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14. ABSTRACT
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Peptide-based protein capture agents with high affinity, selectivity, and stability as antibody replacements in biodetection assays

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ABSTRACT

Current biodetection assays that employ monoclonal antibodies as primary capture agents exhibit limited fieldability, shelf life, and performance due to batch-to-batch production variability and restricted thermal stability. In order to improve upon the detection of biological threats in fieldable assays and systems for the Army, we are investigating protein catalyzed capture (PCC) agents as drop-in replacements for the existing antibody technology through iterative in situ click chemistry. The PCC agent oligopeptides are developed against known protein epitopes and can be mass produced using robotic methods. In this work, a PCC agent under development will be discussed. The performance, including affinity, selectivity, and stability of the capture agent technology, is analyzed by immunoprecipitation, western blotting, and ELISA experiments. The oligopeptide demonstrates superb selectivity coupled with high affinity through multi-ligand design, and improved thermal, chemical, and biochemical stability due to non-natural amino acid PCC agent design.

Keywords: PCC agent, peptide-based sensors, biological detection, antibody replacements

1. INTRODUCTION

The accurate and rapid detection of biological threats outside of the laboratory and in the field is a necessity for truly efficient biodefense analyses and countermeasures. In order to achieve such goals, a dedicated device that is capable of performing analyses under field conditions, i.e. various temperatures and humidity, is essential. As described in Figure 1, a common electronic detection device consists of a highly selective bioreceptor coupled to a transducer. The transducer then transmits a signal which is converted to a readable output. Within this schematic, the bioreceptor is by far the most important aspect of the device, since it must selectively bind to the target of interest and avoid interaction with any interferents. The two main performance characteristics of an ideal bioreceptor are high affinity and high selectivity to the target of interest. While the current gold standard for biodetection are monoclonal antibodies, since they exhibit high affinities and high selectivities, their overall stability is quite limited due to protease susceptibility, thermal shock, and pH instability (affects secondary structure and therefore activity), preventing them from effective incorporation into a fieldable device. Aside from the stability issues, monoclonal antibodies also typically take months to prepare, preventing the crucial rapid creation of bioreceptors for new and emerging threats. Monoclonal antibody alternatives including single chained antibodies [1, 2] and engineered antibodies [3, 4] improve upon thermal stability, but still require months to produce, whereas alternatives such as aptamers [5, 6] require less time to discover, but are not as thermally stable.

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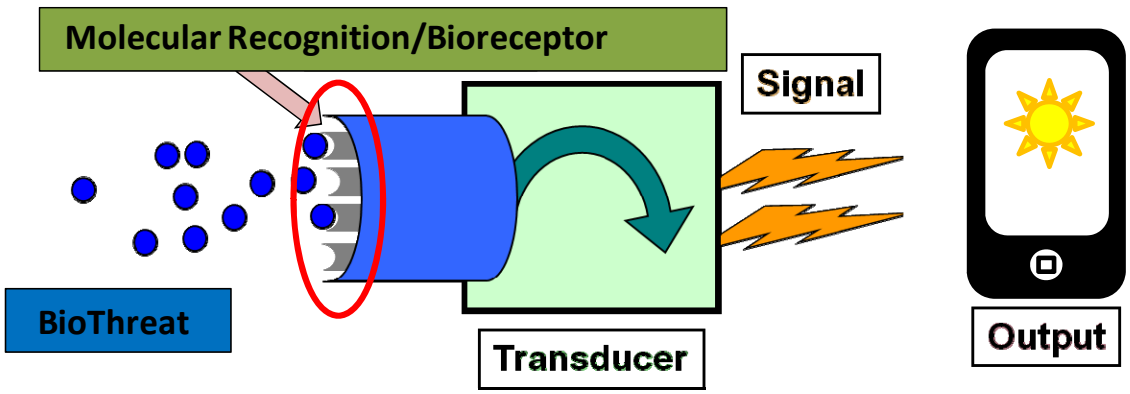

Figure 1. Schematic of a common electronic biosensor. The red circle highlights the bioreceptor portion of the sensor.

