

Electrochemical Aptamer Scaffold Biosensors for Detection of Botulism and Ricin Proteins

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Abstract

Electrochemical DNA (E-DNA) biosensors enable the detection and quantification of a variety of molecular targets, including oligonucleotides, small molecules, heavy metals, antibodies, and proteins. Here we describe the design, electrode preparation and sensor attachment, and voltammetry conditions needed to generate and perform measurements using E-DNA biosensors against two protein targets, the biological toxins ricin and botulinum neurotoxin. This method can be applied to generate E-DNA biosensors for the detection of many other protein targets, with potential advantages over other systems including sensitive detection limits typically in the nanomolar range, real-time monitoring, and reusable biosensors.

Key words Biosensors, Toxins, Electrochemical, Aptamer, Botulism, Ricin, Voltammetry, E-DNA, Gold electrodes, Proteins

1 Introduction

Accurate and rapid detection of biomarkers is useful in many applications, ranging from food safety [1] and environmental sampling to medical diagnostics [2] and small-molecule drug discovery. Biosensors, which are devices that incorporate biological interactions as the basis of their sensing mechanisms [3], are uniquely suited to overcoming challenges associated with detecting a specific biomolecule in dense, complex, biological liquid matrices [4] (e.g., whole blood or river water samples). In addition, biosensors have several other appealing features that allow them to be used successfully in unique and challenging situations, including high specificity of detection, high reproducibility, relative ease of manufacturing and affordability, rapid throughput, direct readout, and minimal invasiveness.

One prominent and successful class of biosensors is electrochemical DNA (E-DNA) biosensors [5]. E-DNA biosensors rely on the changing conformational dynamics of a synthetic

deoxyoligonucleotide (DNA) scaffold containing an aptamer or transcription factor-binding motif that recognizes the target biomolecule [6–9] (Fig. 1). The DNA scaffold is modified with functional groups to enable attachment to an electrode surface (typically through a thiol-gold bond) and to an electrochemically active reporter molecule (e.g., methylene blue) [10, 11]. When the biosensor is subjected to voltammetric analysis, the scaffold conformation changes depending on whether or not it is bound to its target biomolecule, and this affects the dynamics and the position (and thus observed current) of the electrochemically active reporter molecule relative to the electrode surface [12] (Fig. 1). This principle enables E-DNA biosensors to effectively function in complex matrices [4], including real-time monitoring in animal blood [2], and be successfully used against a range of targets, including oligonucleotides [6], small-molecule drugs [13], heavy metals [14], antibodies [15], DNA-binding proteins [16], and protein toxins [9].

In theory, E-DNA biosensors can be designed to detect any molecule for which oligonucleotide-binding interactions are known or discoverable (such as via systematic evolution of ligands by exponential enrichment [SELEX]). Recently, our group generated E-DNA biosensors for the detection of protein toxins responsible for ricin and botulism toxicity [9]. Here we describe the design and use of novel E-DNA biosensors against these and similar targets. The biosensors described here can detect nanomolar concentrations of ricin chain A and botulinum neurotoxin variant A (Fig. 2), with high specificity and negligible off-target signals, and function when challenged with complex matrices such as blood serum albumin or other proteins (Fig. 3).

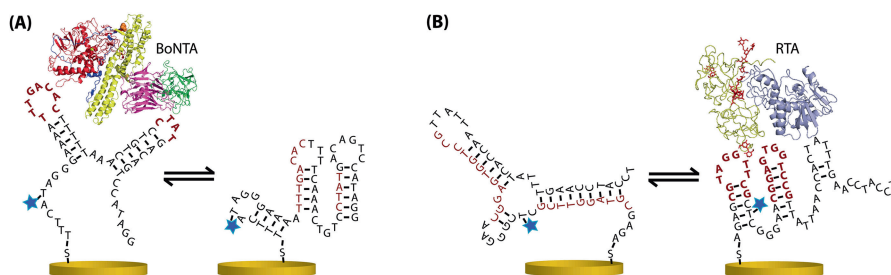


Fig. 1 Schematic of E-DNA biosensor, illustrating the change in position and dynamics of the reporter molecule (methylene blue, represented by a *blue star*) attached to the DNA scaffold in response to binding of the biomolecule target. Shown are biosensors directed toward the biomolecular targets (a) botulinum neurotoxin variant A (BoNTA) and (b) ricin toxin chain A (RTA). The gold electrode surface (*yellow disc*) is passivated with a monolayer of 6-mercapto-1-hexanol (not shown) to prevent nonspecific binding of biomolecules (Reproduced from ref. [9] with permission of the Royal Society of Chemistry)

