	**hit30**	l	r	f	r	r
hit5	h	f	f	f	r	**hit31**	w	k	r	k	k
hit6	s	r	--	r	r	**hit32**	r	r	r	w	s/m
hit7	r	r	w	h	y	**hit33**	r	r	k	f	w
hit8	r	k	w	w	w	**hit34**	r	r	w	r	y
hit9	r	w	s	f	r	**hit35**	w	r	h	y	k
hit10	r	r	G	w	r	**hit36**	r	r	y	f	r
hit11	G	f	r	r	w	**hit37**	w	r	k	w	r
hit12	r	t	r	r	w	**hit38**	w	y	--	r	r
hit13	m	r	w	k	r	**hit39**	y	r	r	r	h
hit14	y	r	k	r	w	**hit40**	y	r	r	r	w
hit15	a	--	--	--	--	**hit41**	p	f	y	w	r
hit16	r	r	i	r	w	**hit42**	k	y	w	r	k
hit17	--	--	k/l	w	--	**hit43**	r	y	w	h	k
hit18	r	w	--	--	r	**hit44**	r	w	h	w	n
hit19	k/l	r	--	w	r	**hit45**	r	h	f	h	h/f
hit20	w	r	f	r	y	**hit46**	r	r	--	h	r
hit21	d/p	y	y	r	r	**hit47**	r	y	r	r	r
hit22	r	y	w	k	k	**hit48**	y	f	h	h/w	w
hit23	k/l	r	r	r	w	**hit49**	r	r	r	w	y
hit24	y	r	r	k	w	**hit50**	w	r	r	r	r/--
hit25	r	k/l	f	y	r	**hit51**	r	w	k	f	h
hit26	r	w	w	k	r						

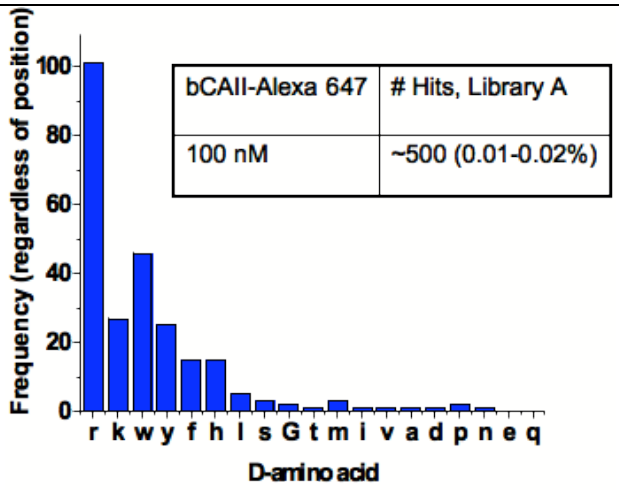


Figure 3.2 Histogram for 51 hit sequences isolated from screen **An1** (first-generation anchor ligand screen)

3.3.2 Re-Screening for Anchor Ligand with a Focused Library. A second, more focused library (**Library B, Table 3.1**) of 117,649 D-peptide compounds was constructed from the most commonly occurring amino acids (r, k, l, w, f, h, y), as identified from screen **An1**, but expanded into a 6-mer peptide, and screened under 50 nM bCAII (**An2a**) and 8 nM bCAII (**An2b**) conditions. The results of the second-generation anchor ligand screens are shown in Tables 3.3 and 3.4. The hit rates were 0.09% and 0.05% for 50 nM and 8 nM, respectively.

Table 3.3 Second-generation anchor ligand screen **An2a** (50 nM) results

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	y	r	w	f	k	f
hit2	h/r	h/r	f	l	l/r	r
hit3	f	r	f	y	y	r
hit4	h/r	f	f	k	l	--
hit5	k	l	f	l	k	l
hit6	l	f	l	w	l	k
hit7	f	f	f	r	y	--
hit8	h/r	f	f	f	r	--
hit9	r	w	w	l	k	f
hit10	h/r	f	f	r	y	y
hit11	l	k	l	f	l	k
hit12	f	r	r	w	w	k
hit13	h/r	y	f	f	k	l
hit14	l	k	f	f	f	k
hit15	h/r	f	f	r	r	--

Table 3.4 Second-generation anchor ligand screen **An2b** (8nM) results

	x_1	x_2	x_3	x_4	X_5	x_6
hit1	h	l	y	f	l	r
hit2	l	k	l	w	f	k

The more stringent screen yielded two hits, **hlyflr** and **lklwfk**. From these two candidates, one peptide **(lklwfk)** was arbitrarily chosen as the starting point for an anchor ligand for use in the multi-ligand screens.

3.4 Binding Measurements for Anchor Ligand by Fluorescence Polarization

The anchor peptide **lklwfk** was then functionalized with an acetylene (-C≡C-H) at the C-terminus and produced in bulk quantities (see Section 2.7 for details) for affinity measurements by fluorescence polarization (using a fluoresceinated peptide). The N-terminus of the anchor ligand was labeled with fluorescein isothiocyanate (FITC) following published protocols.[10] After resin cleavage, the crude fluoresceinated anchor ligand was precipitated with ether and then purified to > 98% 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 varying concentrations of bCAII (0.2 μM 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. Samples were excited at 488 nm (2 nm band-pass), and luminescence spectra were obtained between 500 nm 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 obtained by applying Equation 1.

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \qquad (1)$$

The polarization values were fitted with a sigmodial curve using the logistical equation (Origin 6.1, Northampton, MA),

$$y = \frac{A_1 - A_2}{1 + (x/x_0)^p} + A_2 \qquad (2)$$

where A_1 = initial y value, A_2 = final y value, p = power, and x_0 = center.

By this method, a 500 µM affinity was measured for the fluoresceinated anchor ligand lklwfk-(D-Pra) (Figure 3.3). However this value is an estimate, since weak affinities are hard to quantify.

