**Biosensing with Virus Electrode Hybrids**
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**Abstract**

Virus electrodes address two major challenges associated with biosensing. First, the surface of the viruses can be readily tailored for specific, high affinity binding to targeted biomarkers. Second, the viruses are entrapped in a conducting polymer for electrical resistance-based, quantitative measurement of biomarker concentration. To further enhance device sensitivity, two different ligands can be attached to the virus surface, and increase the apparent affinity for the biomarker. In the example presented here, the two ligands bind to the analyte in a bidentate binding mode with a chelate-based avidity effect, and result in an 100 pM experimentally observed limit of detection for the cancer biomarker prostate-specific membrane antigen. The approach does not require enzymatic amplification, and allows reagent-free, real-time measurements. This article presents general protocols for the development of such biosensors with modified viruses for the enhanced detection of arbitrary target proteins.

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Introduction

This article describes a general protocol for the development of a biosensor aimed at efficiently detecting cancer biomarkers in patient’s biological fluids including blood and urine. The virus electrode biosensors provide highly sensitive, reagent free, real-time detection of the targeted protein (Mohan et al., 2013). M13 filamentous bacteriophage, the biological recognition element, display peptide ligands to a specific biomarker. These viruses and their phage-displayed ligands are incorporated into the biosensor during electrochemical polymerization with a conducting organic polymer, poly-3,4-ethylenedioxythiophene (PEDOT) onto gold electrodes (Weiss and Penner, 2008; Diaz et al., 2008; Yang et al., 2008b, 2006; Arter et al., 2010). Upon binding, a perturbation in the electrode’s electrical impedance results in a quantitative measurement of the target biomarker. In this article, the target analyte is prostate-specific membrane antigen (PSMA), a prostate cancer biomarker (Murphy et al., 1998; Schülke et al., 2003; Chuang et al., 2007).

High sensitivity to PSMA resulted from the synergistic action of two different PSMA ligands on the same virus particle, Figure 1. One ligand, termed the primary recognition ligand, was genetically encoded by a phagemid encapsulated within the virus. The second ligand, termed the secondary recognition ligand, was chemically synthesized as a fusion peptide to oligolysine, i.e., \( K_{CS}-1 \) or \( K_{CS}-2 \) (where \( K_{CS} \) is defined as "lysine [K], Chemically Synthesized"; see Figure 3) for electrostatic wrapping around the phage surface. The dual ligands result in a bidentate binder with dense ligand display for enhanced PSMA detection through both a high concentration of ligands and a chelate-based, avidity effect (Murase et al., 2003). Biosensing with virus-PEDOT films provided a 100 pM limit of detection (LOD) for PSMA in synthetic urine without requiring enzymatic or other amplification (Mohan et al., 2013). The reported approach leverages two ligands with varying target affinities to achieve high sensitivity for the targeted biomarker.
Phage-based biosensors offer a number of key advantages. First, phage and the displayed peptide ligands are extraordinarily stable (Kay et al., 1996). For example, the virus-hybrid surfaces are stable for over 14 hours in rapidly flowing, high ionic strength buffer (Yang et al., 2008b), and the phage retain their binding abilities after 6-weeks at 65 °C (Brigati and Petrenko, 2005). Second, phage form liquid crystals at high concentration, which can maximize the density of packing in the biosensors. We have observed this dense packing, which causes the phage to line-up like match sticks, in atomic force microscopy of our covalent virus surface (Yang et al., 2008b). Third, the “kelp forest architecture” of the covalent virus surface, which we observed by QCM allows rapid kinetics in binding to biomarkers (Yang et al., 2008a). Such multi-point, cooperative binding has been observed by SPR for a phage-based surface (Nanduri et al., 2007).

Most importantly, phage readily allow adaptation to arbitrary molecular targets. Phage display has been successfully applied to a wide range of targets, including proteins (as described here), DNA, and small molecules. The architecture described here, for example, can be applied to the detection of different biomarkers for a broad range of diseases simply by modifying the viral DNA to ligands targeting biomarkers associated with each disease.

**Strategic Planning**

Selection of the targeted biomarkers requires careful consideration. For example, PSMA, a prostate cancer biomarker is shed into the urine sample of cancer patients (Sokoloff et al., 2000). Thus, it serves as a suitable target for non-invasive testing in urine from patients. Phage-displayed peptide libraries were used to select for ligands that selectively bind to PSMA (Kehoe and Kay, 2005; Levin and Weiss, 2006; Arter et al., 2012). Theoretically, the ligands displayed on phage could either be peptides or proteins, but for the secondary recognition ligands, peptides are preferred for dense ligand display. In addition, the high copy number of peptide ligands on the phage surfaces increases their effective concentration in the bioaffinity layer.

A wide range of bioorthogonal chemistries could allow attachment of the secondary recognition ligand. A modular approach is preferred rather than the synthesis of a long peptide to obtain higher yields of the synthetic peptide. Additionally, the oligolysine half of the wrapper can remain constant for all wrappers. Thus, it is advantageous to synthesize the two halves separately and substitute different secondary recognition ligands. Copper-catalyzed azide alkyne-cycloaddition, a ‘click’ reaction, can link the two halves of the wrapper, as the reaction offers a convergent synthesis that proceeds at room temperature in aqueous solution (Rostovtsev et al., 2002).