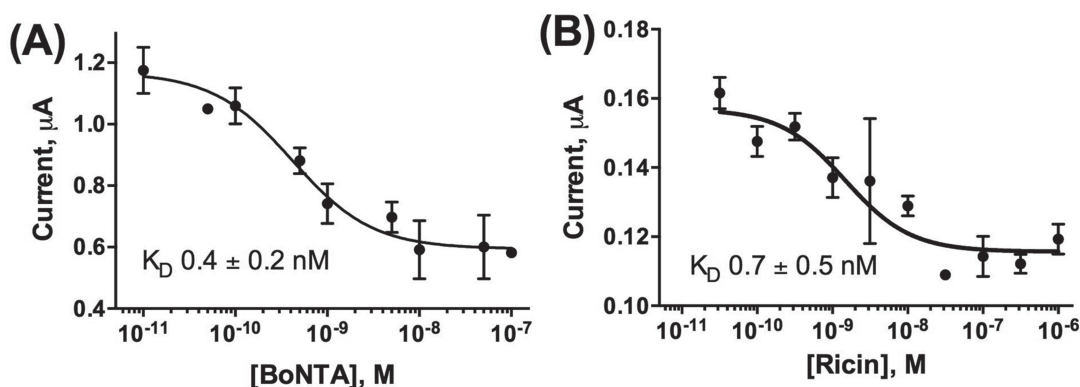


Fig. 2 Representative dose-responsive curves of peak current vs. toxin concentration for botulinum neurotoxin variant A (BoNTA, **a**) and ricin toxin chain A (RTA, **b**). Both E-DNA biosensors display robust equilibrium signal change in response to target concentration, with apparent dissociation constant (K_D) values of 0.4 ± 0.2 nM for BoNTA and 0.7 ± 0.5 nM for RTA (Reproduced from ref. [9] with permission of the Royal Society of Chemistry)

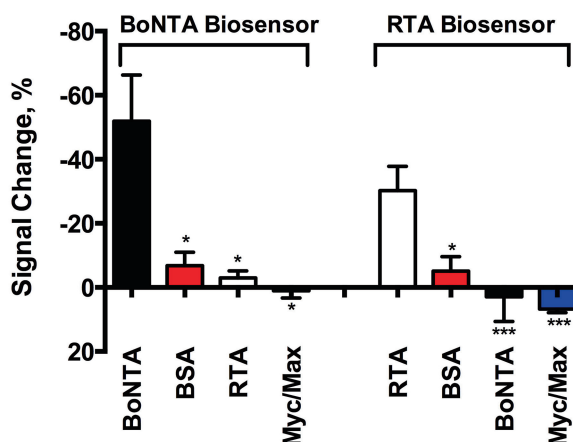


Fig. 3 The botulinum (BoNTA) and ricin (RTA) biosensors display minimal off-target responses when challenged with off-target proteins, including bovine serum albumin (BSA) and other biomolecular targets, such as the unrelated DNA-binding protein complex Myc/Max. Student's *t*-test was performed to compare on-target to off-target response (* for $p < 0.05$, *** for $p < 0.0001$) (Reproduced from ref. [9] with permission of the Royal Society of Chemistry)

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Biosensor Design and Synthesis