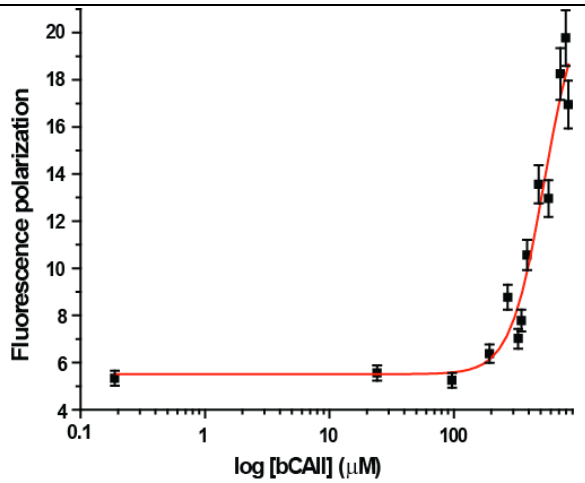
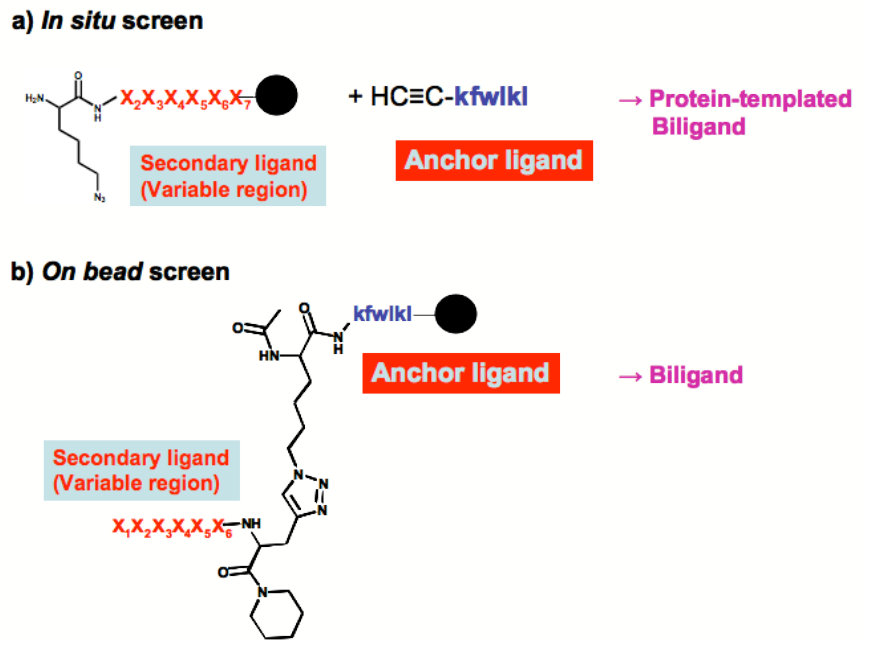


Figure 3.3 Fluorescence polarization binding isotherm for the anchor ligand lklwfk-(D-Pra), showing $K_D \approx 500$ µM.

Surface plasmon resonance (SPR) was also used to measure the affinity of bCAII with lklwfk-(D-Pra), and a similarly low affinity was recorded (at least > 10 µM, data not shown).

3.5 In Situ Click and *On Bead* Biligand Screens and Results

A biligand is constructed of a 2° ligand that is covalently attached, via a 1,2,3-triazole linkage, to the anchor ligand. Two screening strategies, namely *in situ* (Scheme 3.2A) and *on bead* (Scheme 3.2B) screens, were utilized to screen for biligand candidates.



Scheme 3.2 A) *In situ* biligand screen schematic, illustrating the *on bead* biligand synthesis catalyzed by bCAII. B) *On bead* biligand library schematic

3.5.1 First-Generation *In Situ* Biligand Screen. Based upon the *in situ* click chemical reactions reported by the Sharpless group,[11–17] the anchor ligand and protein are in solution, and the library of 2° ligands is on the bead. Only those 2° ligands that bind with bCAII *and* are in close proximity with the anchor peptide, *and* are in the correct orientation, will react to form the 1,2,3-triazole product. In the *in situ* click biligand screen (**Bi1**), a solution of 50 nM bCAII-Alexa Fluor 647 was pre-incubated with the anchor ligand, 100 µM **lklwfk-(D-Pra),** for 2 hours at 37 °C in PBS (pH 7.4) + 1%

DMSO (v/v). The anchor/protein solution was added and incubated with a large and comprehensive, azide-modified OBOC **Library C** (22,284,891 element, 4× sampled, Table 3.1) for 48 hours at 37 °C, with shaking to form an *in situ* biligand. For this screen, excess anchor ligand is supplied so that it is noncovalently attached to its binding site on every protein molecule. The bCAII protein target acts as a catalyst for the click reaction by orienting the anchor ligand and the secondary ligand correctly with respect to each other and the protein surface. The screened beads were washed with 3 × 5 mL PBS (pH 7.4), then 7 × 5 mL water. The beads were imaged for fluorescence using the protocol outlined before. Hits, representing potential biligands formed *in situ*, were manually selected by micropipette and processed with guanidine hydrochloride to remove bound protein. These beads were then sequenced by Edman degradation.

The hit beads (0.007% hit rate) from the *in situ* screen (Scheme 3.2A) were sequenced to identify 2^{o} ligand candidates, from which a biligand could be separately prepared. The results from Edman degradation of the 23 *in situ* hits are presented in Table 3.5.

