Biosensing with Virus Electrode Hybrids

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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 a 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. © 2015 by John Wiley & Sons, Inc.

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INTRODUCTION

This article describes a general protocol for the development of a biosensor aimed at efficiently detecting cancer biomarkers in a 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 (Yang et al., 2006, 2008b; Weiss and Penner, 2008; Diaz et al., 2009; 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 (i.e., phage-**1** or phage-**2**; see Fig. 3). 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 Fig. 3) for electrostatic
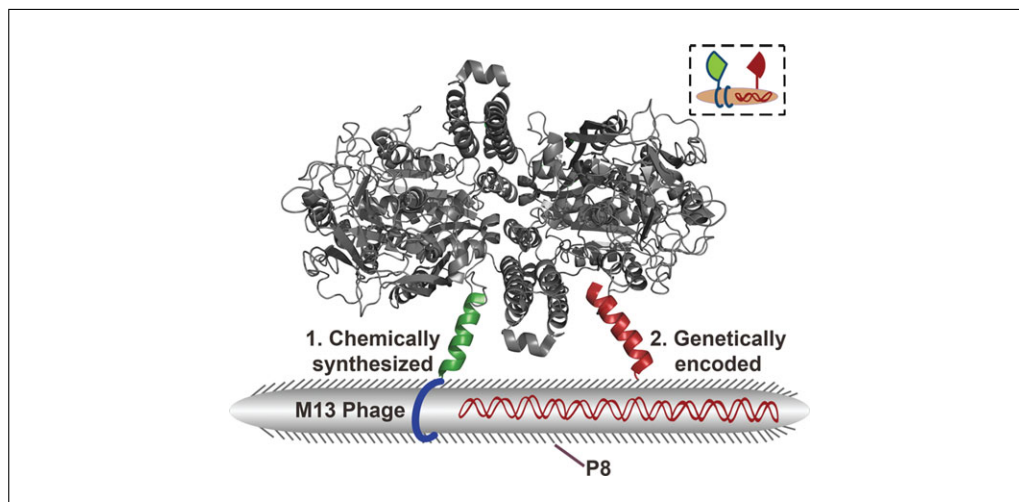
Figure 1 Schematic representation of the bidentate binding mode to PSMA by chemically synthesized (green) and the genetically encoded (red) ligands to PSMA (PDB: 1Z8L). The former ligand wraps non-covalently through non-specific interaction with the negatively charged P8 proteins found on the phage surface and a fused positively charged K_{14} peptide (blue). The inset depicts a simplified version of the schematic appearing in the subsequent figures shown here. Figures 1-8 are being reprinted with permission from Mohan, K., Donavan, K.C., Arter, J.A., Penner, R.M., and Weiss, G.A. 2013. Sub-nanomolar detection of prostate-specific membrane antigen in synthetic urine by synergistic, dual-ligand phage. *J. Am. Chem. Soc.* 135:7761-7767. Copyright (2013) American Chemical Society.

wrapping around the phage surface. The dual display of ligands results in a bidentate binder, and enhanced PSMA detection from both a dense 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, similar virus-hybrid surfaces are stable for over 14 hr 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 quartz crystal microbalance (QCM) allows rapid kinetics in binding to biomarkers (Yang et al., 2008a). Such multi-point, cooperative binding has been observed by surface plasmon resonance (SPR) for a phage-based surface (Nanduri et al., 2007).

Most importantly, phage binding can be readily adapted for the recognition of 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 display 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 two halves 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. When using this approach, the oligolysine half of the secondary recognition ligand can remain constant for many different peptide ligands wrapped onto the phage. Thus, it is advantageous to synthesize the two halves separately to maximize the flexibility of the experimental design and efficiency. The 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 bioaffinity matrix can be affixed to the working electrode of the electrochemical biosensor by adsorption, entrapment, a specific interaction, and covalent attachment. Biomolecular entrapment of the viruses within a conducting polymer matrix provides robust attachment and allows the ligands to remain functional for the recognition of the target molecules (Cosnier, 1999). Additionally, the method is expeditious, and avoids potential phage-degrading steps.