1. Biosensor DNA: Synthetic DNA scaffold with 5' terminal disulfide (e.g., 5'' thio C6 modifier/trityl-6-thiohexyl amidite) and internal thymidine-methylene blue to be used as an electrochemically active reporter molecule (methylene blue succinimidyl ester coupled to amino modifier C6 T amidite/5''-DMT-T[acrylamido-C6-NH-TFA]) (*see Note 1*). Resuspend DNA in ultrapure water at 100 μ M. Store aliquoted at -20°C wrapped in aluminum foil. The ricin biosensor sequence used here is 5'- AGAG CGT AGG TTC G C[T(Methylene Blue)]C GGG AA CGG AGT GGT CCG TTATTA ACC ACT ATTT GAA CCT ACC -3', and the botulinum toxin biosensor sequence is 5'- TTT CA[T(Methylene Blue)] AGG GA AA ATTTGACACT TT TCAAAC T GTCCTATGAC A GTCCA TAGG -3' [9].
2. Quickfold application from the DINAMelt web server, hosted by the RNA Institute at the State University of New York at Albany [17], available at <http://unafold.rna.albany.edu/?q=DINAMelt/Quickfold> (*see Note 2*).
3. OPTIONAL: Fealden DNA biosensor algorithm [8], available at http://www.bonhamlab.com/wp-content/uploads/2016/05/Fealden-0.2_04232016.zip (*see Note 3*).
4. PCR tubes: 0.5 mL flat-cap PCR tubes, RNase- and DNase-free, polypropylene.

2.2 Electrode Preparation

1. Pine Research Instrumentation WaveNano USB Potentiostat (*see Note 4*).
2. Pine Research Instrumentation Compact Voltammetry Cell Grip Mount.
3. Pine Research Instrumentation WaveNano Shielded Cell Cable.
4. Pine Research Instrumentation Compact Voltammetry Cable.
5. Pine Research Instrumentation Ceramic Patterned Gold Electrode.
6. Pine Research Instrumentation AfterMath Scientific Data Organizer Software.
7. Alkaline cleaning solution: 0.5 M NaOH.
8. Acid cleaning solution: 0.5 M H_2SO_4 .
9. Etch solution: 0.1 M H_2SO_4 , 0.01 M KCl.
10. Evaluation solution: 0.05 M H_2SO_4 .

2.3 Biosensor Attachment and Surface Passivation

1. TCEP solution: 1 M Tris(2-carboxyethyl)phosphine hydrochloride. Store aliquoted at -20°C .
2. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 , and pH adjusted to 7.4 with HCl, adjusted to 1 L with ultrapure water.

3. Mercaptohexanol solution: 0.001 M 6-mercapto-1-hexanol in PBS. Prepare and work with solution in a chemical fume hood. Store at 4 °C for up to 1 month.
4. PCR tubes: 0.5 mL flat-cap PCR tubes, RNase- and DNase-free, polypropylene.
5. Petri dish: 100 mm × 15 mm, polystyrene.

2.4 Botulism and Ricin Protein Preparation

1. PBS: *see* Subheading 2.3.
2. Ricin solution: Ricin A chain from *Ricinus communis* (castor-bean or castor-oil-plant, from Sigma-Aldrich). Resuspend at 1 mg/mL in PBS. Store aliquoted at 4 °C.
3. Botulism solution: Botulinum neurotoxin variant A1 atoxic derivative [18]. Resuspend at 1 mg/mL in PBS. Store aliquoted at −80 °C.

2.5 Electrochemical Biosensing Experiment

1. PBS: *see* Subheading 2.3.
2. Target biomolecule solution: *see* Subheading 2.4.
3. Prepared electrode: *see* Subheading 2.3.
4. AnyPeakFinder software program (source code available at <http://www.bonhamlab.com/tools/code/>) or AfterMath program with built-in peak height analysis functions (*see* **Note 5**).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Sensor Design and Synthesis

1. Identify a DNA-binding motif that recognizes your biomolecule target of interest. We have used previously identified transcription factor binding sites [16] or aptamers [2, 9] or aptamers that we identified in-house [9].
2. Identify regions of the motif that are presumed to be “essential” for target binding interactions (Fig. 4). For aptamers, detailed mechanistic binding studies are often available in the literature; the regions of interest will typically be predicted to form “loops” in their secondary structure. Confirmation via Quickfold may be useful.
3. Design a synthetic DNA scaffold that incorporates the motif region(s) identified to be essential for target binding interactions and allows for potential disruption of these binding interactions. To do this, design the essential regions to be flanked on either or both of its 5′ and 3′ ends with deoxyoligonucleotides that are partially complementary to the essential regions, facilitating the formation of secondary structures

