Chapter 16

Synthesis of a Virus Electrode for Measurement of Prostate Specific Membrane Antigen

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Summary

Though relatively unexploited in biosensor applications, phage display technology can provide versatile recognition scaffolds for detection of cancer markers and other analytes. This chapter details protocols for covalent attachment of viruses directly to electrodes for reagent-free detection of analytes in real-time. Customization of binding specificity leverages selections with large phage display libraries prior to covalent attachment of the selected virus to the electrode. The methods described here utilize electrochemical impedance spectroscopy (EIS) to detect molecular recognition between M13 phage bound to a Au electrode and the following analytes: prostate specific membrane antigen (PSMA), positive and negative control antibodies (p-Ab and n-Ab, respectively). Because of a thick layer built on the Au electrode, the real impedance (Z_{re}) increases reliably with S/N ratios upon noncovalent binding to PSMA (~14) and p-Ab (~20).

Key words: Biosensor, Bacteriophage, Surface modification, Electrochemical impedance, Electrode.

1. Introduction

Since its invention in 1985 *(1)*, phage display has found widespread applications in diverse fields ranging from biochemistry to materials science *(2)*. For example, the discovery and engineering of binding motifs has been revolutionized by phage and other molecular display technologies *(3–5)*. The display of vast libraries on the phage surface *(6–8)* is now routine, and current diversities approach ~10^{12} unique variants *(2, 9)*. Multiple rounds of in vitro binding to an immobilized target can tailor the specificity and affinity of the displayed protein. The technique directly links the

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DNA sequence encapsulated by the phage (the genotype) and the polypeptide displayed on the surface (the phenotype).

The enormous diversities afforded by phage-displayed libraries allow selection for binding to essentially any molecular target. However, phage display applications in biosensors remain incompletely explored. For example, previous research has focused upon using phage-displayed proteins as the equivalent of large antibodies to trigger a detection event *(10–15)*. This approach requires use of phage as added reagents. Reagent-free detection requires direct attachment of the phage to the sensor for interrogation of the analyte solution. In theory, real-time sensing can take place, if binding to the phage-coated sensor generates an electrical signal for measurement. We have recently constructed a biosensor through the covalent attachment of phage onto the surface of a gold electrode, and demonstrated direct electronic recognition of the prostate cancer marker, prostate specific membrane antigen (PSMA) *(16)*, a positive antibody (p-Ab) known to bind M13 phage, but not a negative antibody (n-Ab) that fails to bind the phage.

The virus electrode technique described above requires electrochemical impedance spectroscopy (EIS), a reagent and label-free, real-time measurement, with fast response and low cost. Previous applications of EIS include sensors based upon antibodies *(17, 18)*, enzymes *(19, 20)*, and DNA *(21, 22)*. Several approaches can couple an EIS measurement with detection of analytes. First, amplification can leverage a redox probe such as $[Fe(CN)_6]^{3-/4-}$ *(23–27)* or $[Ru(NH_3)_6]^{2+/3+}$ *(28, 29)* to increase electron transfer resistance from the build-up of biolayers upon noncovalent binding. Second, in a similar approach, gold nanoparticles *(30, 31)* can enhance the capacitance of the electrodes during molecular recognition. Third, in a precipitation mode *(32, 33)*, the enzyme horseradish peroxidase (HRP) reacts with target analytes producing an impermeable layer on the electrode, increasing resistance. Fourth, using a nano-gap device *(34, 35)*, the capacitance change upon binding can be detected at very low frequencies in the 100 Hz range.

Our research focuses on the direct EIS measurements in analyte solution without reagent addition using an arbitrarily shaped electrode. Our experiments demonstrate detection by increased resistance at high frequencies from 2 to 500 kHz with useful S/N ratios up to 20-fold.

2. Materials

2.1. Microorganisms and Enzymes

1. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA).
2. *E. coli* CJ236 (New England Biolabs, Beverly, MA, USA).

3. Electrocompetent *E. coli* SS320.
4. M13KO7 helper phage (New England Biolabs, Beverly, MA, USA).
5. T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA).
6. T4 DNA ligase (New England Biolabs, Beverly, MA, USA).
7. T7 DNA polymerase (New England Biolabs, Beverly, MA, USA).

2.2. Chemicals

All chemicals and solvents (>99% purity) were purchased from Fisher Scientific, Merck or Sigma-Aldrich and used as received, unless noted.

1. DMF and ethanol (100% from Gold Shield Chemical Company) were dried with 4 Å molecular sieves obtained from Alfa.
2. Anhydrous methylene chloride (CH_2Cl_2) was filtered through two columns of activated basic alumina and transferred under Ar (g) according to the method described by Grubbs *(36)*.
3. 0.5 N and 0.05 N HCl solutions were made by diluting volumetric standard 2 N HCl with nanopure water.
4. Nanopure water (resistance ≈18 MΩ, Barnstead Inc.) was used in all experiments.
5. QIAprep Spin M13 Kit (Qiagen, Valencia, CA, USA).
6. QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).
7. Ultrapure glycerol (Invitrogen, Carlsbad, CA, USA).
8. Ultrapure irrigation USP water (Braun Medical Inc., Irvine, CA, USA).
9. Carbenicillin (5 mg/mL in water, filter sterilized). All antibiotic stocks were stored at −20°C after filter sterilization and aliquoting.
10. Chloramphenicol (30 mg/mL in ethanol, filter sterilized).
11. Kanamycin (40 mg/mL in water, filter sterilized).
12. Tetracycline (5 mg/mL in ethanol, filter sterilized).
13. Uridine (1 mg/mL in water, filter sterilized).
14. DTT (100 mM) (Sigma, St. Louis, MO, USA).
15. dNTPs (25 mM)*:* Solution with 25 mM each of dATP, dCTP, dGTP, dTTP (Promega Corporation, Madison, WI, USA).
16. ATP (10 mM) (Amersham-Biosciences, Piscataway, NJ, USA).
17. 10% v/v ultrapure glycerol (100 mL ultrapure glycerol in 900 mL ultrapure irrigation USP water, filter sterilized).
18. BSA Fraction V (EM Science, Gibbstown, NJ, USA).
19. Tween-20 (Sigma, St. Louis, MO, USA).