Other alternatives to monoclonal antibodies are peptide-based materials that show promise for usage outside of the laboratory due to their high thermal stability [7]. Since peptides have much lower molecular weights in comparison to antibodies, and have accessible N- and C- termini, they can easily be linked to many surfaces for effective incorporation into devices. Peptides can also be synthesized in large quantities and made on demand. There are effective methods for rapid discovery of peptide binders through the use of screening vast libraries of bioreceptor candidates including phage display [8, 9] and bacterial display technologies [10, 11]. While these screening methods can be successful finding high binding peptides, the selectivity of these peptides in complex matrices can be variable.

To address both the affinity and selectivity aspects of a peptide-based bioreceptor, protein catalyzed capture (PCC) agent technology is a very promising method of antibody replacement discovery [12-14]. We have recently reported the discovery of a highly effective PCC agent for the detection of the Protective Antigen (PA) protein of *Bacillus anthracis* and its performance in an electrochemical binding assay [15]. The PCC agent was constructed from a 15-mer peptide PA binder discovered through a bacterial display screen. The affinity and selectivity of the capture agent was improved by recruiting the PCC agent process to covalently attach a penta-peptide fragment to the originally discovered peptide through click chemistry.

We report additional affinity and selectivity enzyme-linked immunosorbant assay (ELISA) and Surface Plasmon Resonance (SPR) experiments to reiterate the power of the technology. The cross-reactivity SPR experiments were performed against a variety of Department of Defense high priority protein targets. These targets of interest include Protective Antigen (PA) of the Anthrax toxin, the bacteriophage MS2 coat protein (MS2CP), the Rivax vaccine recombinant protein to that of Ricin, the F1 surface protein of *Yersinia pestis* required for effective internalization into cells [ref], the neurotoxin-associated protein HS33A of *Clostridium botulinum*, and the COM1 protein from the *Coxiella burnetii* bacterial pathogen. The resulting data shows how the iterative construction of a bioreceptor by peptide fragments can greatly improve upon the binding characteristics and the power of ELISA and SPR to measure these characteristics.

2. EXPERIMENTAL

2.1 Materials

All chemicals and supplies were purchased from Sigma-Aldrich, Fisher Scientific, Bio-Rad, or Invitrogen and were the highest grade and purity available. All aqueous solutions were prepared with purified nanopure water [16], and solutions were sterile filtered or autoclaved prior to use. Bi-ligand [15] was provided by the laboratory of James R. Heath (California Institute of Technology). All proteins were acquired from the Critical Reagents Program (CRP).

2.2 Methods

ELISA (Figure 2) - 100 µL of 1 µM PA in PBS was added to each of 12 wells of a Black Maxisorp 96-well plate and agitated at 4°C overnight. The solution was removed and 300 µL of Pierce Protein-Free T20 was added to the wells. The plate was agitated for 6 h at 4°C. The wells were washed 3x with 10% Pierce Protein-Free T20 in PBS. 100 µL of serially diluted PA bi-ligand (concentrations ranging from 25 µM to 0.012 µM) in 10% Pierce Protein-Free T20 in PBS was added to each of the 12 wells and allowed to incubated overnight at 4°C with agitation. The wells were washed 3x with 10% Pierce Protein-Free T20 in PBS. 100 µL of a 1:10,000 dilution of Streptavidin Poly HRP in 10% Pierce Protein-Free T20 in PBS was added to each well and agitated for 1 h at 25°C. The wells were washed 4x with 10% Pierce Protein-Free T20 in PBS and then washed 4x with PBS. The wells were developed using QuantaRed Enhanced Chemifluorescent HRP Substrate, following the protocol provided in the kit. The plate was analyzed on a BioTek Synergy HT plate reader at 570 nm. Results were analyzed and fit by a four-parameter sigmoidal algorithm within SigmaPlot 12 analysis software.