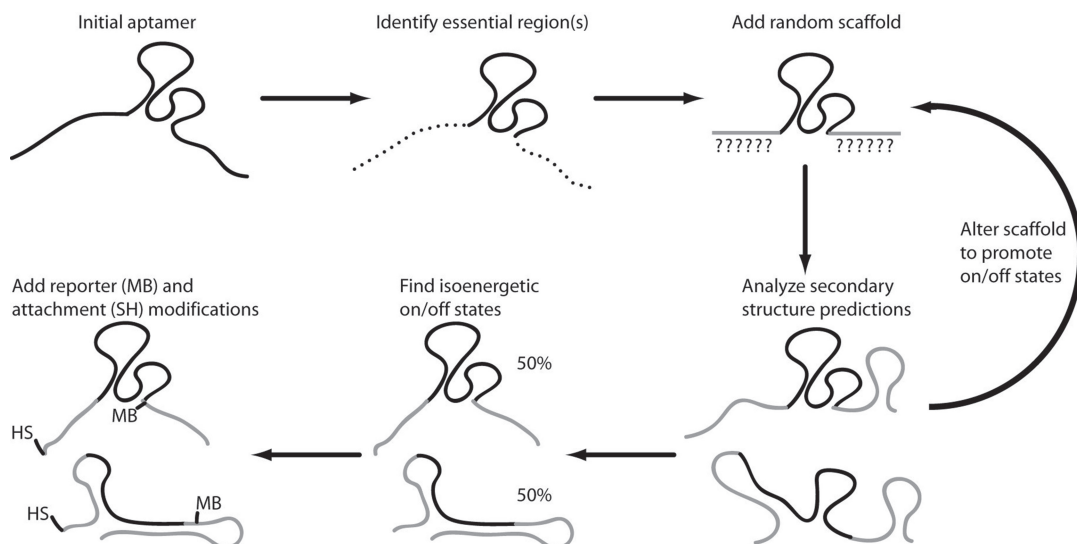


Fig. 4 Schematic of biosensor design workflow process. An initial aptamer is truncated to essential regions and then flanked by a random scaffold of novel oligonucleotides. Secondary structure predictions are used to guide changes to the scaffold sequence to promote the formation of isoenergetic states that either present or obscure the aptamer essential regions. The addition of a reporter molecule (e.g., methylene blue, “MB”) and surface attachment modifications (i.e., thiol-gold bond, “HS”) leads to a completed biosensor design

or folding patterns that likely disrupt target binding. Multiple rounds of confirmation via Quickfold or other secondary structure prediction services may be useful (*see Note 6*).

4. Continue designing the scaffold by iteratively adding, removing, or changing oligonucleotides in the nonessential regions to ultimately create a scaffold with two potential, equally favorable (i.e., isoenergetic) states: one state in which the essential regions are available for target binding interactions (i.e., in their native form) and one in which the essential regions are unavailable due to being base paired with nonessential regions (Fig. 4) [8]. The Fealden DNA biosensor algorithm is a designing tool that may be used to help automate this process.
5. Once a scaffold has been designed with the two desirable isoenergetic states, modify the scaffold design to include an electrode attachment point. A thiol group located at the 5' terminus of the entire scaffold should serve as the attachment point by forming a thiol-gold bond between the scaffold and the gold electrode surface.
6. Further modify the scaffold design to include an electrochemically active reporter molecule; here a methylene blue is used. The methylene blue can be easily covalently appended to a modified thymine. Examine the scaffold's two isoenergetic states to identify a thymine that is nonessential in a significantly different folded environment and has significant distance

change from the 5' terminus between the two folded states (Fig. 1). Again, Fealden may be used to help automate this selection process (*see* **Note 6**).