20. D,L-Thioctic acid (Acros Organics).
21. *N*-Hydroxysuccinimide (Acros Organics).
22. Dicyclohexylcarbodiimide (Lancaster Synthesis, Inc.).
23. 4-(1-Pyrrolidino)pyridine (Lancaster Synthesis, Inc.).
24. *o*-Phenylenediamine dihydrochloride (Acros Organics).
25. Diamond polishing compounds (1 μm and 0.25 μm size, Ted Pella).

2.3. Medium for Growth

1. 2YT media (10 g bacto-yeast extract, 16 g bacto-tryptone, 5 g NaCl; water added to 1 L and pH adjusted to 7.0 with NaOH; autoclaved).
2. LB (5 g bacto-yeast extract, 10 g bacto-tryptone, 10 g NaCl; water added to 1 L; autoclaved).
3. LB agar (5 g bacto-yeast extract, 10 g bacto-tryptone, 10 g NaCl, 15 g granulated agar; water added to 1 L; autoclaved).
4. 2YT/carb/cmp media (2YT, 50 μg/mL carbenicillin, 5 μg/mL chloramphenicol).
5. 2YT/carb/kan/uridine media (2YT, 50 μg/mL carbenicillin, 25 μg/mL kanamycin, 0.25–2.0 μg/mL uridine).
6. LB/carb plates (LB agar, 50 μg/mL carbenicillin).
7. LB/tet plate (LB agar, 5 μg/mL tetracycline).
8. LB/kan plate (LB agar, 25 μg/mL kanamycin).
9. SOC media (5 g bacto-yeast extract, 20 g bacto-tryptone, 0.5 g NaCl, 0.2 g KCl; water added to 1 L and pH adjusted to 7.0 with NaOH; autoclaved; add 5.0 mL of autoclaved 2.0 M $MgCl_2$ and 20 mL of filter sterilized 1.0 M glucose).

2.4. Buffers

1. PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH adjusted to 7.2 with HCl; autoclaved).
2. PEG/NaCl (20% PEG-8000 w/v, 2.5 M NaCl; autoclaved).
3. TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH adjusted to 8.0; autoclaved).
4. TAE/agarose gel (TAE buffer, 1% w/v agarose, 1:5,000 v/v 10% ethidium bromide).
5. TM buffer (10×) (0.1 M $MgCl_2$, 0.5 M Tris, pH 7.5).
6. 0.2% BSA in PBS (pH 7.2). The buffer was stored at 4°C.
7. PBF, pH 7.2 (4.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaF, filter sterilize).
8. PBF wash buffer (0.06% BSA and 0.07% Tween-20) were added to PBF immediately before use. Make fresh as needed.
9. 0.2% BSA in PBF (pH 7.2). Stored at 4°C.

10. PT buffer (PBS, 0.05% Tween-20).
11. PBT buffer (PBS, 0.1% BSA, 0.05% Tween-20). Stored at 4°C.
12. PBF/TW (PBF, 0.05% Tween-20).

2.5. Target Bio-Molecules

1. Anti-M13 antibody (p-Ab) was purchased from Amersham-Biosciences, Piscataway, NJ, USA).
2. Horseradish peroxidase/anti-M13 antibody conjugate (Amersham-Biosciences, Piscataway, NJ, USA).
3. Anti-Flag® M2 (n-Ab) was purchased from Sigma.
4. Prostate specific membrane antigen (PSMA) (*see* **Note 1**).

2.6. Instruments

1. Floor-model centrifuge (Sorvall) with SS-34 and SLA-1500 rotor (or equivalent).
2. 0.2-cm gap electroporation cuvet (BTX, San Diego, CA, USA).
3. ECM-600 electroporator (BTX, San Diego, CA, USA).
4. 96-well maxisorp immunoplates (NUNC, Roskilde, Denmark).
5. Ultrasonic machine: Fisher Scientific FS20.
6. 0.22 μm filter: Fisher Scientific.
7. Electrochemical Measurement: Parr 2263 (Princeton Applied Research Inc.) with software Power CV and Power Sine.
8. pH meter: Fisher Scientific.
9. Shaker: Orbital.
10. Electrode: Circular Au electrode from Bioanalysis System.
11. Microcloth: From Buehler.

3. Methods

3.1. Phage Library Synthesis

3.1.1. Constructing X8 Naïve Library

Libraries of P8 variants are constructed using an optimized version *(4)* of a previously described oligonucleotide-directed mutagenesis method *(37)*. First, a mutagenic oligonucleotide (sequence: GCTACAAATGCCTATGCATAATAATGATGAGGTGGAGGATCCGGCGGA) is used to introduce stop codons at the sites to be randomized; oligonucleotide-directed site-specific mutagenesis protocols are provided in **Subheading 3.1.3**, and can be adapted for small scale mutagenesis to introduce the stop codons (*see* **Note 2**). The resulting "stop template" phagemid can be used as the template for library construction because the presence of stop codons eliminates wild-type (wt) protein display. Uracil-containing ssDNA (dU-ssDNA) stop template

(purified from an *E. coli dut⁻/ung⁻* host) is then annealed to a mutagenic oligonucleotide (sequence: GCTACAAATGCCTATGCANNSNNSNNSNNSNNSNNSNNSNNSGGTGGAGGATCCGGCGGA) designed to replace the stop codons with NNS (N = A/G/C/T, 25% each; S = G/C, 50% each) degenerate codons that encode all 20 natural amino acids. The mutagenic oligonucleotide is used to prime the synthesis of a complementary DNA strand, which is ligated to form a covalently closed circular, double-stranded DNA (CCC-dsDNA) heteroduplex. To complete the library construction, the CCC-dsDNA heteroduplex is introduced into an *E. coli dut⁺/ung⁺* host by electroporation and the mismatch is repaired to either the wild-type or mutant sequence. In an *ung⁺* strain, the uracil-containing template strand is preferentially inactivated and the synthetic, mutant strand is replicated, thus resulting in efficient mutagenesis (> 50%). The use of a template with stop codons at all of the sites to be randomized ensures that only fully mutagenized clones contain open reading frames that can be displayed on phage. The library members can be packaged into phage particles by coinfection of the *E. coli* host with a helper phage.