The protocols in this article present a general method for the detection of an arbitrary disease biomarker, termed a target molecule, using electrochemical biosensors consisting of phage-display ligands wrapped with additional secondary recognition ligands to increase the biosensor's sensitivity for the target molecule (see Fig. 2). Three basic procedures are 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 these steps, the incorporation of peptide ligands into the bioaffinity layer for the detection of PSMA is described here.
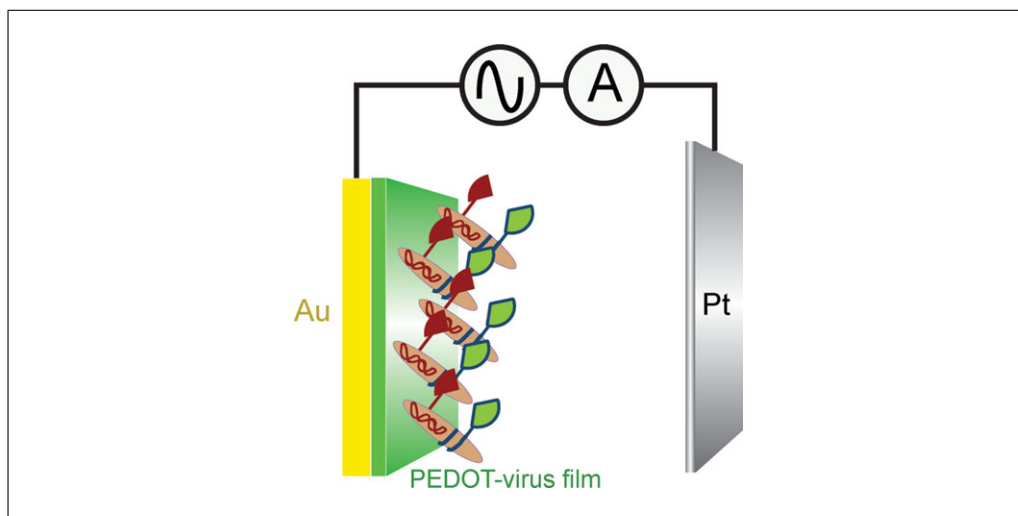

Figure 2 A schematic representation of the biosensor. Binding of the analyte to the phage-displayed peptides in the bioaffinity layer produces a quantifiable change in electrical resistance.

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

Peptide synthesis of ligands

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

BASIC PROTOCOL 1

CLICK CHEMISTRY REACTION: PREPARATION OF K_{CS}-1 AND K_{CS}-2

After the azide- and alkyne-functionalized peptides have been synthesized and purified, the next step is to link them by the click reaction shown in Figure 4. The resultant ligands are listed as K_{CS}-**1** and K_{CS}-**2** in Figure 3. The reaction protocol for the click reaction is modified from the Lumiprobe protocol for oligonucleotides (see Internet Resources section). 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 tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (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 (1 M, pH 7)
HPLC-grade water or Milli-Q water
Azide-functionalized peptide stock solution (200 µM in 1:3 water/acetonitrile mixture)
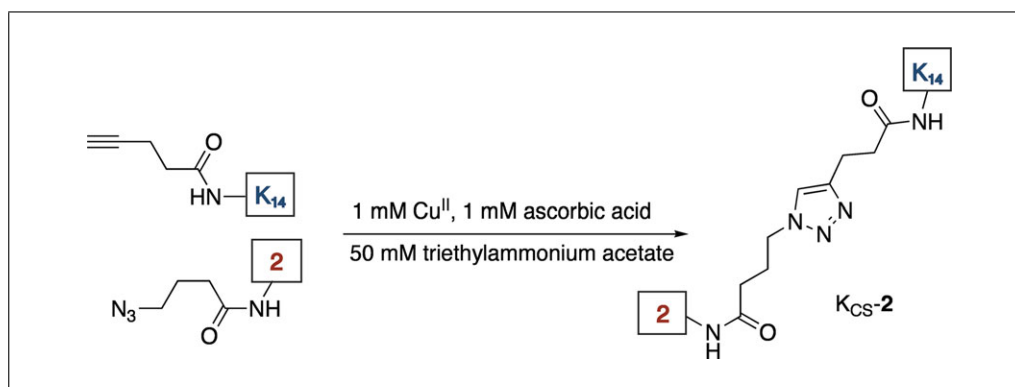Ascorbic acid
Copper sulfate ($CuSO_4$)

Micro concentrator

Figure 4 Cu(I) catalyzed cycloaddition reaction to generate the secondary recognition ligand, K_{CS}-**2**. The boxed **2** and K_{14} indicate the ligand **2** and oligolysine peptides, respectively.

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 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 of the reaction product.

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

4. Add 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 solution.

5. Add 200 µl 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 reaction mixture by bubbling an inert gas (e.g., nitrogen) through the mixture for 30 sec.

7. Add 200 µl ascorbic acid solution. Briefly vortex.

8. Add 10 µl $CuSO_4$ solution (100 mM).

9. Sparge reaction mixture again with inert gas (e.g., nitrogen) for 10 sec.

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

10. Vortex conical tube, and incubate overnight ($\sim$16 hr) at room temperature.

    A precipitate observed after addition of the 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 to attempt resolubilization.

Characterize product formation

11. Combine reactions into one conical tube.

12. Run matrix-assisted laser desorption/ionization time-of-flight (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.