7. Synthesize the designed scaffold using a DNA synthesis company or in-house phosphoramidite deoxyoligonucleotide synthesis.
8. Resuspend the DNA in ultrapure water upon receipt at a concentration of 100 μM , aliquot it into PCR tubes (typically 4 μL per tube), and store aliquots at $-20\text{ }^{\circ}\text{C}$.

3.2 Electrode Preparation

1. Connect the WaveNano USB Potentiostat to a computer via a USB cable.
2. Connect the Compact Voltammetry Cell Grip Mount to the potentiostat using the WaveNano Shielded Cell Cable and Compact Voltammetry Cable, being sure that the alligator clips of the Shielded Cell Cable do not touch each other.
3. Place the Ceramic Patterned Gold Electrode face up in the grip mount, and add a plastic adaptor spacer (included with electrode) at the bottom of the grip mount to ensure solid contact between the grip mount and electrode. Ensure that the black ground electrode of the Shielded Cell Cable is connected to outlet ground (*see* Fig. 5).
4. Power on the potentiostat and ensure that the status light is green.
5. Open and log in to the AfterMath Scientific Data Organizer Software. Ensure that the WaveNano Potentiostat is recognized

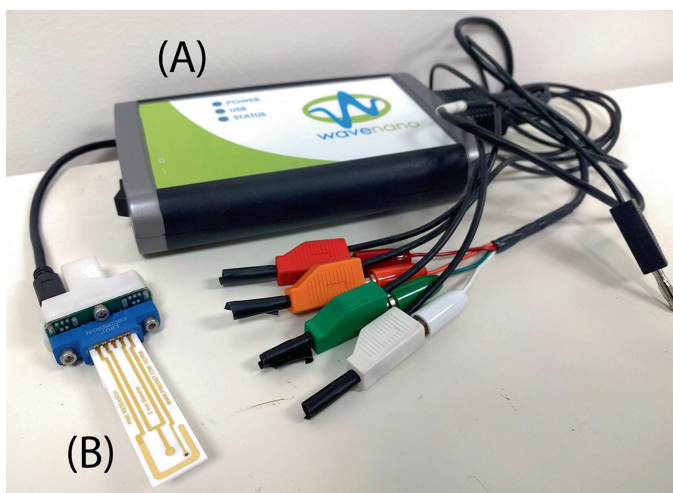


Fig. 5 Image of Pine Research Instrumentation (a) WaveNano instrument with correct cables and (b) ceramic-patterned electrode with exposed gold electrode surfaces. The biosensor attaches to the central, circular gold electrode

and communicating with AfterMath; the potentiostat's status should be listed as "idle" (see AfterMath support site for guidance; <http://wiki.voltammetry.net/pine/aftermath>).

6. Insert the electrode into a 30 mL beaker, and add 15 mL of alkaline cleaning solution, ensuring that the exposed gold surfaces of the electrode are submerged and the grip mount and contacts on the electrode remain dry.
7. Create and run a new cyclic voltammetry experiment to perform 100 scans from -0.4 V to -1.35 V at a sweep rate of 2 V/s. This will reductively desorb any sulfur-linked molecules on the electrode surface.
8. Remove the electrode from the alkaline cleaning solution, rinse with ultrapure water, and repeat **step 6** using 15 mL of acid cleaning solution (instead of alkaline cleaning solution).
9. Create and run a new bulk electrolysis experiment to perform oxidation using 2 V applied for 5 s followed by reduction using -0.35 V applied for 10 s. This will oxidize any organic contaminants and then reduce any gold oxide formed.
10. Create and run a new cyclic voltammetry experiment to perform cyclic oxidation and reduction voltammetric scans, performing 20 scans with a scan rate of 4 V/s, followed by a further 4 scans at 0.1 V/s, from 0.35 V to 1.5 V. This step will sequentially oxidize and then reduce any remaining contaminants on the electrode surface.
11. Remove the electrode from the acid cleaning solution, rinse with ultrapure water, and repeat **step 6** using 15 mL of etch solution (instead of alkaline cleaning solution).
12. Create a new cyclic voltammetry experiment, and perform scans over four different potential ranges, each for ten scans at scan rate of 0.1 V/s: 0.2–0.75 V, 0.2–1.0 V, 0.2–1.25 V, and 0.2–1.5 V. This will etch away the surface layer of the electrode as gold chloride complexes, resulting in a substantially cleaned surface.
13. Remove the electrode from the etch solution, rinse with ultrapure water, and repeat **step 6** using 15 mL of evaluation solution (instead of alkaline cleaning solution).
14. Create a new cyclic voltammetry experiment, and perform four scans from -0.35 V to 1.5 V at a scan rate of 0.1 V/s. This will oxidize a gold oxide layer on the electrode and then completely reduce it. The area under the reduction peak can be used to calculate the available surface area of the electrode [10, 19].
15. Store the cleaned electrode submerged in evaluation solution for up to 1 h before proceeding with using it in Subheading 3.3.