3.1.2. Purification of dU-ssDNA Template

Mutagenesis efficiency depends on template purity, and thus, the use of high purity dU-ssDNA is critical for successful library construction. We use the Qiagen QIAprep Spin M13 Kit for dU-ssDNA purification, and the following is a modified version of the Qiagen protocol. It yields at least 20 µg of dU-ssDNA for a medium copy number phagemid (e.g., pS1607, which contains a pBR322 backbone), and this is sufficient for the construction of one library (*see* **Note 3**).

Purifying dU-ssDNA Template

1. From a fresh LB-antibiotic plate, pick a single colony of *E. coli* CJ236 (or another *dut⁻/ung⁻* strain) harboring the appropriate phagemid into 1 mL of 2YT media supplemented with M13KO7 helper phage (10^{10} pfu/mL) and appropriate antibiotics to maintain the host F′ episome and the phagemid. Shake at 250 rpm and 37°C for 2 h and add kanamycin (25 µg/mL) to select for clones that have been coinfected with M13KO7, which carries a kanamycin resistance gene. Shake at 250 rpm and 37°C for 6 h and transfer the culture to 30 mL of 2YT/carb/kan/uridine media. Shake overnight at 250 rpm and 37°C.
2. Centrifuge for 10 min at 15 krpm and 4°C in a Sorvall SS-34 rotor (27,000 × *g*). Transfer the supernatant to a new tube containing 1/5 volume of PEG/NaCl, mix gently and incubate for 5 min at room temperature. Centrifuge 10 min at 10 krpm and 4°C in an SS-34 rotor (12,000 × *g*). Decant the supernatant. Centrifuge briefly at 4 krpm (2,000 × *g*) and aspirate the remaining supernatant.

3. Resuspend the phage pellet in 0.5 mL of PBS and transfer to a 1.5-mL microcentrifuge tube. Centrifuge for 5 min at 13 krpm in a microcentrifuge (15,000 × g), and transfer the supernatant to a 1.5-mL microcentrifuge tube.
4. Add 0.7 mL of buffer MP (Qiagen) and mix. Incubate at room temperature for at least 2 min.
5. Apply the sample to a QIAprep spin column (Qiagen) in a 2-mL microcentrifuge tube. Centrifuge for 30 s at 8 krpm in a microcentrifuge (6,000 × g). Discard the flow-through. The phage particles remain bound to the column matrix.
6. Add 0.7 mL buffer MLB (Qiagen) to the column. Centrifuge for 30 s at 8 krpm and discard the flow-through.
7. Add another 0.7 mL buffer MLB. Incubate at room temperature for at least 1 min. Centrifuge at 8 krpm for 30 s. Discard the flow-through. The DNA is separated from the protein coat and remains adsorbed to the matrix.
8. Add 0.7 mL buffer PE (Qiagen). Centrifuge at 8 krpm for 30 s and discard the flow-through.
9. Repeat **step 8**. Residual proteins and salt are removed.
10. Centrifuge at 8 krpm for 30 s. Transfer the column to a fresh 1.5-mL microcentrifuge tube.
11. Add 100 µL of buffer EB (Qiagen; 10 mM Tris–Cl, pH 8.5) to the center of the column membrane (*see* **Note 4**). Incubate at room temperature for 10 min and centrifuge for 30 s at 8 krpm. Save the eluant, which contains the purified dU-ssDNA.
12. Analyze the DNA by electrophoresing 1.0 µL on a 1% TAE/agarose gel (*see* **Note 5**).
13. Determine the DNA concentration by measuring absorbance at 260 nm (A_{260} = 1.0 for 33 ng/µL of ssDNA). Typical DNA concentrations range from 200 to 500 ng/µL.

3.1.3. In Vitro Synthesis of Heteroduplex CCC-dsDNA

A three-step procedure is used to incorporate the mutagenic oligonucleotide into heteroduplex CCC-dsDNA, using dU-ssDNA as a template. The oligonucleotide is first 5′-phosphorylated and then annealed to a dU-ssDNA template. The oligonucleotide is enzymatically extended and ligated to form heteroduplex CCC-dsDNA, which is then purified and desalted. The protocol below produces ~20 µg of highly pure, low conductance CCC-dsDNA. This is sufficient for the construction of a library containing more than 10^{10} unique members (*see* **Note 6**).

Oligonucleotide Phosphorylation with T4 Polynucleotide Kinase

1. In a 1.5-mL microcentrifuge tube, combine 0.6 µg of the mutagenic oligonucleotide, 2.0 µL of 10× TM buffer, 2.0 µL of 10 mM ATP, 1.0 µL of 100 mM DTT. Add water to a total volume of 20 µL.

2. Add 20 units of T4 polynucleotide kinase. Incubate for 1.0 h at 37°C (*see* **Note 7**).

Annealing of the Oligonucleotide to the Template

1. To the 20 µL phosphorylation reaction mix, add 20 µg of dU-ssDNA template (*see* **Note 8**), 25 µL 10× TM buffer, and water to a final volume of 250 µL.
2. Incubate at 90°C for 1–2 min, 50°C for 3 min, 20°C for 5 min, and place on ice (*see* **Note 9**).