Purify the cycloaddition reaction product

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

    In general, ten reactions were run and concentrated to 500 to 1000 µl for further purification.

14. Run reverse-phase HPLC for purification.

    The product was purified using multiple runs of analytical scale HPLC. Fractions were analyzed by MALDI-TOF, and the appropriate fractions from each run were combined.

15. Place fractions under speed vacuum to remove solvent.

16. Resuspend product to a final concentration of 1 µg/µl 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.*

PHAGE PROPAGATION, ISOLATION, PURIFICATION, AND QUANTIFICATION

M13 bacteriophages infect Gram-negative bacteria (e.g., *Escherichia coli*), and can be readily isolated from bacterial culture. Within the encapsulated phagemids (plasmid-like vectors), open reading frames (ORFs) encode peptide ligands as fusions to a periplasmic localization signal sequence (stII leader sequence: MKKNIAFLLASMFVFSIATNAYA) and the N-terminus of P8, the major coat protein for fusion on the phage surface. 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 (GGGSGSSSGGGSGGG). 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 applied below also includes an antibiotic-resistance marker to allow selections for its propagation in the presence of carbenicillin. 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 polyethylene glycol (PEG)-sodium chloride (NaCl) precipitation. This protocol details all steps necessary to obtain purified phage.

Materials

E. coli XL1 Blue cells (CaCl$_2$ 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)
LB agar plates
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)
M13 KO7 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 Na$_2$HPO$_4$, 30 mM KH$_2$PO$_4$, pH 7.2 with 0.05% Tween-20 added)
12 mM lithium perchlorate (LiClO$_4$) solution

15-ml Falcon tubes
50- and 250-ml centrifuge bottles
UV transparent plates (cat. no. 3635, Corning)
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 M13 phage-display vectors (phagemids) encoding peptide-**2** fused to P8 into CaCl$_2$ competent *E. coli* XL1 Blue cells.

2. Spread 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 medium into each of two 15-ml Falcon tubes, and add 2 μl carbenicillin and 1 μl tetracycline from the antibiotic stocks to each tube.

   XL1 Blue E. coli carry a plasmid with genes encoding tetracycline resistance and F pili, which are required for phage infection.

4. Pick small quantities from the center of two individual colonies from step 2 to add to the two tubes prepared in step 3 (one colony transfer per tube).

5. Incubate cultures with shaking (250 rpm) at 37°C until each culture reaches log-phase growth (OD$_{600}$ ~0.45 to 0.5*).

   Shaking the cultures with the tubes in a slanted position improves growth as it provides increased aeration. Growing the cultures to log phase is crucial to obtain high infection efficiency (Lowman and Clackson, 2004). Only a few F pili are present per bacterial cell, but a high density of cells (i.e., bacteria grown past log phase) results in decreased expression of the F pili. Thus, the density of the cells is a critical variable in determining helper phage infection efficiency and phage yields.

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

   The 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 OD$_{268}$ of 1 = 5 × 10^{12} particles/ml; for the bacterial cells, OD$_{600}$ of 1 = 1 × 10^9 cells/ml.

7. Incubate cultures with shaking (250 rpm) for 1 hr at 37°C.

Grow overnight cultures

8. Transfer 150 ml of 2YT medium to a 500-ml baffled flask. Add 150 μl carbenicillin and 75 μl 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 (the phagemid packaged into phage with no displayed ligands on their surfaces, used as a negative control) and phage-2, 150-ml and 450-ml overnight culture volumes respectively are recommended. The specific volumes used here were based on the size of the centrifuge bottles available.

10. Incubate 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 hr overnight culture durations respectively were found to be optimal.

Phage precipitation

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

    Pre-cooling of the centrifuge tube is recommended for higher yields. The volume of PEG-NaCl used is ~one-fifth the volume of the overnight culture.

12. Transfer overnight culture to another 250-ml centrifuge tube. Centrifuge at 15,344 × 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 supernatant from step 12 to the centrifuge tube containing PEG-NaCl (step 11). Mix by inverting the tube ten times.

   Care should be taken during transfer to avoid disturbing the cell pellet.

14. Incubate solution on ice for 1 hr.

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

15. Centrifuge at 15,344 × g for 20 min at 4°C. Decant and discard supernatant.

16. Re-centrifuge at 2455 × 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 to 3 min.

18. Resuspend phage pellet in 5 ml PBS-Tween.

   Tween helps to disaggregate the phage particles. Resuspension should be largely accomplished by pipetting. After the addition of PBS-Tween to the pellet, incubation on ice for a few minutes softens the pellet and assists the resuspension. The pipetting steps may be alternated with quick vortexing steps. During resuspension, the phage should be incubated 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 pelletizes the insoluble debris from the cell culture.