3.3 Biosensor Attachment and Surface Passivation

1. While performing the electrode preparation protocol (Subheading 3.2), thaw aliquots of sensor DNA (prepared in Subheading 3.1) and TCEP solution at room temperature. Avoid exposing the sensor DNA to light.
2. To a clean PCR tube, add 3 μL sensor DNA and 3 μL TCEP solution. Allow the sensor DNA/TCEP mixture to react for at least 15 min, until it has changed from light blue to clear in color [10] (*see Note 7*).
3. Mix 44 μL of PBS with the sensor DNA/TCEP mixture.
4. Remove the electrode from the evaluation solution (from **step 15** of Subheading 3.3) and rinse it with ultrapure water. Using a clean, delicate task wiper (e.g., a Kimwipe), dry the electrode by wicking it dry, touching only the ceramic portions of the electrode and taking care not to touch the exposed gold surfaces.
5. Add the entire 50 μL of sensor DNA/TCEP/PBS mixture to the electrode's surface, being careful to cover the entire exposed gold surface. Place the electrode inside a closed petri dish for 60 min, which will minimize evaporation and allow the reaction to proceed. In arid climates, we have found it is important to also add 250 μL of PBS to the bottom of the petri dish and rest the electrode on top of a small, upside-down weigh boat in the dish to help prevent premature drying (*see Note 8*).
6. Using a delicate task wiper, dry the electrode as described in **step 4**. Immediately proceed with the next step to prevent the electrode from completely drying.
7. Add 100 μL of mercaptohexanol solution [20] to the electrode, being careful to cover the entire exposed gold surface. Place the electrode inside a petri dish. Allow the reaction to proceed for 1–24 h at 4 $^{\circ}\text{C}$ (*see Note 9*).
8. Equilibrate the prepared biosensor in PBS for at least 20 min before use (in Subheading 3.5). This can be accomplished by repeating **step 6** of Subheading 3.2 using 15 mL of PBS (instead of alkaline cleaning solution).

3.4 Botulism and Ricin Protein Preparation

1. Remove aliquoted protein solution (either ricin or botulinum solution, depending on desired target biomolecule) from storage and place on ice.
2. In microcentrifuge tubes, prepare a total of approximately ten serial dilutions of the protein solution in PBS, each dilution containing 100 μL , ranging from 0.01 nM to 1 μM . Keep the dilutions on ice and use them within 2 h.

3.5 Electrochemical Biosensing Experiment

1. Following PBS equilibration at the end of Subheading 3.3, remove the electrode from the PBS. Using a delicate task wiper, dry the electrode as described in **step 4** of Subheading 3.3.