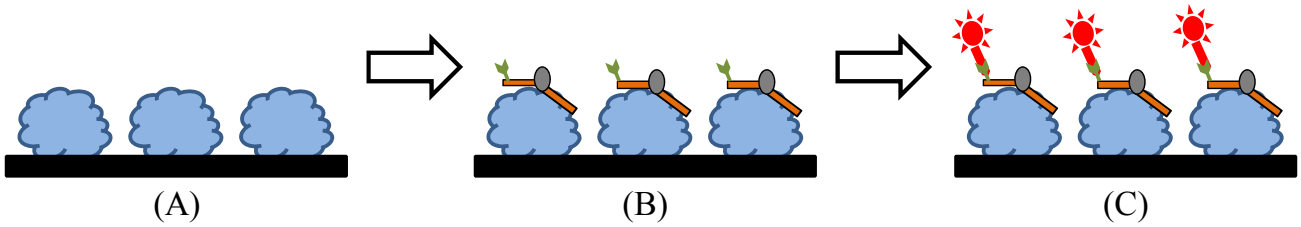

Figure 2. The indirect ELISA method used to probe the affinity of the bi-ligand to PA. (A) The PA protein is laid down in the well overnight followed by (B) incubation with the PA bi-ligand overnight and (C) incubation with streptavidin poly-HRP for 1 h. The poly-HRP substrate is developed using a QuantRed developer kit.

Kinetic Characterization - Using a Biacore T200, 23,000 RU of PA were immobilized to a Biacore CM5 sensor chip using standard amine coupling chemistry. A 1:2 serial dilution of the PA bi-ligand was injected across the immobilized surface and a reference cell at a flow rate of 75 µL/min for 90 seconds, with the highest concentration of bi-ligand being 1 µM and the lowest concentration being 31 nM. All concentrations in the dilution series were done in triplicate with a buffer blank control. Data was referenced to a blank reference channel and fit to a Langmuir 1:1 model using Biacore T200 Evaluation software. The affinity constant was calculated as 450 nM.

Cross-Reactivity Measurements - Specificity of bi-ligand was assessed using Surface Plasmon Resonance (SPR) with a Biacore T200. High levels of recombinant protein (PA, MS2CP, Rivax, F1, COM1, HA33A) were tethered to a Biacore Series S CM5 Sensor Chip (GE BR100530) using standard amine coupling chemistry Surface was primed using 1X HBS-EP running buffer (GE BR100188) for ten minutes at a flow rate of 10 µL/min. Surface was activated with EDC/NHS (GE BR100050) for seven minutes to immobilize protonated ligand, then quenched with an injection of 1.0 M Ethanolamine (GE BR100050). One flow cell was left blank, and used as a reference cell. Using a Manual Run in the Biacore T200 Control software, the sensor surface was stabilized with three subsequent injections of regeneration solution, 10 mM Glycine, pH 3.0 (GE BR100357) followed by a thirty second injection of running buffer. 1uM bi-ligand was injected across the sensor surface for thirty seconds at a flow rate of 30 µL/min. Injections were run in triplicate with each injection being followed by a regeneration and buffer injection to restore the surface.

3. RESULTS AND DISCUSSION

We will discuss the latest results and methods used to study the interactions between our bi-ligand and Protective Antigen. Additionally, the SPR techniques provide effective insight into the cross-reactivity of the bi-ligand with various other protein threats. These methods will be utilized collectively to study the characteristics of future PCC agents.

3.1 Binding Affinity

Two techniques, ELISA and SPR, were employed to elucidate the binding affinity of the bi-ligand to the PA protein target. The ELISA experiment was optimized to allow additional time for protein binding to the wells of the plate, as well as extending the incubation time with the peptide bi-ligand, in comparison to other common ELISA protocols [16]. We found that with increased incubation periods, we can achieve binding measurements for systems, such as short peptides, that can sometimes be problematic due their size and potentially low affinities. The additional use of streptavidin poly-HRP as our secondary, detection element in the ELISA experiment also affords a much higher development signal, providing appreciable separation between the highest and lowest intensities based on the concentrations of added bi-ligand. Figure 3A shows that with this experimental method, we determined the binding affinity for the bi-ligand to be Kd = 379 nM. While the dissociation constant is an order of magnitude or two higher than typical monoclonal antibodies, the superior stability attributes and high selectivity makes the PCC agents a much more viable option for fieldable sensing technologies regardless of the lower affinity.