Enzymatic Synthesis of CCC-dsDNA

1. To the annealed oligonucleotide/template mixture, add 10 µL 10 mM ATP, 10 µL 25 mM dNTPs, 15 µL 100 mM DTT, 30 Weiss units T4 DNA ligase, 30 units T7 DNA polymerase.
2. Incubate at 20°C for at least 3 h. Overnight incubation is preferred.
3. Electrophorese 1.0 µL of the reaction product alongside the dU-ssDNA template. Use a 1% TAE/agarose gel with ethidium bromide for DNA visualization. If the template DNA is still present, the concentration of the mutagenic oligonucleotide can be increased.
4. Affinity purify and desalt the DNA using the Qiagen QIAquick DNA Purification Kit. Add 1.0 mL of buffer QG (Qiagen) and mix.
5. Apply the sample to two QIAquick spin columns placed in 2-mL microcentrifuge tubes. Centrifuge at 13 krpm for 1 min in a microcentrifuge. Discard the flow-through.
6. Add 750 µL buffer PE (Qiagen) to each column. Centrifuge at 13 krpm for 1 min. Discard the flow-through and centrifuge at 13 krpm for 1 min. Place the column in a new 1.5-mL microcentrifuge tube.
7. Add 35 µL of ultrapure irrigation USP water to the center of the membrane. Incubate at room temperature for 2 min.
8. Centrifuge at 13 krpm for 1 min to elute the DNA. Combine the eluants from the two columns. The DNA can be used immediately for *E. coli* electroporation, or it can be frozen for later use.
9. Electrophorese 1.0 µL of the eluted reaction product alongside the dU-ssDNA template. Use a 1% TAE/agarose gel with ethidium bromide for DNA visualization.

3.1.4. E. coli Electroporation and Phage Propagation

To complete the library construction, the heteroduplex CCC-dsDNA must be introduced into an *E. coli* host that contains an F′ episome to enable M13 bacteriophage infection and propagation. Phage-displayed library diversities are limited by methods for introducing DNA into *E. coli*, with the most efficient method being high-voltage electroporation.

1. Chill the 20 µg of purified DNA and a 0.2-cm gap electroporation cuvet on ice. Thaw a 350 µL aliquot of electrocompetent *E. coli* SS320 on ice. Add the cells to the DNA and mix by pipetting several times (avoid introducing bubbles).
2. Transfer the mixture to the cuvette and electroporate. For electroporation, follow the manufacturer's instructions, preferably using a BTX ECM-600 electroporation system with the following settings: 2.5 kV field strength, 125 Ω resistance, and 50 mF capacitance. Alternatively, a Bio-rad Gene Pulser can be used with the following settings: 2.5 kV field strength, 200 Ω resistance, and 25 mF capacitance.
3. Immediately, rescue the electroporated cells by adding 1 mL of ice-cold SOC media and transferring to a chilled 250 mL baffled flask containing 20 mL of ice-cold SOC media. Rinse the cuvette four more times with 1 mL ice-cold SOC media. Incubate for 20 min at 37°C with shaking at 250 rpm.
4. To determine the library diversity, plate serial dilutions on LB/carb plates to select for the library phagemid (in the case of β-lactamase encoding phagemids such as pS1607).
5. Add M13KO7 (4×10^{10} pfu/mL) and incubate for 20 min at 37°C with shaking at 250 rpm.
6. Transfer the culture to a 2 L baffled flask containing 500 mL 2YT media, supplemented with antibiotic for phagemid selection (e.g. 2YT/carb media).
7. Incubate 1 h at 37°C with shaking at 250 rpm and add kanamycin (25 µg/mL). Incubate overnight at 37°C with shaking at 250 rpm.
8. Centrifuge the culture for 10 min at 10 krpm and 4°C in a Sorvall SLA-1500 rotor (16,000 × *g*). Transfer the supernatant to a fresh tube and add 1/5 volume of PEG/NaCl solution to precipitate the phage. Mix gently and incubate for 5 min at room temperature.
9. Centrifuge for 10 min at 10 krpm and 4°C in a SLA-1500 rotor. Decant the supernatant. Re-spin briefly and remove the remaining supernatant with aspiration. Resuspend the phage pellet in 1/20 volume of PBS.
10. Pellet insoluble matter by centrifuging for 5 min at 15 krpm and 4°C in an SS-34 rotor (27,000 × *g*). Transfer the supernatant to a clean tube.
11. Estimate the phage concentration spectrophotometrically (OD_{268} = 1.0 for a solution of 5×10^{12} phage/mL), (*see* **Note 10**).

3.2. Selecting Phage from the X8 Library Against PSMA

Phage from the X_8 library described above is cycled through rounds of binding selection with PSMA *(38)* coated on 96-well Maxisorp immunoplates as the capture target. Phage were propagated in

E. coli XL1-Blue with M13KO7 helper phage for further rounds of selection.

3.2.1. Coating Wells with Target Protein

1. Coat 24 wells of a 96-well Maxisorp plate with 100 µL of a 10 µg/mL solution of the target protein (e.g. PSMA). Shake at room temperature for 1 h (*see* **Note 11**). Discard the solution.
2. To block nonspecific binding to the microtiter wells, add 300 µL of 0.2% BSA in PBS to each well. Shake at room temperature for 1 h. Discard the blocking solution.