2. Place the grip mount and electrode in a horizontal position with the exposed gold surfaces facing up.
3. Cover the electrode's exposed gold surfaces with 100 μL of PBS, being careful to cover the working, counter, and reference elements of the electrode. Allow the electrode to equilibrate for at least 10 min. *Note:* Instead of PBS, bovine blood serum or whole bovine blood may alternatively be used (*see* **Note 10**).
4. Ensure that the Compact Voltammetry Cable and Shielded Cell Cable are correctly attached to the grip mount and that the potentiostat's status in the AfterMath software is shown as "idle."
5. Create a new square wave voltammetry experiment in the AfterMath program, with voltage ranging from -0.5 to 0.1 V, an amplitude of 50 mV, and a step size of 1 mV. The optimal square wave frequency should be experimentally derived as it can dramatically affect the signaling of the biosensor [21]; 100 Hz is a typically useful frequency for a wide variety of biosensors.
6. Run the experiment; a rounded peak in current at approximately -0.3 V should be present, due to the redox potential of the methylene blue modification (*see* Fig. 6). The peak height is proportional to the effective efficiency of electron transfer between the surface and the methylene blue modification [22] (*see* **Note 11**).
7. Rinse electrode with ultrapure water. Using a delicate task wiper, dry the electrode as described in **step 4** of Subheading 3.3.
8. Cover the electrode's exposed gold surface with 100 μL of 0.01 nM ricin or botulinum solution (prepared in Subheading 3.4), being sure to cover the working, counter, and reference

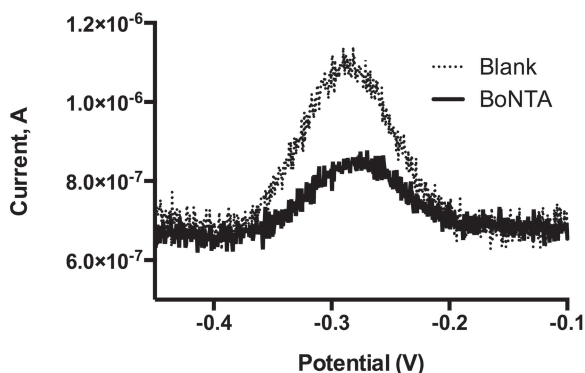


Fig. 6 Square wave voltammograms of BoNTA biosensor equilibrated in PBS with 100 nM BoNTA (BoNTA) or with PBS only (Blank) (Reproduced from ref. [9] with permission of the Royal Society of Chemistry)

elements of the electrode. Allow the electrode to equilibrate for at least 10 min.

9. Create a new square wave voltammetry experiment from -0.5 to 0.1 V with an amplitude of 50 mV and a step size of 1 mV using the experimentally determined optimal frequency.
10. Run the experiment; the methylene blue-derived peak in current at approximately -0.3 V should remain present. The magnitude of any change in peak height reflects changes in biosensor signaling due to the presence of the target biomolecule.
11. Repeat **steps 7–10** using the other prepared serial dilutions (prepared in Subheading 3.4), using them in order of increasing concentration. For each dilution, measure the peak in current using AfterMath's peak height tool, or export the data as a comma-separated value (csv) file format, and use the AnyPeakFinder program to determine the peak heights.
12. Using the peak current observed in **step 6** as the baseline current, calculate the relative change in current for each dilution as a percentage increase or decrease in signal. For example, for each dilution, the baseline peak height could be subtracted from the dilution's peak height, and this value could be divided by the baseline peak height to calculate the percentage change.
13. Use the resulting data to construct a saturation binding curve, allowing visualization of the apparent dissociation constant for the target.
14. Following establishment of the target concentration dependent response, this section's procedure can be repeated using samples of unknown protein concentration to allow for quantification of the protein concentration in solution, enabling biosensing applications.

4 Notes

1. DNA synthesis is performed by standard phosphoramidite coupling on a solid support, which is available from many companies, such as Biosearch Technologies or Integrated DNA Technologies. Briefly, a $5'$ dimethoxytrityl (DMT)-protected deoxynucleotide phosphoramidite is attached to a controlled pore glass support through the $3'$ hydroxyl. Acid treatment is then used to remove DMT, followed by coupling to the next deoxynucleotide phosphoramidite, protective acetylation, and oxidation and then a repeated cycle of deprotection and coupling. Modified deoxynucleotide phosphoramidites can be easily included in this synthesis process.