Table 3.5 *In situ* biligand screen **Bi1** (50 nM) results

	Az_n	**x_2**	**x_3**	**x_4**	**x_5**	**x_6**	**Az_n**
hit1	**Az4**	k	i	w	i	G	
hit2	**Az8**	r	l	w	v	G	**Az4**
hit3	**Az8**	r	r	r	k	r	**Az8**
hit4	**Az4**	l	l	v	i	k	**Az4**
hit5	**Az4**	m	i	l	i	k	
hit6	**Az8**	i	i	i	m	r	**Az4**
hit7	**Az8**	i	i	i	w	r	**Az8**
hit8	**Az4**	n	v	i	i	f	
hit9	**Az4**	i	f	l	v	k	**Az8**
hit10	**Az4**	k	i	w	i	G	**Az8**
hit11	**Az4**	r	r	k	f	r	**Az8**
hit12	**Az4**	r	v	w	l	r	**Az8**
hit13	**Az8**	k	y	r	r	r	**Az4**

hit14	**Az8**	r	r	k	v	w	**Az4**
hit15	**Az4**	i	f	l	v	k	**Az8**
hit16		k	r	k	r	f	**Az4**
hit17	**Az8**	k	i	w	i	k	
hit18	**Az8**	y	r	k	f	k	
hit19	**Az4**	i	f	f	r	v	**Az8**
hit20		a	r	k	k	y	**Az4**
hit 21		r	k	r	t	i	**Az4**
hit 22	**Az8**	k	m	v	f	k	**Az4**
hit23	**Az4**	l	i	m	k	i	**Az4**

An extremely high level of sequence homology is observed. Three almost identical peptides, **Az_nkiwiGAz_n**, are highlighted above. Note also that all of the peptides contain at least one azido group, although, statistically, over 1/3 of the OBOC library does not contain azido groups at the 1 or 7 positions. The high sequence homology, coupled with the persistence of azido groups in the peptide, provides strong evidence that the *in situ* OBOC screen worked to produce a biligand.

3.5.2 First-Generation *On Bead* Biligand Screen. In the second approach, the anchor ligand and cognate library of 2^o ligands are preassembled as complete biligands *on bead*. The protein is screened against the library of biligands in a stringent screen, in order to choose the best biligands. *On bead* (Scheme 3.2B) biligand screens (**Bi2a** and **Bi2b**) were carried out utilizing a focused biligand library (**Library D**; 117,649 peptides, Table 3.1) that was prepared based upon the sequencing results from screen **Bi1**. The 1,2,3-triazole linkage in this library was prepared using the classical click reaction between artificial amino acid **Az4** and **D-Pra** described earlier in Chapter 2. **Az4** was chosen as the optimal azide linker length based on sequence homology found among the *in situ* biligand hits. This library was limited to a smaller size to ensure high purity of full-length biligand sequence on every bead. To block nonspecific protein binding, the library was first incubated in PBS (pH 7.4) + 0.1% Tween 20 + 0.1% bovine serum

albumin (BSA) + 0.05% NaN_3 (PBSTBNaN_3) for 1 hour, with shaking.[18] Following this pre-blocking step, the library was washed with 3 × 5 mL PBSTBNaN_3. The bCAII-Alexa Fluor 647, at 50 nM (**Bi2a)** and 10 nM (**Bi2b**) in 4 mL PBSTBNaN_3, was incubated with the library for 17 hours at 25 °C, with shaking. The screened beads were washed with 3 × 5 mL PBSTBNaN_3, then 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 × 5 mL PBS (pH 7.4). The beads were imaged for fluorescence, and the hits were selected by micropipette. After washing the hits to remove bound protein, their sequences were determined by Edman degradation (Tables 3.6 and 3.7).

Table 3.6 *On bead* biligand screen **Bi2a** (50 nM) results

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	f	k	l	w	i	k
hit2	v	w	l	w	G	G
hit3	f	w	f	w	G	G
hit4	k	w	f	w	G	G
hit5	f	k	l	w	l	k
hit6	k	w	f	w	G	G
hit7	w	w	i	w	G	G
hit8	k	G	w	l	w	G
hit9	k	l	w	i	w	G
hit10	l	w	i	w	G	l
hit11	f	k	G	f	l	i
hit12	f	w	i	w	G	k
hit13	l	w	l	w	G	i
hit14	i	i	v	l	w	k
hit15	l	i	i	f	v	
hit16	v	k	f	i	l	l
hit17	l	G	f	f	w	i
hit18	k	k	l	k	k	l
hit19	f	k	l	w	i	k
hit20	w	i	w	G	G	f
hit 21	f	f	l	l	v	k
hit 22	k	f	k	f	w	k
hit23	l	i	k	l	f	v
hit24	l	w	f	w	G	v
hit25	f	w	f	w	G	i
hit26	G	w	f	w	G	v
hit27	G	w	i	w	G	k

Table 3.7 *On bead* biligand screen **Bi2b** (10 nM) results

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	k	w	i	w	G	w
hit2	k	w	i	w	G	v
hit3	k	w	l	w	G	l
hit4	k	w	i	w	G	l
hit5	k	w	i	w	G	w
hit6	k	w	l	w	G	l
hit7	G	w	i	w	G	i
hit8	k	i	f	k	i	f

The *on bead* **Bi2a** and **Bi2b** screens (0.07% and 0.008% hit rates, respectively) again yielded a striking sequence homology, suggesting the consensus sequence **kwx_3wGx_6** (where x_3 = hydrophobic amino acid, x_6 = any amino acid). Two secondary ligand candidates from the *on bead* screen were repeated several times, namely **kwlwGl** and **kwiwGw**.

From the *on bead* and *in situ* screens, three candidate biligands— **kwlwGl-Tz2-kfwlkl**, **kwiwGw-Tz4-kfwlkl**, and **lklwfk-Tz5-kiwiG** [where **Tz4** = triazole formed between **Az4** (on terminal k) and **D-Pra** (on w), and **Tz5** = triazole formed between **Az4** (on terminal k) and **D-Pra** (on k)]— were synthesized in bulk on 2-chlorotrityl chloride (1.6 mmol/g) resin (Anaspec, San Jose, CA) using Scheme 2.3. The biligand was released either as the fully deprotected peptide by cleavage with 95:5 TFA:water (+ 2 mol equiv TES per side chain protecting group), or as the fully protected peptide by cleavage with 99:1 DCM:TFA (García-Martín et al. 2007).