3.2.2. Phage Library Selections

1. Add a solution of the phage library (~10^{12} phage/mL) in PBT buffer to the wells. Shake at room temperature for 2 h. Wash the plate five times with PT buffer. The stringency of the binding selection can be increased for successive rounds by increasing the number of washes.
2. Elute bound phage, by adding 100 µL of 100 mM HCl to each well. Shake vigorously for 5 min at room temperature.
3. Combine the eluants, and neutralize with one-third volume of 1.0 M Tris–HCl, pH 8.0.

3.2.3. Propagating Phage for the Next round of Selection

1. Add half the eluted phage to ten volumes of log-phase XL1-Blue cells (OD_{600} = 0.5–1.0).
2. Shake at 250 rpm, 37°C for 20 min. Remove 10 µL and determine library diversity by plating 1:10 serial dilutions on LB/carb plates to select for the library phagemid.
3. Add M13KO7 helper phage (10^{10} pfu/mL) and shake at 250 rpm, 37°C for 30 min.
4. Transfer the culture to ten volumes 2YT/carb/kan media and shake overnight at 250 rpm and 37°C.
5. Isolate the phage by PEG/NaCl precipitation, as described in **Subheading "Purifying dU-ssDNA Template" step 2**. Resuspend phage in 1/20 volume of PBS.
6. Repeat the selection process five times, using only half of the eluted phage in each round and changing the blocking agent between each round.

3.3. Screening PSMA Binding Peptide Display Levels by Spot Assay

1. Infect 150 µL of log-phase (OD_{600} ~0.6) *E. coli* XL1-Blue cells, grown from a fresh LB/tet plate, with 1.5 µL of Round 4 eluted phage. Incubate cells for 20 min at 37°C and 250 rpm.
2. Spread 25, 50, and 75 µL of infected cells onto three separate LB/carb plates and incubate overnight at 37°C.
3. The following morning, pick 24 isolated colonies from the plate that is less crowded and place each colony into separate wells of a 96-well block containing 1 mL of 2YT/carb, supplemented with M13KO7 helper phage (10^{10} pfu/mL). Shake overnight at 250 rpm and 37°C.

4. The next morning, coat, and block 24 wells of a 96-well Maxisorp immunoplate as detailed in **Subheading 3.2.1**.
5. Centrifuge the 96-well block containing infected cells for 20 min at 2.5 krpm and 4°C in a Beckman Allegra 6-R centrifuge with swinging trays (1427 × *g*).
6. Transfer 100 µL of the supernatant to the corresponding wells on the 96-well plate.
7. Shake at room temperature for 2 h. Wash the plate five times with PT buffer.
8. Decant the phage solution and wash eight times with PT buffer.
9. Add 100 µL of horseradish peroxidase/anti-M13 antibody conjugate (diluted 5,000-fold in PBT buffer).
10. Shake for 30 min at room temperature.
11. Wash three times with PT, followed by two times with PBS buffer.
12. Develop the wells with 100 µL of *o*-phenylenediamine dihydrochloride (1 mg/mL, 0.02% H_2O_2) solution. Read spectrophotometrically at 450 nm in a microtiter plate reader.

3.4. Growth of Bacteriophage

1. From a fresh LB/tet plate, pick a single colony of *E. coli* XL1-Blue cells harboring the appropriate phagemid into 1 mL of 2YT media supplemented with M13KO7 helper phage (10^{10} pfu/mL), 50 µg/mL carbenicillin (for phagemid maintenance), and 5 µg/mL tetracycline (for F′ episome maintenance).
2. Shake at 250 rpm and 37°C for 6–8 h.
3. Add M13KO7 helper phage (10^{10} pfu/mL) and shake for 30 min at 250 rpm and 37°C.
4. Transfer the culture to 30 mL of 2YT/carb/kan media. Shake overnight at 250 rpm and 37°C.
5. Isolate the phage as detailed in **Subheading "Purifying dU-ssDNA Template" steps 2** and **3**.
6. Determine the phage concentration spectrophotometrically (OD_{268} = 1.0 for a solution of 5 × 10^{12} phage/mL).

3.5. Synthesis of Thioctic NHS-Ester

Thioctic NHS-ester (NHS-TE) is not commercially available, but can be synthesized by the following previously published procedure *(39)*.

1. Add 1.0064g (4.878 mmol) of thioctic acid and 0.7062 g (6.136 mmol) NHS to a 25 mL round bottom flask containing 5 mL of dry DCM.
2. Dissolve 1.7287g of DCC (8.378 mmol) and 0.0639g of 4-pyrrolidinopyridine (0.812 mmol) in 5 mL of DCM (*see* **Note 12**). Mix until the solution is homogeneous.

3. Transfer the DCC solution to the thioctic acid/NHS solution, and purge with N_2 gas. Stir overnight at room temperature. The resultant mixture will be an opaque, light yellow.
4. Vacuum filter the solution, and transfer to a separatory funnel. The filtered solid can be rinsed with DCM to collect as much of the yellow colored product as possible.
5. Add an equal volume of water, and extract into the organic layer. Wash with water a second time.
6. Wash two times with an equal volume of 5% acetic acid, followed by two extractions with an equal volume of water.
7. Dry the organic layer over anhydrous $MgSO_4$, and filter into a 100 mL round bottom flask under reduced pressure.
8. Rinse the filter with 2 mL of DCM, and transfer to a 100 mL round bottom flask.
9. Remove the solvent by rotary evaporation under ambient temperature.
10. Collect the flakey, light yellow solid, and dry overnight under vacuum. The dry product should be packaged in small aliquots, and stored in a light sensitive vial at −20°C in a desiccator.
11. NHS-TE was characterized by NMR and MS: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.56 (m, 2 H), 1.71 (m, 2 H), 1.74 (m, 2 H), 1.93 (m, 1 H), 2.45 (m, 1 H), 2.63 (m, 2 H), 2.84 (s, 4 H), 3.12 (m, 1 H), 3.19 (m, 1 H), 3.58 (m, 1 H); expected mass: 303.06, observed: 304.42 (m/z + 1), 326.38 (major, m/z + Na^+).