The generation of the bioaffinity matrix on the gold electrode may be accomplished by adsorption, entrapment, specific interaction, cross-linking and covalent attachment. Biomolecular entrapment of the viruses within a polymer matrix provides robust attachment and allows the ligands to remain functional (Cosnier, 1999). Additionally, the method is expeditious and avoids potential phage-degrading steps.
The protocols in this article describe a general method for the detection of a biomarker using electrochemical biosensors comprising of phage-display ligands wrapped with additional secondary recognition ligands to increase the sensitivity to the target, Figure 2. Three basic procedures will be described. First, the secondary recognition ligands are synthesized from individual peptides. Second, phage-displayed ligands and control phage are propagated and isolated. Third, the virus-PEDOT films are formed before generation of a calibration curve for target protein detection. As a specific example to illustrate the steps mentioned above, the incorporation of peptide ligands into the bio-affinity layer for the detection of PSMA is described here.

Peptide synthesis of ligands

Conventional solid phase peptide synthesis was used for the generation of alkyne- and azide-functionalized peptides (Figure 3). Next, the peptides were purified by reverse-phase HPLC, and the fractions characterized by MALDI. The relevant fractions were then combined, and their purity analyzed by analytical reverse-phase HPLC.

Figure 3: PSMA ligands, sequence and nomenclature nomenclature. The "X" in the structure describes the entries in the first column, and is the PSMA binding ligand; K₁₄ is defined as a peptide composed of 14 lysines.

| X  | Amino acid sequence | Genetically encoded | Chemically synthesized | Structure of the chemically synthesized peptide |
|----|---------------------|---------------------|------------------------|-------------------------------|
| 1  | CALCEFLG            | phage – 1           | K₁₄–1                  | K₁₄                           |
| 2  | SECVEVFQNSCDW       | phage – 2           | K₁₄–2                  |                               |

Basic protocol 1: CLICK CHEMISTRY REACTION: PREPARATION OF Kcs-1 AND Kcs-2

After the azide- and alkyne-functionalized peptides have been synthesized and purified, the next step is to link them by click reaction, Figure 4, listed as Kcs-1 and Kcs-2 in Table 1. The reaction protocol for click reaction is modified from the Lumiprobe protocol for oligonucleotides. While working with Cu(I), the disproportionation of Cu(I) to Cu(II) or Cu(0) leads to the generation of the non-catalytic Cu states. Ligands such as TBTA are commonly used to stabilize the Cu(I) state. Alternatively, if the reaction proceeds at a rapid rate, the addition of ligands can be avoided. Triethylammonium acetate acts as a buffer.
**Materials**

- Alkyne-functionalized peptide stock solution (200 µM in water)
- Triethylammonium acetate buffer (1M, pH 7)
- HPLC grade water or Milli-Q water
- Azide-functionalized peptide stock solution (200 µM in 1:3 water-acetonitrile mixture)
- Ascorbic acid
- Copper sulphate

**Setting up the cycloaddition reaction**

1. Prepare a 5 mM ascorbic acid solution in HPLC grade water.

   *The solution is unstable and should be discarded after each use. HPLC grade water is recommended, but not required; inconsistent results were observed from reactions performed in Milli-Q water.*

2. Aliquot 200 µL of the alkyne-functionalized peptide solution into a 15 mL conical tube.

   *The concentration of both azide- and alkyne-functionalized peptides in the final reaction mixture will be 40 µM. Higher concentrations resulted in lower yields. Parallel 40 µM, 1 mL scale reactions should be used to obtain higher yields and more product. The reaction mixtures from the parallel reactions can then be combined before characterization.*

3. Add 50 µL triethylammonium acetate buffer solution to a final concentration of 50 mM.

4. Add the required amount of HPLC grade water such that the final reaction volume after the addition of solutions below will be an estimated 1 mL. Vortex the solution.

5. Add 200 µL of the azide-functionalized peptide stock solution. Vortex.

   *If necessary, the azide-functionalized peptide may be used in slight excess as suggested in the Lumiprobe protocol. A 1:1 ratio provided the expected results with the peptides used here.*
6. Sparge the reaction mixture by bubbling through an inert gas (e.g., nitrogen) for 30 sec.

7. Add 200 μL of the ascorbic acid solution. Briefly vortex.

8. Add 10 μL CuSO₄ solution (100 mM).

9. Sparge the reaction mixture again with nitrogen for 10 sec.

   The reaction vessel (conical tube) should be sealed immediately to avoid absorption of oxygen by the solution.

10. Vortex the conical tube, and incubate overnight (~16 hours) at room temperature.

   A precipitate observed after addition of all reagents likely results from a high concentration of azide-functionalized peptide ligand. In addition to decreased concentrations of the azide-functionalized peptide ligand, the solution can be heated to 80 °C for a few minutes.

**Characterization of product formation**

11. Combine the reactions into one conical tube.

12. Run MALDI-TOF mass spectrometry on a sample of the reaction product to confirm product formation.

   α-Cyano-4-hydroxycinnamic acid was used as the matrix for sample preparation.

**Purification of the cycloaddition reaction product**

13. Concentrate the reaction mixture using 2 kD molecular weight cut-off micro concentrators.

   In general, ten reactions were run and concentrated down to 500 μL to 1 mL for further purification.

14. Run reverse-phase HPLC for purification.

   The reactions were run at 40 μM, hence the product concentration is insufficient to run preparative HPLC. The product was purified using analytical scale HPLC. Multiple runs were performed. Fractions were analyzed by MALDI-TOF, and appropriate fractions from different runs were combined.

15. Place the fractions under speed vacuum to remove the solvent.

16. Resuspend the product to a final concentration of 1 μg/uL in 60:40 water: acetonitrile mixture.
HPLC-grade water was used for the final resuspension. The percentage of acetonitrile should be used as needed for solubility.