Perform second precipitation

20. Transfer supernatant to a fresh centrifuge tube. Add a one-fifth volume of PEG-NaCl, and incubate on ice for 1 hr.

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

21. Centrifuge 20 min at 30,996 × g, 4°C. Decant and discard 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. Re-centrifuge 4 min at 2204 × g, 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 phage pellet in 12 mM $LiClO_4$ 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-3,4-ethylenedioxythiophene (EDOT) solution needed for the electrodeposition of the film on the gold electrode uses $LiClO_4$. For other biological assays, the phage can be resuspended in PBS.

Phage quantification

24. Quantify phage by measuring the UV absorbance of the solution: OD_{268} of 1 = 8.31 nM or 5 × 10^9 phage/ml.

For measuring phage concentration, a 1:10 dilution is preferred rather than a 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 to 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 to 12 months. The appearance of phage precipitating from the solution indicates an old and unusable phage stock.

Similarly, the phage overexpression and isolation procedure described above can be repeated to propagate the negative control Stop-4 phage.

BIOSENSING: FORMATION OF THE BIOAFFINITY MATRIX AND ELECTROCHEMICAL DETECTION

After the completion of Basic Protocols 1 and 2, 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 drives phage incorporation into the positively charged PEDOT polymer, forming through electrodeposition 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.

The calibration curve provides the relative changes in resistance obtained over a wide range of PSMA concentrations. Data were collected over a wide range of frequencies and analyte concentrations to determine the optimal frequency to use. For the calibration curve reported previously, the calculations performed and parameters were extracted from the 1000-Hz data (Donavan et al., 2011). As a starting point for measuring a calibration curve, the target concentration should be well above the limit of detection (LOD) for the device. 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 the 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 buffer (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°C)

Piranha solution (optional; sulfuric acid [H$_2$SO$_4$] and 30% H$_2$O$_2$)

1-, 0.5- and 0.25-μm particle size diamond polishing paste (Ted Pella)

Circular gold (Au) electrode (CH Instruments)

Micropolishing cloth (Buehler)

Platinum counter electrode

Ag/AgCl reference electrode

Parstat 2273 potentiostat

POWERCV and POWERSine software (Princeton Applied Research)

Glass vial

Kimwipes

Butane torch

Faraday cage

Preparing the phage-EDOT solution

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

2. Transfer solution prepared in step 1 to a glass vial. Add EDOT via pipet 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 hr.

    The protocol for the preparation of phage-EDOT solution provides the best results when the intermediate cooling steps are included. The LiClO$_4$-EDOT solution is stable for up to 1 week.

4. Transfer 10 to 15 ml solution of 3 nM phage in ~2 mM EDOT to 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). Cut a sheet of micropolishing cloth into smaller pieces ~5.1 cm × 5.1 cm (2 inches × 2 inches). Attach a piece to a work bench, using the adhesive on the reverse surface. 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 neither 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 particle size of the polishing paste smooths out the surface and improves the deposition of the PEDOT-phage film in the subsequent steps.

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

Sonication helps remove any material that might be physically adhered to the glass electrode.

Chemically clean the electrode

11. Optional cleaning with piranha solution: Aliquot 3 ml H_2SO_4 into a glass vial, and carefully add 1 ml of 30% H_2O_2. Gently swirl to mix. Incubate electrode in the solution 10 min. Rinse with DI water.

During the swirling process, the formation of bubbles in the piranha solution should be avoided. H_2O_2 should be added very carefully and slowly to H_2SO_4, and not vice versa. Piranha solution undergoes self-decomposition, hence the solution should be prepared immediately before use. This step may or may not be needed. In our experiments, for example, 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 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 electrode and immerse it in 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 in contact with each other.