3.6 Binding Measurements for Biligand by Surface Plasmon Resonance (SPR)

Immobilization and biligand sensing experiments were performed on a Biacore T100 SPR (California Institute of Technology Protein Expression Center, Pasadena, CA). Two flow cells of the biosensor surface (Biacore CM5) were immobilized with bCAII following standard procedures using NHS/EDC and 0.25 mg/mL bCAII prepared in 10 mM sodium acetate (pH 5.0) buffer.[12] The remaining two flow cells were left underivatized, to correct for changes in bulk refractive index and to assess nonspecific binding. Biligand samples were injected in a concentration series (5 μM to 2 nM) at 100 μL/min flow rate for 120–180 s across the four flow cells.

All three biligands— **kwlwGl-Tz2-kfwlkl**, **kwiwGw-Tz4-kfwlkl**, and **lklwfk-Tz5-kiwiG**— were synthesized in bulk (as described above) and their binding affinities for bCAII were measured using SPR. The binding responses reveal 10^{-6} M affinity for the biligands toward bCAII proving that the bead-based *in situ* screen and the *on bead* biligand library screen converge on similar biligand sequences with similar affinities. The SPR data for the best-binding biligand **kwlwGl-Tz2-kfwlkl** is shown in Figure 3.4B, and its chemical structure is pictured in Figure 3.4A. The biligand affinity fit of $K_D \approx 3$ μM is two orders of magnitude greater than the affinity for the 1^o ligand alone, meeting our goal of affinity enhancement.

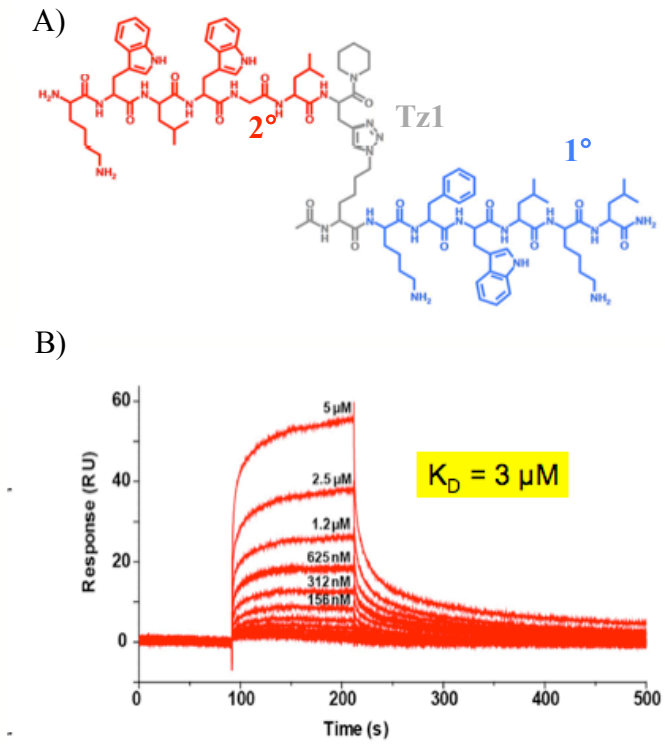


Figure 3.4 A) Biligand chemical structure. B) SPR response sensorgrams obtained with increasing concentration of the biligand kwlwGl-Tz2-kfwlkl (2 nM to 5 µM) demonstrate a 3 µM binding affinity to bCAII.

3.7 In Situ Click and *On Bead* Triligand Screens and Results

A triligand capture agent was identified in a similar way to the biligand, but with two exceptions. First, a modified form of the best-binding biligand **(D-Pra)-kwlwGl-Tz2-kfwlkl** served as the anchor peptide. Second, within the constraints of the OBOC libraries we could reasonably prepare, we attempted a direct comparison of *in situ* (**Tri1**) and *on bead* (**Tri2**) first-generation screens. The notable difference between these screens was the chemical diversity of the OBOC libraries.

3.7.1 First-Generation *In Situ* Triligand Screen. For the first generation *in situ* screen **Tri1**, a solution of 10 nM bCAII-Alexa Fluor 647 was pre-incubated with 100 µM biligand anchor **(D-Pra)-kwlwGl-b-kfwlkl** for 2 hours at 25 °C in PBSTBNaN_3 (pH 7.4)

+ 1% DMSO (v/v). After blocking the library in PBSTBNaN$_3$ (pH 7.4) for 1 hour, with shaking, the anchor/protein solution was added to the bead library **C** (4 g, ~ 2,250,000 beads) and incubated for 15 hours at 25 °C, with shaking, to form an *in situ* triligand. Notice that the same, comprehensive 2×10^7 element OBOC **Library C** that was utilized in screen **Bi1** was applied again here, demonstrating the versatility of this type of general library. The screened beads were washed with 3 × 5 mL PBSTBNaN$_3$, then 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 × 5 mL PBS (pH 7.4). The beads were then imaged for fluorescence. Hits, representing potential triligands formed *in situ*, were selected manually by micropipette. The selected beads were washed to remove bound protein and the sequences of these hits were obtained by Edman degradation (Table 3.8).