Basic protocol 2: PHAGE PROPAGATION, ISOLATION, PURIFICATION, AND QUANTIFICATION

M13 bacteriophages infect gram negative bacteria (e.g., E. coli), and can be readily isolated from bacterial culture. The phagemids (plasmid-like vectors) encode the display of peptide ligands as fusions to a periplasmic localization signal sequence (stII leader sequence: MKKNIAFLASMFVFSIATNAYA) and the N-terminus of P8, the major coat protein. The signal peptide directs the resultant protein into the periplasm. Furthermore, the stII leader sequence also incorporates a signal peptidase site, which is cleaved off leading to the displayed peptide fused to the N-terminus of the P8 coat protein through the Gly-Ser linker (GGGSGSSSGGSGGG). The order of the resultant fusion is stII leader sequence-(N-terminus-peptide ligand)-(Gly-Ser linker)-P8-COOH.

DNA encoding displayed peptides were introduced by mutagenesis (Smith, 1985). The phagemid also includes an antibiotic resistance marker to allow selections for its propagation in the presence of carbenicillin as used below. Other proteins required for phage propagation and assembly are separately provided by co-infecting the cultures with helper phage (M13 KO7). The genome of KO7 has a mutated packaging signal, which decreases its efficiency and ensures preferential packaging of the phagemid DNA. The encapsulated viruses are then secreted from the bacteria and can be precipitated from the culture using PEG-NaCl precipitation. This protocol details all steps necessary to obtain purified phage.

Materials

*E.Coli* XL1 Blue Cells (CaCl₂ competent; e.g. Stratagene)
M13 phage-display vectors (phagemids, Vrisko Limited)
2YT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl; adjust to 1 liter with Milli-Q purified water, pH 7; sterilize by autoclaving)
Carbenicillin stock (50 mg/ml in sterile water, i.e., autoclaved Milli-Q purified water)
Tetracycline stock (5 mg/ml in sterile water, i.e., autoclaved Milli-Q purified water)
Kanamycin stock (40 mg/ml in sterile water, i.e., autoclaved Milli-Q purified water)
Helper phage (GE Healthcare Life Sciences)
PEG-NaCl (2.5 M NaCl, 20% PEG-8000)
PBS-Tween (PBS: 135 mM NaCl, 2.5 mM KCl, 8 mM Na₂HPO₄, 30 mM KH₂PO₄, pH 7.2 with added 0.05% Tween-20)
Lithium perchlorate (LiClO₄)

15-mL Flacon tubes
50- and 250-mL centrifuge bottles
UV transparent plates (Corning, cat. No 3635)
Microtiter plate reader (Bio-Tek)
Note: All buffers and solutions used for phage propagation are sterilized prior to use by autoclaving. Solutions such as PBS-Tween are sterile-filtered after addition of Tween to sterile PBS.

1. Transform the M13 phage-display vectors (phagemids) encoding peptide-2 fused to P8 into CaCl₂ competent *E. coli* XL1 Blue cells following Addgene's protocol for bacterial transformation (see Internet Resources).

2. Spread the transformed cells onto LB agar plates supplemented with 50 µg/mL carbenicillin. Incubate overnight at 37 °C.

**Starter cultures for phage propagation**

3. Aliquot 2 mL of 2YT media into two 15 mL Falcon tubes, and add 2 µL of carbenicillin and 1 µL of tetracycline from the antibiotic stocks to each tube.

   *XL1 Blue* E. coli encode the tetracycline resistance gene on a plasmid with the gene encoding the *F* pili, which are required for phage infection.

4. Add a single colony to each of the two cultures.

5. Incubate the cultures with shaking at 37 °C until each culture reaches log-phase growth (OD₆₀₀ of ≈0.45-0.5).

   *Shaking the cultures in a slanting position improves growth as it provides increased aeration in the Falcon tubes. Growing the cultures to log phase is crucial to obtain high infection efficiency (Lowman and Clackson, 2004). There are only a few *F* pili present per bacterial cell. But, if a high density of cells is achieved by allowing the cells to grow past log phase then the *F* pili expression is lowered. This results in lower infection efficiency. The shaking incubators are set to 250 rpm.*

6. Infect the culture with KO7 helper phage with a multiplicity of infection (MOI) of 4.6.

   *MOI can also be calculated as the ratio of the number of phage particles being added, to the number of bacterial cells in the culture. For the phage particles, an OD₂₆₈ of 1 = 5 × 10¹² particles/mL. For the bacterial cells, OD₆₀₀ of 1 = 1 × 10⁹ cells/mL.*

7. Incubate the cultures with shaking for 1 h at 37 °C.

**Grow Overnight cultures**

8. Transfer 150 mL of 2YT media to a 500 mL baffled flask. Add 150 µL carbenicillin and 75 µL of kanamycin.

   *The kanamycin resistance gene is present in the KO7 helper phage genome. Thus, the antibiotic is added at half strength to the overnight cultures. For overnight
cultures, a desirable ratio of volume of flask to culture is >3:1, for optimal culture aeration.

9. Transfer the two starter cultures to the 150 mL overnight culture prepared in step 8.

A ratio of ≈1:35 between the volumes of starter and overnight cultures works for most phage. Some phage grow better in larger or smaller culture sizes. For Stop-4 phage (phagemid packaged into the phage with no displayed ligands) and phage-2, 150 mL and 450 mL overnight culture volumes are recommended, respectively. The specific volumes used here were based on the size of the centrifuge bottles available.

10. Incubate the cultures overnight with shaking at 37 °C.

As with culture size, the duration of overnight cultures can be an important variable, which may require customization. For Stop-4 phage and phage-2, 16 and 19 h overnight culture durations respectively were found to be optimal.