15. Connect all electrodes to the potentiostat.

16. Cycle 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 from 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
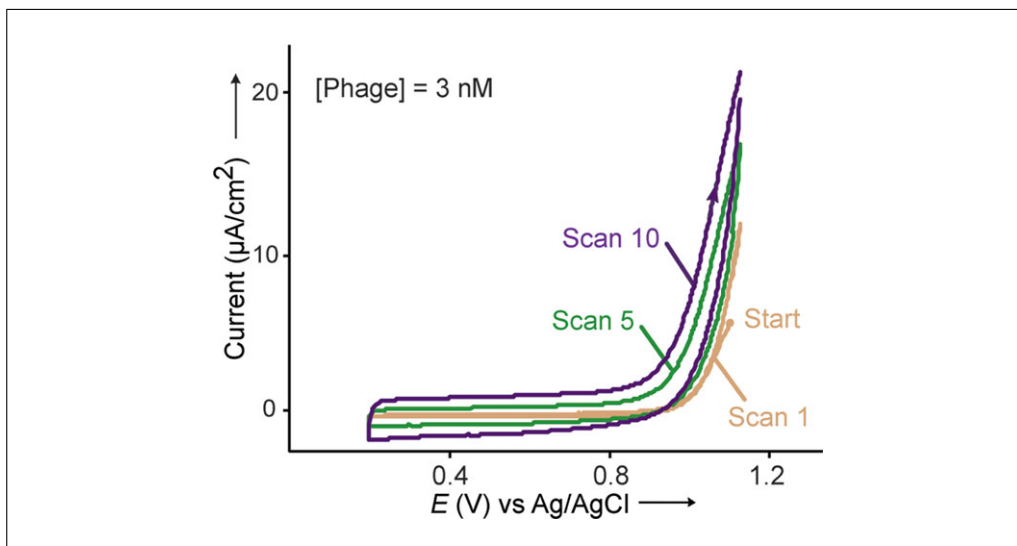
Figure 5 Cyclic voltammogram for the electrodeposition of virus-PEDOT films on the surface of a planar gold electrode.

Figure 5 is not observed, the process failed to deposit a sufficiently thick film; the process should be repeated following the recommendations in the Troubleshooting section (see 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; see Fig. 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 procedure optimization, the solution can be reused two to four times. For the generation of the calibration curve, a fresh solution should be used for each experiment to avoid introducing variation during electrodeposition.

Cyclic voltammogram

17. Export the acquired current versus potential data 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 Microsoft Excel can be used to plot the trace. Each scan can be manually color-coded.

Electrochemical impedance spectroscopy

18. Rinse film with DI water.

19. Fill a glass jar with ∼50 ml PBF-Tween buffer.

    The solution is stable and can be reused for up to 1 week.

20. Rinse electrode with PBF-Tween, and lower it into the PBF-Tween solution from step 19. After rinsing the counter and reference electrodes with DI water, place them in the PBF-Tween solution.

21. Allow 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 5-min equilibration was sufficient for virus-PEDOT films but a 10-min equilibration was necessary for PEDOT films lacking virus. After

22. Connect electrodes to the potentiostat.

23. Acquire five consecutive electro interstitial scanning (EIS) scans: 50 frequency data points spanning 0.1 Hz to 1 MHz with a 10-mV voltage modulation amplitude.

    There should be no significant drift in the resistance or impedance values, and the impedance should be monitored to identify drift (see Troubleshooting section and Fig. 10).

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

Wrap the virus-PEDOT films with additional ligands

24. Rinse electrode and incubate in a 1.5 ml-microcentrifuge tube containing 200 µl of K_{CS}-**1** at a concentration of 0.5 µg/µl. Gently shake at room temperature for 15 min.

    *The interaction between K_{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_{CS}-**1**, the virus-PEDOT films were wrapped after the synthesis of the virus-PEDOT films.*

    *The electrode remains clamped during this incubation. The K_{CS}-**1** solution is reusable, but a fresh solution should be used for each experiment, as described above in step 16.*

25. Rinse 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_{CS}-**1** and PSMA incubation steps.*

26. Optional: Check for resistance changes after phage wrapping by acquiring five consecutive EIS scans, each with 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_{CS}-**1**. However, this possibility should be tested for new peptide wrappers. The dynamic, non-covalent interaction between the K_{CS}-**1** wrappers and the viruses in the films could potentially result in decreased levels of K_{CS}-**1** wrapped on the phage. However, PSMA binding to the film could reduce the possibility of the K_{CS}-**1** wrappers detaching due to its synergistic binding.*

PSMA (analyte) binding

27. Incubate the wrapped virus-PEDOT film with 200 µl PSMA in PBF-Tween buffer (of a desired concentration as shown, for example, in Fig. 8) for 30 min with shaking.

    Typically, higher concentrations are measured first, but random concentrations can also be used.

28. Rinse electrode, equilibrate and acquire five 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. Perform appropriate negative controls.

Analyze data

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

Biosensing with Virus Electrode Hybrids

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

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

    R = Resistance of the film post-PSMA incubation = R_{Re}
    R_o = 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 proportional to the PSMA concentration.

33. Calculate the corresponding error across all frequencies as:

$$\sigma = \sqrt{(\sigma_i^2 + \sigma_f^2)}$$

    where σ_i = Standard deviation of the native film
    σ_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_o. For each PSMA measurement, a new film is being synthesized. This leads to a new R_o 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 σ/R_o across all frequencies.

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

Plot data

36. For full impedance scans, plot $\Delta R/R_o$ 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}-1, and Stop-4 phage targeting PSMA are plotted on the graph in Figure 6. Frequencies >100 Hz could provide a conclusive measurement of $\Delta R/R_o$.