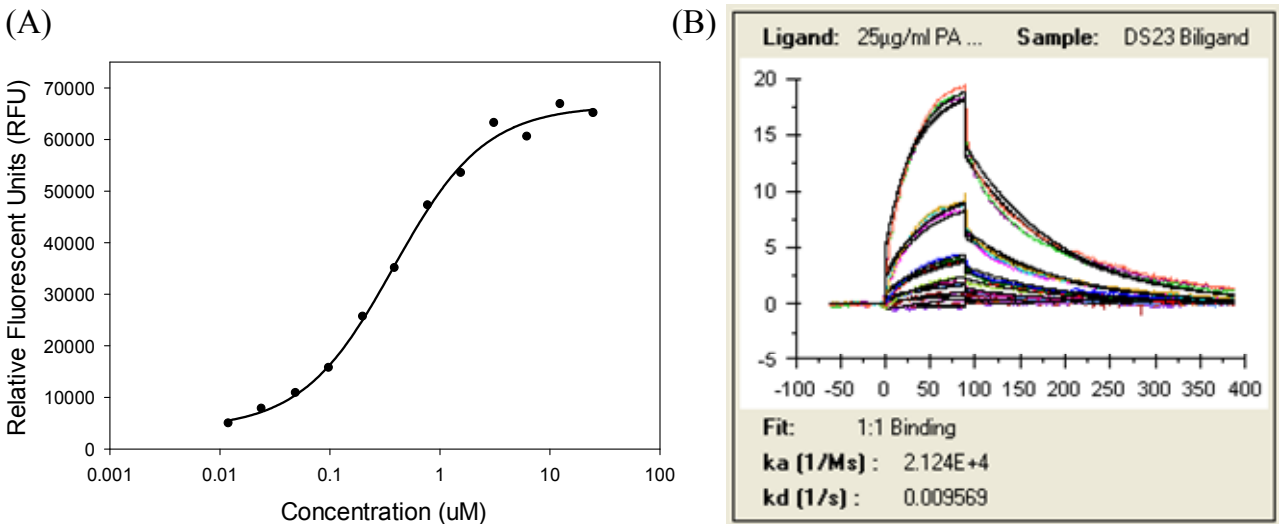

Figure 3. Affinity measurements for bi-ligand binding to PA protein. (A) ELISA results indicate a Kd = 379 nM and (B) SPR measurements provide a Kd of 450 nM.

The SPR experiments were performed in a similar fashion to the ELISA protocol by laying down the protein on the surface first, before incubating with the bi-ligand. The SPR determines the on/off rate of ligand to protein binding and requires less reagent amount for the measurements. Figure 3A shows the sensorgram from the SPR experiment determining a 450 nM dissociation constant for the bi-ligand. These results are very similar to those obtained by the more crude method of affinity determination, ELISA. Based on the results from both binding affinity experiments, we have the ability to rely on either method to ascertain an accurate binding affinity for PCC agents.

3.2 Protein Cross-Reactivity

Another powerful function of the SPR is the ability to perform cross-reactivity studies with the bioreceptor against multiple protein targets and determine its selective capacities. We chose multiple Department of Defense high priority protein targets to test with the bi-ligand in addition to PA, including MS2 coat protein (MS2CP), Rivax, the F1 surface protein of *Yersinia pestis*, HS33A of *Clostridium botulinum*, and the COM1 protein from the *Coxiella burnetii*. It is apparent, as shown in the sensorgram of Figure 4, that the bi-ligand successfully binds to PA. Table 1 shows that after running the bi-ligand against multiple targets, there is no binding to the other proteins of interest, suggesting the high selectivity of the PA PCC agent. Even though there is slight reactivity with COM1, 0.1 RU is negligible overall. It has been observed in previous experiments that the COM1 protein exhibits a heightened interaction with numerous molecules, so this reactivity could be a result of nonspecific binding. It is important to note that the RU value for PA

does not correlate to 5 times better affinity compared to the other proteins, but only that the bi-ligand exhibits binding to PA while the other proteins do not exhibit binding.