Table 3.8 First-generation *in situ* triligand screen **Tri1** (10 nM) results

	Az_n	**x_2**	**x_3**	**x_4**	**x_5**	**x_6**	**Az_n**
hit1	**Az4**	n	i	i	i	v	
hit2	**Az4**	i	i	l	l	k	**Az4**
hit3	**Az4**	n	i	i	v	l	
hit4	**Az4**	n	m	i	f	l	**Az4**
hit5	**Az4**	n	v	l	v	l	
hit6	**Az4**	n	l	i	l	f	**Az4**
hit7	**Az4**	n	l	i	l	f	**Az4**
hit8	**Az8**	r	l	w	i	r	**Az4**
hit9	**Az4**	n	l	i	v	f	**Az4**
hit10	**Az4**	r	m	w	v	k	**Az8**
hit11	**Az4**	i	i	l	l	k	**Az8**
hit12	**Az4**	i	l	v	v	r	**Az4**
hit13	**Az4**	n	l	l	f	l	**Az4**
hit14	**Az4**	n	i	i	v	y	
hit15		m	k	r	k	k	**Az8**
hit16	**Az4**	i	l	i	r	w	**Az4**
hit17	**Az8**	i	i	v	f	r	**Az8**
hit18	**Az8**	y	f	t	r	r	
hit19	**Az4**	n	m	i	i	v	**Az4**
hit20	**Az8**	i	l	i	a	k	**Az4**
hit21	**Az4**	i	l	l	r	w	
hit22	**Az8**	i	v	v	f	r	**Az4**
hit23	**Az4**	l	l	l	v	k	**Az4**
hit24	**Az4**	k	v	w	i	k	**Az4**

3.7.2 First-Generation *On Bead* Triligand Screen. For the *on bead* screen **Tri2**, the difficulty of manually synthesizing a high-purity 22-mer OBOC library meant that we limited the library to a smaller number of amino acids (d, r, s, w, G, f, l), chosen to represent as high a chemical diversity as possible for a limited library size (~ 10^5–10^6 peptides). Screens were conducted using **Library E** (40 mg, ~ 120,000 beads, Table 3.1) in a polypropylene fritted tube. To block nonspecific protein binding, the library was first incubated in PBSTBNaN_3 (pH 7.4) for 1 hour, with shaking. Following this pre-blocking step, the library was washed with 3 × 5 mL PBSTBNaN_3. The bCAII-Alexa Fluor 647, at 10 nM dilution in 4 mL PBSTBNaN_3, was incubated with the library for 17 hour at 25 °C, with shaking. The screened beads were washed with 3 × 5 mL PBSTBNaN_3, then 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 × 5 mL PBS (pH 7.4). The beads were imaged for fluorescence, and the hits were selected by micropipette. After washing the hits to remove bound protein, their sequences were determined by Edman degradation (Table 3.9).

Table 3.9 First-generation *on bead* triligand screen **Tri2** (10 nM) results

	x_2	x_3	x_4	x_5	x_6	x_7
hit1	r	l	w	l	r	f
hit2	r	l	w	l	r	l
hit3	r	f	f	f	r	f
hit4	r	l	f	l	r	f
hit5	l	f	f	w	f	r
hit6	l	w	f	f	f	r
hit7	l	f	l	w	f	r
hit8	l	w	l	f	f	r
hit9	l	f	f	w	l	r
hit10	r	r	r	l	w	r
hit11	r	l	w	l	r	f
hit12	w	r	r	r	r	w
hit13	r	f	r	f	r	w
hit14	f	w	f	f	w	r

There was also a high sequence homology among the triligand sequences. The *in situ* screen **Tri1** yielded a 0.007% hit rate that suggested sequences **Az4-nlilfx$_7$** and **Az4-nlivfx$_7$** (where x_7 = any amino acid). The *on bead* screen **Tri2** resulted in a 3^o ligand candidate, **Az4-rlwlrx$_7$**, whose motif was repeated three times. Since the *on bead* **Tri2** screen was by no means comprehensive, it is not surprising that a somewhat different 3° ligand consensus was reached.

3.7.3 Azide-Free *In Situ* Triligand Screen. To further understand the effect of the azide-acetylene interaction on the *in situ* results, a third *in situ* screen (**TriX**) was conducted under otherwise identical conditions as screen **Tri1**, except for the substitution of azide-rich **Library C** with the azide-free **Library A**, thus prohibiting the formation of a triazole linkage. While this control *in situ* screen **TriX** displayed a nearly identical hit rate (0.007%), a completely different sequence homology was reached as **(d/a/s)wwx$_4$x$_5$** (where x_4,x_5 = any amino acid) (Table 3.10 and 3.11). The remaining hit sequences from screen **TriX** display significant variation and high numbers of repeating arginine residues, indicative of nonspecific anionic-cationic interactions.[19] This argues the importance of the azide-acetylene interaction in the *in situ* library selection for choosing a specific ligand, whose specificity is a consequence of the *in situ* click conjugation.

Table 3.10 Azide-free *in situ* triligand screen **TriX** results (control)

	x$_1$	**x$_2$**	**x$_3$**	**x$_4$**	**x$_5$**
hit1	w	f	r	r	r
hit2	s	w	v	w	G
hit3	p	v	y	f	w
hit4	d	d	y	w	G
hit5	i	w	a	y	w
hit6	d	n	w	G	f
hit7	a	w	w	a	t
hit8	r	f	r	r	f
hit9	d	w	w	h	t
hit10	r	f	r	w	r

hit11	d	e	w	p	h
hit12	a	w	w	l	w
hit13	a	w	w	a	y
hit14	d	k	k	i	y
hit15	d	w	s	i	e
hit16	s	w	w	f	y
hit17	d	w	l	r	y
hit18	s	w	a	f	y
hit19	d	l	f	l	w
hit20	d	w	a	t	w
hit21	f	k	y	r	s
hit22	d	q	r	w	r
hit23	i	w	s	t	h
hit24	l	i	v	m	w

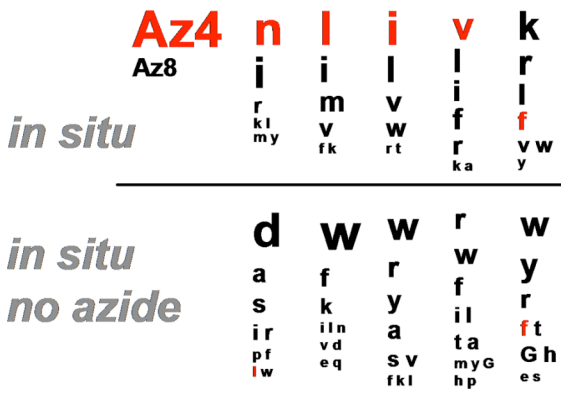


Table 3.11 Position-dependent histograms for the first-generation *in situ* click screens, for peptides with and without an azide-containing amino acid, to generate a triligand. The final, consensus triligand sequence is indicated by red font. For the *in situ* screen, 1/3 of the beads had no azide at the x_1 or x_7 positions, but all hit beads contained an azide. Sample size: *in situ* and *in situ no azide* n=24

3.7.4 Re-Screening for Triligand Ligand with Focused Libraries. Second-generation *in situ* (screen **Tri3**) and *on bead* (screen **Tri4**) triligand screens were conducted using **Libraries F** and **G**, respectively (Table 3.1). Each **Library F** and **Library G** included *all* the high-homology amino acids isolated by the first-generation triligand screens. The second-generation screens were used to determine whether a single

triligand capture agent could be deduced by screening a single, highly focused triligand library.