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

    For this analysis, a specific frequency of 1000 Hz was selected. The $\Delta R/R_o$ 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; see Internet Resources) include n, K_d and Y_{max}.
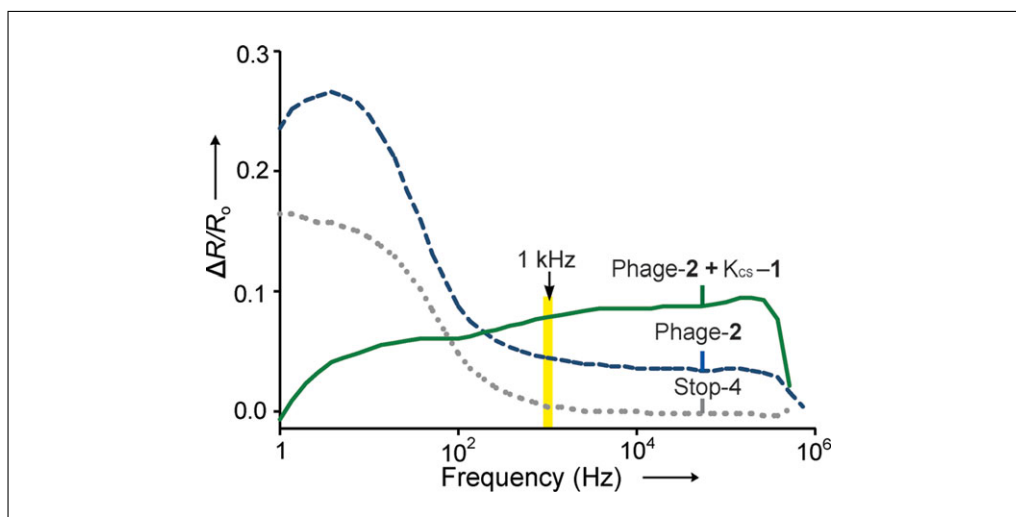
Figure 6 The relative change in resistance obtained, $\Delta R/R_o$, plotted as a function of frequency.

39. Extrapolate the curve to lower concentrations for computing the theoretical limit of detection, using the parameters obtained, n, K_d and Y_{max}.

The experimentally observed LOD is determined as the PSMA concentration for which the $\Delta R/R_o$ is at least three-fold over background. The same principle was applied to the extrapolated data, and provides the theoretical limit of detection.

REAGENTS AND SOLUTIONS
Use Milli-Q water or equivalent in all recipes and protocol steps.

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 HPLC-grade water to a final concentration of $200\,\mu M$, with the percentage of acetonitrile as needed for solubility. The solutions can 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 an 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 (K_{14}) peptide, which wraps around the phage surface due to electrostatic interactions (Lamboy et al., 2008, 2009). Carboxylate-bearing residues near the N-terminus of the P8

coat protein impart a high negative charge to the phage surface. The K_{14} 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:

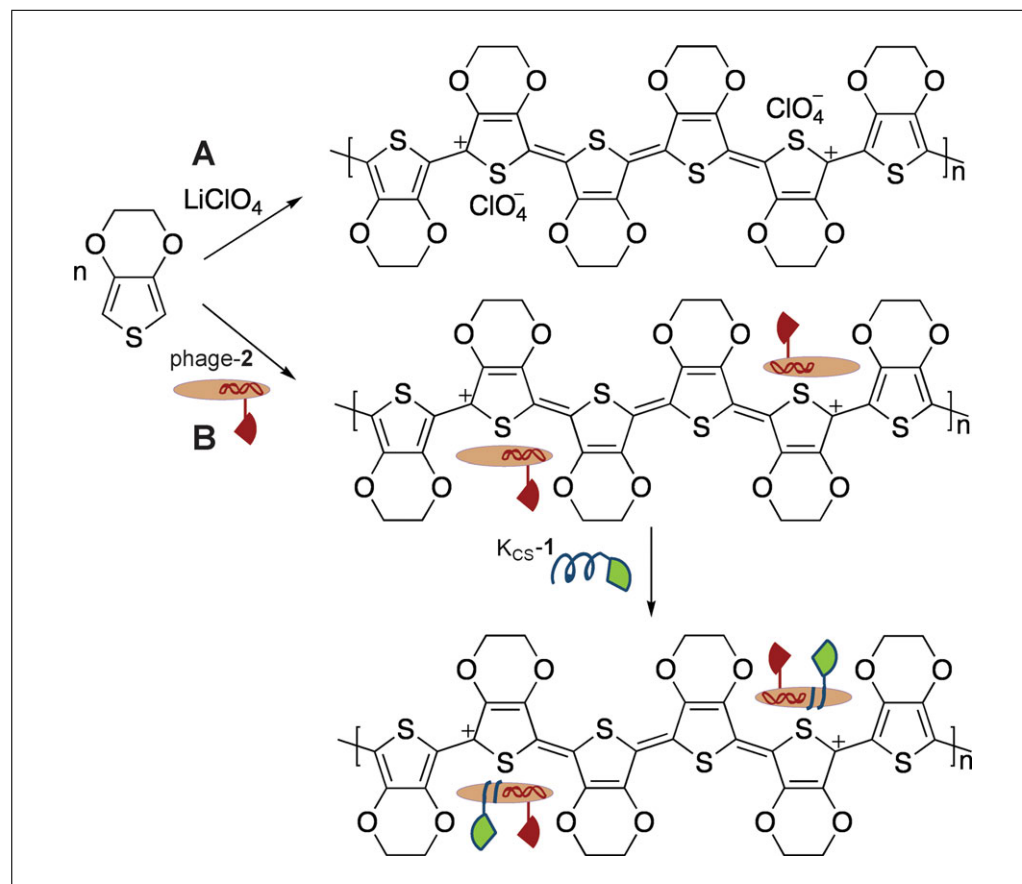$$P(k) = \frac{e^{-m} \cdot m^k}{k!}$$

Equation 2

Figure 7 Polymerization of EDOT in the presence of (**A**) LiClO$_4$ or (**B**) Phage-**2**, followed by wrapping with K_{CS}-**1** (green and blue).

where

$P(k)$ = Probability that a cell will be infected

by 'k' virus particles

= Fraction of cells infected by 'k' virus

particles

If $k = 0$, then,

$P(0)$ = 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 efficiency, 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 (Fig. 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 counterions during deposition.

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):

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

Equation 3

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 the sensitivity of the biosensor. For example, two ligands with negative cooperativity would be poor choices 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 choice of the genetically displayed and chemically synthesized ligand could also be a crucial factor, and the orientation of ligands following bioconjugation should be considered.

Phage propagation

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

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.
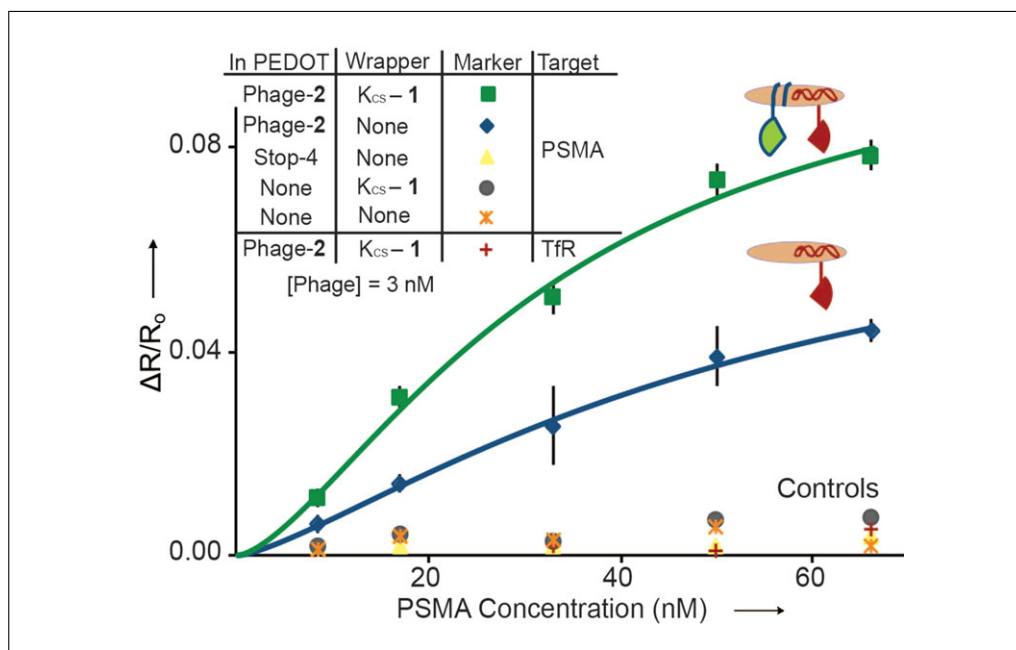
Figure 8 $\Delta R/R_o$ of the film increases with higher concentrations of PSMA.
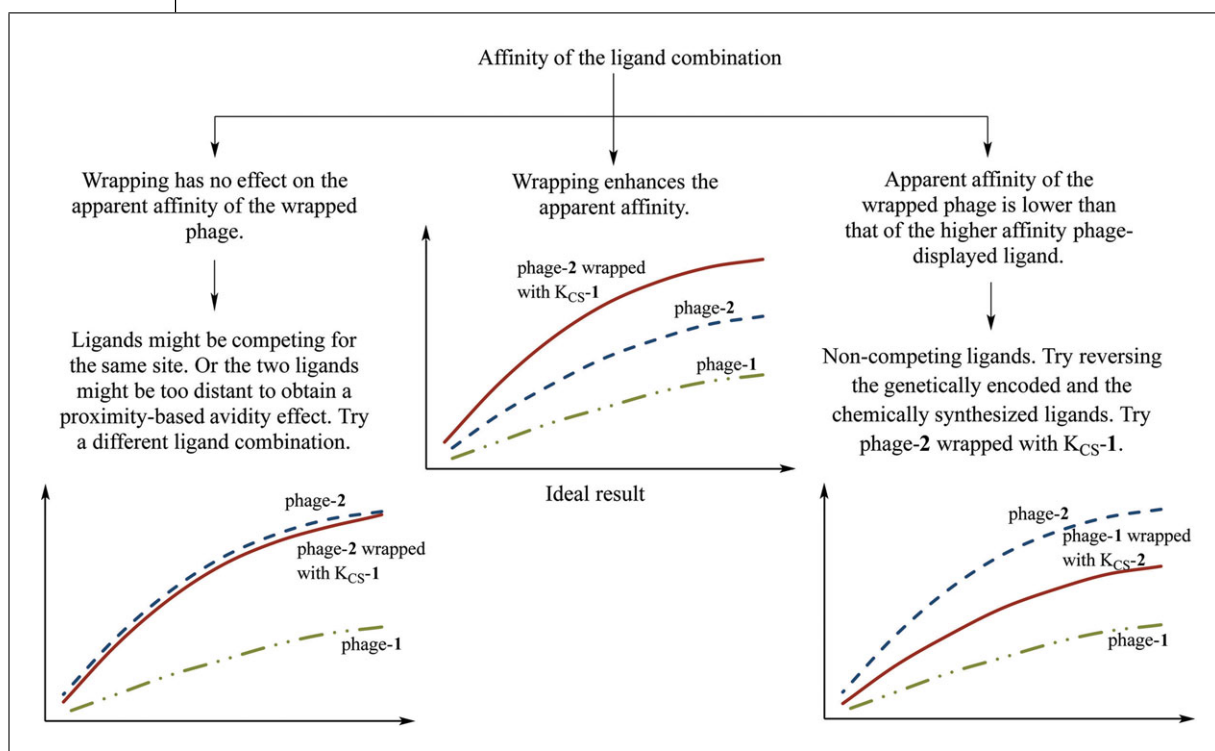