2. The online Quickfold module is convenient, but there are several other tools available that predict DNA secondary structure folding, and any of these other tools should, in principle, be sufficient for the necessary analysis. Examples include RNAstructure from the University of Rochester Medical Center (<http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html>) and Integrated DNA Technologies' OligoAnalyzer Hairpin module (<https://www.idtdna.com/calc/analyzer>).
3. The Fealden software significantly automates the task of evaluating predicted DNA secondary structures for correct biosensor conformational states, but it is optional and may require Python programming experience to customize it for new applications. Fealden requires a UNIX-like environment and has been confirmed to work on Ubuntu Linux and Mac OSX.
4. Potentiostats and analysis software are available from several vendors; here we use Pine Research Instrumentation. Other vendors that could provide suitable instrumentation packages include CH Instruments, Inc., and Metrohm Autolab Nova.
5. Analysis of square wave voltammetric data requires accurately measuring the height of observed current peaks (when plotting current vs. voltage). AfterMath software includes a manual tool for this measurement, and as the data can be exported in csv format, a variety of computational tools can be used to identify and measure current peaks, including Mathematica and Matlab. Our lab provides source code for AnyPeakFinder, a Python program that can automatically read csv formats and extract peak height values; see <http://www.bonhamlab.com/tools/code/any-peak-finder-interactive/>.
6. The core principles of selecting correct regions and optimizing folded structures have been explored in a number of studies [7–9, 23–26] and demonstrate that an iterative, trial-and-error approach can often yield good results. Generally, structures with predicted free energies within 1 kJ/mol are more likely to be meaningful. Minimizing the number of predicted states helps avoid inconsistent results.
7. For sensor DNA solutions, the DNA sensor concentration is known to affect biosensor performance [12, 27]. The added TCEP solution must be sufficient to fully reduce the disulfide modification present in the sensor DNA solution, and consequently in this protocol the amount of TCEP solution added is in high excess. The observed color change is due to reversible reduction of the methylene blue modification. Although this change has no impact on the final performance of the biosensor, it is a convenient marker for the progress of reduction of the solution.

8. This process allows the sensor DNA to attach to the surface in an incomplete monolayer, with average spacing between molecules that minimizes or eliminates interactions between neighboring sensors, which is important for reproducible performance. Optimizations of this surface packing have been previously explored [12, 28].
9. The mercaptohexanol solution addition acts to form a stable, mixed surface monolayer with the attached sensor DNA. While 6-mercapto-1-hexanol is the most common of these “passivation” chemicals, our lab has additionally found success with the use of (11-mercaptopoundecyl)tetra(ethylene glycol), which presents a more biocompatible monolayer for studies in complex matrices. The monolayer formed prevents nonspecific interactions of the biomolecule target with the electrode’s gold surface and provides a more reproducible current response. Typically, 100 μL is added to the electrode surface and allowed to adhere for 1 h. Using larger volumes, such as 200 μL , followed by sealing the electrode in a petri dish and storing it overnight at 4 $^{\circ}\text{C}$, has also been successful. Our lab has also attempted to briefly wash the mercaptohexanol-passivated electrodes with saline sodium citrate (SSC) buffer or 1% bovine serum albumin (BSA) buffer with minimal successes. This was performed by allowing the mercaptohexanol to adhere to the electrode overnight, then removing it with a pipette, and adding 50 μL of either the SSC or BSA. The solution was allowed to sit for 10 min before beginning trials.
10. To serve as a test bed for complex matrices uses of these sensors, we have employed both adult bovine serum and bovine whole blood (citrate stabilized) in place of PBS. In both matrices, sensors still performed well, although the magnitude of current changes is often reduced.
11. The precise voltage where the peak in current is found for methylene blue will vary based on solution conditions (e.g., pH and ionic content). Different reporter dyes will have a different characteristic voltage for peak current.

Acknowledgment

This work would not be possible without ideas from Kevin Plaxco, University of California Santa Barbara, and Ryan White, University of Maryland Baltimore County. Support for this work was provided by the Metropolitan State University of Denver’s College of Letters, Arts, and Sciences Dean’s office, Provost’s office, and the Applied Learning Center.

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Microbial Toxins

Methods and Protocols

Holst, O. (Ed.)

2017, XI, 201 p. 71 illus., 28 illus. in color., Hardcover

ISBN: 978-1-4939-6956-2

A product of Humana Press