**Phage precipitation**

11. Aliquot 30 mL of PEG-NaCl into a 250 mL centrifuge bottle. Place the bottle on ice.

*Precooling of the centrifuge tube is recommended for higher yields. The volume of PEG-NaCl used is ≈1/5th the volume of the overnight culture.*

12. Transfer the overnight culture to another 250 mL centrifuge tube. Centrifuge for 15,334 × g for 10 min at 4 °C.

*The centrifuge should be pre-cooled to 4 °C before centrifugation. At this point, the bacterial cells are contained in the pellet, and the virus particles remain suspended in the supernatant.*

13. Transfer the supernatant from step 12 to the centrifuge tube containing PEG-NaCl (step 11). Mix by inverting the tube 10 times.

*Care should be taken during transfer such that the cell pellet is not disturbed.*

14. Incubate the solution on ice for 1 hour.

*The solution contains phage, and thus the tube should always be completely covered with ice.*

15. Centrifuge at 15,334 × g for 20 min at 4 °C. Decant and discard the supernatant.

16. Recentrifuge at 2445 × g for 4 min at 4 °C.

*After step 15, the phage pellet can appear as a smear along the side of the tube.*
This step helps collect the pellet at the bottom of the centrifuge tube. If the centrifuge tubes are placed in the exact position as before, with the pellet facing away from the center of the rotor, the pellet will be collected at the same spot. Care should be taken not to overdo the centrifugation; otherwise the subsequent resuspension of the phage pellet becomes harder. A 4 min spin is usually sufficient and optimal unless the pellet size is extremely small. If necessary, the centrifugation can be reduced to 2 min.

17. Remove excess supernatant by blotting the centrifuge tubes on paper towels for 1-3 min.

18. Resuspend the phage pellet in 5 mL of PBS-Tween.

_Tween helps disaggregation of the phage particles. Resuspension should be largely performed by pipetting. After the addition of PBS-Tween to the pellet, incubation on ice for a few min softens the pellet, and assists the resuspension. The pipetting steps may be alternated with quick vortexing steps. During resuspension, the phage should still be kept on ice as much as possible._

19. Transfer to a 50 mL centrifuge tube and re-centrifuge at 13,776 × g for 10 min at 4°C.

_This step pellets the insoluble debris from the cell culture._

**Perform Second Precipitation**

20. Transfer the supernatant to a fresh centrifuge tube. Add 1/5<sup>th</sup> volume of PEG-NaCl and incubate on ice for 1 h.

_Repeating the precipitation steps results in a much cleaner phage stock, largely free of impurities._

21. Centrifuge for 20 min at 30,996 × g at 4°C. Decant and discard the supernatant.

_Since the volume of the solution is much smaller compared to the initial culture size, smaller centrifuge tubes (50 mL) and higher speeds can be used for precipitating the phage._

22. Recentrifuge for 4 min at 2204 × g at 4°C. Remove excess supernatant by blotting the centrifuge tubes.

_Blotting removes any residual PEG, which might interfere with future experiments. At this step, the phage pellet should have a white color. The appearance of any discoloration indicates the presence of impurities._

23. Resuspend the phage pellet in LiClO₄ and re-centrifuge at 13,776 × g for 10 min at 4°C.
The amount of 12 mM LiClO$_4$ used should be kept to a minimum to avoid over dilution of the phage stock. The resuspension is done in LiClO$_4$ as the phage-EDOT solution needed for the electrodeposition of the film on the gold electrode uses LiClO$_4$. For other biological assays, the phage resuspension may be performed in phosphate buffer.

**Phage quantification**

24. Quantify the phage by measuring the UV absorbance of the solution. An OD$_{268}$ of 1 = 8.31 nM or $5 \times 10^9$ phage/mL

For measuring phage concentration, an 1:10 dilution is preferred over an 1:100 dilution, as this volume avoids small pipetting errors that could occur with the latter dilution. For long term storage, phage stocks should be diluted to 40-65 nM. The stability at 4 °C varies, but most phage are stable for a few days. For prolonged storage, phage stocks can be flash-frozen and stored at -80 °C. Such phage stocks are stable for 6-12 months. The appearance of phage precipitating from the solution indicates an old and unusable phage stock.

25. Similarly, the experiment can be repeated to propagate the negative control Stop-4 phage.

**Basic protocol 3: BIOSENSING: FORMATION OF THE BIOAFFINITY MATRIX AND ELECTROCHEMICAL DETECTION**

Next, the bioaffinity matrix of the biosensor is prepared for electrochemical detection of PSMA. Incubation and binding of the target analyte, PSMA, to the biological recognition element produces a change in its electrical properties. Specifically, an increase in biosensor resistance is observed upon PSMA binding. As described above, PEDOT is formed by the electrochemical polymerization of EDOT in the presence of LiClO$_4$ and virus particles. The negative charge on the virus surface allows for its incorporation as counter ions to the positively charged PEDOT polymer depositing onto the gold electrode. The concentration of viruses used in the EDOT-LiClO$_4$ solution is an important parameter. Increased virus concentration leads to higher incorporation of viruses into the virus-PEDOT film (Donavan et al., 2012). A 3 nM virus solution provided an optimal working concentration. The sensitivity for PSMA detection was further improved by wrapping the incorporated viruses with additional secondary recognition ligands.

PSMA detection applies electrochemical impedance spectroscopy for monitoring the change in resistance upon PSMA binding. Data was collected over a wide frequency range and analyte concentrations. For the calibration curve, all calculations performed and parameters extracted were at 1000 Hz as previously described (Donavan et al., 2011). As a starting point, target concentrations should not fall below the LOD of the device.
The calibration curve provides the relative change in resistance obtained over a wide range of PSMA concentrations. The negative controls must examine non-specific binding between the target and the components forming the bioaffinity matrix; measurements with target binding to PEDOT films (no virus) provide an important control. Such controls also must test whether wrapping the phage with additional ligands increases resistance and non-specific binding. The data obtained can be analyzed to compute parameters such as the apparent $K_d$ for the interaction between target and the virus-displayed ligands, the LOD, and potential cooperativity.

