Supporting Information for

A Chemically Synthesized Capture Agent Enables the Selective, Sensitive, and Robust Electrochemical Detection of Anthrax Protective Antigen

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Figure S1: Screening protocol and anchor ligand. (a-d), Detailed screening steps for identifying biligand capture agent candidates. The details of this protocol are provided in the Supplementary Methods. (b) Structure of anchor ligand used in the biligand screen. Bacterial display ligand with N-terminal propargyl glycine, C-terminal PEG-11 (EMD Millipore) and l-Biotin.
Table S1: Sequences obtained from the biligand screen. Positions with a strong consensus are shaded.
Figure S2: Immunoprecipitation specificity characterization of biligand screen hits. Each biligand candidate consists of the anchor peptide clicked to a 2° ligand for which the sequence is provided above the individual blot. DMSO is a control, and all biligands are tested in both buffer or 1% serum. The difference in signal between these two media provides a metric for selectivity. The blue spots are a Coomassie stained gel, and show consistent loading of BSA. The biligand candidates are used to precipitate the PA, while the commercial anti-PA antibody (US Biotech) is used for detection.

Figure S3: ELISA specificity characterization of the consensus biligand is shown. For this assay, off-target binding of the anchor and biligand capture agents to other pathogenic proteins is compared against detection of PA. PA – Bacillus anthracis protective antigen, LF – Bacillus anthracis lethal factor, L1r – Vaccinia (Cow pox) virus capsid protein L1.
Figure S4. Optimization results of nanostructured gold electrode, (A) calculation of the active surface area of the working electrode by integrating the current from cyclic voltammograms. (B) Measurement of surface area of the working electrode by AFM. For both plots the x-axis represents the electrochemical deposition time for building the nanostructured gold surface. For both measurement methods, a maximum surface area was observed for a deposition time of around 400 seconds.

<table>
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<th>Bare Electrode</th>
<th>Nanostructured electrode</th>
<th>Bare : Nanostructured Ratio</th>
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Table S2. Comparison of results of calculated active surface area by electrochemistry and average roughness and surface area by AFM.
Figure S5: Representative SEM images of bare gold electrode (A), nanostructured gold electrode (B), and AFM images of bare gold electrode (C), nanostructured gold electrode (D).
Figure S6: Optimization of the formation of the thiolated-ssDNA thin film on a nanostructured gold working electrode surface, as measured by the mean fluorescence intensity of the electrode area patterned with DNA. Fluorescence microscopy images before and after film formation with 200 µM of ssDNA are shown. For these measurements, the surface was incubated overnight with HS-ssDNA, the surface is blocked by mercaptohexanol (MCH), and complementary Cy3-fluorophore labeled cDNA was added to hybridize with the surface bound ssDNA. Optimized concentration of –SH ssDNA is 200 µM. Scale bar is 500µM.
Figure S7. Electrochemical assay for optimizing cDNA-streptavidin concentrations. Results were obtained with optimized 200 µM of –SH ssDNA, 1 µM PCC Agent, 100 nM PA and 200 nM of alkaline phosphatase anti-PA Ab (Ab-ALP, US Biotech). (A) Cyclic voltammograms were obtained, using a scan rate of 20 mV s$^{-1}$, in TBS containing 20 mM p-aminophenyl phosphate (APP) (pH 9.6) after 20 min incubation. (B) Curve of current value at 0.065 V corresponding to the oxidation of APP. Optimized concentration of cDNA-streptavidin is 500 nM.