3.6. Modifying Electrode with NHS-TE

1. Polish a circular gold electrode (3 mm diameter, **Fig. 1C**) with 1 µm first and then 0.25 µm diamond compounds in order on different microcloth pads. Sonicate the electrode three times in nanopure water for 3 min (*see* **Note 13**).
2. Rinse the polished electrode with nanopure water, and dry with N_2 gas.
3. Store the electrode in a 10 mL test tube enclosed by a 250 mL brown glass cylinder with a bottom layer of drierite.
4. While enclosed in a desiccator, incubate the polished electrode for at least 18 h in a NHS-TE solution (16.5 mM in dry DMF).
5. Remove the NHS-TE modified electrode from a desiccator, and rinse with 100% ethanol two times before drying with N_2 gas.

3.7. Constructing the Virus Electrode

1. Suspend the NHS-TE modified electrode in a phage solution (300 µL in PBS, 16–40 nM), and mix for 1 h using an orbital shaker.

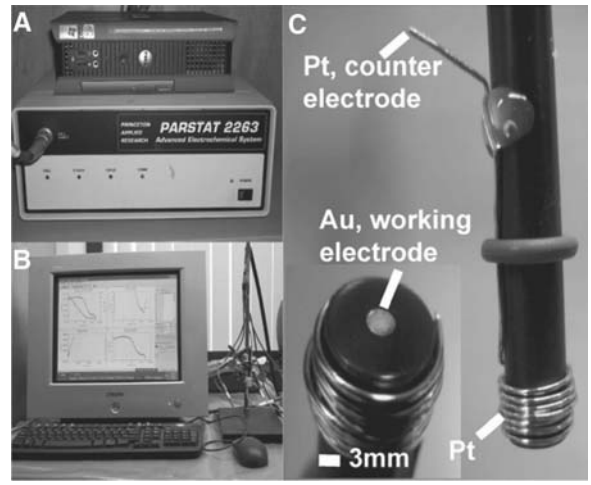

Fig. 1. Potentiostat system and working/counter electrode assembly: (**A**) potentiostat Princeton Applied Research, PAR 2263 controller, and (**B**) computer system installed with PAR Power Suite software, (**C**) *top view* of a Au disk (diameter 3 mm) working electrode and side view, showing the Pt coil counter electrode glued to the body of the working electrode.

2. Rinse the virus-modified electrode 5 min with PBF/TW buffer and then 5 min with wash buffer.
3. Dip the virus electrode in 300 µL 0.2% BSA solution, and mix for 40 min.
4. Rinse 5 min with PBF/TW and then 5 min with wash buffer.
5. Transfer the virus/BSA modified electrode into 300 µL 0.05 N HCl, and shake for another 1 min to wash away nonspecific binding phage.
6. Rinse with PBF/TW and wash buffer.
7. Test the solution used to rinse the electrode with pH paper to ensure pH ~7.2.
8. Dip the virus-modified electrode in wash buffer for further EIS measurements (*see* **Note 14**, construction of virus electrode shown in **Fig. 2A, B**).

3.8. Recognizing Target Biomolecules

1. Thaw analytes (e.g., frozen p-Ab, n-Ab or PSMA aliquots) in an ice-water bath.
2. Mix the target to an appropriate dilution with the wash buffer to provide a 500 µL solution for measurement by the virus electrode.
3. Dip the virus electrode into the analyte solution, and shake for 1 h at room temperature (**Fig. 2C–E** for analytes n-Ab, PSMA, and p-Ab, respectively).

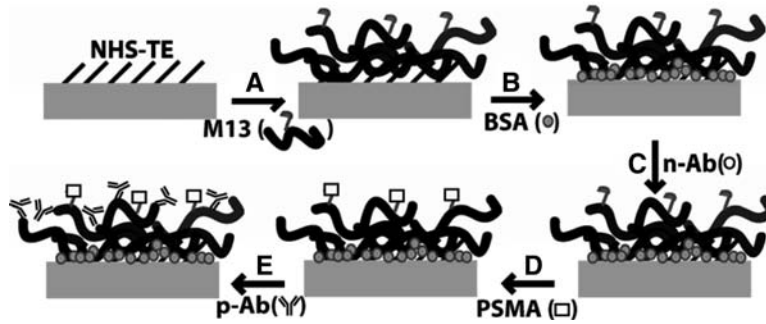

Fig. 2. Virus electrode construction: (**A**) M13 phage is covalently tethered to a self-assembled monolayer of NHS-TE, through formation of amide bonds between free amines on the phage and the activated carboxylate. (**B**) Gaps in the monolayer and unreacted NHS esters are capped with BSA. (**C**) A control experiment is first performed by reacting with n-Ab. (**D**) PSMA is added next to bind molecular recognition scaffolds on the surface of the phage (hooks). (**E**) Last, p-Ab can react with the virus electrode.

4. Rinse the electrode with PBF/TW and wash buffer at least three times.
5. Dip the electrode in 2 mL wash buffer 1 min for further EIS measurements in a 3 mL polypropylene container (*see* **Note 15**).

3.9. Oxidizing Virus Electrode with Cyclic Voltammetry to Test Surface Passivation

1. Rinse the home-made reference electrode, a saturated calomel electrode (SCE), with nanopure water, wipe dry with a kimwipe, and dip into wash buffer solution.
2. Flame anneal the home-made Pt flat counter electrode with a propane torch, and rinse with nanopure water. Assemble, and glue the Pt counter electrode to the virus electrode as depicted in **Fig. 1C**.
3. Dip the counter and working electrode in wash buffer solution immediately, and avoid drying the virus electrode. (*Caution*: if dry, make a new virus electrode.)
4. Immerse virus/counter electrode assembly in fresh PBF buffer (pH = 7.2).
5. Connect labeled alligator clips to the corresponding electrodes (for example: green: working electrode, white: reference electrode, red: counter electrode and gray: not connected).
6. Before opening software, turn on potentiostat power.
7. Click the shortcut to "Power Suite" (if **steps 6** and **7** are reversed, the program will malfunction, and cannot perform the measurement).
8. Select "Power CV" from menu, before changing and saving the file name.
9. Select multiple-cycle waveform (repeat *N* times).