Both triligand screening strategies allowed for isolation of hits at much lower bCAII-Alexa Fluor 647 concentrations, as low as 500 pM for the *in situ* library and 250 pM for the *on bead* library, compared to the biligand screens. In the second-generation *in situ* click triligand screen, a solution of 500 pM bCAII-Alexa Fluor 647 was pre-incubated with 100 μM Biligand Anchor **(D-Pra)-kwlwGl-b-kfwlkl** for 2 hours at 25 °C in PBSTBNaN_3 (pH 7.4) + 1% DMSO (v/v). After blocking the library in PBSTBNaN_3 (pH 7.4) for 1 hour, with shaking, the anchor/protein solution was added to the focused bead **Library F** (3200 D-peptide compounds, 1 mg, ~ 3200 beads) and incubated for 18 hours at 25 °C, with shaking, to form a triligand.

In the second-generation *on bead* triligand screen, screens were conducted using the focused **Library G** (1 mg, ~ 3200 beads) for 18 hours at 25 °C, with shaking. **Library G** was constructed using exactly the same constituent amino acids as **Library F**, but with the biligand anchor covalently attached to the end of the library via the Cu(I)-catalyzed click reaction. In both cases, the screened beads were washed with 3 × 5 mL PBSTBNaN_3, then 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 × 5 mL (PBS pH 7.4). The beads were then imaged for fluorescence. Hits, representing potential triligands formed *in situ*, were selected manually by micropipette. The selected beads were then processed to remove bound protein, and the sequences of these hits were obtained by Edman degradation (Tables 3.12 and 3.13).

Table 3.12 Second-generation *in situ* triligand screen **Tri3** (500 pM) results

	x_2	x_3	x_4	x_5	x_6	x_7
hit1	n	l	I	v	f	r
hit2	n	l	i	v	l	r
hit3	n	i	i	l	l	r
hit4	i	l	f	l	f	r
hit5	n	l	i	v	l	r
hit6	n	i	i	l	w	r
hit7	n	l	i	v	f	r
hit8	n	l	i	v	f	r

Table 3.13 Second-generation *on bead* triligand screen **Tri4** (250 pM) results

	x_2	x_3	x_4	x_5	x_6	x_7
hit1	n	l	i	v	f	r
hit2	n	l	i	v	f	r
hit3	n	i	i	v	f	r
hit4	n	i	i	v	f	r
hit5	n	i	i	l	l	r
hit6	n	l	i	v	l	r
hit7	n	l	i	v	f	r

Both the *in situ* (**Tri3**) and *on bead* (**Tri4**) screens yielded a single 3° ligand sequence of **Az4-nlivfr**. The fact that this same consensus sequence was identified by both an *on bead* and an *in situ* screen (each designed to sample the same chemical space, albeit via different paths) provides further confirmation of the equivalence of the two types of screens.

The triligand— **rfviln-Tz2-kwlwGl-Tz1-kfwlkl**— was synthesized by the click reaction between the fully protected biligand anchor (0.274 g, 0.1 mmol, > 98% HPLC) and bead-bound 3° ligand Az4-nlivfr (0.1 g, 0.07 mmol) using copper iodide (0.021 g, 0.1 mmol) and L-ascorbic acid (0.020 g, 0.1 mmol) in DMF/piperidine (8/2) at 25 °C (Scheme 2.3).

3.8 Binding Measurements for Triligand using SPR

3.8.1 Protein. Human carbonic anhydrase II (hCAII, C6165), from human erythrocytes, lyophilized powder, was purchased from Sigma-Aldrich (St. Louis, MO) and used in affinity and selectivity studies. Both bCAII and hCAII were tested by SDS gel electrophoresis, and confirmed to display a single band corresponding to 29,000 Da.

3.8.2 SPR. The triligand, **rfviln-Tz2-kwlwGl-Tz1-kfwlkl**, (chemical structure shown in Figure 3.5A) was synthesized in bulk and its binding affinity for both bCAII and hCAII was measured using SPR. These affinity measurements utilized a Biacore T100 SPR (California Institute of Technology Protein Expression Center, Pasadena, CA). One flow cell of the biosensor surface (Biacore CM5) was immobilized with bCAII following standard procedures using 0.25 mg/mL bCAII prepared in 10 mM sodium acetate (pH 5.0) buffer and a 1:1 solution of 0.1 mM NHS and 0.4 mM EDC.[20] Similarly, a second flow cell was immobilized with hCAII following standard procedures using 0.25 mg/mL hCAII prepared in 10 mM sodium acetate (pH 5.5) buffer. An immobilization level of 5000 RU was achieved using a flow rate of 100 µl/min over 420 s. The remaining two flow cells were left underivatized, to correct for changes in bulk refractive index and to assess nonspecific binding. The running buffer was prepared to contain 10 mM HEPES + 150 mM NaCl + 0.05% Tween20 + 3% DMSO, and this buffer was used for all experiments.