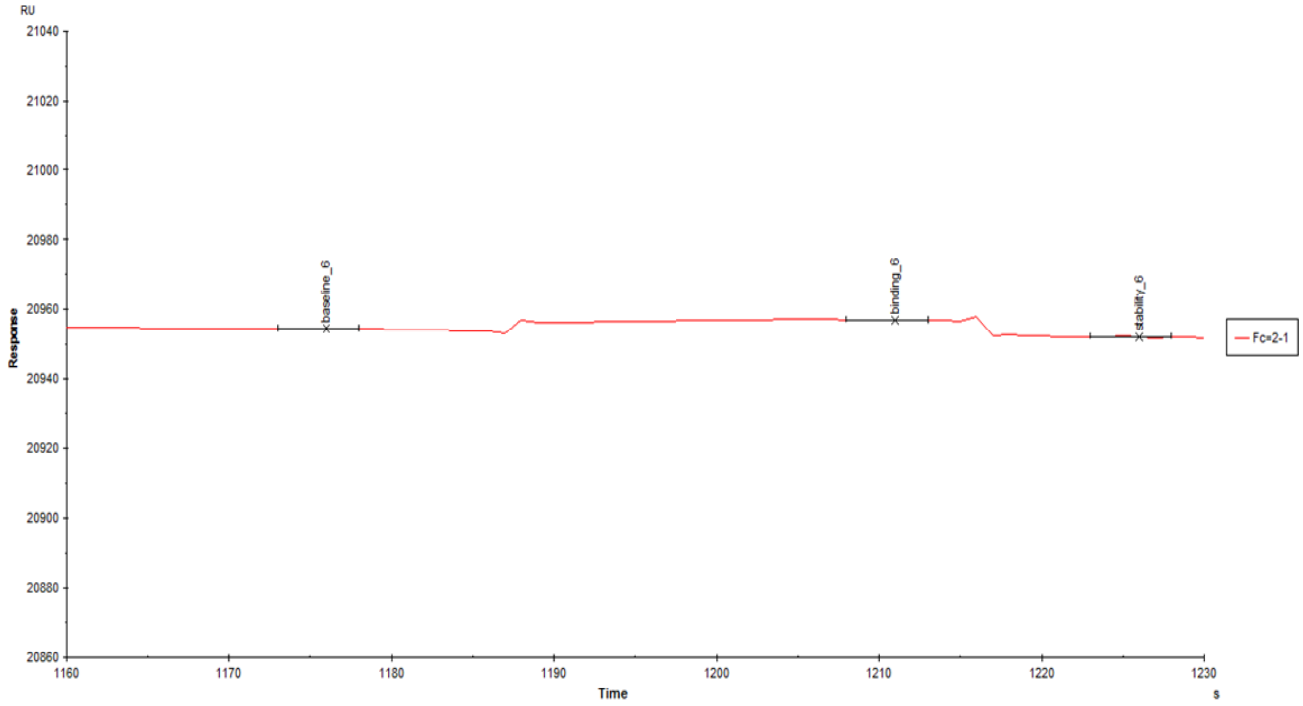

Figure 4. Sensorgram of bi-ligand binding to PA.

Table 1. SPR cross-reactivity results with various Department of Defense high priority targets. A positive R_{max} value indicates successful binding.

Antigen	Immobilization Conditions	R_{max} with 1µM Bi-ligand
Protective Antigen (B. anthracis)	pH 5.0, 20kRU	5RU
COM1 (C. burnetii)	pH 5.0, 9kRU	0.1RU
MS2CP (MS2 bacteriophage)	pH 5.5, 10kRU	0RU
Rivax (Ricin vaccine)	pH 4.5, 13kRU	0RU
F1 (Y. pestis)	pH 4.0, 10kRU	0RU
HA33A (C. botulinum)	pH 4.0, 4kRU	0RU

4. CONCLUSIONS

We have shown that the combination of both a bacterial display library screen and a PCC agent screen can create a strong binding and highly selective bioreceptor for the PA protein. The ELISA and SPR data conclude that the discovered bi-ligand is indeed a strong binder to PA, but unfortunately is still one or two magnitudes lower than other antibodies. However the fact that the PCC agent is highly selective to only PA and the stability of such reagents are much better than its monoclonal antibody predecessors makes it an ideal candidate for incorporation into a fieldable device that can be utilized in any condition.

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