Figure S8. Electrochemical assay for optimizing PCC agent concentration. Results were obtained with optimized 200 µM of –SH ssDNA, 500 nM of cDNA-streptavidin, 100 nM PA, and 200 nM of Ab-ALP. (A) Cyclic voltammograms were obtained (at a scan rate of 20 mV s$^{-1}$) in TBS containing 20 mM APP (pH 9.6) after 20 min incubation. (B) Curve of current value at 0.065 V from CV results. Optimized concentration of peptide is 500 nM.
Figure S9. Result of current values of different incubation time with respect to target concentration from 1 nM to 100 nM. Optimized incubation time is 20 minutes.
Figure S10. Time course detail of PCC Agent stability assay. A. Analytical HPLC traces showing the PCC agent peak is unchanged over 5 days of exposure at 65°C. B. MALDI-TOF spectra of untreated and 5-day thermally treated PCC agent from above. C. MALDI-TOF spectrum of small minority peak of doublet in 5-day sample. This mass spectrum is unchanged from those shown in B, suggesting an isobaric doublet.
METHODS

Peptide Syntheses:

Peptides were synthesized by standard Fmoc solid phase peptide synthesis protocols either manually in 8 mL solid phase reactors, or on a Titan 357 automated peptide synthesizer (AAPPTEC). Libraries were synthesized on 90 µm TentaGel S (NH2) (Rapp Polymere). C-terminal biotinylated peptides were synthesized on Biotin NovaTag resin (EMD Millipore). Side-chain protected peptides were synthesized on Sieber Amide Resin (Anaspec). C-terminal amide peptides were synthesized on Rink Amide MBHA resin (Anaspec). The natural Fmoc-L and unnatural Fmoc-D amino acids were purchased from AAPPTEC or Anaspec. PEG-11 and PEG-5 was purchased from EMD Millipore, and Fmoc-L-propargylglycine and Fmoc-L-Lys(N3) was purchased from Anaspec.

Briefly, resins were swelled in N-methyl pyrrolidone (NMP) and deprotected with 20% piperidine in NMP. 4 equivalents of Fmoc-amino acid (natural L- or unnatural D-, where appropriate), 3.9 equivalents of HATU, and 10 equivalents of DIEA were added (equivalents relative to the loading capacity of the resin). Regular couplings proceeded for 30-45 minutes. L-biotin (Sigma Aldrich) and PEG-x couplings proceeded overnight at 37°C. N-termini were acetylated with 20 equivalents of acetic anhydride and 15 equivalents of lutidine.

Biligand Synthesis:

For synthesizing biligand candidates and scaling up the chosen biligand PCC agent, the biotinylated secondary XXXXX-PEG(4)-Biotin was synthesized according to standard protocols on Biotin Novatag Resin (EMD) (for biligand PCC agent chosen, sequences is ELFHN, all D-amino acids). Following addition of an L-Lys(N3) residue, the resin was washed with NMP and set aside (Fmoc-Lys(N3)-ELFHN-PEG(4)-Biotin). In parallel the anchor ligand (Ac-YGLHPWWKNAPIGQR-CO\textsubscript{NH\textsubscript{2}}) was synthesized on sieber amide resin and cleaved from the resin with side-chain protecting groups intact. This was performed by adding 5mL of 2% trifluoroacetic acid in CH\textsubscript{2}Cl\textsubscript{2} and incubating for 2 minutes. The TFA was quenched by filtration into 250 µL DIEA. The cleavage
was repeated 10 times, the filtrates were combined, and the solvent removed by rotary evaporation. The protected secondary peptides was then purified by C18 RP-HPLC with a dH2O:CH3CN (0.1% TFA) gradient and confirmed by MALDI-TOF (m+H+=1917 Da)

The biligand variants were synthesized by combining 20 mg of resin-bound Fmoc-Lys(N3)-XXXXX-PEG(4)-Biotin with 1.5 equivalents protected anchor peptide (Ac-YGLHPWWKNAPIGQR-CONH2) in the presence of 1.5 equivalents CuI, 5 equivalents L-ascorbic acid, and 20% piperidine with a total volume of 750 µL. The reaction proceeded for 6 hours at room temperature followed by washing in NMP and copper chelation solution (22 mM sodium diethylthio carbamate (trihydrate), 29 mM DIEA, in DMF). The biligands were cleaved from the resin in 95:5:5 TFA:H2O:TES (triethylsilane) for 2 hours, precipitated in diethyl ether, and purified by C18 RP-HPLC with a dH2O:CH3CN (0.1% TFA) gradient and confirmed by MALDI-TOF (m+H+= 3320 Da).