**Materials**

- Lithium Perchlorate (LiClO$_4$)
- Ethylene-3,4-dioxythiophene (EDOT)
- Phage stocks (See basic protocol 2)
- Phosphate-buffered fluoride (PBF)-Tween (PBF, 4.2 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$ and 140 mM NaF, pH 7.2, sterile filtered, with added 0.1% Tween 20)
- K$_{CS}$-1 (see Basic Protocol 1; used as a solution: dissolve K$_{CS}$-1 in a 40:60 acetonitrile/HPLC-grade water solution to a final concentration of 1 µg/µl; K$_{CS}$-1 stock can be stored indefinitely at $-20^\circ$C)
- Piranha solution (optional; sulfuric acid [H$_2$SO$_4$] and 30% H$_2$O$_2$)
- Circular gold electrode (CH instruments)
- Polishing microcloth (Buehler)
- Diamond polishing paste (Ted Pella) having particle sizes of 1, 0.5 and 0.25 µm
- Platinum counter electrode
- Ag/AgCl reference electrode
- Parstat 2273 potentiostat
- POWERCV and POWERSine software (Princeton Applied Research, Oak Ridge, TN)
- Glass vial
- Kimwipes
- Butane Torch
- Faraday cage

**Preparing the phage-EDOT solution**

1. Prepare a 50 mL solution of 12.5 mM LiClO$_4$ in water. Vortex.

2. Transfer the solution prepared in step 1 to a glass vial. Add EDOT via pipette to a final concentration of 2.5 mM in water. Vortex gently.

   *EDOT can adhere to plastic surfaces, hence the solution should be prepared in a glass vial or jar.*

3. Cool the LiClO$_4$-EDOT solution to 4 °C by incubation in a refrigerator for 2 h.
The protocol for the preparation of phage-EDOT solution was found to provide best results if prepared with intermediate cooling steps. This solution is stable for up to a week.

4. Transfer 10 to 15 mL, 3 nM phage in ≈2 mM EDOT solution in a clean glass vial. Refrigerate overnight.

   Aliquot the EDOT solution, and then add phage to a 3 nM final concentration. The glass vial should have a mouth wide enough to hold the three electrodes. Care should be taken to eliminate any traces of ethanol remaining from the vial cleaning step, as ethanol degrades phage samples.

**Physically clean the electrode**

5. Polish a single gold 3 mm diameter electrode (reusable). Take a sheet of micropolished cloth and cut it up into smaller pieces approximately 2” × 2”. Take a piece and paste it onto a work bench; the reverse surface has adhesives on it. Place a small quantity of 1 μm diamond polishing paste on one corner.

   To avoid additional variables, the same electrode was used for the reported measurements.

6. With some paste on the electrode surface, move the electrode in circles on the polishing cloth approximately ten times. Then, apply more paste and slightly rotate the electrode before repeating the polishing step at a fresh spot on the cloth. Repeat this process for a total of ten times.

   Physical cleaning involves grinding and polishing the electrode surface. This crucial step requires some patience. Rushing through this step is not advisable, nor recommended. A smooth and clean electrode surface can make the difference between stable and disintegrating films.

7. Wipe away excess polish from the edges using a Kimwipe. Rinse well with deionized (DI) water. Wipe off excess water using a Kimwipe.

8. Repeat steps 6 and 7 with 0.5 μm diamond polishing paste.

9. Repeat steps 6 and 7 with 0.25 μm diamond polishing paste.

   Decreasing the size of the polishing paste smoothens out the surface and improves the deposition of the PEDOT-phage film in the subsequent steps.

10. Place the electrode in a vial containing DI water, and immerse the setup in a water bath sonicator. Sonicate for ten minutes. Rinse with DI water.

   Sonication helps in removing any material, which might be physically adhered to the glass electrode.
**Chemically clean the electrode**

11. *Optional cleaning with piranha solution*: Aliquot 3 mL H$_2$SO$_4$ in a glass vial, and carefully add 1 mL of 30% H$_2$O$_2$. Gently swirl to mix. Incubate the electrode in the solution for 10 min. Rinse with DI water.

   *During the swirling process, the formation of bubbles in the piranha solution should be avoided. H$_2$O$_2$ should be added very carefully and slowly to H$_2$SO$_4$, and not vice-versa. Piranha solution undergoes self-decomposition, hence the solution should be prepared immediately before usage. This step may or may not be needed. In our experiments, this step proved unnecessary.*

**Cyclic Voltammetry: the three electrode setup**

12. Flame-clean the platinum film electrode with a butane torch. Rinse with water. Clamp the electrode, and immerse it into the phage-EDOT solution.

   *A platinum electrode is used as the counter electrode.*

13. Rinse the Ag/AgCl reference electrode. Clamp the electrode and immerse it into the phage-EDOT solution.

   *The Ag/AgCl electrode serves as the reference electrode.*

14. Clamp and immerse the Au working electrode into the phage-EDOT solution, place the setup in a Faraday cage.

   *The electrodes should not be contacting each other.*

15. Connect all electrodes to the potentiostat.

16. Begin cycling the potential with the following parameters:

   - Initial potential: 1.15
   - Vertex: 0.2
   - Scan rate: 20 mV/sec
   - Cycles: 10

   *For negative controls, the films can be electrodeposited with an EDOT solution lacking phage. After this step, a dark blue film covers the gold electrode, obscuring the original gold color of the electrode. If this color deposition or a cyclic voltammogram as shown in Figure 5 is not observed, the process failed to deposit a sufficiently thick film; the process should be repeated following the troubleshooting below.*

   *The maximum current flowing through the circuit, as monitored by the change in applied potential, increases with every scan. This acts as an indication of increase in the thickness or the surface area of this porous film (i.e., more deposition takes place at the electrode with each scan in Figure 5).*
The phage-EDOT solution can be refrigerated and reused. The limiting factor for the phage-EDOT solution is the concentration of phage in the solution. For all procedure optimization experiments, the solution was reused two to four times. For the generation of the calibration curve, a fresh solution was used for each experiment to avoid introducing variation during electrodeposition.