Prior to the peptide analyte experiment, 8 ‘startup’ cycles (running buffer alone) were completed to ensure that the instrument had a stable baseline. Response data were then collected for biligand samples over increasing concentrations (2 nM to 5 µM) at 100 µL/min flow rate, 120–180 s contact time, and 300 s dissociation phase across the four

flow cells. Similarly, response data were collected for anchor ligands (300 nM to 9.4 μM) and triligand (0.1 nM to 162 nM) over increasing analyte concentrations. After subtracting the background response from the underivatized flow cell, the analyte response data was fitted for 1:1 binding affinity using the BiaEvaluation software.

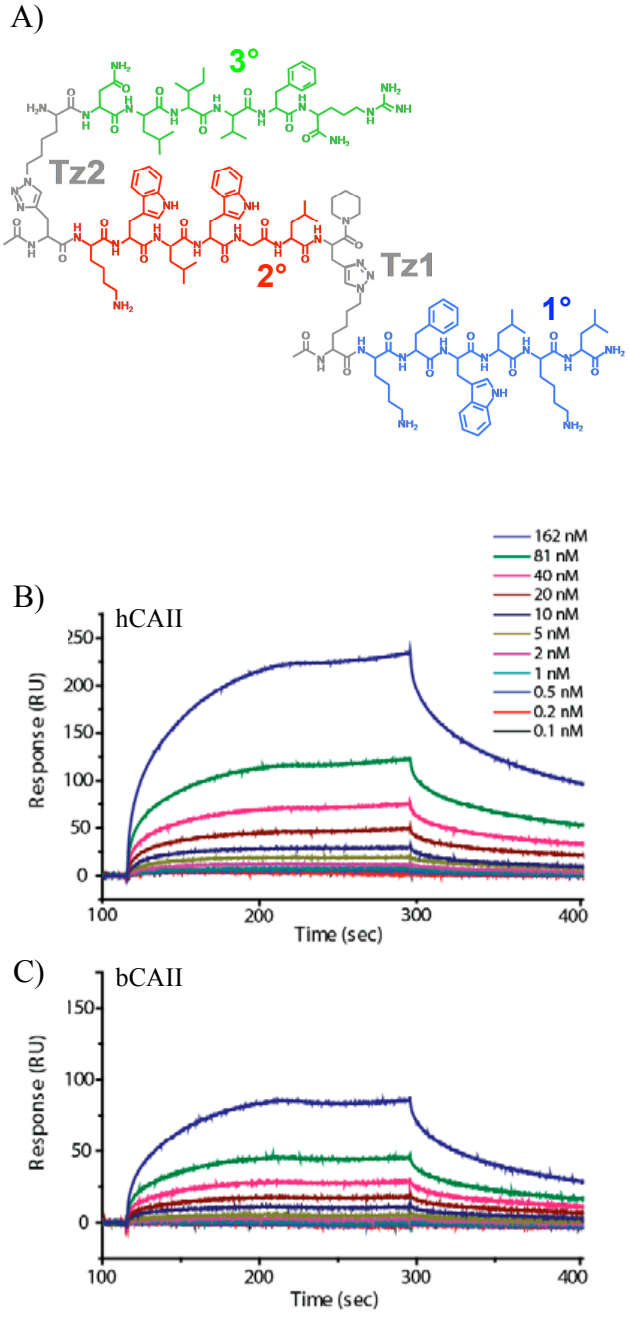


Figure 3.5 A) Triligand chemical structure. B,C) SPR response sensorgrams were obtained with increasing concentration of triligand (0.1 nM to 162 nM) and demonstrate 45 nM and 64 nM affinities for human (B) and bovine (C) CA II, respectively.

Figures 3.5B and 3.5C show the SPR results for the triligand to be $K_D \approx 45$ nM (hCAII) and $K_D \approx 64$ nM (bCAII). This represents a fifty fold affinity enhancement over the biligand. The triligand was also shown to not bind to the enzymatically active site of bCAII by an activity assay using 4-nitrophenyl acetate (4-NPA) as chromogenic substrate (data not shown here).[21]

3.9 Dot Blot Selectivity/Sensitivity Assays in Serum

The sensitivity and selectivity of the multi-ligand (biligand and triligand) capture agents for b(h)CAII in complex environments were demonstrated through the use of dot blot experiments in 10% porcine 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. After resin cleavage, the crude biotinylated multi-ligand was precipitated with ether and then purified to > 98% by C_{18} reversed-phase HPLC. Antigens (bCAII and hCAII) were prepared as 2 mg/mL stocks in PBS (pH 7.4). A dilution series of antigen was applied to a nitrocellulose membrane, typically ranging from 20 µg to 0.4 pg 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 7.4)]. The membrane was then washed with 3 x 5 min TBS (pH 7.4). The biotinylated triligand was prepared at 1 µM in 10% porcine serum in TBS (pH 7.4) + 0.1% DMSO and incubated over the membrane overnight at 4 °C. After washing with TBS (pH 7.4) for 1 hour, 1:3000 Streptavidin-HRP (AbCam, Cambridge, MA) prepared in 0.5% milk/TBS (pH 7.4) was added to the membrane and incubated for 1 hour. After washing with TBS (pH 7.4) for 1 hour, chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Enhancer and

Substrate Solutions, Pierce, Rockford, IL) were incubated over the membrane and then immediately developed on film to determine binding.

Figure 3.6A illustrates that the triligand is a suitable capture agent for detection of ~ 20 ng antigen from 10% serum, and that the capture agent was specific for both the bovine and human forms of the enzyme. Since bCAII and hCAII are 81.2% identical in sequence, similar binding sites are expected (PDB ID: 1CA2, 1V9E). The commercial anti-bCAII was capable of detecting lower antigen (~ 1 ng). However, this result cannot be directly compared to the triligand result, as this antibody is labeled with significantly more biotins per molecule (15–20 biotins/antibody), while the triligand is labeled with only 1 biotin per molecule. When the biligand anchor (D-Pra)-kwlwGl-Tz2-kfwlkl was used as the capture agent in 0.1% serum, the sensitivity was reduced more than ten fold (Figure 3.6B).