Screening Protocols:

All screens used naïve one-bead-one-compound (OBOC) D-pentapeptide libraries on 90 µm polyethylene glycol-grafted polystyrene beads (TentaGel SNH2, 0.28 mmol/g, 2.86 x 10^6 beads/g) (TG) (Rapp Polymere) of the form NH2-Lys(N3)-XXXXX-TG, excluding cysteine and methionine. Libraries were synthesized on a Titan peptide synthesizer (AAPPTEC) using a split/mix method, and couplings were done using standard Fmoc SPPS chemistry in NMP.

Screening for a biligand against Bacillus anthracis PA was carried out in four main steps: pre-clear, target screen, anti-screen, and product screen. Screening against 3D6 was carried out in four main steps: pre-clear, target screen, anti-screen, and product screen. Step 1 preclear: 400 mg (approximately 1.1 x 10^6 beads) of OBOC library was blocked in 5% w/v dry milk in Tris buffered saline (TBS) (25 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl2, pH 7.6) for 2 hr at room temperature, and then incubated with 7.15 uM Anchor-1 peptide and 1:5000 Ms Anti-PA (US Biotech) in 0.5% w/v dry milk in TBS for 1 hr. Then the library was washed and incubated with 1:10,000 alkaline phosphatase-conjugated streptavidin (SA-AP, Promega) and 1:10 000 AP-conjugated Rabbit anti-Ms H&L (Abcam) in 0.5% w/v dry milk in TBS for 1 hr. The
beads were washed in high salt buffer (25 mM Tris-HCl, 750 mM NaCl, 10 mM MgCl₂, pH 7.6) for 1 hr, and then developed with BCIP/NBT (Promega) in BCIP buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, pH 9). After 30 minutes, the reaction was quenched with conc. HCl, and the purple beads were discarded. The remaining library was decolorized in NMP, dried with methanol (MeOH) and dichloromethane (DCM), and then swelled and blocked in 5% w/v dry milk in TBS for 2 hr at room temperature.

**Step 2 target screen**: 4 mL of a solution containing 7.15 µM Anchor-1 peptide and 700 nM PA in 0.5% w/v dry milk in TBS was added to the precleared OBOC library, and the *in situ* click reaction was allowed to proceed overnight at 4°C. Bound target was probed with 1:5,000 MS anti-PA in 0.5% w/v dry milk in TBS for 1 hr at room temperature followed by 1:10 000 AP-conjugated Rabbit anti-Ms H&L (Abcam) in 0.5% w/v dry milk in TBS for 1 hr. The beads were then washed in high salt buffer for 1 hr at room temperature, and developed with BCIP/NBT in BCIP buffer. The reaction was quenched after 30 minutes with conc. HCl, and the purple beads were retained and decolorized in NMP and dried with MeOH and DCM. The hit beads were then stripped of all bound protein by washing with 7.5 M Guanidine-HCl (pH 2) for 2 hr at room temperature, rinsed 10 times with water, and then blocked in 5% w/v dry milk in TBS for 2 hr at room temperature.

**Step 3 serum anti-screen**: A solution of 1% v/v human serum (Omega Scientific) in 0.5% w/v dry milk in TBS was incubated with the hit beads from Step 2 for 1 hr at room temperature. Any off-target serum proteins bound to the beads were probed with 1:50,000 rabbit pAb anti-whole human serum (Abcam) in 0.5% w/v dry milk in TBS for 1 hr at room temperature followed by 1:10 000 Goat anti Rb IgG pAb (Abcam) in 0.5% w/v dry milk in TBS for 1 hr at room temperature. The beads were then washed in high salt buffer for 1 hr, and developed with BCIP/NBT in BCIP buffer. The reaction was quenched after 30 minutes with conc. HCl, and the purple beads were discarded. The remaining beads were decolorized in NMP and dried with MeOH and DCM. The anti-screened beads were swelled in water and washed with Guanidine-HCl for 2 hr at room temperature, rinsed 10 times with water, and then blocked in 5% w/v dry milk in TBS for 2 hr at room temperature.
**Step 4 product screen:** The beads were incubated for 1 hr at room temperature with 1:10,000 SA-AP in 0.5% w/v dry milk in TBS, washed for 1 hr with high salt buffer, and then developed with BCIP/NBT in BCIP buffer for 30 minutes. The reaction was quenched with conc. HCl, and the purple beads were retained and sequenced using a Procise Protein Sequencing System (Applied Biosystems).