10. Input set up parameters: initial potential E_0 (0.0 V), vertex potential E_1 (−0.8 V), E_2 (1 V), scan rate (20 mV/s), number of cycles (20), filter (5 Hz) and max current magnitude (40 μA).
11. Depress "Run" button and record data **(Fig. 3)**.

3.10. Measuring Electrochemical Impedance Spectrum

1. All electrochemical impedance spectra were acquired using two electrodes: The virus electrode and the platinum counter electrode. The four electrode leads from the potentiostat were, therefore, paired as follows: The "sense" electrode and working electrode connections were applied to the virus electrode; the reference electrode and counter electrode were applied to the counter electrode.
2. Click shortcut "Power Suite" and then click software "Power Sine", change and save the file name.
3. Set up parameters: potential amplitude = 10 mV, data quality = 3, acquire 50 points with logarithmic point spacing for each run at over the scanning frequency range from 1 MHz to 0.1 Hz at rest potential.
4. Display four windows during data collection (i) the Nyquist Plot (Z_{im} vs. Z_{re}), (ii) phase angle vs. frequency, (iii) Z_{re} vs. frequency, and (iv) Z_{im} vs. frequency.

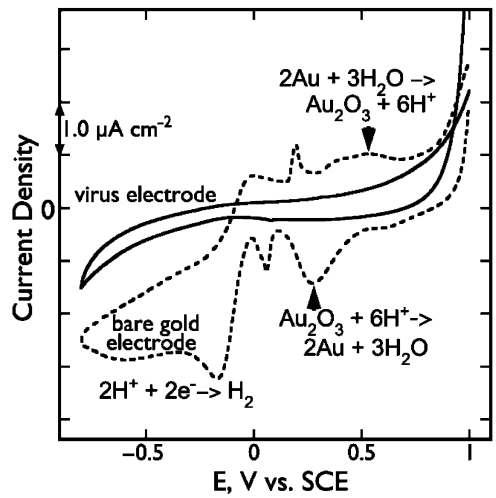

Fig. 3. Cyclic voltammetry of a virus electrode. Cyclic voltammograms at 20 mV/s were used to examine a clean gold electrode (*solid line*) in PBF and a phage-covered biosensor (*dash line*) following experiments to assess the response to n-Ab and p-Ab. Reactions characteristic of exposed gold – including the reduction of protons (at 0.0 V) and the oxidation and reduction of the gold surface (at 0.2 and −0.55 V, respectively) – were suppressed by the covalent virus layer. This layer could be removed, gradually, by scanning the potential of this electrode to +1.0 V vs. SCE revealing the characteristic voltammetric features of the underlying gold surface. Reprinted with permission from ***ref. 16.***

5. Insure the working electrode can pass through the Pt coil counter electrode easily. Do not make it fit too tight to surround the electrode.
6. Flame anneal home-made Pt coil counter electrode with a propane torch, rinse with nanopure water, and assemble and fix the Pt coil to the virus electrode body using glue as before.
7. Rinse the working/counter electrode assembly by dipping it in wash buffer for 1 min.
8. Acquire the following EIS data sets in order (i) virus/BSA, (ii) acid wash (optional), (iii) n-Ab, (iv) PSMA, and (v) p-Ab
9. To initiate each acquisition, depress the "Run" key. Acquire three sets of EIS data for each solution, i–v above ($n = 3$).
10. All these data were analyzed to get average, standard deviation and pool deviation $\delta_{DZ} = \sqrt{\left[\sum_{i=1}^{n}(X_i - \bar{X})^2 + \sum_{j=1}^{m}(\Upsilon_j - \bar{\Upsilon})^2\right] / (m+n-l)}$, if data came from data set X and Υ, then $l = 2$, if three data sets then $l = 3$) to calculate the signal to noise ratio (S/N) and error bar ($2\delta_{DZ}$) (**Fig. 4**).

4. Notes

1. PSMA is stored at 4°C, and should not be frozen.
2. For mutagenesis to introduce the stop codons, the protocols in **Subheadings "Oligonucleotide Phosphorylation with T4 Polynucleotide Kinase," "Annealing of the Oligonucleotide to the Template,"** and **step 1** of **"Enzymatic Synthesis of CCC-dsDNA"** can be scaled down by a factor of ten. The reaction product can be used directly to transform *E. coli* using any standard procedure.
3. The protocol can be scaled up by inoculating a larger overnight culture and purifying the ssDNA with multiple spin columns.
4. Preheating the EB buffer to 50°C prior to use can help to recover more ssDNA during the elution step.
5. The DNA should appear as a predominant single band, but faint bands with lower electrophoretic mobility are often visible. These are likely caused by secondary structure in the ssDNA.
6. All steps can be scaled-up considerably, with the exception of the annealing step. The protocol described here works well with volumes of 250 µL or less.
7. The phosphorylated oligonucleotide should be used immediately in the subsequent steps.