For comparison, the dot blot was completed in parallel using a commercial antibody as the capture agent. The polyclonal rabbit anti-bCAII IgG, biotin conjugate (Rockland Immunochemicals, Gilbertsville, PA) was prepared at 1:4000 dilution and incubated as described above. Both the triligand and commercial antibody were tested for cross-reactivity with 1 μg human IL-2 (BD Biosciences, San Jose, CA) and 1 μg human TNFα (eBioscience, San Diego, CA), and no binding was detected (Figure 3.6C). We also tested BSA as the antigen and similarly found no cross-reactivity.

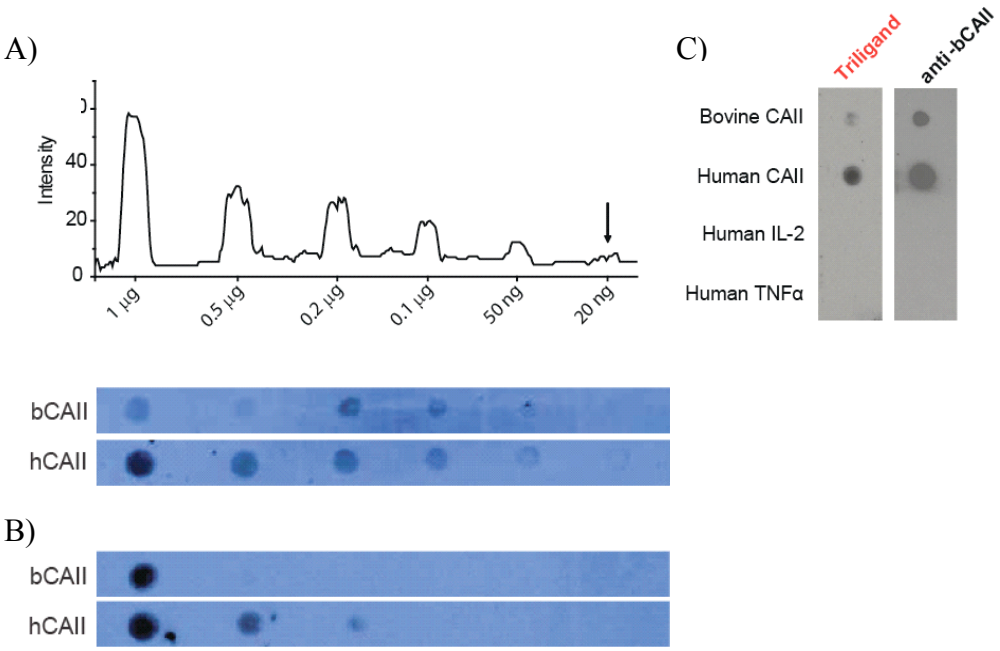


Figure 3.6 A) Dot blot illustrating the limit of detection by the triligand for bCAII and hCAII in 10% serum. B) When the biligand anchor is used as the capture agent in 0.1% serum, the sensitivity is reduced > ten fold. C) Dot blot illustrating the selectivity of the triligand, compared to a commercial antibody

3.10 Conclusion

This thesis demonstrated the development of a peptide capture agent with high affinity and selectivity against CAII through the conjugation of modest affinity peptides using *in situ* click chemistry. An affinity enhancement due to *in situ* click conjugation was apparent starting from the level of the biligand screens. Even for a weakly binding anchor ligand ($K_D \approx 500$ µM), biligand screens yielded high hit homologies and affinities between 3 and 10 µM. Both types of biligand screens, *in situ* and *on bead*, demonstrated this effect, suggesting that although the mechanism of the selection is different, the hits identified are essentially equivalent. When the peptide ligand becomes larger than a 10–15mer, the OBOC library size is practically limited to < 1 million sequences, and the *in situ* screen becomes the only way to sample increasing diversity and length.

Comprehensive *in situ* libraries are also quite valuable in their versatility, as they can be used in more than one screen and with more than one protein target, if synthesized at a large enough scale to accommodate multiple experiments. At the triligand level, a similar concept was explored. While the *in situ* library was still comprehensive, it utilized the basis of the biligand anchor to direct the assembly of the triligand. Based on analysis of sequence homology, we discovered that the final triligand capture agent reflects *in situ* assembly, as the *on bead* triligand library was not comprehensive. The final triligand peptide capture agent was demonstrated to bind to bCAII and hCAII with affinities of $K_D \approx 64$ nM and $K_D \approx 45$ nM, respectively, and it was proven to be a selective binder for the enzyme as illustrated by dot blot. The small size of the triligand peptide capture agent is expected to increase sensitivity of CAII detection in surface-based diagnostic assays.

The advantage of this approach is multifold. First, each multi-ligand is comprised of two or more ligands, and each ligand is a peptide-like molecule, comprised of natural, artificial, or nonnatural amino acids and other organic molecule building blocks. Chemical and biochemical stability, water solubility, thermal stability, and other desired characteristics can be custom designed into the multi-ligand. Also, multi-ligand capture agents produced in this way do not denature, as antibodies do, since their affinity and selectivity are not contingent upon their folded structure. Next, as the number of ligands comprising the multi-ligand protein capture agent is increased, the selectivity and affinity of the multi-ligand for the protein of interest rapidly increases. Tetraligands, pentaligands, etc., are all possible using the same concepts. Moreover, the target protein itself is utilized as a catalyst to assemble its own multi-ligand capture agent. The

individual ligands themselves are specifically designed for this catalytic process. No previous knowledge is needed of the capture agent and the protein of interest. Furthermore, amino acid building blocks are readily available, and chemical synthesis of peptides in relatively large quantities using conventional amide coupling chemistries is not difficult. This implies that the peptide capture agents may be produced in multi-gram quantities at low cost. Since straightforward chemistries are implemented at every stage in the process, we expect to use this as a general and robust platform for high-throughput capture agent discovery making an *in vitro* diagnostic device possible and inexpensive.

3.11 References

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