**PCC Agent binding ELISA assays out of Buffer**

Biotinylated anchor or biligand peptides were immobilized on streptavidin-coated 96-well plates (Thermo Scientific), at a concentration of 500nM in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the peptides were incubated with various concentrations of PA in 0.5% w/v dry milk in TBS at room temperature for 3 hours (100 nM PA for all single point ELISAs in buffer). Bound protein was probed with 1:10 000 Ms anti-PA mAb (US Biotech) as a primary, and 1:10 000 AP-conjugated Rb anti-Ms H&L (Abcam) as the secondary antibody both diluted in 0.5% w/v dry milk in TBS for 1 hr at room temperature. The colorimetric assay was developed with TMB substrate (KPL), then quenched with 1M H$_2$SO$_4$ and absorbance was recorded at 450 nm.

**PCC Agent competitive binding ELISAs out of diluted human serum**

Biotinylated anchor or biligand peptides were immobilized on streptavidin-coated 96-well plates (Thermo Scientific), at a concentration of 500nM in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the peptides were incubated with 100nM of PA in 0%, 1% and 5% human serum diluted into 0.5% w/v dry milk in TBS at room temperature for 4 hours. Bound PA protein was probed with 1:10 000 Ms anti-PA mAb (US Biotech) as a primary, and 1:10 000 AP-conjugated Rb anti-Ms H&L (Abcam) as the secondary antibody both diluted in 0.5% w/v dry milk in TBS for 1 hr at room temperature. The colorimetric assay was developed with TMB substrate (KPL), then quenched with 1M H$_2$SO$_4$ and absorbance was recorded at 450 nm.
Similar ELISA assays were performed with incubation of *Bacillus anthracis* lethal factor (LF), and Vaccinia L1 envelope protein as monitors of selectivity of the peptide ligands (Figure S3).

**Immunoprecipitation assays with PCC Agents out of diluted human serum**

Biotinylated PCC agents were immobilized on Streptavidin-agarose (Invitrogen) by adding 10 equivalents of PCC agent stock in DMSO to 50 µL of 50% v/v SA-agarose slurry after thorough washing and resuspension in 200 µL of TBS (equivalence is with respect to average loading of streptavidin monomers on agarose resin). The resin-bound PCC agent was added to a Spin-X filter unit (Sigma), and washed with TBS to remove unbound peptide. The resin was then blocked with 10 mg/mL BSA in TBS for 2-3 hours at room temperature to decrease non-specific binding to streptavidin-agarose.

To this was added diluted human serum (0%, 1%, 5%) in 1 mg/mL BSA in TBS doped with 150 nM of PA to a final volume of 100 µL. Binding occurred at 4°C for 12 hours with agitation. The resins were washed three times in 10 mg/mL BSA blocking buffer, three times with TBS, and three times with high-salt TBS. The bound protein was eluted by adding 50 µL 3x SDS-PAGE loading buffer and heated at 100°C for 10 minutes. A portion of each elution was run on duplicate AnyKD SDS-PAGE gels (Biorad). One gel was stained with Coomassie, while the other was transferred to nitrocellulose, blocked in 5% m/v dry milk, and probed overnight with Ms anti-PA antibody (US Biotech) followed by Rb anti-Ms pAb HRP secondary antibody (Abcam). The primary antibody was used at 1:1000 dilution, while the secondary was at 1:10 000, both in 0.5% m/v dry milk. The blots were developed with Pico West Dura ECL substrate (Thermo Scientific) and imaged with film.