Figure 9 Flowchart describing the process of designing and planning a ligand combination for the detection of analyte with increased sensitivity. The commonly observed phenomena are listed here alongside possible solutions, including troubleshooting the ligand combination using biological assays such 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.

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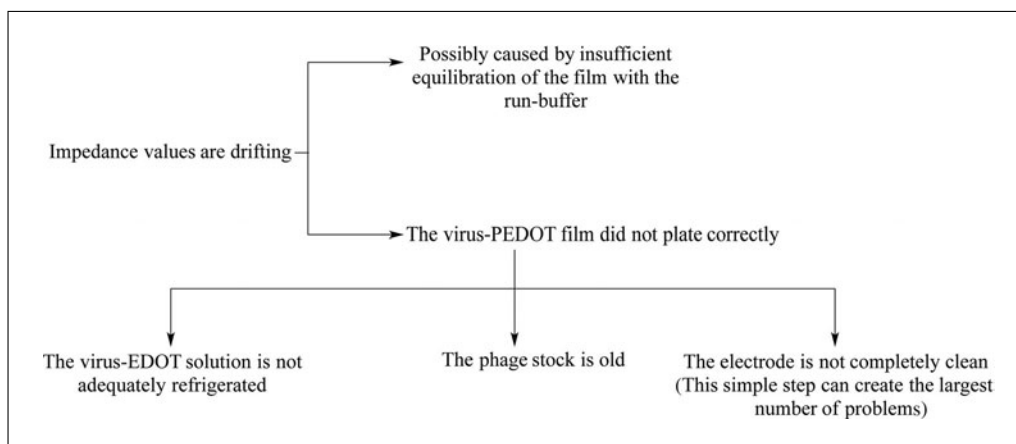
Figure 10 Flowchart listing the possible problems, the corresponding sources of errors, and the recommended troubleshooting steps.

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 to 4 days. Basic Protocol 2 takes a total of 3 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 electrode cleaning to measuring the final resistance upon PSMA incubation requires ∼2.5 hr. 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

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.

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., Higgins Professor of Microbiology & Immunology in the College of Physicians and Surgeons of Columbia University, discusses the concept of multiplicity of infection from a statistical standpoint.