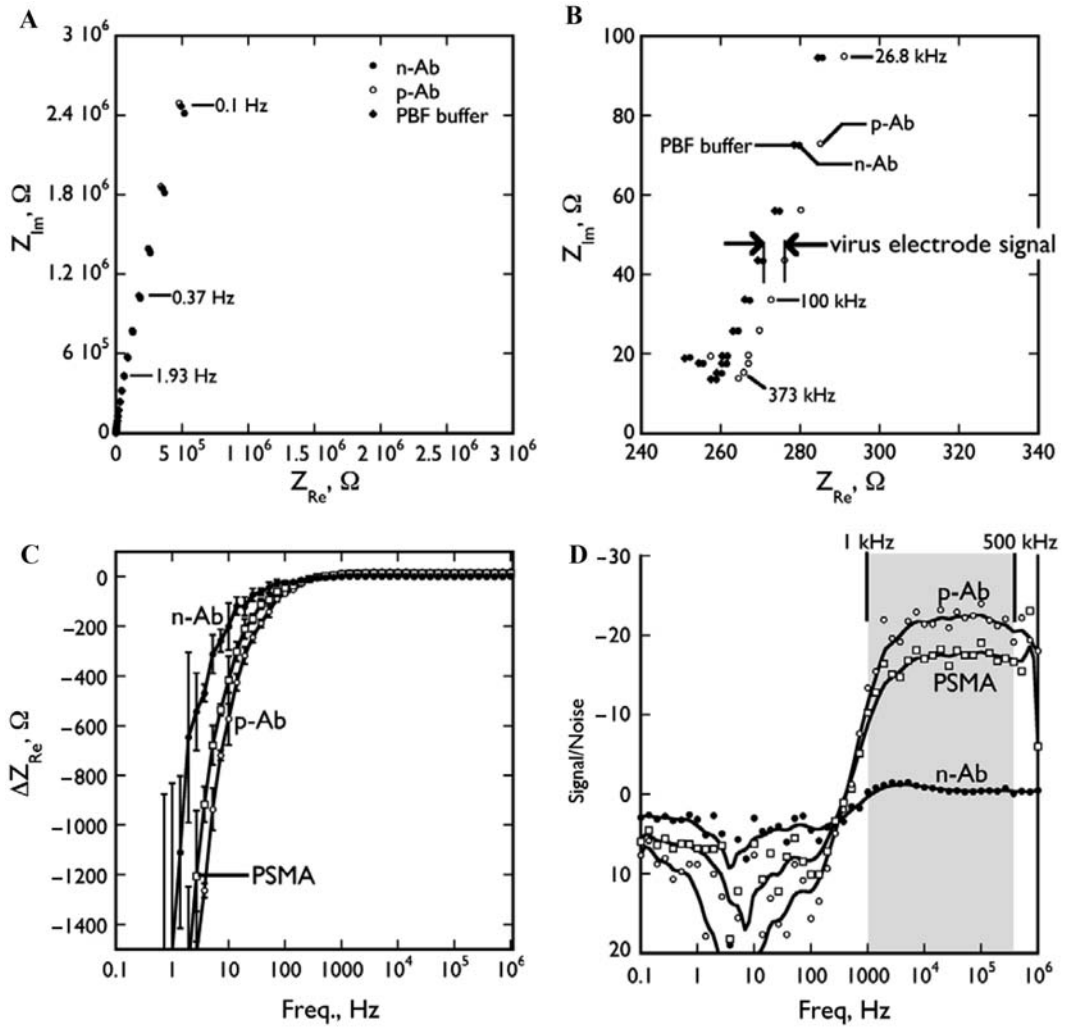

Fig. 4. Electrochemical Impedance Measurement. (**A**, **B**) Nyquist plots (Z_{re} vs. Z_{im}) for the frequency range from 0.1 Hz to 1.0 MHz. Shown are three data sets: PBF buffer (*filled diamond*) is the baseline against which the impedance in n-Ab (*filled circle*) and p-Ab (*open circle*) solutions are measured. The impedance "spectrum" of this covalent virus electrode was first measured in PBF buffer, then it was measured in PBF buffer containing n-Ab, it was rinsed, and finally it was measured in PBF buffer containing p-Ab. As shown in (**B**), we define the virus electrode signal, ΔZ_{re}, to be Z_{re} measured against Z_{re} in PBF buffer. (**C**) ΔZ_{re} for solutions of three molecules – p-Ab (*open circle*), PSMA (*open square*), and n-Ab (*filled circle*) – are plotted here as a function of frequency. Error bars were obtained by repeating each impedance measurement three times and calculating the standard deviation. ΔZ_{re} and the standard deviation of ΔZ_{re} are both large for frequencies below 1 kHz. Above this frequency, ΔZ_{re} is less than 20 Ω but the reproducibility of the measured values is much better, proportionately, than at lower frequencies. To highlight this fact, one can divide ΔZ_{re} by the standard deviation, σ_{re}, to obtain an estimate of the signal-to-noise ratio (*S*/*N*). These *S*/*N* ratios, showing the appreciable values of 10–20 in the frequency range from 2 to 500 kHz, are plotted in (**D**). Reprinted with permission from ***ref** 16.*

8. The dU-ssDNA template can be heated to 90°C for 2 min, then cooled slowly to room temperature to remove any secondary structures that may interfere with the annealing of the mutagenic oligonucleotide.
9. To scale up this reaction, run multiple annealing reactions of 250 µL each.
10. Use the library immediately or add glycerol to a final concentration of 10% and store at –80°C. Some displayed proteins can be denatured by freezing, which could render them unusable for selections. In general, it is best to use libraries immediately.
11. Wells can be coated overnight at 4°C with shaking on an orbital shaker.
12. DCC can be dissolved gently with a heat gun if it does not initially go into solution. Alternatively, more DCM can be added drop-wise until the solid DCC fully dissolves after swirling.
13. Do not dip the electrode into solutions more than 1 cm above the Au disk. The junction between wire and gold electrode is quite fragile, and should not be exposed to water particularly during sonication.
14. The first measurement provides a baseline for comparison with analytes PSMA and antibodies.
15. To avoid cross-contamination, use a new container for each EIS measurement.

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