**Cyclic voltammogram**

17. The acquired current versus potential data can be exported to any computing software of choice.

> If no parameters are computed from the cyclic voltammogram, the plot merely shows an increase in the maximum current flowing through the circuit. As a result, a graphing program such as MS Excel can be used to plot the trace. Each scan can be manually color-coded.

**Electrochemical impedance spectroscopy**

18. Rinse the film with DI water.

19. Fill a glass jar with ≈50 mL of PBF-Tween buffer.

> The solution is stable and can be reused for up to a week.

20. Rinse the electrode with PBF-Tween, and lower it into the PBF-Tween buffer from step 19. Also, move the counter and reference electrodes to PBF-Tween after rinsing with DI water.

21. Allow the virus-PEDOT film to equilibrate in PBF-Tween for 10 min.

> The virus-PEDOT film is porous, and the equilibration step is crucial to avoid current disturbances. It was observed that a five min equilibration was sufficient for virus-PEDOT films but a 10 min equilibration was necessary for PEDOT films lacking virus. After 10 min, the electrode can be lifted and placed back in the solution to avoid any concentration gradients forming in the electrode micro-environment.

22. Connect the electrodes to the potentiostat.

23. Acquire 5 consecutive EIS scans: 50 frequency data points spanning 0.1 Hz to 1 MHz with a 10 mV voltage modulation amplitude.

![Figure 5: Cyclic voltammogram for the electrodeposition of virus-PEDOT films on the surface of a planar gold electrode.](image)
There should be no significant drift in the resistance or impedance values, and the impedance should be monitored to identify drift.

This acquisition provides the native resistance ($R_0$) of the native film, the virus-PEDOT film with no analyte bound.

**Wrapping the virus-PEDOT films with additional ligands**
24. Rinse the electrode and incubate in a 1.5 mL eppendorf tube containing 200 µL of K\textsubscript{CS-1} at a concentration of 0.5 µg/µL. Gently shake at room temperature for 15 min.

The interaction between K\textsubscript{CS-1} and the virus particles is electrostatic. Since the negative charges on the virus surface are required for incorporation into the PEDOT films and wrapping with K\textsubscript{CS-1}, the virus-PEDOT films were wrapped after the synthesis of the virus-PEDOT films.

The electrode remained clamped during this incubation. The K\textsubscript{CS-1} solution is reusable, but a fresh solution was used for each experiment, as described above in step 16.

25. Rinse the electrode with DI water and then with PBF-Tween.

Care should be taken to wipe off any excess wash liquid adhered to the insulator on the electrode, to avoid any changes in concentration especially for the K\textsubscript{CS-1} and PSMA incubation steps.

26. Optional: check for resistance changes after phage wrapping by acquiring 5 consecutive EIS scans 50 frequency data points spanning 0.1 Hz to 1 MHz with a 10 mV voltage modulation amplitude.

In our experiments, the resistance of the films did not undergo any significant change upon phage wrapping with K\textsubscript{CS-1}. However, this aspect should be tested for new peptide wrappers. The dynamic, non-covalent interaction between the K\textsubscript{CS-1} wrappers and the viruses in the films could potentially result in decreased levels of K\textsubscript{CS-1} wrapped on the phage. However, PSMA binding to the film could reduce the possibility of the K\textsubscript{CS-1} wrappers detaching due to its synergistic binding.

**PSMA (analyte) binding**
27. Incubate the wrapped virus-PEDOT film with a 200 µL solution (of a desired concentration as shown, for example, in Figure 8) of PSMA in PBF-Tween buffer for 30 min with shaking.

Typically higher concentrations are measured first, but random concentrations can also be used.
28. Rinse the electrode, equilibrate and acquire 5 EIS scans as previously described in steps 20 through 23.

The resistance measurement obtained here is the final resistance of the film (R); this measurement indicates the change in resistance due to PSMA binding.

29. Repeat the above experiment with a different PSMA concentration or an alternative target. Also, perform appropriate negative controls.

**Analyze Data**

30. Export the data from the 5 scans for both the native film and the film after exposure to PSMA.

Any computing software capable of doing simple calculations and plotting graphs will suffice for the analysis presented here. MS Excel was used for our analysis.

31. Calculate the average resistance and standard deviation values across the 5 scans corresponding to each frequency for both the films.

\[
R = \text{Resistance of the film post PSMA incubation} = R_{Re}
\]

\[
R_o = \text{Resistance of the native film}
\]

Each measurement will provide a set of frequencies, and their corresponding \(R_{Re}\) and \(R_{Im}\) values, the real and imaginary components of the impedance, respectively.

32. Calculate the change in resistance caused by PSMA binding across all frequencies as:

\[
\Delta R = R - R_o
\]

This value is found to be proportional to the PSMA concentration.

33. Calculate the corresponding error across all frequencies as:

\[
\sigma = \sqrt{\sigma_i^2 + \sigma_f^2}
\]

where \(\sigma_i\) = standard deviation of the native film

\(\sigma_f\) = standard deviation of the film post PSMA incubation

More than one variable is being used to compute the above parameter, and thus, propagation of error is required for the determination of uncertainty.

34. Calculate the relative increase in resistance caused by PSMA binding across all frequencies as \(\Delta R/R_o\).
This step is essential to remove the dependence on $R_0$. For each PSMA measurement, a new film is being synthesized. This leads to a new $R_0$ for each measurement. To eliminate this variable across different films, the increase in resistance is normalized to the native resistance of the film.

35. Next, calculate the relative error as $\sigma/R_0$ across all frequencies.

Relative error is required for the calculation in step 34 with interdependent variables.

**Plot Data**

36. For full impedance scans, plot $\Delta R/R_0$ as a function of frequency.

This analysis can be done for any concentration of PSMA. The resultant plot can suggest the optimal frequency range for plotting the calibration curve. Phage-2, phage-2 wrapped with $K_{cs}$<sub>-1</sub>, and Stop-4 phage targeting PSMA were plotted on the graph in Figure 6. Frequencies >100 Hz could provide a conclusive measurement of $\Delta R/R_0$.

37. For a calibration curve, plot $\Delta R/R_0$ as a function of PSMA concentration.

For this analysis, a specific frequency of 1000 Hz was selected. The $\Delta R/R_0$ values at 1000 Hz corresponding to each PSMA concentration were plotted. It is essential to run all potential negative controls to test the validity of the data.

**Extracting key parameters**

38. Fit the data from the calibration curve to the Hill equation.

The equation used is for ‘non-linear regression and specific binding with Hill slope.’ The parameters computed by the software (GraphPad Prism) include $n$, $K_d$ and $Y_{max}$.

39. The parameters obtained, $n$, $K_d$ and $Y_{max}$, were used to extrapolate the curve to lower concentrations for computing the theoretical LOD.

The experimentally observed LOD is determined as the PSMA concentration for which the $\Delta R/R_0$ is at least 3-fold over background. The same principle was applied to the extrapolated data, and provides the theoretical LOD.
Reagents and solutions

Use Milli-Q water for the preparation of all solutions and buffers unless specified.

Azide- and alkyne-functionalized peptides

The lyophilized peptides can be stored indefinitely at -20 °C. Or dissolve the lyophilized peptides in a solution of acetonitrile and water (HPLC grade) to a final concentration of 200 µM, with the percentage of acetonitrile as needed for solubility. The solutions can also be stored indefinitely at -20 °C.

COMMENTARY

Background Information

M13 bacteriophage viruses

M13 bacteriophage viruses serve as the scaffold for the receptors in the bioaffinity matrix of the biosensor. The viruses have a ssDNA genome, which is encased in a protein coat comprising ≈2700 copies of a single major coat protein (P8), and five copies each of the four minor coat proteins (Welsh et al., 1998; Lowman and Clackson, 2004). The encapsulated DNA can be modified to display peptides or proteins on the surface of the phage as a fusion to the termini of the coat protein. For phage-2, the gene encoding the peptide ligand is fused to the N-terminus of the P8 coat protein. Furthermore, the phage propagation protocol could also be modified to simultaneously display two genetically encoded ligands on phage (Mohan and Weiss, 2014). In each case, the copy number of the displayed peptide ligands on the surface of the phage is much lower in comparison to the total number of P8 coat proteins. Hence, the phage are wrapped with additional chemically synthesized ligands to increase the apparent concentration of ligands in the bioaffinity matrix without increasing the phage concentration. Wrapping phage with additional ligands provides a quick method to increase the affinity and sensitivity of the device due to the enhanced avidity effect.

For the generation of the secondary recognition ligands, the wrappers are synthesized in two parts. The first half consists of an oligolysine (K₁₄) peptide, which wraps around the phage surface due to electrostatic interactions (Lamboy et al., 2008, 2009). The residues near the N-terminus of the P8 coat protein impart a high negative charge to the phage surface. The K₁₄ peptide is coupled to pentynoic acid during the synthesis process, providing an alkyne functionality for the click reaction. The second component of the wrapper is the peptide ligand to PSMA. The peptide ligand is similarly synthesized and functionalized with an azide moiety by coupling to 4-azidobutanoic acid. The two components are subsequently linked together by the click reaction.

Phage infectivity

A key step in the phage propagation protocol is the infection of the bacterial culture with the helper phage. The helper phage encodes the proteins necessary for
propagation, and the assembly of the virus. The phagemids encode an antibiotic resistance gene different from the helper phage. The addition of both antibiotics to the culture ensures the presence of both phagemid and helper phage DNA in each bacterial cell. For further details of phage propagation and design, we refer the reader to various books on this topic (Kay et al., 1996; Lowman and Clackson, 2004).

**Multiplicity of infection**

The infection of KO7 phage into the bacterial cell proceeds through the F pili, which are the receptors for phage infection. The number of F pili present per bacterial cell are few and limited (Lowman and Clackson, 2004). Thus, the multiplicity of infection (MOI) ensures high infection efficiency (equation 1). Additionally, the density of bacterial cell culture at the time of infection is also crucial. Cultures grown past log phase have high cell density, and the expression of pili decreases, which results in lower infection efficiency.

\[
MOI (m) = \frac{\text{number of virus particles}}{\text{number of bacterial cells}}
\]

**Equation 1**

The number of virus particles actually infecting a cell can be estimated by Poisson distribution as:

\[
P(k) = \frac{e^{-m} \cdot m^k}{k!}
\]

**Equation 2**

Where,

- \(P(k)\) = Probability that a cell will be infected by ‘\(k\)’ virus particles
- \(P(k)\) = Fraction of cells infected by ‘\(k\)’ virus particles

If \(k = 0\), then,

\[P(0) = \text{fraction of uninfected cells} = e^{-m}\]

For 99% infection efficiency, the fraction of uninfected cells is:

\[P(0) = 0.01 = e^{-m}\]

Solving for \(m\):

\[m = 4.6\]

Thus, for 99% infection, the ideal MOI is 4.6.

**Biosensing Theory**

The bioaffinity matrix in the biosensor consists of a composite film of a conductive organic polymer (PEDOT) and viruses deposited on a planar gold electrode. EDOT undergoes electrochemical polymerization in the presence of LiClO\(_4\) to form cationic PEDOT units, which associate with perchlorate ions while depositing onto the electrode, Figure 7 (Sharma et al., 2012). Polymerization of EDOT in the presence of
phage particles leads to the generation of the virus-PEDOT film due to the incorporation of phage particles as the counter ions during deposition, Figure 7.

Ligand binding interactions are enhanced due to the cooperative effect of the two ligands present on the surface of the phage, one genetically encoded and the other chemically synthesized. This bidentate binding mode combined with the saturation binding effect can be quantified, and its parameters computed using the Hill equation (Equation 3) (Hill, 1910):

\[
Y = \frac{Y_{\text{max}} \cdot [L]^n}{K_d^n + [L]^n}
\]

Where,
- \( Y = \Delta R/R_o \)
- \( L = \) PSMA concentration
- \( n = \) Hill coefficient
- \( K_d = \) Dissociation constant

The Hill coefficient is a measure of cooperativity present in the binding interaction. A value of 1 indicates no cooperative binding interactions. A value >1, as seen in Figure 8 indicates positive cooperativity, whereas a value <1, indicates negative cooperativity. The bidentate binding mode of ligands 1 and 2 (arising from the combination of phage-2 and K_{CS-1}) results in a Hill coefficient of 1.5, which demonstrates the synergy of the two ligands in cooperatively binding to PSMA.

**TROUBLESHOOTING**

*Optimizing the combination of ligands*

The choice of ligands coating the phage surface might require careful selection to optimize sensitivity of the biosensor. For example, two ligands with negative cooperativity would be a poor choice for biosensor development. Fortunately, phage display selections typically result in large numbers of possible ligands. Various scenarios, their causes, and suggestions for improvement are summarized in the Figure 9 flowchart.
For non-competing ligands, the identity of the genetically displayed and chemically synthesized ligand could still be a crucial factor due to the inversion of the ligand structure on the phage surface during the synthesis of the wrapper.

**Phage propagation**

Acquiring a phage stock free from contamination is very important for obtaining high sensitivity and specificity for analyte detection. During the process of phage propagation, it is essential to meticulously follow all the steps listed above. Possible sources of error and points of concern have been incorporated in the notes for each step in the basic protocol 2. Close attention should also be paid to the yields obtained for phage-displayed peptide. Unforeseen circumstances such as errors with MOI calculations etc., could lead to the propagation and packaging of KO7 phage, but this results in exceptionally high phage yields.

Figure 9: Flowchart describing the process of designing and planning a ligand combination for the detection of analyte with increased sensitivity. The commonly observed phenomena are listed here alongside possible solutions, including troubleshooting the ligand combination using biological assays such as enzyme-linked immunosorbent assay. The figures provide representative data for each scenario. Phage concentrations are plotted along the x-axis and the assay response along the y-axis.
**The cycloaddition reaction**

The choice of azide- and alkyne-functionalized peptides governs the identity of the solvent used for the reaction. If precipitation is observed in the reaction mixture, different temperature conditions (e.g., heating) or solvents could be tried. If the reaction results in low yields, Cu(I) stabilizing ligands such as TBTA could be used.

**Biosensing**

A common problem encountered with biosensing is drift observed in the impedance values during EIS. The possible causes leading to drifting values and the corresponding solutions have been summarized as a flowchart in Figure 10. Non-specific binding observed during the biosensing experiments could be attributed to impurities in the materials or deteriorated reagent stocks. Phage and peptide purity is essential for low background measurements.

![Flowchart](image)

Figure 10: Flowchart listing the possible problems, the corresponding sources of errors, and the recommended troubleshooting steps.

**Anticipated results**

Basic protocols 1 and 2 provide the materials necessary for the generation of the bioaffinity layer. These materials can then be applied to the detection of the cancer biomarker, as described in basic protocol 3. Once a clinically relevant limit of detection has been obtained for biosensing with virus-PEDOT films, the assay setup can be used for clinical research with biological fluids from cancer patients.

**Time considerations**

Once the purified azide- and alkyne-functionalized peptides have been synthesized and purified, basic protocol 1 can be completed in 3-4 days. Basic protocol 2 takes a total of three days including the transformation step. The majority of the effort required for phage propagation occurs on day 3 with the phage precipitation. For basic
protocol 3, each experiment from the cleaning of the electrode to measuring the final resistance upon PSMA incubation takes ~2.5 hours. With previously prepared, equilibrated and calibrated phage-PEDOT films in device form, five redundant measurements could theoretically be completed in minutes or less.

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**Internet resources with annotations**

http://www.lumiprobe.com/protocols/click-chemistry-dna-labeling

*Lumiprobe protocol: Click-Chemistry Labeling of Oligonucleotides and DNA.*

https://www.addgene.org/plasmid-protocols/bacterial-transformation/

*Addgene protocol: Heat-shock transformation of plasmids/phagemids into chemically competent bacterial cells.*

http://www.graphpad.com

*GraphPad Prism software has been used for biosensing data analysis. GraphPad software was founded by CEO Dr. H. J. Motulsky, located in San Diego, CA.*
http://www.virology.ws/2011/01/13/multiplicity-of-infection

Prof. Vincent Racaniello Ph.D., Professor of Microbiology & Immunology in the College of Physicians and Surgeons of Columbia University, discusses the concept multiplicity of infection from a statistical standpoint.